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PLATELET AND ENDOTHELIAL CELL INTERACTIONS *IN VITRO*

Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in the faculty of Science of the University of Adelaide

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CONTENTS

DECLARATION

The work described in this thesis was carried out in the department of Clinical and Experimental Pharmacology, University of Adelaide and the Division of Haematology, Institute of Medical and Veterinary Science under the joint supervision of Professor F. Bochner and Dr J.V. Lloyd. This thesis does not contain any material which has been submitted for a degree at any other institution and to the best of my knowledge contains no material that has been previously published except where due reference is cited.

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ABBREVIATIONS

ACE	angiotensin converting enzyme
ADP	adenosine 5'-diphosphate
ATP	adenosine triphosphate
ANOVA	analysis of variance
Ca^{2+}	ionised calcium
cAMP	cyclic adenosine 3', 5'-phosphate
cGMP	cyclic guanosine 3', 5'-phosphate
CCM	complete culture medium
DAG	diacylglycerol
EDRF	endothelium-derived relaxing factor
G-protein	guanine nucleotide binding protein
Hb	oxyhaemoglobin
HBSS	Hank's buffered salt solution
Hepes	N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid.
HETE	hydroxy-5,8,10,14-eicosatetraenoic acid
HHT	12-hydroxy-5,8,10-heptadecatrienoic acid
13-HODE	13-hydroxyoctadecadienoic acid
HPETE	hydroperoxy-5,8,10,14-eicosatetraenoic acid
IC_{50}	concentration required to inhibit response by 50%
IP_3	inositol 1,4,5-trisphosphate
MDA	malondialdehyde
NO	nitric oxide
PA	phosphatidic acid
PAF	platelet activating factor (1-o-alkylglycerophosphocholine)

ABBREVIATIONS (continued)

PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PFP	platelet free plasma
PG	prostaglandin
PGI_2	prostacyclin
PIP	phosphatidylinositol-4-phosphate
PIP_2	phosphatidyl-4,5-bis-phosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PRP	platelet rich plasma
RIA	radioimmunoassay
TX	thromboxane
vWF	von Willebrand factor

ANNOTATION

α	alpha
β	beta
γ	gamma
1°	primary
2°	secondary
3°	tertiary
4°	quaternary
$t_{1/2}$	half life
%	percentage
x	times

ABSTRACT

The studies described in this thesis involved the evaluation of an *in vitro* experimental system which was designed to assess functions of platelets and cultured endothelial cells when they were incubated either independently or in combination.

Initial studies involved the characterisation of human umbilical vein endothelial cells (HUVECs) and their prostacyclin generation responses (measured as 6-keto-$PGF_{1\alpha}$) to a range of stimuli. Investigations proceeded to determine the responses of washed platelets (shaken in the absence or presence of HUVEC monolayers) to various stimuli. Platelet activation was evaluated in terms of their ability to generate thromboxane A_2 (measured as TXB_2) and undergo dense granule release (quantified through their release of ^{14}C-serotonin). Both platelet and HUVEC responses were evaluated in the wells of 12 or 24 well culture plates. The developed system allowed the simultaneous assessment of responses of both cells independently and in combination.

The majority of studies performed examined platelet and HUVEC responses to collagen (5μg/ml) and thrombin (0.05u/ml). In the absence of HUVECs platelet serotonin release in response to collagen was thromboxane-dependent, whilst that in response to thrombin was thromboxane-independent. HUVECs caused inhibition of activated platelet responses (both thromboxane generation and serotonin release), and thromboxane-independent mechanisms of inhibition of serotonin release were demonstrable when thrombin was used as a stimulus.

In the absence of platelets, thrombin but not collagen stimulated prostacyclin generation from HUVECs. The presence of platelets was associated with enhanced prostacyclin generation responses and it was established that this phenomenon could not be satisfactorily explained on the basis of platelets donating endoperoxides to HUVECs. When thrombin was used as a stimulus, the enhancement seemed to be be associated with a stable platelet-derived product, although attempts were not made to identify this mediator. For collagen, the platelet-associated enhancement was reduced (but not significantly) upon inhibiting platelet thromboxane generation or blocking TXA_2/PGH_2 receptors (using SQ29548). This implied that platelet-derived thromboxane A_2 may be capable of stimulating prostacyclin generation from HUVECs through occupying TXA_2/PGH_2 receptors, but this possibility was not pursued.

The effects of two platelet thromboxane synthetase inhibitors were examined and their inhibition of platelet thromboxane generation coincided with apparent enhancement of prostacyclin generation from HUVECs in the presence of collagen (but not thrombin) stimulated platelets. Part of this enhancement was clearly an artefact of cross reactivity of the RIA antiserum for 6-keto-$PGF_{1\alpha}$ with elevated levels of platelet-derived prostanoids. Platelet serotonin release in response to collagen but not thrombin was inhibited by these agents and ridogrel (or R68070, which can also antagonise TXA_2/PGH_2 receptors) was more effective than dazoxiben.

Investigations were made to determine the participation of prostacyclin and/or endothelium-derived relaxing factor (EDRF) in the platelet inhibitory effects of HUVECs. Prostacyclin inhibition (by aspirin) together with the inhibition of EDRF effects (using haemoglobin) or synthesis (using N^{ω}-nitro-L-arginine) eliminated the platelet inhibitory effects of HUVECs. A similar effect was produced by methylene blue (10μM) which was used to inhibit the effects of EDRF, but also prevented prostacyclin generation. Prostacyclin and EDRF therefore accounted for all the platelet inhibitory effects of HUVECs. For collagen, prostacyclin alone could maintain the majority of typical platelet inhibition but for thrombin both prostacyclin and EDRF were required to maintain such inhibition, also, platelet inhibition by EDRF could be enhanced by superoxide dismutase. When non-endothelial cells lines were examined, it appeared that platelet inhibitory effects of EDRF were not confined to endothelial cells.

An adaptation of the system to allow the assessment of platelet adherence was evaluated but this method required modification as it assessed platelet deposition rather than just adherence.

In conclusion, the findings of these studies revealed that *in vitro*, factors derived from both platelets and endothelial cells could influence each others' functions. Interactions between these cells could be manipulated by a variety of pharmacological agents which affected either prostacyclin generation and/or the effects of EDRF. Systems such as the one described in this thesis may prove to be useful research tools or screening techniques for evaluating the effects of newly developed agents which may affect interactions between these cells. They may also provide valuable information regarding the potency and potential effects of such agents *in vivo*.

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Chapter 1

General Introduction
Platelets and the vascular endothelium

The activation and aggregation of platelets in response to vascular trauma are important events in normal, protective haemostatic processes. Although the vascular endothelium was once thought to play a minor role in such processes, being little more than an inert coating for the interior of blood vessels, over the past twenty years our knowledge of the diverse functions of the vascular endothelium has increased dramatically. The endothelium plays a role in the regulation of vascular permeability, vascular tone, haemostasis and fibrinolysis, and thus has been recognised as an important participant in a wide range of physiological responses (Bull & Machin, 1986; Sixma, 1987; Vane, Angaard & Botting, 1990), and a mediator in some pathological conditions (Key, 1992; Moncada, Palmer & Higgs, 1991). During thrombosis the extent of platelet activation and vascular endothelial cell functions are strongly affected by each other, and the consequences of their interactions can influence the course and extent of thrombotic events. The studies described in this thesis were carried out to provide further information regarding the nature of such interactions, and the factors (physiological and pharmacological) which may influence them.

Platelets

Current therapeutic strategies for the treatment of both bleeding and thrombotic disorders have been established through an appreciation of the mechanisms involved in haemostasis and thrombosis. Our understanding of physiological and biochemical events associated with haemostasis has led to the development of new pharmacological agents with highly specific and previously inconceivable mechanisms of action.

It is known that many mediators (blood borne and originating from the vessel wall) contribute to normal haemostatic processes. Platelets normally play a protective role in haemostasis through their activation in response to vascular damage. Following vascular trauma, platelets adhere to exposed vessel wall constituents and subsequent platelet aggregation results in the formation of a haemostatic plug. This platelet activation and aggregation in conjunction with the involvement of other blood cells, coagulation factors and the deposition of fibrin leads to the consolidation of the haemostatic plug and the cessation of bleeding (Sixma, 1987).

Deficiencies in platelet adhesive or aggregatory functions *in vivo* may be reflected by clinical manifestations ranging from an increased bruising tendency to a haemorrhagic disorder (White, 1987; Sixma, 1987; Nievelstein & De Groot, 1988). The pathology of arterial thrombotic disease can stem from events in which platelets become activated and aggregate or give rise to platelet-rich thrombi which lodge in vascular regions such as the coronary or cerebral circulation where they may cause vasospasm, reduced perfusion or even vessel occlusion, resulting in myocardial infarction or stroke respectively. The participation of platelets in the aetiology of arterial thrombotic conditions has been exemplified by the successful use of anti-platelet agents in the secondary prevention of arterial thrombosis. The results of clinical trials have demonstrated that agents with platelet inhibitory activity such as aspirin, sulphinpyrazone and dipyridamole can be used in the prophylactic treatment of unstable angina, and the prevention of secondary myocardial infarction, cerebral transient ischaemic attacks and stroke (Turpie, 1988; Harker & Gent, 1987). There is also evidence that platelet activation and interactions with subendothelial vascular tissue increase with the development of atherosclerosis and can contribute to the development of intimal thickening and stenosis associated with the progression of atherosclerosis (Scharf & Harker, 1987; Mustard, Packham & Kinlough-Rathbone 1990; Stemerman, 1987b; Badimon *et al,* 1993).

Over the past 30 years the effects of many endogenous factors and xenobiotics on platelet functions have been examined. We are also increasingly familiar with platelet structure, physiology and the internal biochemical messenger systems which regulate platelet activation.

Platelet structure

Platelets are anucleate, cellular fragments and are discoid in their resting state. They are derived from megakaryocytes, and are approximately 1μm in depth and 3-4μm in diameter. Platelets normally have a circulating life span of between 8 and 11 days. They have an external coat, rich in glycoproteins (GPs) many of which act as receptors and allow platelet adhesion to subendothelial vascular constituents including collagen, fibronectin, laminin and von Willebrand factor (vWF)(Ruggeri & Ware, 1992; Tuffin, 1991). Platelets also express specific surface receptors for platelet stimuli such as adenosine 5'-diphosphate (ADP), serotonin and thrombin

(Colman, 1990; Tuffin, 1991). There are still other receptors such as GPIIb/IIIa (or fibrinogen receptors) which become exposed only following platelet activation and can bind vWF, fibrinogen and fibronectin (Tuffin, 1991; Ruggeri & Ware, 1992; Ruggeri, 1993). Inside this external glycocalyx lies a phospholipid-rich unit membrane which is continuous with the platelet open canalicular system. The open canalicular system is a tubular network which traverses the platelet cytoplasmic matrix and allows communication between the internal and external environments. It also serves as a means by which internal platelet constituents, such as granule contents can be released or externalised by platelets. Inside the unit membrane lies a filamentous layer which is important for maintaining platelet shape, and is also involved in platelet shape change and the extension of pseudopodia which occurs following activation. The platelet matrix contains actin and myosin filaments which upon platelet activation interact to cause shape change, granule centralisation, platelet contraction and secretion. There is also a circumferential microtubule band which is involved in maintaining platelet shape rather than in secretory activity.

Platelet organelles include electron dense granules, alpha (α)-granules, peroxisomes, lysosomes, glycogen granules, and mitochondria. The latter two organelles are involved in supplying the metabolic energy required for platelet activation. The dense granules are a storage site for many releasable constituents including calcium, serotonin, adrenaline and adenine nucleotides including ADP (adenosine diphosphate) and ATP (adenosine triphosphate). Alpha-granules store platelet specific proteins including beta (β)-thromboglobulin, platelet factor 4, platelet-derived growth factor, vWF, fibrinogen and coagulation factors. The release, or externalisation of these granular contents occurs via the open canalicular system following platelet activation. Other granules are involved in the storage of non-releasable metabolically active adenine nucleotide pools and enzymes. Finally, the platelet dense tubular system (which represents the residual smooth endoplasmic reticulum from the parent megakaryocyte) is associated with calcium storage. Release of calcium from this pool contributes to the increase in internal calcium concentrations in platelets observed in association with platelet activation. The active calcium sequestration by this system is pivotal in regulating such activation. Accounts of platelet ultrastructure have been given by White (1987), and Crawford & Scrutton (1987).

Platelet activation

A variety of stimuli which occur *in vivo* can cause platelet activation and aggregation. Platelets have receptors for many such endogenous stimuli including ADP ($P_{2\gamma}$-purinoreceptors) serotonin ($5HT_2$ or S_2), adrenaline (α_2) collagen, vasopressin (V_1), 1-o-alkyl-2-acetyl glycerophosphocholine or platelet activating factor (PAF) and thrombin (Tuffin, 1991). Receptors also exist for arachidonic acid metabolites such as thromboxane A_2 (TXA_2) and cyclic endoperoxides such as PGH_2 can also occupy these receptors and stimulate platelet activation (Tuffin, 1991; Jaschonek & Muller, 1988). Some of these mediators, such as ADP, are released from damaged vascular tissue or damaged erythrocytes at sites of vessel injury. Fibrillar collagen (types I and III) which represents a major constituent of medial vascular tissue can also activate platelets, with type III showing the greater activity (Tuffin, 1991). Other stimuli such as serotonin, adrenaline, ADP and TXA_2 are liberated from platelets themselves following activation, and these may act on platelet receptors to enhance aggregation responses. Following activation, platelets can also bind coagulation factors which can lead to their participation in intrinsic coagulation processes, resulting in the activation of factor Xa, or in the formation of a prothrombinase complex (consisting of activated factors V and X)(Nesheim *et al,* 1993). This can act as a catalytic site for the local activation of thrombin (Tracy, 1988; Nesheim *et al,* 1993).

A number of morphological changes occur in platelets following their activation (Crawford & Scrutton, 1987; White, 1987; Scrutton & Athayde, 1992). Activated platelets undergo a shape change from discs to spheres which project pseudopodia. This shape change is accompanied by the exposure of fibrinogen receptors and platelet adherence to each other or to specific vascular elements exposed upon vascular injury. The initial or primary phase of platelet aggregation is reversible, but with sufficient stimulation, platelets synthesise and release factors which facilitate the activation and recruitment of other platelets and promote secondary or irreversible platelet aggregation. *In vivo*, during the formation of a haemostatic plug, other cell types including leukocytes and erythrocytes may also become incorporated and the involvement of coagulation factors can lead to fibrin formation, deposition, and the consolidation of the plug.

Assessments of platelet activation

A number of techniques have been developed to assess platelet aggregation responses *ex vivo* and these procedures have been invaluable in determining platelet reactivity to a wide range of agonists. Light transmission or turbidometric aggregometry was developed by Born in 1962. This technique records the alteration in light transmission through (or turbidity of) platelet suspensions during aggregation (Born, 1962; Born & Cross, 1963). Upon the addition of a stimulus, platelet shape change typically causes a modest reduction in light transmission, while increasing platelet aggregation results in an increase in transmission. Such changes are recorded, then quantified as % aggregation based on the assumption that maximal light transmission occurs upon 100% aggregation. Because of its simplicity, this method has gained increasing popularity for routine clinical assessments of platelet function. The technique is also attractive because it provides a means of continuously monitoring and recording platelet aggregation responses, and both primary and secondary waves of aggregation are frequently identifiable.

Another method preferred by some investigators to quantify platelet activation responses is whole blood or impedance aggregometry, initially developed by Cardinal & Flower (1980). This method allows assessment of the cumulative deposition of platelets on an electrode. An advantage of this technique is that it allows the participation of many other cell types and blood borne elements contained in whole blood which may regulate platelet activation. A limitation of this method is that it cannot detect platelet shape change or platelet disaggregation responses.

Particle counting has also been employed in some laboratories and uses the disappearance of single platelets from blood samples to determine their inclusion in aggregates. The method is considered by some groups to be more sensitive than light transmission aggregometry, particularly for assessing the effects of weak stimuli upon platelet aggregation (Crawford & Scrutton, 1987). Despite such possible advantages however, the technique has a considerable disadvantage in that (unlike light transmission aggregometry or impedance aggregometry) the continuous assessment of platelet aggregation is not possible.

Other methods which do not produce visual recordings of platelet aggregation responses have also been useful in increasing our knowledge with respect to stimulus-response relationships in platelets (Ludlam, 1987). The extent of platelet activation can for example be determined through the measurement of platelet-derived products. Measurement of platelet-derived serotonin, ADP and ATP which may be released from platelet dense granules upon platelet aggregation, represents one means of quantifying platelet activation. Similarly, the release of β-thromboglobulin or platelet factor 4 from platelet α-granules can also be quantified as an index of whether or not platelets have been stimulated. Biochemical mediators which are synthesised by stimulated platelets, such as thromboxane A_2 (TXA_2), can also be measured as a means of quantifying platelet activation. In addition, intracellular biochemical indices of platelet activation or inhibition can also be monitored. These include measuring rises in intracellular calcium or assessing phospholipid metabolism observed during platelet activation, or rises in cyclic adenosine 5'-adenosine monophosphate (cAMP) and cyclic guanosine 5'-monophosphate (cGMP), typically associated with the inhibition of platelet activation. Such techniques and others (Adams, 1985a; Ludlam, 1987) have provided insight into the stimulus-response mechanisms associated with the regulation of platelet activation.

Biochemical pathways involved in platelet activation

The methods described above have provided an increased understanding of the effects of many mediators which stimulate or inhibit platelet activation. In addition, our current knowledge of stimulus-response coupling in platelets encompasses an understanding of the biochemical messengers involved in platelet activation and its regulation. Comprehensive and detailed analyses of such biochemical pathways appear in numerous reviews (Zucker & Nachmias, 1985; Hawiger, Steer & Salzman, 1987 and Crawford & Scrutton, 1987). A number of more recent reviews highlight facets such as the involvement of G-proteins in receptor-mediated responses (Kroll & Schafer,1989; Seiss, 1991; Scrutton & Athayde, 1991 and Tuffin, 1991; Brass, Hoxie & Manning, 1993). A brief summary of the biochemical pathways known to participate in the regulation of platelet activation is given below and summarised schematically in Figure 1a (page 11).

G-proteins

Platelets react to many stimuli, and although non-receptor-mediated factors (such as shear stress) can cause activation (Ruggeri, 1993), platelets generally react in response to the occupation of specific receptors. Following occupation of receptors, a membrane bound guanine nucleotide binding protein (or G-protein) is involved in signal-transduction. This transduction involves the alteration of the G-protein which in the resting state consists of three subunits (α,β and γ). Receptor occupation causes the hydrolysis of guanosine diphosphate (GDP) from the α-subunit of the G-protein and the subsequent binding of guanosine triphosphate (GTP) in its place. Upon GTP binding, the α-GTP subunit dissociates from the beta, gamma ($\beta\gamma$)-dimer. The α-GTP subunit can then activate the enzyme associated with the G-protein. This leads to either the stimulation or inhibition of platelet activation depending upon the G-protein involved. Three distinct G proteins are known to function in platelets distinguished on the basis of differing α subunits. Two G-proteins possess molecular characteristics typical of G_s and G_i forms (Jaschonek & Muller, 1988; Kroll & Schafer, 1989; Brass, Hoxie & Manning, 1993), whilst the third, responsible for PLC activation remains to be characterised but has previously been annotated G_p (Jaschonek & Muller, 1988; Kroll & Schafer, 1989; Seiss, 1991; Tuffin, 1991 and Scrutton & Athayde, 1991; Brass, Hoxie & Manning, 1993). The G_p and G_i forms stimulate platelet activation, whilst the G_s form inhibits platelet activation.

Receptor occupation and subsequent G_p-protein mediated activation of membrane bound phospholipase C (PLC) represents the most recognised mechanism of receptor mediated activation of platelets. Activation of PLC results in the conversion of phosphatidylinositol-4,5-bis-phosphate (PIP_2) to the second messengers inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) (Kroll & Schafer, 1989; Seiss, 1991; Scrutton & Athayde, 1991; Brass, Hoxie & Manning, 1993).

Second messengers involved in platelet activation

As a result of PLC activation, the liberation of IP_3 acts to evoke the release of calcium from the dense tubular system. As with many cells, platelets actively maintain low internal concentrations of ionised calcium (Ca^{2+}) and an increase in internal Ca^{2+} concentrations is an essential step in cellular activation. In platelets, an increase in platelet internal Ca^{2+} concentrations from resting levels of less than 100nM to 1-2μM Ca^{2+} is required for such activation. IP_3 formation and the release of Ca^{2+} from internal calcium stores, serves in part to elicit this increase. Receptor occupation can also directly stimulate the opening of receptor operated calcium channels possibly through the involvement of an as yet unidentified G-protein (G_x). Consequently the influx of Ca^{2+} from the external environment (where the Ca^{2+} concentration is approximately 1.2mM) contributes to the increased internal Ca^{2+} concentration required to evoke platelet activation (Scrutton & Athayde, 1991).

The second product of PLC activity, DAG, activates protein kinases which are responsible for the phosphorylation of proteins (e.g. 20K myosin light chain proteins and 40-47K proteins). Upon phosphorylation, these proteins become actively involved in platelet shape change, expression of fibrinogen receptors and secretion responses including the liberation of both α- and dense granule contents (Seiss, 1991; Scrutton & Athayde, 1991; Brass, Hoxie & Manning, 1993).

Although excitatory platelet stimuli generally elicit platelet activation through a mechanism involving G_p-proteins, some stimuli (including ADP, thrombin, adrenaline and possibly TXA_2) can occupy receptors which are associated with G_i-proteins (Seiss, 1991; Jaschonek & Muller, 1988; Crawford & Scrutton, 1987). Occupation of these receptors causes the inhibition of the membrane bound enzyme adenylate cyclase, the activity of which regulates concentrations of cAMP in platelets. The mechanisms by which cAMP inhibits platelet activation will be discussed shortly, but it is recognised to inhibit the rise in intracellular platelet Ca^{2+} concentrations typically associated with activation. Consequently inhibition of adenylate cyclase results in the loss of an important inhibitory mechanism in platelets and therefore can facilitate or augment the effects of stimuli which evoke rises in internal platelet Ca^{2+}.

Such a rise in internal Ca^{2+} results in the activation of phospholipase A_2 (PLA_2) which acts on membrane phospholipids (principally phophatidylcholine and phosphatidylethanolamine) to liberate 20-carbon fatty acids, predominantly arachidonic acid (or eicosatetraenoic acid; C20: 4ω6). In addition, arachidonic acid can be liberated through the dual activity (on phosphatidylinositol) of phospholipase C and diglyceride lipase (Marcus, 1987).

Metabolites of arachidonic acid

Arachidonic acid is converted by the enzyme complex cyclooxygenase to form the highly labile eicosanoid PGG_2 which in turn is converted by hydroperoxidase to PGH_2. In platelets PGH_2 is predominantly converted to thromboxane A_2 (TXA_2) by platelet thromboxane synthetase; and 17-hydroxy-5,8,10-heptadecatrienoic acid (17-HHT) and malondialdehyde (MDA) are also formed as by-products. There is also minor conversion of PGH_2 to other eicosanoids including PGD_2, PGE_2 and $PGF_{2\alpha}$. The amounts of such eicosanoids formed upon platelet activation are generally considered to be too low to exert any biological effects although some pharmacological agents enhance their generation from activated platelets (Bertele *et al,* 1984; De Clerck *et al*, 1989a). The conversion of arachidonic acid by 12-lipoxygenase to form 12-hydroperoxy-5, 8,10,14-eicosatetraenoic acid (12-HPETE) also occurs in activated platelets. This is then converted to 12-HETE, which possesses leukocyte chemotactic properties and has been reported to act as a feedback control to inhibit platelet aggregation (Fonlupt, Croset & Lagarde, 1991). In contrast, others have reported that 12-HETE and its precursor 12-HPETE may augment platelet activation by other stimuli (Buchanan & Brister, 1993). Additionally, many platelet stimuli cause changes in internal platelet pH (alkalinisation) associated with changes in Na^+/H^+ exchange. This may enhance calcium mobilisation and TXA_2-dependent amplification of platelet activation, although the exact role of Na^+/H^+ exchange in platelet activation remains uncertain (Scrutton & Athayde, 1991; Siffert & Akkerman, 1989).

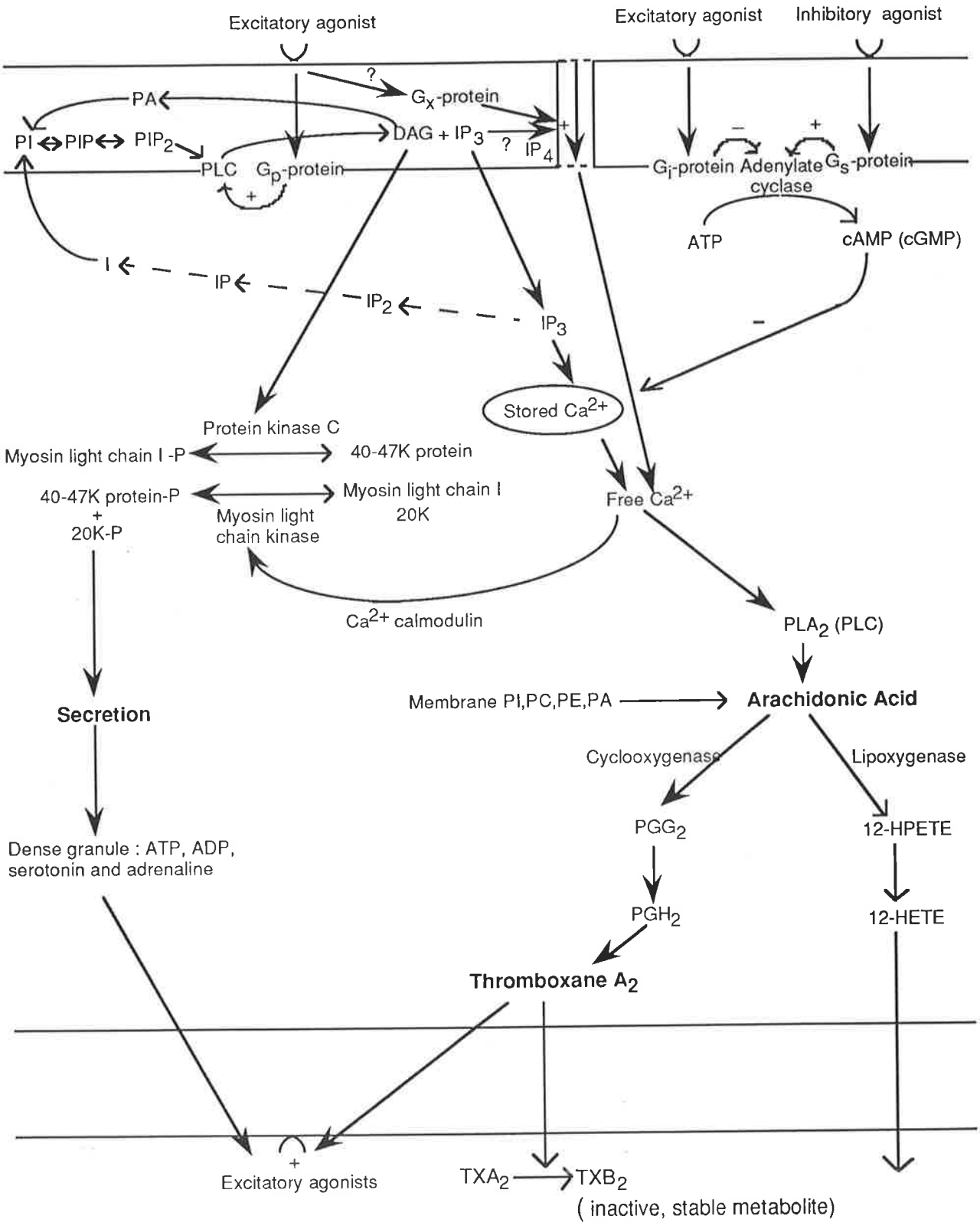
TXA_2 is well recognised as a strong platelet aggregatory stimulus and vasoconstrictor (Hamberg, Svesson & Samuelson, 1975). Consequently platelet TXA_2 generation together with secretion of dense granule contents results in a powerful positive feedback mechanism which can transform a reversible platelet aggregatory response into an irreversible one. Indeed, the role of TXA_2 generation in stimulating platelet aggregation (Emms & Lewis, 1986) and granule release (Ambler *et al,* 1985; Best *et al,* 1980) as well as the mechanisms by which TXA_2 evokes platelet activation (Jaschonek & Muller, 1988) have been studied by several groups.

Figure 1a (overleaf) illustrates some of the known pathways involved in platelet activation, and also indicates the biochemical pathways which may regulate or inhibit platelet activation. Although the abbreviations used in the figure were indicated earlier, relevant abbreviations are repeated below.

ADP	adenosine 5'-diphosphate
ATP	adenosine triphosphate
cAMP	cyclic 3',5'-adenosine monophosphate
cGMP	cyclic 3',5'-guanosine monophosphate
G-protein	guanine nucleotide binding protein
IP_3	inositol 1,4,5-trisphosphate
IP_4	inositol 1,3,4,5-tetraphosphate
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PIP	phosphatidylinositol-4-phosphate
PIP_2	phosphatidyl-4,5-bis-phosphate
PLC	phospholipase C
PLA_2	phospholipase A_2

Figure 1a

Biochemical pathways involved in platelet activation



Regulation of platelet activation

Regulation of platelet responses to some stimuli may occur by platelet receptor desensitisation to those stimuli. For example, the platelet stimulatory activity of thrombin, PAF, vasopressin, and TXA_2 is reduced with prolonged receptor occupation by means of receptor uncoupling (Crouch & Lapetina, 1989). One mechanism by which this is achieved may involve a PKC-mediated reduction in the ability of G_p-proteins to activate PLC although a second PKC-independent mechanism may also exist (Crouch & Lapetina, 1989). Alternatively, platelet desensitisation to adrenaline, PGD_2 and ADP has been shown to occur via a reduction in their receptor densities (Crawford & Scrutton, 1987; Scrutton & Athayde, 1991).

Other mechanisms by which platelet activation is regulated rely on the control of internal calcium concentrations. Cyclic AMP can inhibit the typical increases in internal platelet calcium concentrations associated with platelet activation and exerts its effects by causing increased calcium efflux from platelets and increased internal sequestration of calcium (Scrutton & Athayde, 1991; Hawiger, Steer & Salzman, 1987). This impairs the many calcium-dependent mechanisms involved in platelet activation, including the activation of PLA_2 and therefore the release of arachidonic acid, and the activation of contractile proteins. Cyclic AMP can also cause calcium-independent inhibitory effects by activating a specific cAMP-dependent protein kinase, PKA (Crawford & Scrutton, 1987). One site of phosphorylation by this protein kinase is myosin light chain kinase, and its phosphorylation inhibits its activation and thus impairs platelet secretion responses (Kroll & Schafer, 1989). Phosphorylation events by PKA may also be involved in stimulating an increase in Ca^{2+}, Mg^{2+}-ATP'ase activity and thus causing enhanced re-uptake of calcium into the dense tubular system and possibly enhanced efflux from platelets, resulting in platelet inhibitory effects (Scrutton & Athayde, 1991). In addition, it appears that cAMP can inhibit the effect of platelet stimuli by impairing PLC activity and thus the formation of the second messengers IP_3 and DAG. It is suggested that G_p-proteins themselves may also be specifically inhibited by a cAMP-dependent phosphorylation event which increases GTP'ase activity, and therefore opposes G_p-proteins remaining in their active state (Scrutton & Athayde, 1991).

The role of internal cAMP in inhibiting platelet activation is probably best exemplified by the effect of prostacyclin (PGI_2). The ability of this endogenous platelet inhibitory mediator to strongly stimulate cAMP synthesis in platelets was first shown by Tateson, Moncada & Vane (1977). Occupation of platelet prostacyclin or DP receptors (to which platelet inhibitory PGE_1 can also bind) stimulates signal transduction by way of a G_s -protein (Tuffin, 1991; Crawford & Scrutton, 1987). This results in the activation of platelet adenylate cyclase and an elevation in cAMP. This elevated cAMP then opposes the effects of excitatory platelet stimuli. Other less potent platelet inhibitors which act by a similar mechanism include PGD_2 and adenosine, which act on IP and A_2 receptors respectively (Tuffin, 1991). Adrenaline can similarly cause increases in cAMP through occupying β receptors, although the overall effect of adrenaline is platelet excitatory because of the effects adrenaline imparts through occupation of the more numerous α_2 receptors (Tuffin, 1991, Crawford & Scrutton, 1987) .

Another platelet inhibitory cyclic nucleotide which can be formed in platelets is cGMP. Agents which generate NO such as nitroprusside or s-nitrosothiols are assumed to be able to inhibit platelets by stimulating guanylate cyclase and thus the generation of cGMP (Salvemini *et al*, 1990; Mellion *et al,* 1981; Lieberman, O'Neill & Mendelsohn, 1991). Similarly, endothelium-derived relaxing factor (EDRF) (which is generally accepted to be nitric oxide (NO); Gillespie & Sheng, 1988; Ignarro *et al*, 1988, Palmer, Ferrige & Moncada, 1987) can also inhibit platelet activation by causing increases in platelet cGMP. The mechanisms by which raised concentrations of cGMP inhibit platelet activation have been proposed to be similar to those of cAMP (Crawford & Scrutton, 1987; Hawiger, Steer & Salzman, 1987; Scrutton & Athayde, 1991). Certainly similarities have been demonstrated by reports that EDRF or NO (through the effects of cGMP) may exert its inhibitory effects against receptor mediated platelet activation through inhibiting PLC, thus prohibiting the production of the calcium liberating second messenger, IP_3 and also preventing DAG formation and subsequent events associated with platelet secretion (Nguyen, Saitoh & Ware, 1991; Durante *et al*, 1992). Radomski Palmer & Moncada (1987d) observed however that EDRF (which raises platelet cGMP) but not prostacyclin (which raises platelet cAMP) can inhibit platelet adhesion to collagen. Thus

differences must exist with respect to the platelet inhibitory effects of cAMP and cGMP. It has also been proposed that the capacity of cGMP to inhibit cAMP metabolism (Maurice & Haslam, 1990) may explain the capacity of EDRF and prostacyclin to inhibit platelet activation in a synergistic manner (Radomski, Palmer & Moncada, 1987b; Alheid, Frolich & Forsterman, 1987; Macdonald, Read & Dusting, 1987). In addition, Radomski, Palmer & Moncada (1990) have identified the capacity of platelets to generate low levels of NO, and this capacity may represent a weak self-regulatory mechanism involved in dampening or opposing platelet activation to contact induced-platelet activation.

Recognition of the platelet inhibitory capacity of the endothelium

The role of the endothelium in regulating platelet functions was first appreciated following the finding by Moncada and colleagues in (1976) that endothelial cells could produce the potent platelet inhibitory factor and vasorelaxant, PGX , now termed prostacyclin (Tateson, Moncada & Vane, 1977). Since that time the properties of many endothelial cell derived factors on platelets have been investigated, including the vasorelaxant EDRF which was first shown to inhibit platelet activation in 1986 (Azuma, Ishikawa & Sekizaki).

During the late sixties, it was demonstrated that a wide range of non-steroidal anti-inflammatory drugs possessed platelet inhibitory activity (O'Brien, 1968) and in the early seventies, the therapeutic potential of such platelet inhibitory agents in patients suffering from arterial thrombotic tendencies came under investigation. From the extensive clinical trials (involving aspirin and sulphinpyrazone) undertaken and published from 1974 to 1980, it was demonstrated that daily administration of aspirin (then given at doses of between 975mg and 1300mg per day) could offer benefits for specific patients with a tendency towards arterial thrombosis (Harker & Gent, 1987; Turpie, 1988).

The mechanism by which aspirin exerted its effects was also investigated during the seventies. In 1971 it was found that aspirin inhibited prostaglandin (eicosanoid) synthesis in platelets (Smith & Willis), and in 1975 Roth and Majerus published the finding that aspirin acetylated a

particulate enzyme in platelets. The specific effect of aspirin, which involved the irreversible acetylation of the platelet enzyme cyclooxygenase, was not however described until 1978 (Roth & Siok), two years after the identification of prostacyclin.

With the discovery of prostacyclin in 1976, the synthesis of which was dependent on vascular cyclooxygenase, and the elucidation of the mechanism of action of aspirin, it was realised that the effect of aspirin on vascular cells may compromise its own antithrombotic efficacy. Because of its non-platelet specific mechanism of action, aspirin could inhibit vascular as well as platelet cyclooxygenase and could therefore compromise the ability of the vascular cells (the most efficient in the synthesis of prostacyclin being vascular endothelial cells) to generate prostacyclin. This finding led to a revision of the accepted dosing regimens of aspirin for prevention of arterial thrombosis. Investigations were undertaken to evaluate whether the administration of low-dose aspirin and slow release formulations could provide complete platelet inhibition whilst sparing vascular cyclooxygenase, and thereby optimise effects of aspirin in the prevention of arterial thrombotic events (Bochner & Lloyd 1986). In addition, the desire to modify platelet function through inhibition of the production or effects of TXA_2, whilst sparing vascular cell PGI_2 generation, has led to the development of alternative drugs with greater specificity of action. Some of these agents will be addressed in more detail in a later section.

The events which led to revision of the way in which aspirin was used to prevent thrombosis emphasised that limits existed with respect to the information which could be gained through the assessment of platelets (and platelet inhibitory drugs) alone. A logical progression of *in vitro* studies into the effects of antithrombotic agents came when attention focussed upon the role of the vessel wall, including the vascular endothelium in thrombotic events. Since that time, a great deal of information has accumulated regarding the mechanisms by which the vessel wall and the vascular endothelium participate in haemostasis and thrombosis. Particular attention has focussed upon the vascular endothelium, and a number of techniques have been developed to allow the assessment of platelets and endothelial cell interactions *in vitro*. Although once considered an inert coating for blood vessels, the vascular endothelium has become recognised as a highly reactive regulator of platelet function, coagulation and fibrinolytic events as well as

vascular tone. The dynamic situation where the functions of blood elements and the endothelium are influenced by each other has become a subject of immense interest to physiologists and pharmacologist alike. Research in this area has continued to provide information which is pertinent to the development of strategies to prevent thrombosis. Many such strategies are now directed towards influencing platelet and endothelial interactions rather than the function of a single blood element such as platelets.

The morphological structure of the endothelium and vessel wall has been well characterised. In contrast, our knowledge regarding the biological functions of the vascular endothelium and the mechanisms involved in the regulation of endothelial cell activation is clearly incomplete. Nevertheless, our current level of knowledge demonstrates that vascular endothelial cells react to a wide range of stimuli and exhibit a diverse array of functions.

The Vascular Endothelium

Vascular physiology

All vessels contain adventitial, medial and intimal layers. The outer adventitial layer consists of connective tissue, lymphatic vessels and nerves as well as (in larger vessels) conduit blood vessels and their associated smooth muscle cells. The media consists predominantly of smooth muscle cells, connective, elastic and collagen fibres, and this layer varies in width depending on the blood flow dynamics for the vessel in question. In larger vessels an internal elastic lamina divides the media from the intima. This consists of a layer of connective tissue and in the case of arteries (but not venules or capillaries) may contain smooth muscle cells. The intima consists of a subendothelial basal lamina (lamina lucida and lamina densa) commonly termed the basement membrane. This typically contains a number of products of endothelial cell origin. These include von Willebrand factor, laminin, fibronectin, and various collagens, established as important in platelet adhesion (and possibly, activation). The subendothelium may also contain heparan sulfate and thrombomodulin (able to activate protein C), involved in maintaining the non-thrombogenic nature of this layer (De Groot & Sixma, 1990; Sixma, 1987 and Sixma *et al* 1991). The innermost layer of the intima consists of an unbroken monolayer of endothelial cells and their associated extracellular matrix. Detailed analysis of the physiology of blood vessels is

given by Stemerman (1987a), Shepro & Dunham (1987), Kefalides (1987) and Pittilo (1988).

Endothelial cell physiology

Endothelial cells are typically 15μm in width by 25-50μm in length and *in vivo* their greatest length lies in parallel with the direction of blood flow. *In vitro* they grow to a polygonal shape such that at confluence a cobblestone appearance is observed. They vary in thickness from several microns at the point where the nucleus is located to less than 1μm at their periphery. The endothelial cell plasma membrane expresses at its luminal surface a glycocalyx consisting of many proteins, glycoproteins, sialoconjugates, and sulfated proteoglycans, the predominant one being heparan sulfate (Nievelstein & De Groot, 1988). Many such integrins act as receptors, but other receptors only become exposed in response to appropriate stimuli. Endothelial cell receptors include those which bind coagulation factors, lipolytic enzymes, insulin, a wide range of vasoactive mediators (Giltay, 1988; Simionescu & Simionescu, 1986) and receptors which bind other cell types such as polymorphonuclear neutrophils (Hoover & Karnovsky, 1984; Vadas & Gamble, 1990; Gimbrone & Buchanan, 1982). The properties of the surface glycocalyx and the extracellular matrix are important facets of the endothelium which are involved in the regulation of haemostasis, and are discussed in greater detail in the following section (Sage, 1986; Jaffe, 1987; Bull, 1988; Sixma, 1987, Sixma *et al*, 1991).

In vivo, appositional endothelial cells are linked by two junctional networks. Occluding or tight junctions represent the physical barrier between cells which impedes the passage of molecules between adjacent cells. Gap junctions are cytoplasmic links between appositional cells, and act as a means of cellular communication between adjacent endothelial cells. *In vivo,* the density of gap and tight junctions between endothelial cells differs for different regions of the circulation with the most complex junctional networks occurring in arterioles and similar but less extensive networks occur in arteries. Still less organised networks are apparent in capillaries, veins and venules (Pittilo, 1988). *In vitro*, it has been shown that cultured endothelial cells may not express comparable junction formation (e.g. the formation of tight junctions) to that seen *in vivo* (Hoek, 1992).

The endothelial cell cytoplasm contains a large number of vesicular structures, some of which span the endothelium and are proposed to be associated with transendothelial cell communication between the luminal and the subendothelial vascular surface. Endothelial cells also contain a number of typical cellular organelles including the nucleus, mitochondria, rough and smooth endoplasmic reticulum, golgi bodies and lysosomes. Weibel-Palade bodies are cellular organelles which are specific to endothelial cells and are spread throughout the endothelial cell cytoplasmic matrix. They are approximately 0.1 x 3μm in size and consist of 6-20 tubular or rod shaped structures, each of which is approximately 150Å in width (Gimbrone *et al,* 1974, Jaffe, 1987). These organelles are the site of storage of von Willebrand factor (Wagner, Olmsted & Marder, 1982, Reinders *et al,* 1988; Ruggeri & Ware, 1993) and P-selectin (Ruggeri & Ware, 1993). The endothelial cell cytoplasm also contains actin and myosin filaments which are proposed to assist in the maintenance of endothelial cell conformation and to be involved in endothelial cell mobility and migration. Other filaments referred to as stress fibres are observed in endothelial cells which have been exposed to high or turbulent blood flow situations *in vivo* and in addition intermediate filaments and microtubules constitute typical components of the endothelial cell cytoplasmic matrix (Pittilo, 1988).

Biological functions of the endothelium

An extensive number of reviews identify the biological functions of the endothelium. One such function is the ability of endothelial cells to alter vascular permeability (Renkin & Curry, 1982; Shepro & Dunham, 1987; Bundgaard, 1988). This area of endothelial cell function remains a topic of ongoing investigation (Hoek, 1992) and is of particular relevance in the pathology of inflammatory conditions. In addition to its barrier function, the endothelium possesses many properties which regulate haemostasis. These can be considered in terms of the surface properties of the endothelium and the extracellular matrix, the metabolic activity of the endothelium and the synthetic and the secretory functions of the endothelium.

Surface properties of the endothelium, the extracellular matrix and subendothelium

Anticoagulant and procoagulant properties of the endothelium

In vivo, the healthy endothelium expresses a non-thrombogenic surface. Part of this capacity has been attributed to a negative surface charge associated with the expression of sialoconjugates, proteoglycans and glycoproteins at the luminal surface of the endothelium. These present an electro-repulsive surface to similarly charged blood elements, including platelets (Simionescu & Simionescu, 1986; Nievelstein & De Groot, 1988; Thilo-Korner, Heinrich & Lasch, 1983). The ability of endothelial cells to express heparan sulfate and other sulfated glycosaminoglycans (dermatan and chondroitin sulfate) on their luminal surface is also important in the ability of endothelial cells to express anticoagulant properties. The endothelium can express and bind plasma antithrombin III at its surface (Jaffe, 1987). In association with surface heparans antithrombin III can efficiently inhibit intrinsic pathways of coagulation by the rapid inactivation of factors IXa, and XIIa. In addition, it can inactivate factor Xa and thrombin itself. The endothelium can also express thrombomodulin at its surface. It can bind thrombin and *in vivo*, the thrombin-thrombomodulin complex can cause rapid activation of protein C. Protein C then inhibits intrinsic coagulation processes through inactivating factor VIII, and by inactivating factor V, involved in the final common pathway in both the extrinsic and intrinsic coagulation processes (Jaffe,1987; Bull & Machin, 1987; Preissner, 1988; Nievelstein & De Groot, 1988; Thilo-Korner, Heinrich & Lasch, 1983).

The surface of the endothelium can also express procoagulant activity in response to appropriate stimuli including interleukin-1 (IL-1), tumor necrosis factor and endotoxin (or lipopolysaccharide). In response to these stimuli, endothelial cells can initiate coagulation via the extrinsic pathways through expressing tissue factor (equivalent to thromboplastin). Since endothelial cells can also bind factors IX, VIII and X, the activated tissue factor/ VIIa complex may then initiate intrinsic coagulation processes though activating factor IX which in conjunction with factor VIII can activate surface bound factor X (Wu, Frasier-Scott, & Hatzakis, 1988; Stern, Kaiser & Nawroth, 1988). As endothelial cells can also synthesise and bind factor

V (Bull & Machin, 1987)(a mediator in the final common pathway of coagulation), this in association with surface bound factor Xa forms a prothrombinase complex, able to efficiently convert prothrombin to thrombin (Stern, Kaiser & Nawroth, 1988; Wu, Frasier-Scott & Hatzakis, 1988; Bull & Machin, 1987; Nievelstein & De Groot, 1988).

Properties of the extracellular matrix and the subendothelial basement membrane

Endothelial cells constitutively secrete a number of substances which are incorporated into the endothelial cell extracellular matrix and the underlying basement membrane. These substances assist in endothelial cell attachment, and after de-endothelialisation facilitate spreading, migration and proliferation of endothelial cells during re-endothelialisation. The substances secreted by endothelial cells include collagen (of various types), fibronectin, laminin, elastin, von Willebrand factor and thrombospondin (Bull, 1988; Sage, 1986; Sixma, 1987). Upon vascular trauma and de-endothelialisation, exposure of some of these substances also promotes platelet adhesion. Although laminin and elastin do not display this capacity, platelets do possess receptors for fibronectin and subendothelial cell fibronectin is a recognised mediator of platelet adherence. Under high shear conditions, as may occur in stenosed arteries or at arterial bifurcations, von Willebrand factor has gained particular attention as a mediator of platelet adhesion (Sixma, 1987, Sixma *et al,* 1991, De Groot & Sixma, 1990; Ruggeri & Ware, 1992; Ruggeri & Ware, 1993). Collagen is also synthesised by endothelial cells and is incorporated into the basement membrane and extracellular matrix. The predominant form is type IV which can support platelet adherence under conditions of low shear. However, *in vitro*, the types of collagen synthesised by endothelial cells appear to differ between endothelial cells from different sources and over time during cell culturing (Sage, 1986) and other forms of collagen (such as types I and III) may also be generated (Sage, 1986; Bull, 1988; Sixma, 1987; Sixma *et al,* 1991).

Metabolic functions of the endothelium

The apical or luminal surface of endothelial cells is also the site of considerable enzymic activity. Heparan sulfates can bind lipoprotein lipase which can metabolise di and tri-acyl glycerol, very low density lipids and chylomicrons (Simionescu & Simionescu, 1986; Jaffe, 1987). Surface heparan sulfate can also bind extracellular superoxide dismutase type C, which can offer protective effects against endothelial damage by oxygen radicals (Abrahamsson *et al*, 1992). The endothelium can also regulate the activation or deactivation of vasoactive proteins and platelet stimuli. For example, endothelial cell angiotensin-converting enzyme (ACE) can inactivate kinins such as the vasorelaxant bradykinin and convert angiotensin I into its active product, the vasoconstrictive angiotensin II. Substance P is another mediator which is inactivated by endothelial cells (Johnson, 1984).

Endothelial cells can also inactivate (through ectonucleotidase activity) platelet stimuli such as ATP and ADP to the weak platelet inhibitor AMP which can be further metabolised to adenosine, which is a platelet inhibitor and vasorelaxant. Since adenosine can be internalised and metabolised by endothelial cells and erythrocytes to inosine, the biological effects of adenosine formed through ectonucleotidases may be limited *in vivo* (Pearson & Gordon, 1984; Marcus *et al*, 1991). The pulmonary endothelium is well recognised for its contribution to clearing many circulating vasoactive mediators either through surface enzyme activity or by way of active uptake and degradation. Substrates include prostaglandins PGE_2 and $PGF_{2\alpha}$ and PGD_2, serotonin and to some extent noradrenaline. Prostacyclin, adrenaline and histamine are spared (Fishman, 1982; Johnson, 1984). This high clearance capacity may in part be associated with the large endothelial cell surface area of the pulmonary endothelium and/or due to specialisation of endothelial cells in this vascular region (Pearson & Gordon,1984; Small, Macarak & Fisher (1976).

Synthetic properties of the endothelium

Factors affecting fibrinolysis

As well as participating in coagulation processes which regulate the generation of thrombin, and thus fibrin formation, the endothelium also participates in fibrinolytic activity. Endothelial cells can synthesise tissue plasminogen activator (and urokinase-type plasminogen activator). At the surface of endothelial cells, the combination of tissue plasminogen activator and plasminogen results in the synthesis of active plasmin (Thilo-Korner, Heinrich & Lasch, 1983). Endothelial cells can also synthesise tissue plasminogen activator inhibitor; however the ratio of the activator to the inhibitor generally favours expression of the fibrinolytic potential. Interestingly, the stimuli endotoxin, tumor necrosis factor and IL -1, which induce endothelial cells to express procoagulant activity, also reduce the fibrinolytic capacity of the endothelium. These stimuli reduce the endothelial cell generation of tissue plasminogen activator and stimulate plasminogen activator inhibitor generation (Nachman & Hajjar, 1991; Key, 1992). In light of these effects, it is not surprising that patients with high circulating concentrations of endotoxin (e.g. suffering gram-negative sepsis) or high circulating monokine concentrations (e.g. during viral infections and neoplastic diseases) also suffer prothrombotic tendencies (Key, 1992).

Endothelium-derived contracting and relaxing factors

The endothelium can release products at its apical (luminal) and basolateral (abluminal) surface which gives it the ability both to affect haemostatic events within the vessel and regulate subendothelial vascular tone. Such regulation of vascular tone can (through altering flow conditions within vessels) affect haemostatic processes. Prostacyclin is the best known vasorelaxant factor synthesised by the endothelium. In addition, two other vasoactive mediators synthesised by the endothelium in response to many stimuli have been identified. These include the vasorelaxant, endothelium derived relaxing factor EDRF (or NO), and the vasoconstrictive peptide endothelin-1 (Yanagisawa *et al* 1988; Angaard, Botting & Vane, 1990; Rubanyi & Botelho, 1991; Luscher *et al,* 1993; Tanner, Boulanger & Luscher, 1993).

Endothelin-1 is the only one of the three endothelins identified which is known to be released from vascular endothelial cells. In addition to its potent vasoconstrictive capacity, like a number of endothelium derived growth factors (Bull, 1988), it also possesses mitogenic activity. *In vivo* endothelin may inhibit platelets through stimulating the release of PGI_2 and EDRF by the endothelium (Rubanyi & Botelho,1991; Angaard, Botting & Vane, 1990). These mediators may in turn down-regulate its formation (Luscher *et al*, 1993; Tanner, Boulanger & Luscher, 1993). Other endothelium-derived vasoconstrictors such as superoxide anions (which may be generated following reperfusion of ischaemic tissue) and vasodilators such as endothelium-derived hyperpolarising factor have also been identified (Vanhoutte, Luscher & Graser, 1991; Luscher *et al,* 1993) but the regulation of their synthesis and biological significance remain to be clarified.

EDRF inhibits both platelet adhesion (Radomski *et al* 1987c,d; Herbaczynska-Cedro *et al* 1991; De Graaf *et al* 1992; Venturini, Del Vecchio & Kaplan, 1989) and platelet aggregation, reported initially by Azuma, Ishikawa & Sekizaki (1986) and subsequently by many others. A wide range of cells contain constitutive EDRF synthetase, including neuronal cells, leukocytes and vascular endothelial cells. This enzyme converts the substrate L-arginine to L-citrulline, with the simultaneous liberation of EDRF or NO. The N group is derived from the N-guanadino terminal of L-arginine, and the O has been postulated to be derived from molecular oxygen (Moncada Palmer & Higgs, 1991; McCall & Vallance, 1992). Endothelial cells liberate continuous low concentrations of NO (via a constitutive nitric oxide synthetase enzyme) and this may be assisted by endothelial cells recycling L-citrulline into L-arginine (Wu & Meininger, 1993). *In vivo* due to the short half life of EDRF such production is considered important in the maintenance of normal vascular tone and may also be involved in the regulation (inhibition) of vascular smooth muscle cell proliferation (Scott-Burden & Vanhoutte, 1993). Endothelial cells can also, in response to some stimuli (e.g. tumor necrosis factor, IL-1 and endotoxin), express an inducible (calcium independent) form of nitric oxide synthetase, and produce abnormally high levels of EDRF (Moncada, Palmer & Higgs, 1991). *In vivo*, such induction may result in excessive vasorelaxation, platelet inhibitory effects and possibly cellular toxicity (Bassenge, 1992; Moncada, Palmer & Higgs, 1991; Moncada, 1992; Stoclet, 1993; Palmer *et al*, 1992).

The biological implications of endothelial cell mediators which affect vascular tone and/or platelet functions have been extensively reviewed (Vanhoutte, 1989; Vane, Angaard & Botting, 1990; Vanhoutte, Luscher & Graser, 1991; Tanner, Boulanger & Luscher, 1993; Radomski & Moncada, 1993).

Products of arachidonic acid metabolism

Following endothelial cell activation the liberation of arachidonic acid occurs and it is metabolised via cyclooxygenase or lipoxygenase to form lipid endoperoxides which are subsequently converted into a wide range of biologically active products.

Cyclooxygenase metabolites

Cyclooxygenase converts arachidonic acid to the cyclic endoperoxide PGG_2 which is converted by hydroperoxidase to PGH_2. In endothelial cells PGH_2 is predominantly converted via prostacyclin synthetase into prostacyclin, or PGI_2. Because of its potency as an inhibitor of platelet activation (through raising platelet cAMP; Tateson, Moncada & Vane, 1977) and as a vasorelaxant, prostacyclin is considered the most biologically significant product of endothelial cell arachidonic acid metabolism. The biological significance of PGI_2 *in vivo* has been reviewed extensively since its identification (as PGX) in 1976 (Moncada, 1982; Weksler & Jaffe, 1986). It is known that endothelial cells, like platelets can also produce minor amounts of PGD_2, PGE_2 and $PGF_{2\alpha}$ (Buchanan, Crozier & Haas, 1988; Smith, 1986) but normally these mediators (except possibly for PGE_2 generation in capillaries; Smith; 1986) are produced at levels too low to produce biologically significant effects on vascular tone or platelet functions. Other by-products of cyclooxygenase activity include hydroxy-heptadecatrienoic acid (HHT) and malondialdehyde (MDA).

Prostacyclin

The platelet inhibitory effects of prostacyclin have been studied both *in vivo* and *in vitro* by many investigators. The platelet inhibitory potency of prostacyclin *in vitro* may be affected by factors which alter its binding status and stability, such as the presence or absence of plasma factors. *In vivo*, the biological effects of prostacyclin may also be reduced through its

inactivation by erythrocytes (Willems *et al*, 1983) which may explain the short half life of prostacyclin *in vivo*, and the low circulating concentrations of ~1-3pg/ml (Vane Angaard & Botting, 1990; Gresele *et al,* 1991). The ability of prostacyclin to inhibit platelet aggregation is highly dependent upon the strength or concentration of the platelet agonist used (Krishnamurthi Westwick & Kakkar 1984). *In vitro* in both platelet rich plasma (PRP) (Whittle and Moncada,1985; Krishnamurthi Westwick & Kakkar 1984) and washed platelets (Radomski, Palmer & Higgs, 1987a; Jakubowski, Thompson & Deykin, 1984) platelet responses to ADP and low concentrations of collagen and thrombin are inhibited by concentrations of prostacyclin of 1-5nM. In contrast, in response to strong stimuli such as high thrombin concentrations, platelet responses clearly become irreversible such that prostacyclin even at 150nM does not inhibit them (Krishnamurthi Westwick & Kakkar 1984).

Lipoxygenase metabolites

Upon activation of endothelial cells, arachidonic acid which is not metabolised by cyclooxygenase is predominantly converted by 15-lipoxygenase and results in the production of minor amounts of 15-HPETE which are subsequently converted to 15-HETE (Buchanan, Crozier & Haas, 1988). The biological importance of this product remains unclear although it has been reported to enhance thrombin induced platelet activation (Setty *et al,* 1992). In 'resting' endothelial cells, 13-lipoxygenase has been reported as important in the metabolism of endogenous triglycerides such as linoleic acid. The final product from linoleic acid metabolism is 13-hydroxy-octadecadienoic acid (13-HODE) (Buchanan *et al* 1985). This product has been proposed to inhibit platelet adhesion and consequently its production has been linked with the thromboresistant properties of the endothelium (Buchanan, Crozier & Haas, 1988; Buchanan & Brister, 1993).

Factors affecting arachidonic acid metabolism in endothelial cells and the biological effects of the products, are reviewed by Buchanan, Crozier & Haas, 1988 and more recently by Vane, Angaard & Botting, (1990), Vane, Gryglewski & Botting, (1990) and Buchanan & Brister, (1993).

Biochemical pathways involved in endothelial cell activation

Stimuli and endothelial cell activation

Amongst the stimuli known to activate endothelial cells are those which cause cell injury (Brox & Nordoy, 1982) for example hypoxia and exposure to free radicals (Hiebert & Liu, 1990; Busse *et al,* 1993) and exposure to proteases such as trypsin (Weksler, Ley & Jaffe, 1978). Physical stimuli such as cell deformation and shear stress also cause endothelial cell activation and are recognised stimuli of EDRF generation (Lamontagne, Pohl & Busse, 1991; Shen *et al,* 1992; Busse *et al*, 1993; Davies, 1993).

To examine specific stimulus-response coupling mechanisms, many investigators have chosen to evaluate receptor mediated responses by endothelial cells which result in prostacyclin or EDRF generation. These particular stimulus-response transduction mechanisms have been assessed for a number of reasons. Both responses are recognised to be of considerable biological significance. In addition, these endothelial cell responses were identified prior to other responses, such as the production of endothelin. Other reasons include the ease with which such responses can be quantified (by direct or indirect methods), the wide range of stimuli which elicit such responses and their rapid onset following cell stimulation. It is probable that in contrast to such rapid responses in endothelial cells, more complex stimulus-response coupling mechanisms also exist, and remain to be elucidated; for example, the responses to cytokines which are delayed and require time for enzyme induction and protein synthesis (Endo, Akahoshi & Kashiwazaki, 1988; Pohlman & Harlan, 1989)

Endothelial cells are known to possess membrane receptors for bradykinin (Bk_2), histamine (H_1 and H_2), adrenaline (α_2), thrombin, adenine nucleotides such as ADP and ATP (i.e. $P_{2\gamma}$-purinoreceptors), serotonin ($5HT_1$ or S_1), acetylcholine, angiotensin 11, calcitonin, vasopressin, PAF, cytokines (including IL-1 and 2 and tumor necrosis factor), substance P, platelet-derived growth factor, insulin and TXA_2/PGH_2. Many stimuli, including histamine (H_1), bradykinin, thrombin, and adenine nucleotides are known to stimulate both prostacyclin and EDRF production from endothelial cells (Vanhoutte, 1989; 1990; Levin *et al,* 1984; Weksler & Jaffe, 1986; Kelm *et al*, 1993). Others, such as serotonin and acetylcholine and adrenaline are

recognised stimuli of EDRF generation but not of prostacyclin (Ignarro, 1989; Moncada, Palmer & Higgs, 1991; Vanhoutte, 1989; 1990; Liao & Homcy, 1992; Tanner, Boulanger & Luscher, 1993). Evidence also suggests that occupation of TXA_2/PGH_2 receptors in transformed bovine aortic endothelial cells stimulates prostacyclin but not EDRF (Clesham *et al,* 1992). Some agents also stimulate other endothelial cell functions; for instance adrenaline, angiotensin II and thrombin are able to stimulate the production of endothelin-1 from endothelial cells (Angaard, Botting & Vane, 1990; Luscher *et al,* 1993; Tanner, Boulanger & Luscher, 1993) and agents such as histamine and thrombin are well recognised for causing increased endothelial cell permeability (Hoek, 1992).

Receptor mediated signal transduction mechanisms

Following specific receptor occupation, the generation of endothelial cell derived products such as EDRF and prostacyclin depends upon the activation of second messengers. Some of the biochemical second messengers of cellular activation (such as increased intracellular concentrations of Ca^{2+}) are common to most cell types including platelets. Many biochemical pathways involved in endothelial cell activation have been elucidated; however, work in this area has not progressed sufficiently to provide a complete picture of the activation and response coupling mechanisms. These investigations have employed both vessel sections and cultured endothelial cells from a wide range of sources. Evidence indicates that endothelial cells from differing species and vascular regions possess different functional characteristics; for example in their capacity to generate prostacyclin (Czervionke *et al,* 1979a, Revytak, Johnson & Campbell, 1987). The functions of isolated endothelial cells may also change with time following isolation (Christofinis *et al,* 1979; Ager *et al,* 1982; Chesterman, Ager & Gordon, 1983) and in cultured endothelial cells, different culturing conditions or subculturing procedures may also affect receptor expression (Sung, *et al,* 1989; Tracey & Peach, 1991; Durante *et al,* 1992). Such effects have probably contributed to some discrepancies in the views held between various workers with respect to the biochemical mechanisms involved in endothelial cell activation. Despite such discrepancies, a number of consistent observations have been made.

Typical of most cells, G-proteins are involved in the transduction of receptor occupation to cellular responses. G-proteins have been identified for a number of stimuli (e.g. histamine and bradykinin: Voyno-Yasunetsakaya *et al,* 1989; Buckley *et a*l, 1991, Liao & Homcy, 1992) and the transduction mechanisms involved in the stimulation of PLC appear similar to those identified in platelets. Studies involving specific inhibitors or stimuli of G protein activation are still being carried out to define the exact nature of the transduction mechanisms and the G proteins involved in endothelial cell activation and its regulation.

Second messengers involved in the activation of endothelial cells

It has been established that following receptor occupation many processes involved in endothelial cell activation are similar to those involved in platelet activation. Receptor occupation, results in PLC activation with the subsequent hydrolysis of membrane PIP_2 to form IP_3 (and the simultaneous production of DAG and subsequently PA). The appearance of IP_3 has been shown to correlate with increases in intracellular Ca^{2+} following its release from the endoplasmic reticulum (Adams *et al,* 1989). Such responses (increases in intracellular IP_3 or Ca^{2+}) have been confirmed in various endothelial cell lines to occur for bradykinin, (via the B_2 receptor: Derian & Moskowitz 1986, Schini *et al,* 1990) histamine (via the H_1 receptor: Rotrenson & Gallin, 1986) vasopressin, angiotensin II, PAF, serotonin (Grigorian *et al,* 1989) the TXA_2/PGH_2 mimetic U46619 (Grigorian *et al,* 1989; Kent *et al,* 1993), acetylcholine (Grigorian *et al* 1989), thrombin (Grigorian *et al,* 1989; Ngaiza & Jaffe, 1991; Durante *et al,* 1992), ADP and ATP (Boeynaems & Pearson, 1990; Lustig *et al* , 1992).

Following endothelial cell activation, increases in intracellular Ca^{2+} concentrations can occur through calcium being released from internal stores or through the influx of extracellular calcium. In response to receptor occupation, IP_3 formation can result in the mobilisation of internal Ca^{2+} from stores and can lead to a reported rise in internal Ca^{2+} concentrations from 50-100nM up to ~ 2-4μM within about 10-15 seconds (in human umbilical vein endothelial cells in response to ADP or ATP; Carter *et al,* 1990) or according to others, within 30-50 seconds (in porcine aortic endothelial cells in response to bradykinin: Buckley *et al,* 1991).

Following this peak, internal Ca^{2+} concentrations fall over 2-3 minutes until a plateau phase (at a concentration between the resting and peak concentration) is reached. The maintenance of this plateau concentration is dependent upon maintained receptor occupation and a continuing influx of external Ca^{2+}. This influx allows sustained Ca^{2+}-dependent cellular responses to stimuli including ADP, ATP, thrombin, histamine and bradykinin (Adams *et al*, 1989; Boeynaems & Pearson 1990; Himmel, Whorton & Strauss, 1993). Such ongoing entry of Ca^{2+} is terminated with the removal of the stimulus and (in bovine aortic endothelial cells) appears to be linked to Ca^{2+} levels in intracellular stores returning to their resting level (Dolor *et al* 1992). In addition, bradykinin has been shown to directly activate receptor operated calcium channels (opened by a specific G protein regulated mechanism) in porcine aortic endothelial cells (Graier, Schmidt & Kukovetz, 1992). Endothelial cell calcium channels which are activated in response to stretch or calcium leakage have also been proposed to be involved in increases in intracellular Ca^{2+}, particularly in response to endothelial cell injury. There is also evidence to suggest that changes in Na^+/H^+ exchange and internal pH may also regulate calcium influx and intracellular Ca^{2+} concentrations (Ghigo *et al* 1988; Siffert & Akkerman, 1989).

For EDRF generation, a calmodulin-dependent process, Ca^{2+} released from internal stores may support such generation for a brief period following endothelial cell activation. However to provide continuous generation of EDRF, extracellular influx of Ca^{2+} is essential (Lopez-Jaramillo *et al,* 1990, Moncada, Palmer & Higgs, 1991; Busse *et al,* 1993) and intracellular concentrations in excess of 200nM have been reported to be necessary to maintain such EDRF generation (Parsaee *et al*, 1992). In contrast, the production of prostacyclin from endothelial cells may not be greatly impaired by the absence of extracellular Ca^{2+} (White & Martin ,1989; Carter *et al*, 1988) or may be reduced but not eliminated (Brotherton & Hoak, 1982). Thus, the release of internal endothelial cell calcium stores which is associated with short lived peak concentrations of 2-4μM Ca^{2+} appears sufficient to allow arachidonic acid liberation and supports prostanoid production even without external Ca^{2+}. Certainly in human umbilical vein endothelial cells, in response to $P_{2\gamma}$ receptor activation, a critical internal Ca^{2+} concentration of only 0.8-1μM has been reported to allow PLA_2 activity and prostacyclin release (Carter *et al,*

1988) and others (using transformed bovine aortic endothelial cells) report that the critical concentration required to allow prostanoid generation may be even lower, or 350nM (Parsaee *et al,* 1992). The reported reductions in endothelial cell prostacyclin generation responses in the absence of external Ca^{2+} do however imply that optimal arachidonic acid liberation may require the influx of external Ca^{2+}(Brotherton & Hoak, 1982). The role of Ca^{2+} as an intracellular messenger in endothelial cell activity is reviewed by Adams *et al* (1989), Rubanyi *et al* (1988) and Himmel Whorton & Strauss (1993).

DAG is produced simultaneously with IP_3 (as in platelets) and mediates PKC activation in a similar manner to platelets. A number of other protein kinases also appear to become activated, although the roles of subsequent calcium-dependent, protein phosphorylation events are still to be elucidated (Boeynaems & Pearson, 1990).

Regulation of endothelial cell activation

It has been postulated that endothelial cell activation is regulated by number of feedback mechanisms. Following endothelial cell activation rises in both cGMP and cAMP can occur and have been proposed as potential regulators of such activation. Prostacyclin generation by endothelial cells is accompanied by rises in endothelial cell cAMP and, in the presence of phosphodiesterase inhibition, levels of cAMP stimulated by PGI_2 have been reported to reach concentrations sufficient to inhibit PGI_2 generation (Brotherton & Hoak, 1982). It is unlikely that, in the absence of phosphodiesterase inhibition, these increases in cAMP are sufficient to regulate endothelial cell activation (Schafer, Gimbrone & Handin, 1984) although they have been linked with receptor desensitisation effects (Boeynaems & Pearson, 1990). Evidence also suggests that in the absence of phosphodiesterase inhibition, the rises in cGMP associated with EDRF generation (Boulanger *et al*, 1990) are unable to alter basal release of EDRF generation from cultured calf or rabbit endothelial cells (Marczin, Ryan & Catravas 1992a). Furthermore, others have shown that neither cAMP nor cGMP alter basal or stimulated EDRF generation in bovine aortic endothelial cells (Kuhn *et al,* 1991), and thus the role of these cyclic nucleotides as regulators of endothelial cell activation seems questionable.

Other intracellular mechanisms have also been proposed to play a role in down-regulating endothelial cell activation in response to receptor occupation. For example, PKC-mediated inactivation of PLC by a phosphorylation reaction has been suggested as one means by which endothelial cells may become refractory to activation by some stimuli (e.g. $P_{2\gamma}$-purinoreceptors: Boeynaems & Pearson, 1990).

A number of regulatory mechanisms specifically affecting the generation of endothelial cell PGI_2 have also been reported. *In vitro*, upon stimulating endothelial cell prostacyclin generation, subsequent restimulation evokes a reduced response. For stimuli such as thrombin or ionophores, such refractoriness was postulated to be linked to the exhaustion of releasable arachidonic acid (Dejana *et al,* 1983). In light of the evidence that such refractoriness also develops where arachidonic acid is used as the first stimulus, alternative explanations have been favoured (Brotherton & Hoak, 1983; Dejana *et al,* 1983). Cyclooxygenase is apparently susceptible to inactivation by the accumulation of metabolites formed as a result of its own metabolism of arachidonic acid (Yardumian & Machin, 1986). Endothelial cells which are made refractory to stimulation with arachidonic acid can however still generate prostacyclin via prostacyclin synthetase if PGH_2 is used as a substrate, and cyclooxygenase is therefore bypassed (Brotherton & Hoak, 1983). Upon cyclooxygenase inactivation, the synthesis of new cyclooxygenase in endothelial cells provides a mechanism by which these cells can rapidly recover from such effects (Dejana *et al,* 1983).

In addition to cyclooxygenase inactivation prostacyclin synthetase is also susceptible to inactivation by some oxidative species (Vane, Gryglewski & Botting, 1987; Salvemini & Botting, 1993). For example prostacyclin synthetase is susceptible to inactivation by the lipid hydroperoxide 15-hydroperoxy-eicosatetraenoic acid (15:HPETE), derived during lipoxygenase metabolism of arachidonic acid (Weksler & Jaffe, 1986; Salvemini & Botting, 1993). This evidently inhibits prostacyclin synthetase either directly or through the liberation (via hydroperoxidase activity) of superoxide anions or other oxidants derived from superoxide (Martin *et al,* 1983; Yardumian & Machin, 1986). With the development of atherosclerosis, the enhanced formation of such lipid hydroperoxides is thought to be causal in the inhibition of

enzymes responsible for prostacyclin generation (Vane, Gryglewski & Botting, 1987). Similarly, oxidized low density lipoproteins found in association with atherosclerotic lesions may also impair the generation or release of EDRF (Tanner, Boulanger & Luscher, 1993; Luscher *et al,* 1993).

Loss of regulated endothelial function and pathological conditions

As indicated earlier, a number of stimuli, with recognised pro-inflammatory activity, including endotoxin and the cytokines, tumor necrosis factor and IL-1, can affect endothelial cell functions. These stimuli can induce endothelial cells to express increased adhesiveness towards leukocytes (Ward, 1991), abnormal surface procoagulant activity and can also affect endothelial cell mediated fibrinolytic functions through altering their expression of mediators including plasminogen activator and its inhibitor (Key, 1991). Endotoxin, tumor necrosis factor and IL-1 induce endothelial cell protein synthesis (Pohlman & Harlan, 1989) and stimulate the expression of the inducible form of nitric oxide synthetase in vascular endothelial cells (Moncada, 1992). Similarly, they can induce increased prostacyclin generation from endothelial cells (dependent upon protein synthesis) (Endo, Akahoshi & Kashiwazaki, 1988) possibly as a result of enhanced expression of cyclooxygenase (Wu, Frasier-Scott & Hatzakis, 1988). The effects of increased EDRF (and prostacyclin) generation may result in excessive vasorelaxation and platelet inhibitory effects *in vivo*. *In vitro*, following induction, even EDRF mediated cytotoxicity towards endothelial cells themselves has been reported (Palmer *et al,* 1993).

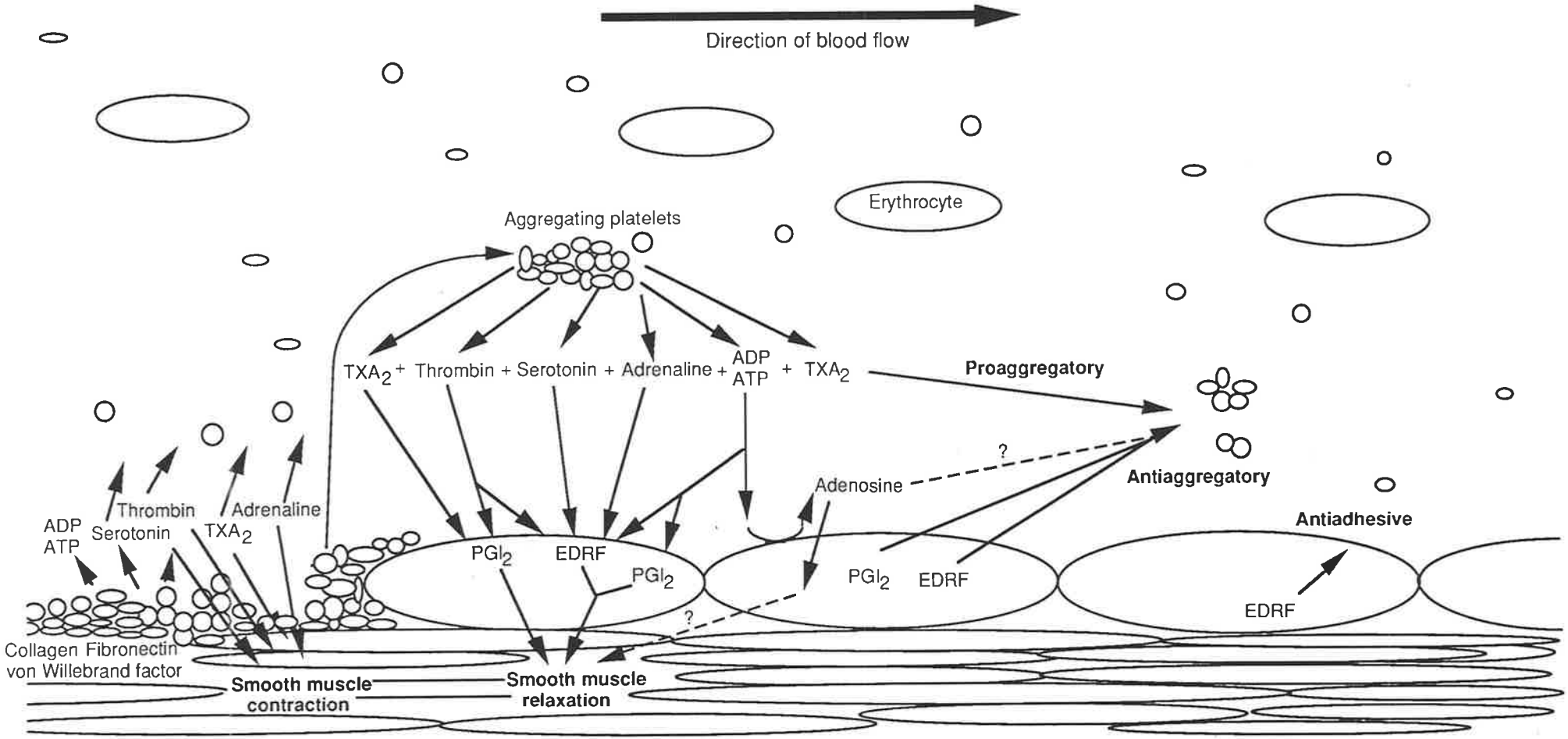
The combined effects of these mediators in altering normal endothelial cell function may contribute to the pathogenesis of conditions including gram-negative sepsis, thrombotic disorders associated with neoplastic diseases (Key, 1992), disseminated intravascular coagulation and endotoxic shock (Moncada, 1992; Moncada, Palmer & Higgs, 1991; Stoclet *et al,* 1993). In contrast, other cytokines (with anti-inflammatory activity), such as transforming growth factor-β, IL-8, IL-10, platelet-derived growth factor (Moncada, 1992) and possibly interferon-γ (Key, 1992) may inhibit such responses. It is evident that in the future, the complex mechanisms associated with the effects of mediators which produce delayed effects on endothelial cell function, will gain increasing interest.

Stimuli affecting both platelets and endothelial cells

Many of the stimuli or factors described in previous sections provide immediate rather than delayed effects on both platelets and endothelial cells and their interactions during acute thrombotic events are still being clarified. To highlight the complexity of such interactions, the following diagram (Figure 1b) illustrates just a few mediators and their activities upon platelets and the vasculature. For the sake of simplicity, only endothelium-derived prostacyclin and EDRF generation are highlighted because of their recognised influence on platelet function. Other stimuli which are illustrated are serotonin, ADP, ATP, TXA_2, adrenaline and thrombin, all of which are released from platelets (or other cell types) or formed at sites of vascular trauma.

The majority of the indicated stimuli have effects on both platelets and endothelial cells with the exception of collagen which possesses platelet-specific effects. The other mediators can act as platelet stimuli and can also stimulate prostacyclin generation (e.g. ADP, ATP and thrombin and TXA_2) or EDRF generation (e.g. ADP, ATP, serotonin, adrenaline and thrombin) from endothelial cells. Many of the stimuli can also act directly on smooth muscle cells to cause vasoconstriction (e.g. TXA_2, serotonin and thrombin; Vanhoutte, 1989; 1990; Fenton, 1988). Adenosine is also indicated, as a metabolite of ADP and ATP, and if not taken up by erythrocytes or endothelial cells may cause both platelet inhibition and vasorelaxation. *In vivo* the local, combined effects of such mediators on platelets, the vascular endothelium and vascular smooth muscles may all contribute to haemostatic or thrombotic events.

Figure 1b


Direction of blood flow
Erythrocyte
Aggregating platelets
TXA2 + Thrombin + Serotonin + Adrenaline + ADP ATP + TXA2
Proaggregatory
Antiaggregatory
Antiadhesive
Adenosine
?
ADP ATP
Serotonin
Thrombin
TXA2
Adrenaline
PGI2
EDRF
PGI2
PGI2
EDRF
EDRF
?
Collagen Fibronectin von Willebrand factor
Smooth muscle contraction
Smooth muscle relaxation

Additional stimuli of platelets and/or endothelial cells

Amongst the platelet-derived mediators not included in Figure 1b are vasopressin and PAF. Although both these stimuli can evoke platelet aggregation responses (and vasopressin can cause endothelium-dependent relaxation), the concentrations of these platelet-derived mediators *in vivo* are considered to be unlikely to mediate any endothelium-dependent effects (Vanhoutte, 1989). The participation of other cell types such as erythrocytes and leukocytes would occur to some extent *in vivo*. Consequently, the factors released from such cells including ADP or adenosine from erythrocytes and PAF, cytokines, EDRF and lipoxygenase products from leukocytes, may also influence thrombotic events. Also omitted from the figure are prostanoids other than prostacyclin (such as PGE_2 and PGD_2). Although typically generated by platelets at low concentrations, at higher concentrations such prostanoids may influence vascular tone and influence platelet aggregation (e.g. PGD_2, antiaggregatory and PGE_2, weak proaggregatory: Gray & Hepinstall, 1991).

Other stimuli not included in Figure 1b are those which are known to influence endothelial cell but not platelet functions. These include circulating biogenic amines such as bradykinin and histamine. Bradykinin and histamine can act on endothelial cell receptors (Bk_2 and H_1) to stimulate prostacyclin and EDRF generation (Levin, 1984; Ignarro, 1989; Vanhoutte, 1989). Histamine also stimulates increases in endothelial cell permeability (Hoek, 1992). Finally, acetylcholine, although not a circulating hormone, is also frequently cited as a specific stimulus for EDRF generation and was the first agonist identified to possess this property (Furchgott & Zawadzki, 1980). Interestingly, in more recent studies using cultured bovine aortic endothelial cells, acetylcholine has also been reported to stimulate PGI_2 generation (Jaiswal, Jaiswal & Malik, 1991).

Much of our knowledge of how platelets and endothelial cells interact has been provided through using experimental systems which have been developed specifically to assess such interactions and some of these are the subject of the next section.

Methods for the assessment of platelet and endothelial cell interactions *in vitro*

Since the early seventies many *in vitro* systems have been developed to assess interactions between platelets and endothelial cells. These systems have been developed as an alternative to *in vivo* experimental systems and possess the advantages of their simplicity and reproducibility. They also allow experimental aims to be approached with superior specificity, and under well controlled conditions difficult to achieve using *in vivo* models. Such *in vitro* systems have been used to assess a variety of endothelial cell (and platelet) functions. These have included studies to assess how platelet-derived factors may influence endothelium-dependent regulation of vascular tone. They have also been used to determine factors which may affect the normal thromboresistant nature of the endothelium and sub-endothelium and to assess which endothelium-derived factors influence platelet adherence and aggregation responses. The effects of pharmacological agents on all these parameters have been studied and the diverse aims of the studies in this area of research have meant the systems used to measure platelet and endothelial cell interactions have been equally diverse. Some of these systems will be summarised in the next section.

Isolated vascular segments and the effects of platelets on vascular tone

Investigators frequently use isolated vessel sections to assess the effects of endothelium derived-mediators such as EDRF (Furchgott & Zawadzki, 1980; Ignarro *et al,* 1988; Palmer Ferrige & Moncada 1987) or arachidonic acid metabolites (Bunting *et al,* 1976; Katusik & Vanhoutte, 1989; Miller & Vanhoutte, 1985) on vascular tone. With the inclusion of aggregating platelets, such studies have enabled assessments of the effects of platelet-derived mediators on endothelium-dependent modulation of vascular tone. An example can be seen in the study of Forsterman *et al* (1988) where aggregating washed platelets were added to human coronary artery segments (with or without endothelium) to determine their effects on vascular tone. Work by Houston and co-workers and by Cohen and colleagues followed similar methods and some of their results have been reviewed by Vanhoutte (1989; 1990).

Isolated vascular segments and platelet adherence

Many investigators have considered the effects of the endothelium on platelet adhesive activity to be of primary interest. Platelet adhesion to components of the subendothelial cell extracellular matrix and basement membrane is a recognised event following de-endothelialisation (Nievelstein & De Groot, 1988; Bull, 1988; Sixma, 1987, Sixma *et al*, 1991). Platelet adhesion to endothelial cells activated by some stimuli such as thrombin (Kaplan, *et al,* 1989; Tloti *et al,* 1991) or ADP (Reininger, Reininger & Wurzinger, 1993) has also been reported. Platelet adhesion alone does not necessarily result in thrombus formation. However where platelet activation is also promoted, such sites of platelet adhesion naturally act as the focus for platelet aggregation and for the initiation of thrombotic events (Sixma *et al,* 1991). Consequently *in vitro* techniques to assess this particular aspect of platelet and endothelial cell (or the subendothelial) interactions have become common. A detailed account of all the methods developed since these investigations began will not be given; however a brief summary of some of the techniques developed is in order. Some of these methods have also been adapted to investigate polymorphonuclear leukocyte adhesion to the endothelium (Gimbrone & Buchanan, 1982; Hoover & Karnovsky, 1984) but such studies lie beyond the scope of this overview.

Some studies of platelet adherence have involved the use of vascular segments, such as everted rabbit aortae which are superperfused with washed platelets or erythrocytes (^{51}Cr-labelled) (Menys & Davies, 1985). Unmanipulated isolated vessels such as human umbilical veins perfused with ^{99}Tc-labelled platelets (Lewalle *et al* 1991) or isolated sheep pulmonary artery sections incubated in the presence of ^{111}In-labelled platelets (Kaplan *et al*, 1989) have also been used. In these studies, adherence has been determined through the association of radiolabel with the segments, although in some studies morphological assessments of thrombus formation has also been reported (Korbut *et al,* 1990). Others have used more elegant experimental systems and have used vessel segments fixed in a flow chamber apparatus. The vessels have been perfused with platelets and adherence has been measured either as percentage coverage by morphometric analysis (Tschopp *et al,* 1979) or by measurement of ^{51}Cr-labelled platelet coverage (Sakariessen & Baumgartner, 1989).

The use of cultured endothelial cells

A major advance in our capacity to specifically assess platelet and endothelial cell interactions without the interference of undefined variables (which may occur when using whole vessels) has come about with the development of cell culturing techniques. The impact of cell culturing techniques with respect to the assessment of endothelial cell functions is reminiscent of the impact that Born and Cross had with the introduction of platelet aggregometry (1962, 1963). Their development of the light transmission platelet aggregometry technique allowed the assessment of isolated platelet aggregation activity *in vitro* and has subsequently become a mainstay in the assessment of platelet functions.

In 1973 and 1974, Jaffe and colleagues and Gimbrone and associates independently published papers giving detailed methods for culturing human umbilical vein endothelial cells (HUVECs) (Jaffe *et al,* 1973; Gimbrone, Cotran & Folkman, 1974). Their cell lines were extensively characterised by morphological and immunological criteria. Although numerous cell culturing techniques for various cell lines have since been adopted, in the culturing of HUVECs the methods described by these authors remain among the most cited investigations from which current endothelial cell culturing techniques have evolved. Since the development of such cell culturing techniques, data have been generated assisting in the elucidation of many endothelial cell functions. Furthermore, cell culturing techniques have facilitated the development of assays in which we can specifically assess platelet and endothelial cell interactions *in vitro*.

Comment on the use of cultured endothelial cells

In some instances, some functions of the endothelium expressed *in vivo* may not be expressed or the expression may alter, once the cells are isolated or cultured *in vitro*. Some of these discrepancies have been attributed to the differing characteristics of cells derived from differing species and different vascular beds (Smith, 1986; Fishman, 1982, Pearson & Gordon, 1984). There is however reasonable evidence to suggest that some characteristics of endothelial cells are lost following isolation simply because of their removal from their *in vivo* situation. Some investigators have reported that when endothelial cells were grown in culture, a loss of receptor

expression was caused by a loss of mRNA expression over time (Tracey & Peach, 1991). Others have reported the loss of endothelial cell surface enzymic (Chesterman, Ager & Gordon, 1983) or active uptake (Shepro *et al,* 1975) functions over time in culture. Alterations in basal prostanoid (Christofinis *et al,* 1979; Ingerman-Wojenski & Silver, 1988; Ager *et al,* 1982) or stimulated EDRF generation (Durante *et al,* 1992) have also been reported over time following isolation. Although some investigators have used various harvesting culturing and subculturing techniques (such as non-proteolytic cell harvesting procedures) to avoid these changes in endothelial cell functions, loss of responses has still been reported to occur over time when cells are cultured (Chesterman, Ager & Gordon, 1983).

Endothelial function *in vivo* is continuously responding to modulatory mediators derived from its external environment. It would be optimistic to expect that cultured human endothelial cells, following possibly weeks in isolation in an artificial environment (lacking in many factors to which the endothelial cells would normally be exposed in their *in vivo* milieu), would possess exactly the receptor density and all the cellular functions of the untraumatised endothelium *in vivo*. Despite such limitations most investigators can accept that cultured endothelial cells offer a convenient means for determining factors which influence specific and well defined endothelial cell functions for a particular cell line, under a particular set of experimental conditions. On establishing typical functions of the cells in use, they can then assess factors which affect or regulate those specific functions and draw conclusions on this basis. The key to the validity of their findings lies not in the assumption that the endothelial cells in culture are identical to those *in vivo*, but that they are able to identify clear changes in well characterised or defined endothelial cell responses in their experimental systems. Some of the systems which have utilised cultured endothelial cells to investigate specific aspects of platelet and endothelial cell interactions are outlined in the next section.

Cultured endothelial cells and platelet adherence

In 1982, Curwen *et al* described a platelet adherence assay where cultured HUVECs grown on coverslips were incubated with ^{3}H adenine labelled platelets in citrate anticoagulated PRP. Following standardised washing procedures, platelet adherence to the monolayer was assessed by determining radioactivity associated with the coverslips. This was a modification of a system described by the same group where the role of PGI_2 upon platelet adherence was investigated (Curwen, Gimbrone & Handin, 1980) and it was adapted by Buchanan *et al* (1987) to assess platelet adherence to HUVEC subendothelial matrix proteins, or coverslips coated with specific proteins.

Using a similar approach, Radomski, Palmer & Moncada (1987c) assessed the effects of EDRF on platelet adherence, once again utilising cultured endothelial cells. Bovine aortic endothelial cells were cultured in 24 well culture plates and the cells underwent various treatments before the addition of ^{111}In-labelled washed platelets. Following aspiration of platelets, the monolayer was washed and the radioactivity associated with the cells was determined. Other systems have been developed along similar lines, including methods described by Czervionke and co-workers (1978,1979b), Shatos, Doherty & Hoak (1991), Kaplan *et al,* (1989) and Venturini *et al*,1989; more recently used by Tloti e*t al*, (1991). These have involved the use of cultured endothelial cells grown on coverslips or in wells and interacted with radiolabelled platelets either statically, or following rocking or shaking for a period of time. Wells or coverslips have been washed and the remaining level of platelet adherence determined by solubilising the endothelial cells in the wells to determine the radiolabel associated with them. The investigations have assessed many factors including the platelet-adhesive or anti-adhesive properties of the subendothelium and its known constituents, and the role of endothelial cells and platelet stimuli in regulating such adherence.

Some investigators have preferred to assess platelet adherence under flow conditions and elegant flow chamber models have been developed where cultured endothelial cells have been incorporated into the systems and platelets or blood has been superperfused over the cells (Sakariessen & Baumgartner, 1989; De Graaf *et al* ,1992: Jandak, Steiner & Richardson, 1989

and Reininger, Reininger & Wurzinger, 1993). The importance of factors such as von Willebrand factor in platelet adherence under flow conditions has been highlighted through the use of these systems (Hardistry, 1985). Reviews of platelet adherence studies involving flow chamber techniques (as well as non-perfused methods) and their associated findings have been published by Adams (1985b); Sixma (1987) and De Groot & Sixma (1990)

Vascular segments and platelet aggregation

Assessments of platelet and endothelial cell interactions have frequently placed emphasis on determining the effects of endothelial cells on platelet activation and aggregation rather than adherence. Once again as with adherence models, some techniques have utilised vascular segments. Some of these studies have assessed the effects of whole vessels rather than the endothelium itself on platelet aggregation. In these studies, the effects of vascular fragments (Lalanne *et al,* 1990) or even vascular tissue homogenate extracts (Furlong *et al,* 1987) have been placed in platelet rich plasma (PRP) or washed platelets respectively in order to assess their effects on platelet aggregation. Others interested in the effects of platelets on arachidonic acid metabolism in vascular cells have assessed prostacyclin generation from vascular fragments incubated in the absence or presence of whole blood (Schror & Seidel, 1988), washed platelets (Papp *et al*, 1986; Mayeux, Kadowitz & McNamara, 1989) or PRP (Bunting *et al,* 1976; Hornstra, Haddeman & Don, 1979).

Endothelial cell perfusates and platelet aggregation

Perfusion cascade techniques using vascular segments

Models where the specific effects of the endothelium on platelet activation and aggregation have been assessed have sometimes involved perfusion cascade techniques. Azuma, Ishikawa & Sekizaki (1986) used perfusates from endothelialised or de-endothelialised rabbit aortic segments in which cyclooxygenase had been inhibited. They transferred the perfusates into aggregating platelets and thereby assessed the platelet inhibitory effects of EDRF (analysed using light transmission platelet aggregometry).

Perfusion cascade techniques using cultured endothelial cells

Endothelial cell cultures as well as being used for assessing endothelial cell effects on platelet adherence have also been used to assess their effects upon platelet activation and aggregation. The use of cultured cells has brought specificity, convenience and reproducibility to studies which assess such platelet and endothelial cell interactions. The particular conditions under which they have been used have varied enormously depending on the investigating group. Some groups have performed studies where perfusates generated from cultured endothelial cells have been added to platelets in aggregometry systems. Busse, Luckhoff & Bassenge, (1987) and Loeb, Peach & Gear (1989) described the use of a perfused column of bovine aortic endothelial cells (cultured on microcarriers). Effluent from the column was transferred into washed platelets to determine the effects of endothelial cell mediators on aggregating platelets responses, assessed in an aggregometer. Similarly, Radomski, Palmer & Moncada (1987a and 1987b) used porcine endothelial cells grown on microcarrier beads to generate a perfusate and this group also assessed the effects of the perfusate on aggregating platelets.

In all of the above studies, endothelial cells and platelets remained isolated from each other; however many studies using cultured endothelial cells have assessed the two in conjunction, possibly reflecting their interactions *in vivo* more accurately.

Cultured endothelial cells and platelet aggregometry

Alheid, Frolich & Forsterman (1987 and Alheid, Reichwehr & Forsterman, 1989) used HUVEC coated (or uncoated, control) microcarriers, and stirred these with washed platelets prior to the addition of bradykinin. This was followed by the addition of thrombin to stimulate aggregation which was assessed by light transmission aggregometry. A similar method (without the use of bradykinin) was described by Mollace e*t al* (1991) and Macdonald, Read & Dusting (1988) in which bovine aortic endothelial cells, grown on microcarrier beads, were added to a suspension of washed platelets and thrombin (PAF or ionophore A23187) was used to stimulate aggregation. Durante *et al* (1992) also used a similar method of interacting platelets and endothelial cells but used either HUVEC or bovine aortic endothelial cell coated microcarriers.

Such methods have been particularly useful in the assessment of the platelet inhibitory effects of EDRF as they have allowed high levels of contact between platelets and endothelial cells such that the short half-life of EDRF has not prevented the detection of its platelet inhibitory effects. Unfortunately, such systems are likely to produce a certain degree of damage to endothelial cells and presumably also cellular activation and in such studies these factors may have influenced the inhibitory effects that endothelial cells exerted on the platelet aggregation responses assessed.

Other studies in which endothelial cells have been mixed with platelets in an aggregometer have been described by Marcus *et al* (1980, 1991), Schafer, Crawford & Gimbrone, 1984, Durante *et al,* 1992 and Bertolino *et al* (1990). These investigators have added resuspended HUVECs, or bovine aortic endothelial cells to washed platelets (or PRP in the latter instance). In Bertolino's study the effects of erythrocytes in the system were also assessed. Once again, the effects of endothelial cells on platelet aggregation were measured by light transmission platelet aggregometry, and endothelial cells were exposed to the mechanical stresses of stirring. Broekman *et al* (1991) used an adapted cuvette system with resuspended endothelial cells which were separated from platelets but again both cell types were subjected to stirring. In studies where resuspension procedures have been used in harvesting endothelial cells, the cells would be activated during resuspension even before being exposed to aggregating platelets or specific stimuli, and this may have influenced the platelet inhibitory effects of cells in such studies.

Endothelial cell monolayers and platelets

This brings one to experimental systems which may most accurately resemble platelet and endothelial cell interactions *in vivo*. These systems are based upon the use of endothelial cell monolayers, which are interacted directly with platelets.

Marcus and associates (1980), in addition to undertaking studies using washed platelets co-incubated with HUVEC suspensions, also performed studies in which platelets were interacted with HUVEC monolayers. Their studies were undertaken to evaluate the effects of platelets upon endothelial cell prostacyclin generation and in particular, to assess the ability of endothelial

cells to use platelet derived endoperoxides for prostacyclin synthesis (i.e. endoperoxide steal). Where monolayers of HUVECs were used, washed platelets were layered over the surface of the cell monolayer and placed on a rotating platform for 5 minutes prior to removing the platelet supernatant for assessments of arachidonic acid metabolites. Similar aims were approached by Baenziger, Becherer & Majerus (1979) using cultured monolayers of HUVECs, smooth muscle cells or fibroblasts, which were co-incubated with washed platelets. Likewise, Hechtman *et al,* 1991, used cultured smooth muscle or rat aortic endothelial cell monolayers with washed platelets. Others (Jorgensen *et al*,1986; Kishi & Numano, 1989) have also described systems in which HUVEC or bovine aortic endothelial cell monolayers were overlaid with washed platelets and stimulated with thrombin or collagen. In these particular studies however, the damage to or detachment of endothelial cells was assessed.

Other investigators have examined the effects of cultured endothelial cell monolayers upon activated platelet responses, rather than the reverse. Murata *et al* (1988) described a system in which the role of thrombomodulin in the platelet inhibitory activity of endothelial cells was explored. For these studies, a monolayer of HUVECs was grown on the inner surface of a standard glass cuvette. Washed platelets were added to seeded or unseeded (control) cuvettes and stirred as in a typical aggregometer. Upon the addition of a stimulus (thrombin) platelet aggregation was assessed by light transmission platelet aggregometry. A similar system was described by Altorjay, Kirchmaier & Breddin (1989) except that HUVECs were grown in specialised rotating cuvettes rather than standard cuvettes. Again platelets (PRP) were added to the cuvette (seeded or unseeded) before the addition of a stimulus (ADP, adrenaline, collagen, or thrombin) and again platelet aggregation was assessed by changes in light transmission. In both these studies, HUVEC-mediated inhibition of platelet aggregation was assessed by comparing platelet responses in seeded and unseeded cuvettes. Predictably, it was observed in both studies that when varying platelet to HUVEC ratios were used (~560 up to 3000:1), the greatest endothelial cell-mediated platelet inhibition was observed at the lower ratio.

Pharmacological agents affecting platelet and vascular/endothelial cell functions and interactions

The methods described in the previous section have been invaluable in increasing our understanding of how platelets interact with vascular cells, or more specifically, with endothelial cells. However, the potential of these models for investigations of such interactions has been greatly enhanced through their use in conjunction with a range of pharmacological agents. Many of these agents have only been used as pharmacological tools to allow the investigation of specific aspects of platelet and vascular cell interactions, whilst others have already found clinical applications.

Arachidonic acid metabolism and platelet and vascular cell interactions

In vivo, the level of platelet activation can be strongly affected by the opposing influences of the platelet stimulatory effects of thromboxane A_2 (generated from platelets) and the platelet inhibitory effects of prostacyclin (generated by vascular cells). Both factors are well recognised regulatory mediators in haemostatic and thrombotic events. Therefore it is not surprising that regulation of arachidonic acid metabolism in both platelets and vascular cells when these cells interact, has been an area of considerable interest. In addition, numerous pharmacological agents have been used and some have been specifically developed to assist in the evaluation and the manipulation of these interactions. The next section will introduce some of these agents and will review some of the investigations which have made particular impact in determining the influence of interactions between platelets and vascular cells which affect their metabolism of arachidonic acid.

Before the use of cultured endothelial cells became widespread, many studies examined the influence of platelets on arachidonic acid metabolism in the whole vessel rather than any specific vascular cell type. Such studies occasionally used the inhibition of the enzyme cyclooxygenase in order to block arachidonic acid metabolism by either platelets, vascular cells or both. Invariably, the agents employed for this purpose were non-steroidal inflammatory agents.

Cyclooxygenase inhibitors

An extensive range of non-steroidal anti-inflammatory agents (including aspirin and indomethacin) possess the ability to inhibit cyclooxygenase (Vane, 1971). The platelet inhibitory effect of these agents is due to this inhibition which prevents platelets from generating thromboxane A_2.

Aspirin is frequently used for *in vitro* investigations in preference to reversible cyclooxygenase inhibitors (such as indomethacin; Stanford *et al,* 1977) because of its capacity to irreversibly (Roth & Majerus, 1975; Roth & Siok, 1978) inhibit in a concentration and time-dependent fashion, both platelet (Sils *et al*, 1988) and/or endothelial cell (Baenziger, Becherer & Majerus,1979) cyclooxygenase. Even in viable endothelial cells (capable of replacing inhibited cyclooxygenase) complete inhibition of cyclooxygenase with aspirin has been reported, with no detectable recovery for up to 90 minutes (Marcus *et al,* 1980).

The inhibition of vascular cell cyclooxygenase in studies in which platelet and vascular cell interactions have been assessed has provided evidence that vascular cells are able to utilise endoperoxides (PGH_2) derived from platelets to produce prostacyclin. It is of interest to see how this single postulated phenomenon of endoperoxide steal has been either confirmed or refuted depending upon the experimental method used to assess platelet and vascular cell interactions.

The use of in vitro methods to clarify the role of platelet derived endoperoxides in vascular prostacyclin synthesis

The concept of endoperoxide steal was first postulated by Bunting *et al* in 1976. This group demonstrated that rabbit mesenteric arterial rings could generate PGX (now termed prostacyclin) when exposed to arachidonic acid, or the endoperoxide PGH_2. These investigators found the conversion efficiency of arachidonic acid to prostacyclin was extremely low (0.5-1%) whereas the conversion of endoperoxides was 80-90%. It was also observed by this group that the incubation medium derived from rabbit mesenteric arterial rings could inhibit ADP induced

platelet aggregation in PRP, but if the tissue had been taken from indomethacin pre-treated rabbits or had been pretreated (*in vitro)* with indomethacin (to inhibit cyclooxygenase) the incubate could not prevent aggregation. This finding demonstrated that the platelet inhibitory activity of the tissue's incubation medium was dependent upon the cyclooxygenase activity in the tissue. In contrast, where indomethacin treated or untreated arterial rings were placed directly into PRP, platelet aggregation to ADP was inhibited irrespective of the indomethacin pre-treatment. This finding illustrated that when platelets and vascular tissue were co-incubated, the platelet inhibitory effects of the tissue could be maintained despite inhibition of the tissue's cyclooxygenase enzyme. It was concluded that the presence of platelets and platelet-derived endoperoxides may be responsible for the platelet inhibitory effects of the treated tissue. That is, platelet-derived endoperoxides may have been utilised by the prostacyclin synthetase of the vascular tissue to form prostacyclin. The pathways of arachidonic acid metabolism implicated in this phenomenon, later termed endoperoxide steal, are illustrated in Figure 1c (page 54). Since this pioneering study by Bunting and colleagues, a number of studies have provided evidence both supporting and opposing the hypothesis that platelet-derived endoperoxides can be converted by vascular tissue or by specific vascular cell types (e.g. endothelial cells) to form prostacyclin.

Needleman, Wyche & Raz (1979) addressed this question by attempting to demonstrate that platelet-derived endoperoxides could be used by bovine aortic tissue to generate prostacyclin. They found that microsomal preparations of this tissue could utilise ^{14}C-PGH_2 (in preference to arachidonic acid) to form prostacyclin, consistent with the prior reports by Bunting *et al,* 1976 and later, Marcus, Weksler & Jaffe, 1978. In conflict with Bunting's findings however, when activated platelets (pre-labelled with ^{14}C-arachidonic acid) were co-incubated with such microsomes or intact aspirin treated vessels, the vascular tissue displayed no capacity to use platelet-derived endoperoxides to form prostacyclin.

Hornstra, Haddeman & Don (1979) also produced evidence which cast doubt upon the original hypothesis of endoperoxide steal proposed by Bunting. This study employed rat aortic

segments stirred in the presence of PRP or platelet poor plasma (PPP). The extent of prostacyclin synthesis was assessed by the platelet inhibitory effects of the resultant plasma. Prostacyclin synthesis from the segments was determined to be the same when the vessels were incubated with activated platelets (PRP) or in their absence (i.e. in PPP) and the concept of platelet-dependent prostacyclin generation was therefore questioned. It was also demonstrated by this group (as by Bunting *et al*, 1976) that following indomethacin pretreatment of the vessels, the segments could still exert platelet inhibitory effects when exposed to PRP, but they reached a different conclusion as to why this was observed. Hornstra's group (1979) suggested that both in their own studies and in those performed by Bunting *et al,* (1976) a reversal of the inhibition caused by the indomethacin pretreatment probably occurred during the course of the incubation (3 minutes), and that the tissues used were still capable of generating prostacyclin through their own cyclooxygenase activity.

Despite the negative evidence described above, in more recent experiments using rabbit aortic tissue or cultured rat aortic smooth muscle cells (Papp *et al* in 1986 and Hechtman *et al,* 1991, respectively) supportive evidence for endoperoxide steal has been obtained. Both groups found that cell types other than endothelial cells (e.g. smooth muscle cells) could utilise platelet derived endoperoxides and generate prostacyclin synthesis. The second group was able to show that the degree of platelet-dependent enhancement of prostacyclin generation was proportional to the platelet count used, and importantly, that the effects of platelet-derived TXA_2 or PGH_2 on smooth muscle cell TXA_2/PGH_2 receptors was not responsible for stimulating prostacyclin generation. Finally, also through assessments of vascular tissue, Mayeux, Kadowitz & McNamara (1989) demonstrated that bovine coronary arterial rings could generate prostacyclin in response to PGH_2, and again this prostacyclin generation was not dependent upon the presence of the endothelium. They also gained results which suggested that aspirin pretreated platelets could utilise endoperoxides generated from vascular tissue to generate thromboxane, and therefore concluded that bi-directional endoperoxide shunt could occur between platelets and vascular cells.

In order to address the question of how endoperoxides derived from platelets specifically interact with the endothelium, many studies have used cultured endothelial cells. Marcus, Weksler and Jaffe (1978) demonstrated that cultured HUVECs could utilise exogenous radiolabelled arachidonic acid and the endoperoxide PGH_2 to synthesise prostacyclin (measured as 6-keto-$PGF_{1\alpha}$). The conversion efficiency of PGH_2 was 11.9%, once again substantially higher than that observed for arachidonic acid (1.6%). Marcus *et al* (1980) also published evidence which demonstrated that in response to A23187, thrombin and collagen, endoperoxides generated from washed platelets could be utilised by aspirin treated HUVEC suspensions to generate prostacyclin. The use of techniques to radiolabel arachidonic acid in platelets allowed the investigators to demonstrate that approximately 50% of the prostacyclin generated by HUVECs in the presence of platelets was derived from platelet arachidonic acid metabolism.

Schafer, Crawford & Gimbrone in 1984 also found that endoperoxide steal occurred in mixed suspensions of bovine aortic endothelial cells with washed platelets or citrate anticoagulated PRP. They gained evidence to suggest that the direction of endoperoxide donation was uni-directional, that is from platelets to endothelial cells. In addition they found that prostacyclin generation did not occur at the expense of platelet thromboxane synthesis; rather, the endothelial cells utilised excess endoperoxides to generate prostacyclin.

Marcus and colleagues (1980) also observed that endoperoxide steal occurred when HUVEC monolayers (considered to be more physiologically representative than suspensions) were incubated with platelets. However endoperoxide steal was less efficient than in experiments where platelet and HUVEC suspensions were mixed. Baenziger and co-workers (1979) also attempted to evaluate endoperoxide steal using platelets incubated with cell monolayers. This group assessed a number of cell types including cultured fibroblasts, smooth muscle cells and HUVECs. In response to arachidonic acid, HUVECs demonstrated the greatest capacity (followed by smooth muscle cells) to produce prostacyclin, but in contrast to Marcus, endoperoxide steal from platelets could not be demonstrated. Indeed when monolayers of HUVECs were incubated with washed platelets (stimulated with arachidonic acid) they actually produced less prostacyclin than in the absence of platelets.

Others (Chesterman *et al,* 1986) who have investigated the role of endoperoxide steal between platelets and endothelial cells have used columns of HUVEC coated microcarrier beads and have perfused these with washed platelets. It was found that collagen stimulated platelets could provide endoperoxides to HUVECs, which resulted in the formation of prostacyclin, and this was not prevented by treating HUVECs with indomethacin. It was also observed that this platelet-dependent generation continued over prolonged periods of time (greater than 15 minutes), prior to platelet elution.

Many factors should be considered when interpreting the results obtained in the studies described above. It cannot be ignored that the various systems used for such studies provided highly variable contact levels between platelets and endothelial cells, which as emphasised by Marcus *et al* (1980) and Chesterman *et al,* (1986) may have influenced the results substantially. These authors suggested that the high level of contact between platelets and endothelial cells favoured the demonstration of endoperoxide steal. Notably however, contact alone is not apparently sufficient to ensure the occurrence of endoperoxide steal as shown in the studies of Needleman's group (1979), where using vascular segments, 30% platelet adherence was observed, but platelet-dependent prostacyclin generation was not demonstrated.

The ratio of platelets to endothelial cells also influences results. This is not surprising as the ratio of platelets to endothelial cells should affect the ratio of platelet thromboxane synthetase to endothelial cell prostacyclin synthetase, and this in itself may affect the utilisation of available endoperoxides. Marcus *et al* (1980) determined that in capillaries, the ratio of platelets to endothelial cells would approach 1 to 1. They demonstrated that endoperoxide steal occurred with a ratio of platelets to endothelial cells of 33 or 66 to 1 and found that with increasing platelet to endothelial cell ratios up to 1069 to 1 (more reflective of a ratio which may exist in larger vessels), greater generation of prostacyclin was observed from aspirin treated HUVECs. Importantly these authors also noted that with increasing platelet to HUVEC ratios, the ratio of platelet thromboxane generation to prostacyclin generation also increased to the point where (at the highest ratio) the platelet inhibitory effects of the aspirin pre-treated HUVECs were

compromised. Similarly, Schafer's group (1984) which also demonstrated endoperoxide steal, used platelet to endothelial cells ratios of 1:1 up to 500:1. Like Marcus *et al* (1980), they demonstrated that the greater the number of platelets, the greater the generation of prostacyclin from aspirin pre-treated endothelial cells. In addition, they observed that the ratio of thromboxane generation to prostacyclin generation also rose with increasing ratios of platelets to endothelial cells. On the assumption that high platelet to endothelial cell ratios may provide greater amounts of PGH_2 to endothelial cells and favour the demonstration of endoperoxide steal, in Baenziger's study (1979) a ratio of 10^4-10^5 platelets to one endothelial cell (estimated to be 3-8 times physiological levels) was used. In these studies endoperoxide steal was not demonstrated and the presence of platelets actually appeared to reduce prostacyclin generation. Thus factors other than platelet to endothelial cell ratios evidently affect whether endoperoxide steal occurs. In Chesterman's study (1986), a ratio of only 300:1 was generally used, and endothelial cell endoperoxide steal was demonstrable. However, the authors placed greater emphasis on the contribution of direct platelet and endothelial cell contact in their experimental system, rather than the ratio of platelets to endothelial cells used.

That endoperoxide diffusion is not impeded by the membranes of platelets (Marcus *et al*, 1980), endothelial cells (Schafer Crawford & Gimbrone, 1984) or other vascular cell membranes (Needleman *et al*, 1979) obviously favours endoperoxide steal. Similarly the conversion efficiency of PGH_2 to prostacyclin by endothelial cells is far greater than that of arachidonic acid (Bunting *et al*, 1976; Needleman, Wyche & Raz, 1979; Marcus *et al*, 1978), possibly due to poor membrane permeation of this fatty acid (Smith,1986), which also suggests that endoperoxide steal may occur. Other factors which would also be likely to affect whether endoperoxide steal occurs (and its direction) include such factors as the efficiency of endoperoxide conversion by the enzymes of platelets and vascular cells, their cellular compartmentalisation, the relative stability of PGH_2 in various experimental systems, and the amounts of PGH_2 formed following platelet or endothelial cell activation (Smith,1986).

Many of the investigations described above were designed to demonstrate that endoperoxide steal could occur, and therefore used extremely high stimulus concentrations such as 20μM-

50μM arachidonic acid, 30-50μg/ml collagen, 5u/ml thrombin or strong and non-physiological agonists such as the ionophore A23187 (1μM). Clearly the investigators desired a high level of liberation of endoperoxides from platelets and Marcus *et al* (1980) showed that platelet-dependent prostacyclin generation from aspirin pretreated HUVECs was greatest in the presence of platelets which produced the greatest platelet thromboxane generation. Under less contrived experimental conditions, using less potent platelet stimuli or lower concentrations of them, endoperoxide steal may not have been demonstrable.

It has also been demonstrated (Hunt *et al*, 1992; Clesham *et al,* 1992; Kent *et al*, 1993), that occupation of TXA_2/PGH_2 receptors on cultured endothelial cells can stimulate prostacyclin generation, and platelet derived TXA_2 or PGH_2 may act upon endothelial cell receptors to stimulate prostacyclin generation. As suggested by Kent *et al,* (1993) this phenomenon in addition to endoperoxide steal may contribute to platelet-dependent enhancement of endothelial cell prostacyclin generation. The recent development of stable TXA_2/PGH_2 receptor agonists and antagonists mean such mechanisms of platelet-dependent prostacyclin generation can be evaluated. For example in the investigation by Hechtman *et al* (1991) where it appeared that endoperoxide steal from platelets to smooth muscle cells was occuring, it was confirmed (using the TXA_2/PGH_2 mimetic, U44619) that this effect was not exerted by receptor mediated effects on smooth muscle cell TXA_2/PGH_2 receptors.

Thromboxane synthetase inhibitors

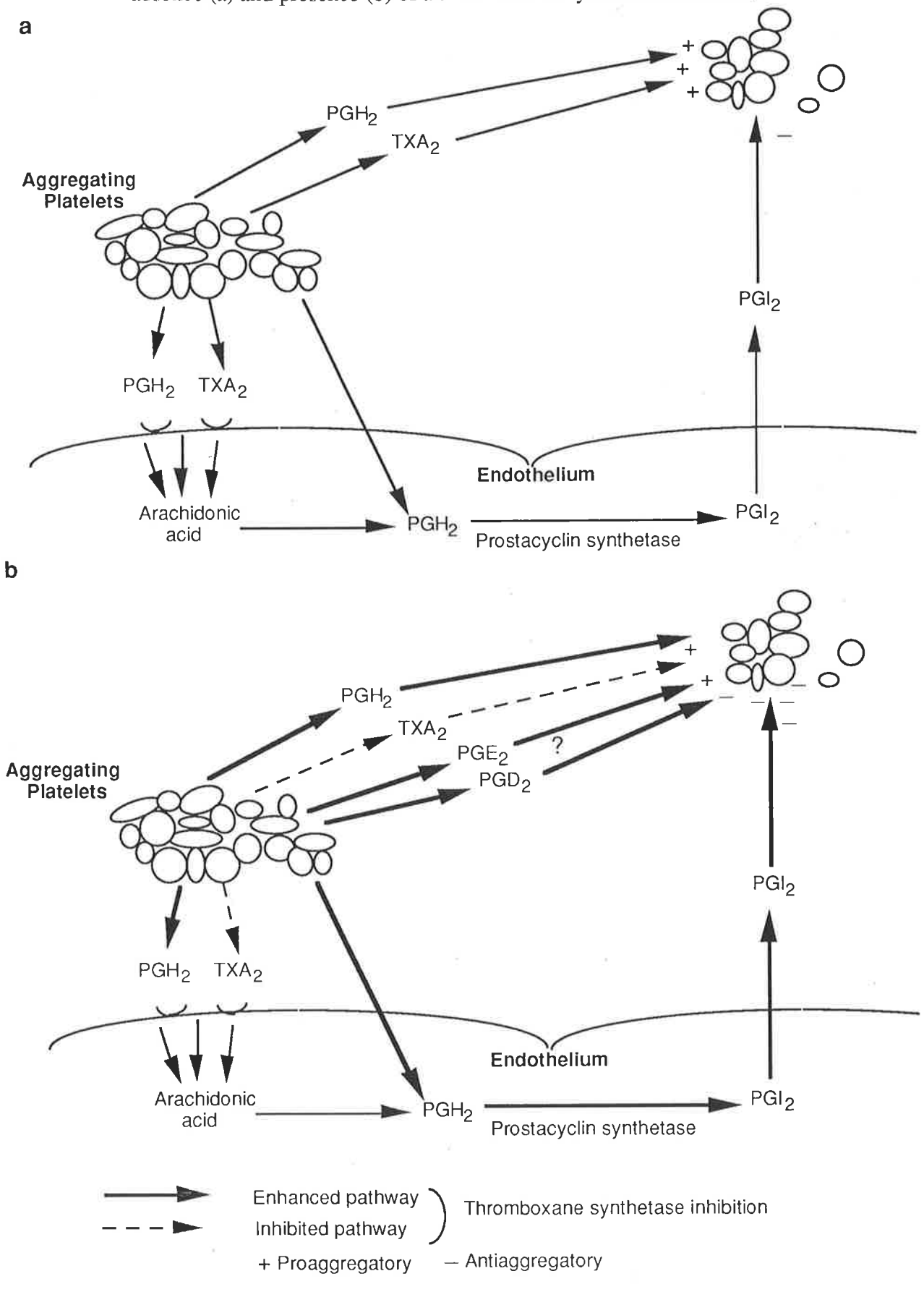
The thromboxane synthetase inhibitors dazmagrel, imidazole and dazoxiben (UK-37248) were developed specifically to inhibit platelet TXA_2 generation at the level of TXA_2 synthetase without affecting cyclooxygenase, thus avoiding *in vivo* the inhibition of vascular cell PGI_2 generation. In the *in vitro* studies described earlier (irrespective of whether endoperoxide steal could be demonstrated between platelets and vascular cells alone) enhancement of vascular prostacyclin generation was consistently observed in the presence of platelets and thromboxane synthetase inhibition. Marcus *et al*, 1980 (with suspensions and monolayers) demonstrated that in the presence of imidazole and stimulated platelets, an apparent 10-14 fold increase in

prostacyclin generation from aspirin pretreated endothelial cells was observed. In the study by Baenziger Becherer & Majerus (1979) no evidence of endoperoxide steal was gained until a thromboxane synthetase inhibitor was introduced, and it was observed that in the presence of imidazole treated platelets, a two fold enhancement in prostacyclin generation from aspirin pretreated endothelial cells occurred. Needleman's group (1979), which also failed to demonstrate endoperoxide steal in the absence of thromboxane synthetase inhibition, also obtained results which indicated the phenomenon was apparently demonstrable in the presence of such inhibition. Finally, Mayeux, Kadowitz & McNamara (1989) also found that an 11-12 fold enhancement of platelet-dependent prostacyclin generation from coronary artery rings occurred in the presence of the thromboxane synthetase inhibitor CGS13080.

The mechanism these investigators used to explain the effects of these thromboxane synthetase inhibitors was based on the assumption that when platelet thromboxane synthetase was inhibited, platelet-derived endoperoxides from activated platelets accumulated. These could then be utilised by endothelial cell (or other vascular cell) prostacyclin synthetase, to cause the enhanced prostacyclin generation observed.

Figure 1c (overleaf) illustrates metabolites of arachidonic acid and the pathways associated with endoperoxide steal. The diagram illustrates the ability of thromboxane synthetase inhibition to enhance the liberation of not only platelet endoperoxides (PGH_2) but also of other platelet prostanoids including PGD_2 (antiaggregatory) and PGE_2 (weakly proaggregatory: Gray & Hepinstall, 1991). Platelet lipoxygenase metabolites do not appear to be affected by thromboxane synthetase inhibition (De Clerck *et al*, 1989a) and are not illustrated. The proaggregatory mediators TXA_2 and PGH_2 are also shown and evidence suggests that endothelial cell TXA_2/PGH_2 receptor occupation may stimulate prostacyclin generation (Hunt *et al,* 1992; Clesham *et al,* 1992; Kent *et al*, 1993).

Figure 1c
Metabolites of arachidonic acid and their effects in platelets and endothelial cells in the absence (a) and presence (b) of a thromboxane synthetase inhibitor.



The specific mechanism of action of thromboxane synthetase inhibitors and their *in vitro* effects have made these agents attractive as potential therapeutic agents. It was hoped that *in vivo*, as *in vitro,* they may inhibit platelet function through blocking thromboxane generation and concomitantly enhance prostacyclin liberation from vascular cells.

Clinical trials studying the effects of dazoxiben provided evidence that, with the inhibition of serum thromboxane generation by dazoxiben, circulating levels of prostacyclin rose only 2-3 fold over basal circulating levels (FitzGerald & Oates, 1984; FitzGerald, Reilly & Pedersen, 1985). It has become apparent that such minor increases in circulating PGI_2 concentrations may be of limited biological significance. Certainly with infusions of prostacyclin, FitzGerald *et al* (1979) demonstrated that a 20-40 fold increase in prostacyclin concentrations over basal levels was necessary to achieve platelet inhibitory effects. From clinical trials, *ex vivo* assessments of platelet responses to thromboxane-dependent stimuli have also demonstrated that such agents display inferior platelet inhibitory effects compared to more established antithrombotics such as the cyclooxygenase inhibitor aspirin (FitzGerald, Reilly & Pedersen, 1985, Gresele *et al*, 1991, De Gaetano, Bertele & Cerletti, 1987a,b). For example in one trial, successful inhibition of platelet aggregation in response to arachidonic acid was only observed in 4 of 16 subjects (Bertele *et al,* 1984). It has also been shown that *in vitro*, platelet aggregation in response to collagen is inhibited more effectively by aspirin than by the thromboxane synthetase inhibitor CGS13080 (Hoet *et al*, 1990). Despite such negative results *ex vivo*, or *in vitro*, it is generally agreed that *in vivo*, at sites of thrombotic activity where activated platelets interact with vascular tissue, these agents (as shown with *in vitro* models) may favour redirection of platelet derived endoperoxides towards prostacyclin generation. Thus these agents acting at local sites of platelet activation (rather than systemically) may still provide antithrombotic effects (FitzGerald & Oates, 1984; Gresele *et al,* 1991).

One possible explanation for lack of inhibitory effects of these agents *ex vivo* was postulated to lie in the demonstrated ability of thromboxane synthetase inhibition in platelets to cause a re-direction of arachidonic acid metabolism towards PGE_2, PGD_2, and $PGF_{1\alpha}$, prostanoids which are produced to a negligible extent in untreated platelets (De Clerck *et al*, 1989a). *In vitro* PGD_2

and PGE_2 (>10μM), in conjunction with other platelet stimuli, have been shown to cause diminution or enhancement of platelet aggregation responses respectively (Bertele *et al,* 1984; Gray & Hepinstall, 1991). Unfortunately in the study performed by Bertele's group (1984), the non-responsive subjects did not differ in their platelet generation of PGE_2 or PGD_2, eliminating these prostaglandins as a cause of the anomaly. In addition, since thromboxane synthetase inhibition has no effect on platelet lipoxygenase (12-HETE) product formation (De Clerck *et al,* 1989a), the modified formation of these products cannot be considered in explaining non-responders.

The most convincing reason for the lack of efficacy of this type of agent is the suggestion that thromboxane synthetase inhibitors may cause a build up of unconverted PGH_2 endoperoxides. Although less potent than TXA_2, PGH_2 can cause platelet activation through the occupation of platelet TXA_2/PGH_2 receptors (Fitzpatrick & Gormon, 1979; Jaschonek & Muller, 1988) and such effects of PGH_2 would oppose the desired platelet inhibitory effects of thromboxane synthetase inhibition. Evidence in favour of this hypothesis came from combining thromboxane synthetase inhibitors and TXA_2/PGH_2 receptor antagonists. *Ex vivo*, following the administration of dazoxiben the additional presence of such receptor antagonism has been shown to convert non-responders into responders (Bertele *et al*, 1984). In addition, *in vitro,* greater platelet inhibitory activity has been demonstrated with thromboxane synthetase inhibitors and TXA_2/PGH_2 receptor antagonists in combination, than with either agent alone (Bertele & De Gaetano, 1982).

TXA_2/PGH_2 receptor antagonists

The potential use of specific TXA_2/PGH_2 receptor antagonists has gained increasing interest in recent years. A range of TXA_2/PGH_2 antagonists has become available, such as EIP, AH23848, Bay U 340 , BM13177, GR32191 and SQ 29,548. All have been investigated for their binding characteristics to TXA_2/PGH_2 receptors on platelets and/or their anti-platelet effects against various stimuli (Bertele & De Gaetano, 1982; Bertele *et al,* 1984; Brittain *et al*, 1985; Theis *et al,* 1992; De Clerck *et al* 1989a,b; Watts *et al,* 1991; Hedberg *et al,* 1988 and Ogletree *et al,* 1985).

The effects of TXA_2/PGH_2 receptor antagonists on vascular tissue have also been investigated. Most of these studies have assessed the capacity of TXA_2/PGH_2 receptor antagonists to inhibit smooth muscle contraction in response to thromboxane or thromboxane mimetics (Ogletree & Allen, 1992) or have concentrated on the apparent heterogeneity of TXA_2/PGH_2 receptors between platelets and vascular smooth muscle (Furci, FitzGerald & Garrett, 1991). The effects of platelet-derived TXA_2 on smooth muscle cells and vascular tone (and the antagonism of these effects) has ramifications in the regulation of haemodynamics at sites of platelet activation. It is surprising however, that relatively little attention has been paid to how TXA_2/PGH_2 mimetics and TXA_2/PGH_2 receptor antagonists may affect endothelial cell responses. Indeed it remains uncertain how at sites of thrombotic activity, high levels of TXA_2 (or PGH_2) may influence endothelial cell functions such as prostacyclin generation. One study by Sung, Arleth & Berkowitz (1989) found that in membrane preparations derived from bovine pulmonary endothelial cells, the TXA_2/PGH_2 mimetic, U44619 inhibited basal and bradykinin stimulated prostacyclin generation. In direct conflict with this study, Hunt *et al* (1992) and Clesham *et al* (1992) demonstrated that in a transformed bovine aortic endothelial cell line, U44619 stimulated the release of prostacyclin. Recently, also in transformed bovine aortic endothelial cells, it was confirmed that U44619 was again able to cause the liberation of prostacyclin (but not EDRF) from such cells (Clesham *et al,* 1992). Human umbilical vein endothelial cells have also been found to possess such TXA_2/PGH_2 receptors (Kent *et al,* 1993) which on occupation cause increases in intracellular calcium concentrations and prostacyclin generation. Clearly further studies are needed to clarify how TXA_2/PGH_2 receptor occupation affects endothelial cell functions.

Combined thromboxane synthetase inhibitors and TXA_2/PGH_2 receptor antagonists

As indicated, TXA_2/PGH_2 receptor antagonism can improve the anti-platelet effects of thromboxane synthetase inhibitors *ex vivo* and *in vitro*. Drugs have been developed with both thromboxane synthetase inhibitory activity and TXA_2/PGH_2 receptor blocking capacities. The effects of such agents have been reviewed by Gresele *et al* 1991. Picotamide (Violi *et al* 1988) is

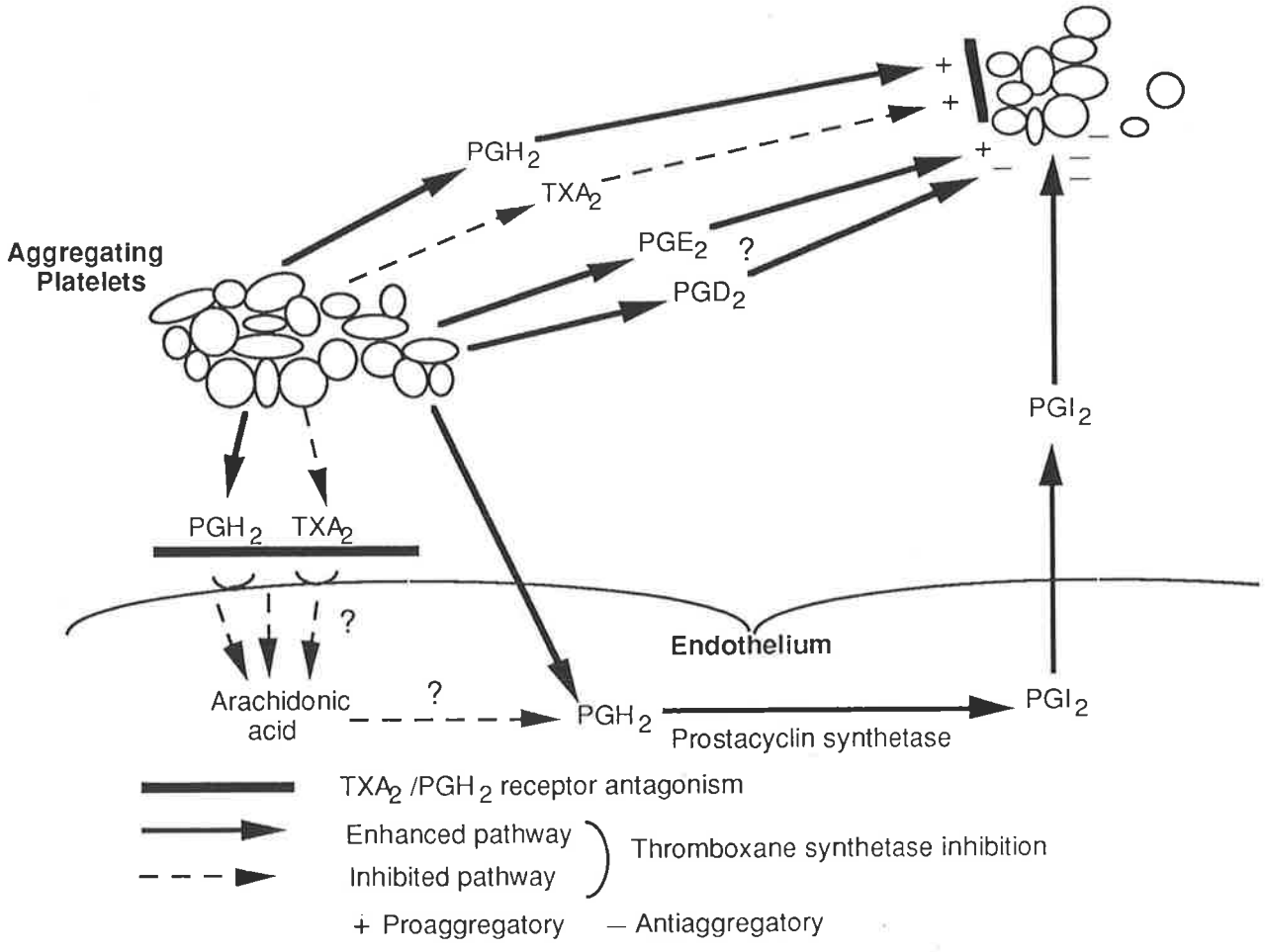
one such agent and ridogrel (i.e. R68070) is another which remains under investigation (De Clerck *et al*, 1989a,b). It has been claimed that ridogrel presents some effects not observed in pure thromboxane synthetase inhibitors or cyclooxygenase inhibitors. Evidence that ridogrel possesses platelet TXA_2/PGH_2 receptor antagonistic effects has been provided by observations that ridogrel can cause reduced aggregation to the TXA_2/PGH_2 mimetic U46619 *in vitro* (De Clerck *et al*, 1989a) and *ex vivo,* for 1-12 hours following administration of 400mg (De Clerck *et al,* 1989b). In addition ridogrel (400mg) has been reported to produce extended bleeding times greater than those obtained following administration of 400mg (Hoet *et al,* 1990) or 1 gram (De Clerck *et al,* 1989b) of aspirin. In addition, *in vivo* in rats, ridogrel increased bleeding times to a level comparable to that caused by the combination of dazoxiben and the TXA_2/PGH_2 antagonist BM 13177 (agents which independently caused lesser increases in bleeding times)(De Clerck *et al,* 1989b). It was noted in studies by Watts *et al* (1991), that at higher concentrations where ridogrel achieved TXA_2/PGH_2 receptor antagonistic effects as well as thromboxane synthetase inhibition, it also produced non-specific effects on platelet cyclooxygenase activity.

It remains to be established whether drugs such as ridogrel may provide greater clinical efficacy against arterial thrombotic events compared to pure thromboxane synthetase inhibitors or more established anti-platelet agents such as aspirin. Certainly in a recent trial (involving 907 patients), evidence suggested that ridogrel offered no advantages over aspirin as an adjunct to thrombolytic therapy in patients with acute myocardial infarction (RAPT Investigators, 1994). From this study however, it was suggested that, following such thrombotic events, ridogrel may be superior to aspirin in preventing new ischaemic episodes. Trials to address this question are still required. Furthermore, *in vitro*, information is required from experimental systems where specific platelet and endothelial cell interactions are monitored, to identify how agents such as ridogrel differ in their effects when compared to pure thromboxane synthetase inhibitors.

Figure 1d illustrates how pharmacological manipulation of platelet and endothelial cell derived arachidonic acid metabolism may affect platelet and endothelial cell interactions. The figure indicates how thromboxane synthetase inhibition may enhance prostacyclin generation from the endothelium and therefore the platelet inhibitory effects of the endothelium. The proaggregatory mediators TXA_2 and PGH_2 are also shown as are the effects of blockade of TXA_2/PGH_2 receptors which may prevent the proaggregatory effects of platelet-derived endoperoxides. Since there is evidence to suggest that TXA_2 and PGH_2 are amongst the platelet-derived mediators which may stimulate prostacyclin generation, then TXA_2/PGH_2 receptor blockade on endothelial cells may specifically compromise prostacyclin generation via this pathway. This effect may affect prostacyclin generation to only a minor extent under these conditions, but such specific effects of thromboxane synthetase inhibitors with TXA_2/PGH_2 receptor antagonistic properties have not been investigated.

Figure 1d

Metabolites of arachidonic acid and their effects in platelets and endothelial cells in the presence of a thromboxane synthetase inhibitor and TXA_2/PGH_2 antagonist.



Prostacyclin analogues and agents which increase prostacyclin generation

Pharmacological agents which substitute for prostacyclin have already found a place in clinical practice. Specific and relatively stable prostacyclin analogues (Whittle & Moncada, 1985; Szczeklik *et al,* 1985) have been developed which have potent platelet inhibitory effects and also *in vivo,* express cytoprotective, vasorelaxant and fibrinolytic activity (Gryglewski *et al,* 1987). These agents have been used in the treatment of a number of conditions including ischaemic heart disease. Unfortunately they possess too many vasorelaxant related side effects, including reduced blood pressure and increased heart rate, to be used for long term therapy (FitzGerald *et al* 1979). Furthermore, the development of platelet refractoriness to the effects of prostacyclin and its analogues during periods of infusion, limits the use of such agents (MacDermot *et al,* 1987).

Due to the recognised platelet inhibitory activity of prostacyclin, pharmacological agents which may stimulate the production of vascular prostacyclin have been considered as potential therapeutic agents for reducing the risk of thrombosis. Nafrazatrom (a lipoxygenase inhibitor: Martin *et al,* 1983) has gained some recognition as a stimulant of prostacyclin generation, as have some thiazide diuretics (Yardumian & Machin, 1988). The apparent ability of such agents to stimulate prostacyclin synthesis may actually be attributable to their ability to delay the metabolism of prostacyclin by inhibiting vascular 15-hydroxydehydrogenase, responsible for metabolising prostacyclin. Alternatively, as with nafrazatrom, by inhibiting lipoxygenase and promoting the reduction of 15-HPETE to 15-HETE, such agents may reduce the inhibitory effects of 15-HPETE on prostacyclin synthetase (Yardumian & Machin, 1988). The mechanism of action of agents such as defibrotide, which also appears to enhance prostacyclin generation, remains undefined (Berti *et al,* 1988). However, flavonoids which *in vitro* have been shown to produce only modest direct platelet inhibitory effects (Gryglewski *et al,* 1987; Tzeng *et al,* 1991) may favour prostacyclin generation, possibly through acting as antioxidants and preventing oxidant induced inhibition of enzymes responsible for prostacyclin generation.

Dietary supplements and platelet/vascular cell interactions

Dietary approaches have also been used to increase the circulating levels of PGI_2 through favouring the production of prostacyclin. With the development of atherosclerosis there are associated increases in lipid peroxides which are known to have inhibitory effects on enzymes responsible for prostacyclin generation (Vane, Gryglewski & Botting, 1987). In rabbits fed atherogenic diets both elevated plasma lipid peroxides and reduced prostacyclin generation have been shown to be partially reversed using vitamin E supplements. This is presumed to be due to the antioxidant activity of vitamin E reducing lipid peroxidation, thus favouring prostacyclin generation (Szczeklik *et al,* 1985). Others have also shown vitamin E intake may inhibit platelet adhesion (Jandak, Steiner & Richardson, 1989) but as yet the therapeutic potential of such agents remains uncertain.

In other approaches involving dietary manipulation, investigators have attempted to substitute products of arachidonic acid metabolism such as prostacyclin with products of eicosapentaenoic acid metabolism. The use of eicosapentaenoic acid (C20:5ω3) (fish oil) supplemented diets relies on the ability of cellular membranes to take up this fatty acid such that on cellular activation it can be liberated and can compete with arachidonic acid (C20:4ω6) as a substrate for cyclooxygenase. The metabolism of eicosapentaenoic acid rather than arachidonic acid in various cells, means the end products following enzymic conversion differ from those which would be produced if arachidonic acid were the substrate. In the case of platelet-derived TXA_2, the end product becomes TXA_3 which possesses only minor platelet stimulatory or vasoconstrictive activity. In the case of vascular prostacyclin (PGI_2) the end product is PGI_3 which retains the potent anti-platelet aggregatory activity and vasorelaxant properties of PGI_2. Such diets are expected to result in the retention of the anti-thrombotic properties of the vascular endothelium whilst compromising the thromboxane activating pathway in platelets (Buchanan Crozier & Haas, 1988). Unfortunately, eicosapentaenoic acid is not metabolised by cyclooxygenase with the same efficiency as arachidonic acid, and these diets result in the overall inhibition of fatty acid metabolism by cyclooxygenase. They nevertheless have been shown to produce modest reductions in the risk of re-occurrence of heart attacks (Vane & Botting, 1991).

Pharmacological agents and EDRF

The biological effects of EDRF include its capacity to cause vasorelaxation, and the inhibition of platelet adhesion and activation, discussed in previous sections. The capacity of the endothelium to generate EDRF and its recognised influence over platelet and endothelial interactions has meant that pharmacological manipulations of its synthesis and/or effects have been of considerable interest

With the discovery of EDRF, and the elucidation of the mechanism (through raising cellular concentrations of cGMP) by which it obtains its biological effects, the mechanism of action of a number of therapeutically used vasorelaxants was clarified. For example glyceryl trinitrate and other nitrates cause vasodilation when metabolised by smooth muscle to form EDRF (via nitrosothiol intermediates) (Ignarro *et al*, 1981). Not surprisingly, since EDRF was identified many pharmacological agents have been found which influence its formation, activity and elimination. At the present time such agents are used principally as pharmacological tools in various experimental systems. In addition (both *in vivo* and *in vitro*) these agents are frequently used to determine indirectly the presence or absence of EDRF in situations where actual measurement is not required.

Pharmacological agents affecting the stability of EDRF

In buffers and biological fluids, EDRF is subject to rapid oxidation by molecular oxygen to nitrite and nitrate. It is also highly susceptible to inactivation by superoxides which can be generated in *in vitro* systems. This known lability of EDRF has often been used to advantage to infer the presence or absence of EDRF. By providing conditions of high or low oxidant stress the biological effects thought to be occurring through EDRF can be diminished or enhanced.

The activity of enzymes such as endothelial cell cyclooxygenase and lipoxygenase can lead to superoxide generation when their products are converted via hydroperoxidase to lipid peroxides, and in closed experimental systems this generation may compromise the activity of EDRF (Katusic & Vanhoutte, 1989). Pharmacological agents can also promote superoxide generation. These include pyrogallol, some lipoxygenase inhibitors (Read & Dusting, 1987; Marshall &

Kontos, 1990; Moncada, Palmer & Gryglewski, 1986; Katusic & Vanhoutte, 1989) and more recently, methylene blue (Marczin , Ryan & Catravas, 1992b).

Logically, because superoxide anions reduce the half-life of EDRF, agents which inhibit the formation of superoxide anions also increase the half-life of EDRF and therefore enhance its effects. As discussed by Marshall & Kontos, (1990), the generation of superoxides appears to be the major cause of EDRF elimination *in vitro*. Consequently, in many experimental systems, antioxidants and superoxide dismutase have been used to enhance effects proposed to be mediated by EDRF. Superoxide dismutase but not catalase or reductions in glutathione have been shown to increase the effects of EDRF in closed (non-perfused) *in vitro* systems, and such effects emphasise the participation of superoxides in the elimination of EDRF (Mugge *et al*, 1991). In contrast *in vivo,* EDRF effects appear to be affected not only by superoxides but also by the conversion of superoxides and hydrogen peroxide to hydroxy radicals, as catalase and deferoxamine are both effective in increasing its effects (Marshall & Kontos, 1990).

Pharmacological agents which inhibit the activity of EDRF

Another inhibitor of EDRF is oxyhaemoglobin (subsequently referred to as haemoglobin). This agent avidly binds to EDRF (NO) with greater affinity than O_2 (Gibson & Roughton, 1957). In contrast, the oxidised counterpart of haemoglobin, that is methaemoglobin (with haem containing oxidised iron (Fe^{3+})) binds neither O_2 nor EDRF. Haemoglobin, because of its large molecular weight (~ 64,500) cannot penetrate cellular membranes. Thus in experimental systems it is used as a specific scavenger of extracellular EDRF. Because it has extracellular effects, haemoglobin should not inhibit normal intracellular feedback effects in cells which generate EDRF and thus raised cGMP (frequently used as an indirect indicator of the effects of EDRF) should still occur with EDRF generation. The presence of haemoglobin can however indirectly influence intracellular concentrations of EDRF by creating a cross cell-membrane concentration gradient, resulting in the diffusion of EDRF out of cells (Boulanger *et al*, 1990; White *et al*, 1993). *In vivo*, circulating concentrations of haemoglobin are maintained at a low level but local increases in such concentrations due to erythrocyte lysis during thrombotic processes may alter platelet or vascular functions through reducing the effects of EDRF.

EDRF acts by causing the activation of cellular guanylate cyclase with the subsequent formation of cGMP. Thus the effects of EDRF can also be inhibited by agents which inhibit guanylate cyclase. Methylene blue, an oxidative cellular stain which penetrates cell membranes, is reported to be such an agent (Gruetter *et al,* 1980 and Gruetter, Kadowitz & Ignarro, 1980). It has been proposed that this effect may occur through oxidation by methylene blue of the haem group of guanylate cyclase (Martin *et al*, 1985) and there is also evidence that such effects may be irreversible (Gruetter *et al*, 1980; Martin *et al*, 1985). The specificity of the pharmacological effects of methylene blue has recently been questioned with evidence indicating its effects may be associated with its capacity to stimulate the generation of superoxides in experimental systems (Marczin, Ryan & Catravas, 1992b). Studies by Wolin *et al,* (1990) also concluded that methylene blue may inhibit the effects of EDRF by two mechanisms. The first involved the ability of methylene blue to promote the formation of superoxides which resulted in the direct oxidation of EDRF, and was reversed in the presence of superoxide dismutase. The second involved the inactivation of guanylate cyclase. There is also increasing evidence to suggest that even the enzymic inhibition produced by methylene blue may not be specific to guanylate cyclase alone (Martin, Drazan & Newby, 1989; Mayer, Brunner & Schmidt, 1993).

Pharmacological agents which inhibit the synthesis of EDRF

The most recently employed pharmacological agents used to assist in characterising the role of EDRF in experimental systems are the L-arginine analogues. These agents have effectively superseded some of the agents already mentioned to inhibit the activity of EDRF, and have added specificity to the characterisation of EDRF. Their use became possible only with the determination of the biochemical pathways associated with the formation of EDRF. It is now known that nitric oxide synthetase generates EDRF through the conversion of L-arginine to L-citrulline, with the simultaneous liberation of EDRF or NO. The N group is derived from the N-guanadino terminal of L-arginine and L-arginine analogues with substitutions made at their N-guanadino terminal effectively compete with L-arginine and block the formation of EDRF.

One of the best known L-arginine analogues is N^G-monomethyl-L-arginine (commonly referred to as LNMMA). Others such as N^G-nitro-L-arginine (LNNA) appear to act more rapidly and

possess a greater potency than LNMMA (Moore *et al,* 1990). Differences in their activities may be related to their differing effects on L-arginine uptake, or their stability, with the potency of LNMMA possibly being reduced due to its metabolism, eventually resulting in the formation of L-arginine itself (Hecker *et al,* 1990b). Nevertheless, these analogues are effective both *in vivo* and *in vitro* in inhibiting postulated biological effects of EDRF. For example, *in vivo* such agents are able to inhibit the endothelium-dependent (EDRF-mediated) reductions in blood pressure caused by infusions of acetylcholine (Parker & Adams, 1993).

Clinically, the inhibition of EDRF generation *in vivo* may occasionally be desirable. Certainly, the pathology of conditions such as disseminated intravascular coagulation and septic shock may in part be attributable to the activity of inducible nitric oxide synthetase and enhanced generation of EDRF (McCall & Vallance, 1992, Moncada, Palmer & Higgs, 1991, Moncada, 1992; Stoclet *et al,* 1993; Parker & Adams, 1993). Although steroids used prophylactically may prevent the induction of nitric oxide synthetase, where such induction has occurred nitric oxide synthetase inhibitors can be used to reduce excessive EDRF generation and its effects. Indeed recently, LNMMA has been used successfully in patients with severe sepsis to treat the hypotension associated with this condition (Petros *et al,* 1994). Unfortunately a lack of selectivity of these agents for constitutive or inducible forms of the enzymes means such agents have limited applications in clinical practice (Warren, Pons & Brady, 1994). Meanwhile they continue to serve as useful pharmacological tools in *in vitro* experimental systems.

Pharmacological agents used to enhance the synthesis or mimic EDRF

There is evidence that in the development of atherosclerosis, the release or effects of EDRF are reduced (McCall & Vallance, 1992; Meredith *et al,* 1993, Tanner, Boulanger & Luscher, 1993). Consequently interest has arisen as to whether L-arginine may be able to increase the generation of EDRF. In man, plasma L-arginine concentrations are ~100μM, and in healthy subjects such concentrations do not change with L-arginine administration (Warren, Pons & Brady, 1994; McCall & Vallance, 1992). It seems unlikely that limited availability of L-arginine could limit EDRF generation, although it has been shown that in hypocholesterolaemic patients, L-arginine administration reverses the impaired EDRF-mediated haemodynamic effects (Drexler *et al,*

1991). Clearly the effects of such supplementation, particularly where EDRF generation may be impaired, requires further investigation.

As with prostacyclin, interest has focussed on agents able to mimic the effects of EDRF. Clinically glyceryl trinitrate and other nitrates used as coronary vasodilators possess some capacity to mimic the effects of EDRF, at least on vascular tone. Nitrate metabolism by smooth muscle cells to form NO or s-nitrosothiol intermediates results in raised smooth muscle cell cGMP and associated vasorelaxation (Ignarro *et al*, 1981). Unlike EDRF however, organic nitrates do not possess strong platelet inhibitory effects due to the inability of platelets to efficiently metabolise them. In contrast, pharmacological agents which in aqueous solutions spontaneously generate NO (e.g. nitroprusside) and even synthetically generated s-nitrosothiols (such as s-nitroso-N-acetylpenicillamine and s-nitrosocysteine) are able to mimic the effects of EDRF in both vascular tissue and platelets (Ignarro, 1989). Unfortunately the clinical applications of such agents are limited by their lability and (like prostacyclin analogues) the lack of specificity of their effects (Radomski & Moncada, 1993).

Other pharmacological agents affecting platelets and vascular cells

Many of the pharmacological agents which have been discussed have found therapeutic applications through their ability to reduce thrombotic tendencies associated with platelet activation. Some of these agents also utilise or enhance the natural platelet inhibitory effects of the vascular endothelium. Although they lie beyond the scope of this overview, there are many more agents which can influence platelet and vascular cell interactions. Such agents include receptor antagonists such as the serotonin (S_2) antagonist ketanserin, which may prevent platelet-derived serotonin from enhancing platelet activation and causing vasoconstriction, yet still allow serotonin to stimulate endothelial cell EDRF generation (via S_1 receptors) (De Clerck, Van Neuten & Reneman, 1984). Fibrinogen (GPIIb/IIIa) receptor antagonists may also prove to be powerful platelet inhibitory agents, affecting platelet adherence not only to each other but also to subendothelial cell constituents (Topol & Plow, 1993; Simoons *et al*, 1994). The mechanism of action and effects of many more agents are reviewed by De Gaetano, Bertele & Cerletti (1987a); Kelton & Hirsh (1987) and Yardumian & Machin (1988).

Therapeutic antithrombotic strategies and future directions in research

Much research into pharmacological agents which may affect arterial thrombotic events no longer merely targets the anti-platelet effects of these agents. Research continues to indicate that the effects of established anti-platelet strategies may involve or affect endothelium-dependent functions. Such findings have assisted in the revision of how some of these agents should be used to optimise their efficacy. In addition, with an increasing knowledge of factors which affect platelet and endothelial cell interactions, new pharmacological agents are being developed which are specifically designed to influence these interactions. *In vitro* methods to determine the mechanisms of such agents are now considered a necessity since so many potential strategies rely on the manipulation of these interactions. Consequently, methods to specifically monitor such interactions have never been in greater demand.

Many of the *in vitro* methods which have been developed to assess platelet and endothelial cell interactions have been designed to assess either the effects of platelets on endothelial cell functions or the effects of endothelial cells on platelet responses. Frequently experimental systems are designed to examine only specific characteristics of platelet and endothelial cell interactions. For example, in systems where the phenomenon of endoperoxide steal and its effects upon endothelial cell prostacyclin generation are examined, investigators may not examine in any depth how this phenomenon may affect the platelet inhibitory effects of endothelial cells. Alternatively, where the platelet inhibitory effects of endothelial cell-derived mediators such as EDRF or prostacyclin *are* examined, investigators do not evaluate how platelets may affect endothelial cell generation of such factors. Without considering the complexity of platelet and endothelial cell interactions such methods are effectively limited in their potential applications. Within a single experimental system, few methods characterise the mutual influence of platelets and endothelial cells upon each other, or the multiple mechanisms by which each can influence the other's functions.

For this reason, it was determined to develop an *in vitro* system which evaluated both platelet and endothelial cell functions alone and in combination. It was considered desirable under a single set of conditions to characterise the effects of each cell type upon the other. Emphasis was placed upon assessing a number of previously reported mechanisms by which these cells affect the other cell type. This included examining whether activated platelet-derived endoperoxides or other platelet-derived products affected endothelial cell prostacyclin generation. It also involved characterising the mechanisms by which endothelial cells affected activated platelet responses, including determining the role of prostacyclin or EDRF in any evident inhibitory effects of endothelial cells. It was not the intention of the studies undertaken in this thesis to manipulate experimental conditions to demonstrate any single feature of endothelial cell and platelet interactions, but rather to investigate and characterise under a single set of experimental conditions the important or less important factors involved in these interactions. Thereby, the best applications for the system could be exposed and its rational use for assessment of the effects of pharmacological agents (designed to alter these interactions) could be determined.

In assessing platelet and endothelial cell interactions, it was desirable to use a physiologically relevant system. Therefore monolayers of cultured endothelial cells were employed, and platelet and endothelial cell responses to physiological stimuli were examined. Prostacyclin generation from endothelial cells was assessed, and as some platelet stimuli can stimulate prostacyclin generation whilst others may not, it was desirable to examine both types of stimuli in the system. In selecting end-points of platelet activation (as in endothelial cells) some examination of platelet responses which were indicative of their arachidonic acid metabolism was desirable, therefore platelet thromboxane generation was assessed as one end-point of platelet activation. As platelet aggregation is not necessarily dependent upon platelet thromboxane generation, the assessment of platelet dense granule release (i.e. serotonin release) was considered as a second end-point which could be employed to assess platelet activation. Characterising possible relationships between these two end-points of platelet activation was naturally of interest.

Aims

The primary aim of the investigations described in this thesis was to evaluate *in vitro* the responses of platelets and endothelial cells (alone and in combination) to various stimuli. It was of particular interest to evaluate how endogenous mediators derived from these cells affected the responses of the other cell type. To expose the influence of such mediators, pharmacological agents known to inhibit the synthesis or effects of these mediators were employed.

Cultured human umbilical vein endothelial cell (HUVEC) monolayers were utilised in these studies and consequently such cell lines had first to be established and characterised by standard morphological assessments and immunohistochemical techniques. The capacity of HUVECs monolayers to generate the platelet inhibitory mediator prostacyclin also had to be confirmed using an RIA for its stable metabolite (6-keto-$PGF_{1\alpha}$).

Methods also had to be developed to quantify platelet activation to a range of stimuli such that the effects of HUVECs upon these responses could be evaluated. Techniques to quantify platelet thromboxane A_2 generation (using an RIA for its stable metabolite TXB_2) and dense granule release (though assessing ^{14}C-serotonin release) therefore had to be established and validated.

To characterise platelet and HUVEC responses (to a range of stimuli) and determine the mediators which influenced the responses of these cells both in isolation and in combination, it was decided to examine:

a) Technical factors which influenced platelet and/or HUVEC responses to a range of stimuli.

b) Relationships between assessed parameters of platelet activation (i.e. thromboxane generation and serotonin release).

c) Whether the phenomenon of endoperoxide steal, or platelet thromboxane generation itself, could influence prostacyclin generation from HUVECs.

d) Whether endoperoxide steal could be established or enhanced by the use of platelet thromboxane synthetase inhibition and to compare the effects of a pure thromboxane synthetase inhibitor with that of a newer agent with additional TXA_2/PGH_2 receptor antagonistic properties.

e) The platelet inhibitory effects of prostacyclin, and EDRF, using pharmacological agents to prevent their production (or effects).

f) Whether the established system could be adapted to evaluate other parameters of platelet and endothelial cell interactions, such as platelet adherence, and the HUVEC-derived mediators which may affect this parameter .

Chapter 2

General methodology

This chapter outlines the techniques employed in the development of a method to assess platelet and endothelial cell interactions *in vitro*. A number of factors which may have affected the validity of the techniques described were evaluated prior to their routine use. The results of some of these control experiments are described in this chapter, while others are presented in Chapters 3 and 4.

Isolation and culture of human umbilical vein endothelial cells (HUVECs)

All cell isolation procedures and subsequent cell manipulations were performed under sterile conditions. Solutions were routinely warmed to 37°C prior to use. Solutions described in % terms were prepared on a weight/volume (w/v) or volume/volume (v/v) basis and are indicated as their final concentration. The composition and methods of preparation for all solutions and buffers are given in Appendix 1.

HUVEC isolation

HUVECs were isolated, cultured and subcultured by an adaptation of techniques previously described by Jaffe *et al* (1973) and Gimbrone Cotran & Folkman, (1974). Untraumatised human umbilical cords at least 15cm long were obtained (from the Department of Surgery, Royal Adelaide Hospital), stored at 4°C and used within 36 hours of collection.

For each cord, end sections (approximately 1cm) of cord were removed prior to the dilation of the vein at each end of the cord. A sterile female 1/8" tubing adaptor (Becton-Dickinson, Division of Becton, Dickinson and Co., Rutherford, New Jersey. U.S.A.) was inserted at each end and attached by sterile cotton ties. Sterile three-way stop-cocks (Connecta, Viggo. A.B. Helsingborg, Sweden) were inserted into the tubing adaptors for perfusion manipulations.

Veins were perfused with Hanks Balanced Salt Solution (HBSS: see Appendix 1) to remove all traces of blood from the vein lumen. Following draining, the lumen was filled with 10-20ml of 0.1% collagenase (Worthington Biochem. Corp, Freehold, New Jersey U.S.A.) prepared in HBSS as described in Appendix 1. Filled cords were then incubated in 0.9% w/v saline at 37°C for 7 minutes. The cord was removed and gently massaged for between 30 seconds and 1 minute. The vein was then flushed with serum free culture medium or HBSS (10ml) followed by complete culture medium (CCM: see 'culturing conditions')(10ml). These rinses were collected into a 50ml plastic centrifuge tube (Falcon, Blue Max 2070, Becton and Dickinson Labware, Becton Dickinson and Co. Lincoln Park, New Jersey. U.S.A.) and centrifuged at 200g for 5 minutes. Following aspiration of the supernatant the cell pellet was resuspended by gentle trituration in 5ml CCM and the cell suspension was seeded into pre-gelatinised 25cm^2 tissue culture flasks. (Costar, Cambridge, MA. U.S.A.). Flasks were gelatinised using 1% gelatin (Eastman Kodak Co. Rochester, New York, U.S.A; prepared in 0.9% w/v saline as described in Appendix 1) and excess gelatin was removed by aspiration.

Cells were grown at 37°C under 5% CO_2 in air in a Kevatron (Model 102) CO_2 cabinet. Twenty four hours after plating, cultures were rinsed twice with HBSS to remove non-adherent cells and fresh CCM was added. Thereafter, CCM was replaced with fresh medium every two to three days. Lines were not maintained if any visible contamination of the HUVEC population with atypical cells was apparent.

From seven randomly selected cords, primary cell yields were determined and were standardised to a 20cm cord length. For this length, the average (±sem) cell harvest was $1.90 \pm 0.34 \times 10^6$ cells, ranging from $0.5\text{-}3.4 \times 10^6$ cells. Primary culture cells generally reached confluence within 4 days of seeding and lines were terminated (Chapter 5 onwards) if confluence was not achieved in 6 days following seeding.

Culturing Conditions

For initial lines, the culture medium in which HUVECs were grown was based upon RPMI 1640 containing 20mM Hepes (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid) and was prepared and sterilised in the Institute of Medical and Veterinary Science (Adelaide, S.Australia). The majority of cell lines (see Chapter 5 onwards) were cultured in CCM based on medium 199 with Earle's salts and containing 20mM Hepes (prepared and sterilised as described in Appendix 1).

To produce CCM (prepared in 50ml batches), the basic culture medium was supplemented with foetal calf serum (20%), L-glutamine (2mM), sodium pyruvate (1mM), amphotericin B (2.5µg/ml), 1 x non-essential amino acids, penicillin (100u/ml) and streptomycin (100µg/ml). Sodium bicarbonate was added (1.5µg/ml) to assist in maintaining optimal pH conditions for HUVEC growth. All culture medium constituents were purchased from Flow labs., North Ryde, N.S.W., Australia or Irvine, Scotland, U.K. All CCM supplements were aliquoted into appropriate volumes under sterile conditions and stored at 4°C or -20°C, as recommended by the manufacturer, until use. No growth factors were incorporated into the culture medium. The routine addition of heparin to CCM was made only when it was confirmed that it produced no detectable effects on prostacyclin generation by HUVECs. These studies were performed following reports that heparin may inhibit prostacyclin synthesis (Hasegawa, Yamamoto & Yamamoto, 1988). More recent evidence has however confirmed that only heparin in combination with growth factors produces this effect (Minter, Dawes & Chesterman, 1992). Heparin alone was therefore added to CCM (10u/ml CCM (~100µg/ml)) to inhibit smooth muscle cell proliferation (Castellot, Rosenberg & Karnovsky, 1984) and to assist in the maintenance of pure HUVEC cultures.

Subculturing: passaging and seeding

Confluent HUVECs were subcultured following detachment of the cells using 0.05% trypsin-0.02% EDTA (Flow labs). After washing the cell monolayer twice (1-2 minutes/wash) with pre-warmed HBSS or basic serum free medium, 2.5ml trypsin-EDTA/25cm^2 flask was added to cause cell detachment (achieved within 1-2 minutes). The extent of cell detachment was observed by phase contrast microscopy and when complete, detached cells were immediately transferred into 10ml sterilised centrifuge tubes containing an equal volume of CCM. Following centrifugation at 200g for 5 minutes, the supernatant was aspirated and the cell pellet resuspended by trituration in CCM. The cell population was split (1:2) during passaging and seeded into fresh 25cm^2 pre-gelatinised tissue culture flasks (Costar). Each passage therefore involved dividing the total cell population into two, prior to seeding into fresh flasks.

Typically, on the third passage, (generally made within 10 days of initial cell isolation) HUVECs from a single line were harvested from four 25cm^2 flasks and were seeded into pre-gelatinised wells of 12 or 24 well tissue culture plates (Costar). Cell counts were made by haemocytometer (Freshney, 1987). This allowed appropriate calculations and adjustment of cell counts/ml to provide the desired seeding density/well such that in either 12 or 24 well culture plates an equivalent number of cells were seeded per base surface area (4 or 2cm^2/well). Following seeding, CCM was changed at 24 hours following seeding and cells were routinely used at 48 hours post-seeding. Specific variations from this routine are indicated in the relevant chapters.

Characterization of HUVECs by morphological criteria and by the presence of factor VIII related antigen

Methods described in this section are relevant to results reported in Chapter 3.

Morphological assessments and photography

Endothelial cells (cultured using RPMI 1640 based CCM) were identified on the basis of their morphology by phase contrast microscopy using an Olympus CK 2 inverted microscope. Photographs were taken using an Olympus OM 20 camera and using Kodak 35mm, 100 ASA film .

Immunofluorescent staining and photography

Further characterization of endothelial cells was made by direct immunofluorescent staining of factor VIII related antigen, based on indirect methods described previously (Hoyer, De Los Santos & Hoyer, 1973, Booyse *et al,* 1981). Endothelial cells (from 2 separate cell lines which had been passaged 2 and 3 times respectively) were seeded into wells of 12 well culture plates containing gelatinised glass coverslips at a seeding density of 1.2×10^5 cells/well. At 48 hours following seeding, coverslips were removed and rinsed for 2 minutes in warm, then for 2 minutes in cold phosphate buffered saline pH 7.2 (PBS; see Appendix 1). Cells (confluent and semi-confluent) were then fixed by immersion in cold acetone for 30 minutes, after which two further two minute rinses in cold PBS were performed to remove all acetone. Excess PBS was drained from coverslips and they were then placed cell side up on moist tissue paper. Two drops of fluorescein isothiocyanate-conjugated antiserum against factor VIII related antigen (Atlantic antibodies, Scarborough, ME 04074, U.S.A.: diluted 1/10 in PBS) was added to the surface of each coverslip. They were then incubated at 37°C in a humidified chamber for 30 minutes. Following incubation, coverslips were washed a further 3 times (2 minutes/wash) in warm PBS and drained between each rinse to remove all excess antiserum. Following draining, 2 drops of 10% glycerol in PBS were added to the cell covered surface of coverslips prior to their inversion onto ethanol cleaned microscope slides.

Microscopy involved use of an Olympus BH-2 system. Both phase contrast (BH2-PC) and reflected light fluorescence (BH2-RFL) capacities were available in this system. This allowed photomicrographic assessments of cell morphology and (under fluorescent light) fluorescein isothiocyanate localisation in the same cell population. As a consequence, photomicrographs of identical cell populations were obtained showing both their morphological characteristics and the precise distribution of factor VIII related antigenic staining.

Radioimmunoassays (RIAs)

Materials

Buffers and solutions (Details on preparation appear in Appendix 3)

Phosphate buffered saline, (PBS) pH7.5 ; gelatin-Tris buffered saline, (GTBS) pH7.3; dextran coated charcoal (in PBS, pH7.5).

6-keto-$PGF_{1\alpha}$ RIA

^{3}H-6-keto-$PGF_{1\alpha}$ (150-180Ci/mmol, in 250µl acetonitrile:water, 9:1) was purchased from Amersham Int. (Buckinghamshire, U.K). 6-keto-$PGF_{1\alpha}$ used in the preparation of standards and 6-keto-$PGF_{1\alpha}$ antiserum were purchased from Advanced Magnetics Inc.(Cambridge, MA, U.S.A; formerly Seragen Inc., Boston, MA.,USA). Cross reactivity between the RIA antiserum for 6-keto-$PGF_{1\alpha}$ and other prostanoids possibly present in the samples was a consideration and the antiserum from Advanced Magnetics was selected (in preference to Sigma Chemical Co. St Louis, MO, U.S.A. and Cayman Chemicals, Denver, Colarado, U.S.A.) on the basis of the low cross reactivity reported by the manufacturers towards other prostanoids, particularly $PGF_{2\alpha}$ (2.2%), PGE_2 (0.6%) and PGD_2(<0.1%) and TXB_2(<0.1%).

Thromboxane B_2 (TXB_2) RIA

^{3}H-TXB_2 (180-210Ci/mmol, 250µl in ethanol) was purchased from Amersham Int. TXB_2 used in the preparation of standards was purchased from Cayman Chemical Co. and TXB_2 antiserum was a gift from Dr. M. James (Department of Rheumatology, Royal Adelaide Hospital, S.Australia). Cross reactivity with 6-keto-$PGF_{1\alpha}$,, $PGF_{2\alpha}$ and PGE_2 were all <0.1% (personal communication from Dr. M. James).

Methods

The RIA method for 6-keto-$PGF_{1\alpha}$ was based upon that described by Maclouf (1982). The RIA method for TXB_2, adapted from that used previously by Herd *et al* (1987), was based upon the method described by Fitzpatrick (1982).

For the 6-keto-$PGF_{1\alpha}$ assay, specific antiserum binding to the analyte was assessed in a dose-dependent fashion, and a titre was selected for routine use in assays which gave ~30% binding. For the TXB_2 assay, specific antiserum binding of the radiolabelled analyte was set to a binding level of ~50%.

All standards and quality controls (QCs) were stored at -70°C. Standards were prepared as described in Appendix 3 and batches of working standards for both TXB_2 and 6-keto-$PGF_{1\alpha}$ (0.5ng/ml and 5ng/ml) were prepared every 6-9 months. When fresh batches were prepared, experimental samples were analysed using standard curves derived from old and fresh stocks and the values obtained from both were compared to ensure the new standards were correctly prepared. This was also confirmed by comparing QC values calculated before and following the preparation of fresh standards. QCs displayed minimal (<4%) deterioration during their use over the entire period of analyses (3 years). Samples were stored at -70°C until assay, generally undertaken within 2 weeks of collection; however there was no indication of sample instability when samples were re-assayed up to 6 months from their collection date.

An automatic diluter was used to dilute working stock standards of 0.5ng/ml and 5.0ng/ml to produce concentrations for the standard curve namely 1, 2.5, 5, 10, 25, 36.1, 50,100, and 250 pg/100µl for the 6-keto-$PGF_{1\alpha}$ assay and 5, 10, 25, 50, 100, 250, and 500pg/100µl for the TXB_2 assay. Examples of standard curves for both assays are shown in Appendix 3. QCs were created by pooling samples generated from platelet and HUVEC interaction studies.

For RIAs, samples to be assessed for their TXB_2 or 6-keto-$PGF_{1\alpha}$ content were diluted into GTBS to fall within the standard curve range. The dilution factors ranged from 1/100 to 1/2.

These dilution factors meant sample assay tubes contained in a volume of 100µl from 1µl up to 50µl of the actual sample under analysis. Each tube also contained 100µl ^{3}H-labelled analyte (prepared as described in Appendix 3), 100µl standard or diluted sample, 100µl anti-serum, and an additional 200µl GTBS. The exceptions were the 'total binding' and non-specific binding (NSB) tubes which were prepared in the absence of antiserum and the blank tubes, which were prepared in the absence of standard (volumes were substituted with 100µl GTBS). All tubes were prepared in duplicate and all (except the'total binding' tubes to which an additional 300µl GTBS was added) contained a final volume of 500µl.

Samples and standards were stored for 24 hours at 4°C prior to precipitating unbound antiserum. The precipitation procedure required the removal of the assay tubes from the refrigerator and (within a period of 5 minutes) the addition of 300µl/tube dextran coated charcoal (prepared in 4°C PBS) to all except the pair of 'total binding' tubes. All tubes were then centrifuged at 2500g at 4°C for 30 minutes and the supernatant was transferred to glass scintillation vials (20ml). Eight millilitres of ACS11 (Amersham Corp., Arlington Heights, IL, U.S.A.) scintillation fluid was added and counts per minute (cpm)/tube were recorded using a Beckman Liquid Scintillation Counter, LS6800. A defined window setting (using channels 10-400) was used to measure the energy spectrum for tritium (^{3}H) and exclude radioactivity associated with ^{14}C in the samples. Investigations were performed to study the effects of the presence of ^{14}C-serotonin on the measurement of ^{3}H associated cpm in samples. These indicated that with the use of this specific counting window ^{14}C counts were effectively eliminated without marked (<3%) reduction of ^{3}H counts, thus retaining the accuracy of the RIA.

^{3}H cpm of the 'total binding' tubes were generally 8000-10000 cpm (mean of duplicates) and cpm in the NSB tubes (mean of duplicates) was less than 7% of the total bound cpm. For all the standard, blank and sample tubes, the obtained cpm values were corrected by the mean cpm value associated with the NSB tube in order to obtain specifically bound cpm values. These specifically bound cpms associated with each of the standard and the blank tubes (all in

duplicate) were then processed using a four parameter logistic function curve fitting program (Rodbard, 1978). When sufficient samples had been analysed, appropriate weighting coefficients were incorporated into the programme. The % of specifically bound ^{3}H 6-keto-$PGF_{1\alpha}$ or ^{3}H TXB_2 was plotted against the relevant standard concentrations. The fitted curve included 95% confidence intervals and indicated any standards which had been rejected. The linear portion of the standard curve was used to determine concentrations of the relevant analyte in the samples. Concentrations of the analyte were determined from the mean of the duplicate tubes. This value was however rejected if duplicates varied more than 5% from their mean, and the sample was re-assayed in a subsequent RIA.

A number of parameters associated with the standard curve were calculated for each RIA.

EC_{50} : the calculated concentration of the analyte required to displace 50% of specifically bound cpm from the antiserum.

Slope : the calculated slope of the standard curve over its linear range.

MDC : the extrapolated minimum detectable concentration, or the minimum concentration of the analyte which could cause detectable displacement of the specifically bound labelled analyte from the antiserum.

NSB : cpm of the analyte where no antiserum was added to the assay tube. Expressed as a % of the 'total binding' cpm.

Blank : cpm associated with the blank tube (corrected by NSB cpm) where no analyte was added to the assay tube. Expressed as a % of the total cpm (also corrected by NSB cpm) and indicative of the maximum level of specifically bound analyte in the assay.

Reproducibility and accuracy

Reproducibility of the RIAs was verified initially by monitoring the consistency of all of the parameters just described. When sufficient samples from experiments (containing platelet resuspension buffer (RB)) were generated, these were pooled to provide 2 QCs which were used in all subsequent RIAs. Appropriate dilution of QCs was made such that final concentrations fell near the upper and lower standard limits for the assays. When sufficient assays had been made, QC concentrations obtained were plotted against the mean value (determined from the first 10 assays) and for each subsequent assay the obtained concentrations for QCs were required to remain within 2 standard deviations (SDs) of this mean. If one fell outside these limits, other parameters of the assay were checked to determine whether the assay was acceptable for use. If both fell outside the accepted limits (which occurred in ~5% of assays performed), the assay was considered invalid.

When the assay had been established, reproducibility between assays was assessed by observing the variability of the QC values. In addition, in 13 independent assays the calculated concentrations of standards were determined. Mean (and SD) values were calculated and were compared to expected (nominal) values. Within assay variation was determined by assessing a single sample 4 or 6 times within a single assay run. Results from these studies are provided in Appendix 3.

Interference assessments

Assessments were made to determine whether the constituents (of buffers) in the samples generated from experiments would lead to inaccurate concentration estimations or loss of assay sensitivity. These parameters were assessed after spiking potential interfering buffers (equivalent to those generated experimentally) with the analyte (either TXB_2 or 6-keto-$PGF_{1\alpha}$) prior to their assay, or by assessing standard curves in the absence or presence of the buffer. It was found that none of the buffers (HBSS, RPMI 1640 or platelet resuspension buffer (RB)) altered the specific binding levels or the sensitivity of the RIAs. In addition, their presence in spiked samples or standard curves did not affect the accuracy of the assays. Two other

potentially interfering compounds used in experiments (methylene blue and oxyhaemoglobin) were also assessed for interference and again their presence was not found to affect assay accuracy or sensitivity. The potential interference of cpm from ^{14}C-serotonin in platelet derived RB samples was avoided by using a specific counting window, as described earlier.

Initial assessments of prostacyclin generation from HUVECs

Methods described in this section are relevant to results reported in Chapter 3.

HUVECs for these initial studies were cultured using CCM (based upon RPMI 1640) and were seeded into pre-gelatinised 12 well culture plates with a seeding density (unless otherwise indicated in the results section) of 1.2 x 10^5 cells/ well.

Experimental protocol

Prior to use, HUVECs were washed twice (5 minutes/wash) with 1-2ml of either HBSS or RPMI 1640 (serum free with no added supplements) which had been pre-warmed to 37°C. Cells were finally incubated with 950µl of the same buffer to which 50µl of a stimulus solution was added. Following a 10 minute incubation at room temperature, the supernatant was removed and stored at -70°C for later 6-keto-$PGF_{1\alpha}$ determination, expressed in these studies as ng/ml. Primary cells or cells which had been passaged 1-6 times were used, and where relevant, passage numbers are indicated in the results section. In initial experiments, incubations were not performed under controlled temperature or CO_2 conditions. Consequently, although (with pH stick assessment) no large shifts in the pH of incubates occurred over the time course of incubations, slight alterations in the pH of the HBSS and RPMI 1640 incubates may still have occurred.

In a limited number of preliminary experiments, factors which may affect prostacyclin generation responses were examined. These included the effects of seeding density, the supernatant used, the effects of the time required to process 12 well plates and the effects of the

time between seeding and using the cells. In addition, between and within cell line variability and the effects of passaging on HUVEC responses to a variety of stimuli were also considered. It was also confirmed that HUVECs (either unstimulated or stimulated) did not produce detectable thromboxane generation (as assessed by RIA for TXB_2).

The 6-keto-$PGF_{1\alpha}$ generation by HUVECs was determined in response to various stimuli and stimulus concentrations. Stimuli included arachidonic acid, thrombin and adrenaline, ADP, histamine, platelet activating factor (PAF), bradykinin and the buffer (or control) used in the preparation (and dilution) of the stimuli. Methods for preparation of the stimuli are given in Appendix 2.

Platelet isolation, labelling with ^{14}C serotonin and measurements of serotonin release

Methods described in this section are relevant to results reported in Chapter 4 and subsequent chapters.

Platelet washing procedures

Washed platelet suspensions were prepared in a modified Hepes-Tyrode's buffer (see platelet washing and resuspension buffers, Appendix 1) according to methods described previously by Klinlough-Rathbone *et al* (1977) which were adapted from those initially used by Mustard *et al* (1972). Three modifications to the described protocol were made. Firstly, although all washing and resuspension buffers were based upon the Tyrode's buffer described by Mustard *et al* (1972), Hepes (3.78mM) was added to the basic buffer to improve buffering capacity. Secondly, heparin was included in only the first washing buffer to avoid possible carry-over into the final platelet suspension. Finally, unless otherwise specified, for all buffers, calcium chloride was added to give a final 1.2mM concentration (not 2mM) and final ionised calcium concentrations were confirmed to be approximately 1.2mM using a Ciba-Corning 634 Ca^{2+}/pH analyser. Details of the platelet washing protocol follow.

Following clean venepuncture, venous blood was collected into 50ml syringes (Terumo Medical Corp. Elkton, MD, USA) using a 21G butterfly (Terumo, Surflo®). Blood was immediately transferred into 50ml plastic centrifuge tubes (Nunc Inc. Naperville IL, U.S.A or Falcon, Becton Dickinson and Co.) containing acid citrate dextrose anticoagulant to give a blood-anticoagulant ratio of 7/1 (v/v) and final pH of ~6.5. Following gentle mixing, the blood was centrifuged at 200g for 13 minutes at room temperature. The supernatant platelet-rich-plasma (PRP) was transferred by plastic transfer pipette into 15ml plastic centrifuge tubes. These were centrifuged at 1000g for 17 minutes and the supernatant, platelet-poor plasma was transferred back to the initial 50ml centrifuge tubes and re-mixed with the red blood cells. The second yield of platelets was obtained by re-centrifuging the 50ml tubes at 200g and transferring the second resultant yield of PRP into 15ml tubes. These were then centrifuged at 1000g for 17 minutes, as was the first yield, and the supernatant platelet-poor plasma was removed and discarded. For all 15 ml tubes, when the platelet-poor plasma had been removed from the platelet pellet, the sides of the tubes were cleared of residual plasma (using a cotton bud) and the platelet pellets were resuspended in 1-2ml of washing buffer (pH 6.5, pre-warmed to 37°C) by gentle trituration using a siliconised (Coatasil, AJAX chemicals Pty. Ltd., Auburn, NSW, Australia) pasteur pipette. The platelets of the first and second yield were pooled and finally resuspended in 20ml of washing buffer. The pooled platelets were centrifuged at 1000g for 17 minutes and washing buffer was discarded (the last traces were removed using a cotton bud) and the platelets were resuspended in 20ml of fresh washing buffer (free of heparin) and incubated at 37°C for 10 minutes prior to centrifugation at 1000g for 17 minutes. The second washing buffer was removed prior to resuspending the platelets in the final resuspension buffer (RB: 20ml, also free of heparin, pH 7.4) A platelet count was obtained (by Coulter counter) and the final count was corrected to 300,000/µl (generally within 5%) by the further addition of resuspension buffer. The platelet suspension was then stored at 37°C in air tight plastic syringes, to maintain a pH of 7.4 for the duration of the experiment.

Platelet labelling with ^{14}C-serotonin

Platelet serotonin release was used in experiments to assess platelet activation in response to a range of stimuli. To allow these assessments, platelets were prelabelled prior to experiments with ^{14}C-serotonin (actively taken up and stored in dense granules). To label platelets, following platelet washing and the correction of platelet counts, platelets were incubated with 5-hydroxytryptamine-3'-creatinine sulphate-[^{14}C] (Amersham/Searle, Arlington Heights, III, 57mCi/mmol). The label, 50μCi/ml was diluted in platelet RB 1/10 and then added to the final washed platelet suspension to give 0.02μCi/ml (or 0.35μM) in washed platelets. Platelets were then stored at 37°C in an airtight plastic syringe until used. An equilibration time of 20 minutes was allowed prior to use. Platelet ^{14}C-serotonin uptake was defined in percentage terms as the total platelet suspension dpm corrected by the platelet-free background dpm, all divided by the uncorrected total dpm of the platelet suspension. In initial studies background levels of ^{14}C-serotonin were assessed over time following labelling and it was found these levels did not alter substantially over 4 hours. In addition, when variation in the extent of uptake between experiments was assessed, platelet uptake was consistent between experiments with (from 10 experiments) a mean uptake±sem, of 94.6±0.44% (C.V. 1.5%).

Calculation of % serotonin release

After the final incubations during experiments, platelets were immediately transferred from culture plate wells (or in aggregometry studies, from cuvettes) to Eppendorf centrifuge tubes and centrifuged at 8400g for 1 minute. The addition of an EDTA and formaldehyde mixture to stop aggregation and prevent centrifugation induced release of ^{14}C-serotonin (Krishnamurthi, Westwick & Kakkar, 1984) was avoided to prevent likely interference of the mixture with RIAs, and because a correction factor for background release was applied in the determination of % serotonin release. Following centrifugation 100μl or 50μl, (depending on the total supernatant volume) of the platelet-free supernatant was transferred into vials for liquid scintillation counting, the remainder being stored in fresh Eppendorf tubes (at -70°C). Scintillation counting was undertaken using a Beckman Liquid Scintillation counter, model LS3801 which calculated ^{14}C disintegrations per minute (dpm) associated with samples. The % serotonin release for

samples was determined to be equal to the sample dpm expressed as a percentage of the total dpm (after both had been corrected by the background dpm).

Total and background values

To obtain total and background values for each experiment, ^{14}C-serotonin dpm representative of total and background values were obtained from 100µl or 50µl aliquots of appropriate control samples. The total dpm value was obtained from an aliquot (~3000dpm/100µl) of the (non-centrifuged) platelet suspension, taken directly from the storage syringe. The background dpm were determined from an aliquot of platelet-free supernatant, obtained following centrifugation (at 8400g) of unstimulated platelets. In the experiments described in Chapter 4, the background dpm value was gained using unstimulated platelets, taken directly from the storage syringe. For subsequent experiments a mean background dpm value was obtained following centrifugation of platelets removed from the control (RB) wells.

The ability of the scintillant (Ready Value, Beckman Instruments Inc. Fullerten, CA, USA) to completely solubilise platelets in the 'total' tube was confirmed by comparing % release results obtained using the Beckman scintillation cocktail with those gained when using a solubilising step prior to adding scintillant. The latter method involved processing samples using a 2 hour solubilisation step with soluene (Beckman) prior to addition of a toluene based scintillation fluid (see Appendix 1 for details on preparation of POPOP-PPO scintillation fluid). No difference in the final serotonin release results was observed using these two methods, indicating both provided complete platelet solubilisation. Following this finding, the Beckman scintillation fluid was used for future studies.

Use of a fibrinogen source

For Chapters 4 and 10, a crude fibrinogen source was added to washed platelets at 5µl/ml just prior to their use. This fibrinogen source or platelet free plasma (PFP) was prepared from citrate anticoagulated blood (as described in Appendix 1). From aggregometry studies, a PFP volume of 5µl/ml washed platelets was determined to be the minimum volume which supported extensive washed platelet aggregation responses and to ADP.

Platelet aggregometry studies

Methods described in this section are relevant to results referred to in Chapter 4.

Washed platelets were prepared in the presence or absence of added (1.2mM) $CaCl_2$ and labelled with ^{14}C-serotonin as described previously and were used with or without the addition of a fibrinogen source (PFP; 2-20µl/ml washed platelets). Turbidometric platelet aggregometry was performed as described previously (by Herd *et al,* 1987 and Wilson *et al,* 1990). The only adaptations were that percentage (%) aggregation was assessed on the basis of light transmission calibrated against unstimulated, unstirred washed platelets (0%) and platelet-free resuspension buffer (100%), rather than PRP and PFP respectively. In addition a 950µl volume of platelets and a 50µl volume of stimulus were used. Following platelet aggregation, assessed over 5 minutes, cuvette contents were removed and centrifuged at 8400g. Aliquots of the supernatants were used to determine % platelet serotonin release, as described under 'Calculation of % ^{14}C-serotonin release'. For some studies, following labelling, platelets were were stored in the presence of 0.5µM fluoxetine (a specific inhibitor of serotonin uptake).

Investigations to assess uptake of ^{14}C-serotonin by HUVECs

Experimental protocol

In studies which examined platelet responses in the presence of HUVECs, the accuracy of platelet serotonin release results (as an indicator of platelet activation) may have been compromised if HUVECs had actively taken up serotonin which may have been released from platelets. Studies were therefore undertaken to determine the extent of HUVEC uptake of serotonin.

Platelet free resuspension buffer (RB) containing ^{14}C-serotonin with dpm approximating those of background, and a 50% release response (i.e.~0.01µCi) was incubated with HUVEC monolayers or in non-seeded wells for periods of 10 up to 30 minutes. Incubations were carried out at 37°C and under 5% CO_2 in air and plates were agitated using a Titertek titre plate shaker (DSG Titertek/4, Flow Labs;setting 3). Cells were either incubated without the addition

of a stimulus, or were stimulated with thrombin (0.5u/ml) and in some instances co-incubated with a monoamine oxidase inhibitor, nialamide (20μg/ml: 70μM) to prevent metabolism of any internalised serotonin by HUVECs. Following the removal of the supernatant and two subsequent washes, ^{14}C-serotonin counts associated with the cells or the plastic were assessed. These were compared to the dpm actually added to and retrieved from the wells. Dpm associated with the cells or plastic were obtained by incubating the cells (or plastic) with either 0.1M NaOH in 2% Na_2C0_3 solution (used by Czervionke, Hoak & Fry, 1978 to solubilise HUVECs)(at 37°C for 2 hours), to achieve complete loss of HUVEC integrity and the release of internal constituents.

From two experiments, HUVEC or plastic associated ^{14}C-dpm ranged from 0.4-5% of the total added and HUVEC-associated ^{14}C-dpm were not consistently greater than plastic associated ^{14}C-dpm. Furthermore, ^{14}C-dpm in the supernatant recovered from the wells ranged from 95-106% of those added. Neither the presence of thrombin, nor the monoamine oxidase inhibitor nialamide, nor different incubation times (up to 30 minutes) produced any effects which were consistent with active uptake of ^{14}C-serotonin by HUVECs. That is, neither the stimulation of HUVECs, nor an inhibitor of serotonin metabolism led to any detectable accumulation of serotonin within HUVECs over time. From these results, it was established that of the added ^{14}C-serotonin dpm only minor levels became associated with HUVECs or plastic and these were not considered sufficient to strongly influence the accuracy of platelet serotonin release results. Also, this association could be attributed entirely to non-specific binding to HUVECs or plastic, and serotonin uptake by HUVECs (which could have affected the accuracy of platelet serotonin release results in the presence of HUVECs) was not evident. The results of these studies were confirmed in two further experiments where actual platelet free RB, derived from stimulated platelets was used, rather than RB spiked with ^{14}C-serotonin. Possible uptake over prolonged incubation times was not investigated as it was considered irrelevant to the studies of interest.

Interactions Studies with Platelets and HUVECs

Methods described in this section are relevant to results of Chapter 4 and subsequent chapters. Following the described preliminary and control studies to assess responses of platelets and HUVECs independently, a system was developed to allow assessment of the interactions of these cells. Four initial investigations were made, in which various experimental conditions were assessed. It was confirmed that gelatin pre-treatments of culture plate wells did not result in platelet activation. In addition, neither unstimulated nor activated platelets generated detectable levels of prostacyclin (assessed by RIA for 6-keto-$PGF_{1\alpha}$). From these studies, the protocol used for the experiments reported in Chapter 4 was established. Further minor modifications to the method were implemented and are summarised in Table 2a, on page 94. The basic study design used for interaction studies follows.

Preparation of HUVECs

HUVECs were isolated, cultured and subcultured as described previously. Following their third passage, HUVECs from a single line were seeded into culture plate wells and any unseeded wells were filled with sterile water to maintain humidified conditions. Cells were added to 12 well clusters in 0.8-1.0 ml/well and to 24 well clusters in 0.4-0.5ml/well. For the experiments described in Chapter 4, cells were seeded into 12 well culture plates (base surface area/well: $4cm^2$) at 1.2×10^5 cells/well. From Chapter 5 onwards, cells were seeded into 12 wells culture plates at $1.8\text{-}2.0 \times 10^5$ cells/well and into 24 well culture plates (base surface area/well: $2cm^2$) at $\sim 1.0 \times 10^5$/well. Thus HUVECs were seeded at $\sim 0.5 \times 10^5$ cells/cm^2. Following seeding, the culture medium over the cells was changed at ~24 hours and the cells were used at ~48 hours.

Preparation of washed platelets

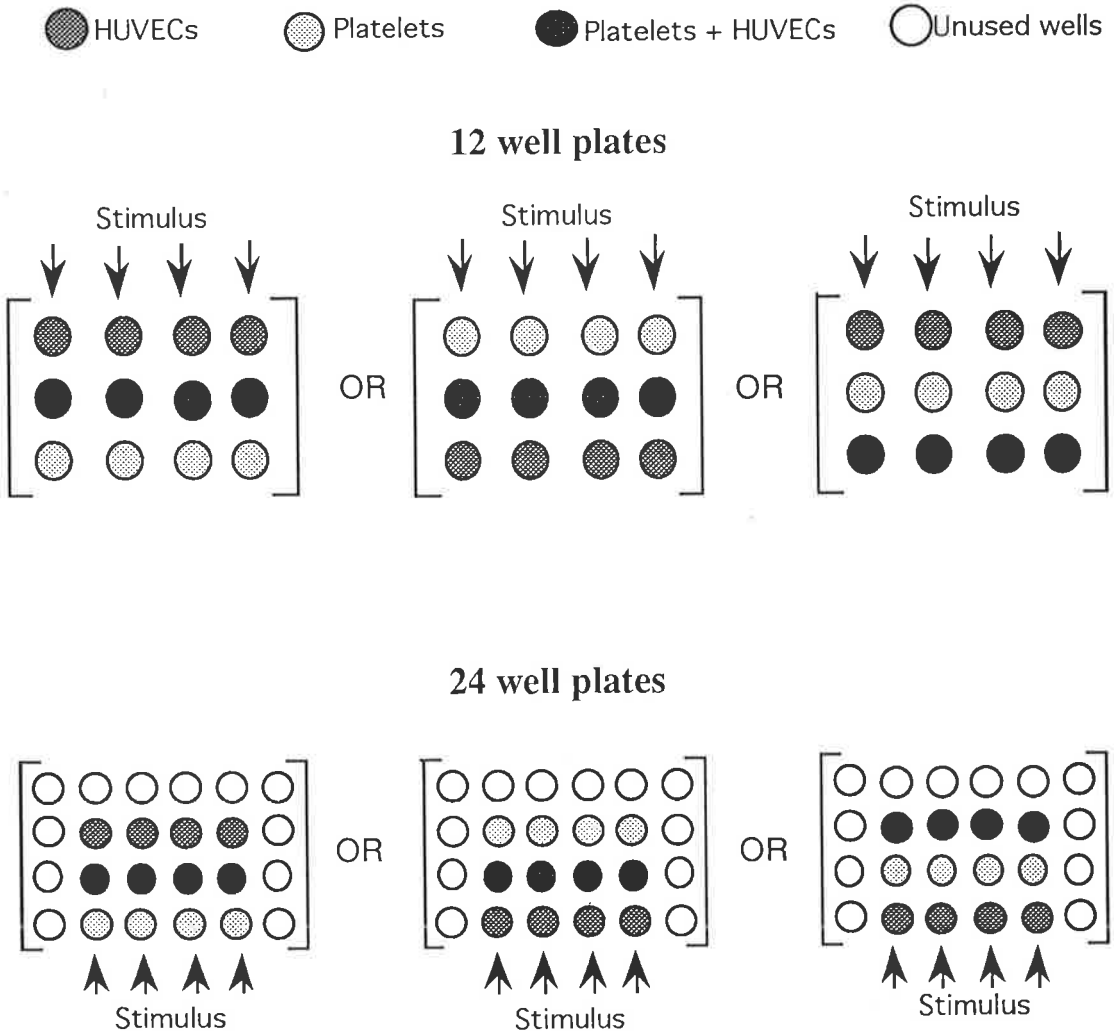
Washed and ^{14}C-serotonin-labelled platelets were prepared and stored as described previously. For some studies, it was necessary to divide the first pooled yield of platelets (in the first washing buffer) in order to allow different populations of platelets to be washed under different conditions (e.g. ± 1.2mM $CaCl_2$), or to allow pretreatment with aspirin. For these experiments, each population of platelets was labelled independently, and their respective total and background dpm (used for correction purposes) were also derived independently. Differing preparation conditions did not cause any detectable alteration in levels of ^{14}C-serotonin uptake.

Experimental protocol for platelet and HUVEC interaction studies

Typically, culture plate wells in which HUVECs were cultured were seeded in a manner diagramatically represented in Figure 2a (below) and for experiments washed platelets or platelet free resuspension buffer (RB) was also added to the wells as indicated. Some experimental protocols required the incorporation of additional wells. However, in order to allow processing of plates within a reasonably consistent time period, 16 wells of 24 well culture plates was the maximum number of wells used in experiments. From the time HUVEC interaction studies were initiated, experimental protocols were completed within ~1.5 hours (3 plates) or up to ~3 hours (5 plates).

Figure 2a

Platelet and HUVEC interaction studies



Experimental protocol for platelet and HUVEC interaction studies (continued)

For a single treatment, and/or stimulus, the protocol simultaneously provided data, on platelet and HUVEC responses under the following three conditions:

1- HUVECs alone (supernatant buffer=platelet RB)

2- Washed platelets alone (no HUVECs)

3- Washed platelets + HUVECs

Prior to use, all experimental wells (with and without HUVECs) were typically preincubated with serum-free culture medium for 15 minutes then with platelet resuspension buffer (RB) for a further 5 minutes. Variations from this preincubation protocol (Chapter 4) are indicated in Table 2a or relevant chapters. The final experimental incubation was timed to 5 minutes. All incubations were performed at 37°C under 5% CO_2 in air.

For the final incubation (Chapters, 4,5,6, and 8) in 12 well culture plates, washed platelets or platelet-free resuspension buffer (950µl) were aliquoted into wells (with or without HUVECs) and a stimulus (or paired stimuli) was added (50µl) such that the final well volume was 1ml. Alternatively (Chapters 6,7,9 and 10) 24 well culture plates, were used and because the well volume in 24 well plates was half that of 12 well plates, the incubate and stimulus volumes used were halved to 475µl and 25µl respectively. Stimuli were prepared as described in Appendix 2. The experimental wells, with and without HUVECs, were randomised between different plates and different experiments. Similarly, the order in which the stimuli were applied to wells was also randomised between independent experiments. For every experiment, control wells were included where the 'stimulus' was substituted with RB. These were used to determine baseline levels of activation of platelets alone, HUVECs alone, and platelets and HUVECs together.

For the final incubation, plate agitation was achieved using a Titertek titre plate shaker (DSG Titertek/4, Flow Labs.). In preliminary studies shaker speeds of 3 to 3.5 were used but it was observed that stronger and more consistent platelet activation and serotonin release responses were observed with increasing shaker speeds. A final shaker speed of 4 was therefore implemented for interaction studies described in Chapter 4 and all subsequent studies. This

speed produced an agitation amplitude (diameter of circle) of 5mm and an agitation frequency of 560-580 cycles per minute.

Collection of data

Platelet serotonin release

Following shaking, supernatants were immediately removed from all wells and placed in Eppendorf centrifuge tubes. Those supernatants containing washed platelets were centrifuged at 8400g for 1 min. The resultant platelet-free supernatant was then transferred into fresh Eppendorf tubes and prior to storage at -70°C, an aliquot was removed for liquid scintillation counting and the determination of ^{14}C-serotonin dpm. Aliquots representative of total and background dpm were also processed. Calculations of % ^{14}C-serotonin release were then performed using the total, background and sample dpm values, as described previously.

For the studies described in Chapter 4, total and background ^{14}C-serotonin dpm were derived from aliquots of washed platelets taken directly from the relevant syringe(s). In studies described in Chapter 5 and subsequent chapters, the background dpm (used for the correction of all samples and the total) was determined from the mean of the control (or RB) wells, containing platelets. These control wells contained unstimulated platelets incubated with and without HUVECs. When described as a % of the total dpm, mean±sem background values in these wells was 6.0 ±1.4% (Chapter 5, n=5). Background ^{14}C-serotonin dpm were generally less in the wells containing HUVECs, but the difference in background dpms between seeded and unseeded wells was negligible. Indeed where this background correction procedure was first used (Chapter 5, n=5), the final corrected platelet serotonin release (mean ±sem) in the two control wells was only ± 0.16 ± 0.11 % (−/+HUVECs).

RIAs

All supernatants derived from experimental wells were stored at -70°C prior to RIA for TXB_2 or 6-keto-$PGF_{1\alpha}$. The samples were generally assayed within 2 weeks of collection. Platelet thromboxane generation, measured as TXB_2, was standardised against the platelet count for each experiment and expressed as pg/10^6 platelets. HUVEC counts were not performed on wells in initial experiments (Chapter 4) and consequently 6-keto-$PGF_{1\alpha}$ was expressed in ng/ml. In subsequent studies (Chapter 5 onwards) prostacyclin generation was standardised to ng/10^6 cells.

Cell counts

From Chapter 5 onwards, to obtain a representative cell count for each experiment, wells (at least 10% of the total experimental wells containing HUVECs) were selected from different plates for counting purposes. Despite no evidence of thrombin induced cell detachment, wells in which thrombin had been used as a stimulus were excluded due to the reported ability of thrombin (at > 0.4u/ml) to cause HUVEC detachment (Jorgensen *et al,* 1986). When experimental procedures were completed on the selected wells, the cells were covered with serum free medium 199 (and stored at 37°C in the CO_2 cabinet) until the end of the experiment. HUVECs were then resuspended using trypsin-EDTA and counted to determine a representative estimation for cells/well for the experiment. This count was then used for standardising 6-keto-$PGF_{1\alpha}$ results to ng/10^6 cells.

Expression of data and statistical analyses

For initial experiments described in Chapters 3 and 4, non-quantifiable concentrations of TXB_2 or 6-keto-$PGF_{1\alpha}$ were described as 0 pg/10^6 platelets or 0 ng/ml respectively. For studies described in Chapter 5 onwards, samples containing non-quantifiable concentrations of TXB_2 or 6-keto-$PGF_{1\alpha}$ were given arbitrary values, based on approximately half the RIA quantification limit and calculated using typical platelet or cell counts. For TXB_2 this arbitrary value was 0.1pg/10^6 platelets; for 6-keto-$PGF_{1\alpha}$, this was 0.2ng/10^6 cells. These arbitrary values allowed the application of log transformations to data where non-quantifiable concentrations of TXB_2 or 6-keto-$PGF_{1\alpha}$ were evident.

The arbitrary values were chosen to lie below but close to lowest concentration values which could be obtained from the RIAs such that upon log transformation the arbitrary values did not substantially affect (add skew to) the distribution of the data collected.

Data derived from 5 repeated experiments (Chapter 5) were assessed to address the normality of the distribution of the data and associated variance. Variance was assessed using Bartlett's test for homogeneity of variance and for these data, significant heterogeneity of variance was found in all assessed parameters. Visual analyses of the distribution of variance against the mean values led to the application of log transformation to TXB_2 and 6-keto-$PGF_{1\alpha}$ data. These log (base 10) transformations reduced or eliminated heterogeneity of variance and normalised variance distribution. Data for % platelet serotonin release were left untransformed as log and even arcsin transformation (frequently applied to percentile data; Bolton, 1990b) failed to overcome the heterogeneous nature of these data .

All 6-keto-$PGF_{1\alpha}$ and TXB_2 data acquired from experiments described in Chapter 5-9 were log transformed and statistical analyses were performed following transformation. These data were represented graphically using geometric means (gm) and geometric standard errors (gse). In contrast, no transformation was applied to any % data and results were represented graphically using arithmetic means and standard errors of the mean (sem).

Details on statistical analyses are provided in relevant chapters. However, where complete and balanced data were obtained, parametric analyses of variance (ANOVA) were performed, and specific sources of variation were assessed using Tukey's Honestly Significant Difference tests (Tukey's HSD tests; Daniel, 1983). Occasionally such tests were performed on data where some heterogeneity of variance was evident. In these instances, significant effects between paired groups, as determined using Tukey's HSD tests, were confirmed using either Student's paired t tests or Wilcoxon's matched-pairs, signed-rank tests. Non-parametric ANOVA and the identification of sources of variation by differences in the sum of the ranks between paired groups (Bolton, 1990c) were not reported, as when performed, they frequently failed to detect highly significant effects (as determined by specific paired non-parametric comparisons).

Adaptations of the conditions/protocol for platelet and HUVEC interaction studies

Table 2a below, summarises the experimental conditions used for platelet and HUVEC interaction studies and highlights the minor differences between methods used in Chapters 4 and 5. Any further changes made after Chapter 5 are specified in relevant chapters.

Experimental parameter	Chapter 4	Chapter 5
HUVECs		
Culturing conditions	CCM based on RPMI 1640	CCM based on medium 199
Plates used	12 well	12 well
Final seeding density	~1.2 x 10^5cells/well	1.8-2.0 x 10^5 cells/well
Cells used	48 hours post-seeding	48 hours post-seeding
Platelets		
Washing procedures	Prepared in buffers ± 1.2mM$CaCl_2$	Prepared in buffers +1.2mM$CaCl_2$
Used	with 5µl PFP/ml	without PFP
Experimental protocol		
Preincubation 1	RPMI 1640 (10-15 minutes)	Medium 199 (15 minutes)
Preincubation 2	RB±$CaCl_2$ (5 minutes)	RB +$CaCl_2$ (5 minutes)
Final incubation	Platelets±HUVECs and HUVECs + RB (2, 5 or 15 minutes)	Platelets ±HUVECs and HUVECs + RB (5 minutes)
Shaker speed	4	4
Cell counts from wells	not determined routinely	determined in 10% of wells
Data collection		
Platelet serotonin release	^{14}C-serotonin dpm Total and background dpm from syringe aliquot	^{14}C -serotonin dpm Total dpm from syringe aliquot and background from average of control (RB)wells
Platelet thromboxane generation	RIA for TXB_2	RIA for TXB_2
HUVEC prostacyclin generation	RIA for 6-keto-$PGF_{1\alpha}$	RIA for 6-keto-$PGF_{1\alpha}$
Data expression		
Serotonin release	% serotonin release	% serotonin release
Transformation	none	none
TXB_2	pg/10^6 platelets	pg/10^6 platelets
Non-quantifiable concentrations	0	0.1pg/10^6 platelets
Transformation	none	log (base 10)
6-keto-$PGF_{1\alpha}$ measurements	ng/ml	ng/10^6 cells
Non-quantifiable concentrations	0	0.2ng/10^6 cells
Transformation	none	log (base 10)

Interaction studies using L and FL cells

Methods described in this section are relevant to results reported in Chapter 9.

A number of experiments were performed to determine how platelets interacted with cells other than HUVECs. Two certified transformed cell lines were kindly provided by Ms Sarah Robertson, (Department of Obstetrics and Gynaecology, University of Adelaide). L cells (with fibroblast-like morphology, derived from mouse connective tissue) and FL cells (with epithelial-like morphology, derived from human amnion) were cultured and subcultured under near identical conditions to HUVECs. Conditions included culturing the cells in CCM based upon M199 with Earle's salts with 20mM Hepes (but in the absence of added heparin) under 5% CO_2 in air at 37°C.

For interaction studies, both FL and L cells were seeded as recommended at ~5 x 10^5 cells/well in 8 of 24 culture plate wells and appeared confluent 24 hours following seeding. They were then used in studies with platelets in an identical fashion to the interaction studies using platelets and HUVECs.

It was ascertained that in the presence of ^{14}C-serotonin, there was no greater association of serotonin with the cells (compared to plastic) over the incubation time (5 minutes) to be used in the interaction studies with platelets. This demonstrated that (as in HUVEC studies) serotonin released from platelets would not become associated with these cells. Thus platelet ^{14}C-serotonin release could be used as an index of platelet activation even in the presence of these cell lines and % serotonin release could be calculated as for platelet and HUVEC interaction studies.

Method for the assessment of platelet deposition

In the studies reported in Chapter 10, platelet and HUVEC interaction studies were performed using an adaptation of the protocol described earlier. Following standardised washing procedures, estimates of platelet adherence were evaluated by quantifying ^{3}H-labelled platelets found in association coverslips alone or coverslips which had been seeded with HUVECs. Scanning electron microscopic examinations of coverslips, following exposure to platelets, indicated aggregates of platelets still remained following washing procedures. Hence, the term platelet deposition rather that platelet adherence was considered more appropriate to describe the results from these studies. Platelet activation was also determined in these studies though assessments of % ^{14}C-serotonin release, as described earlier.

Platelet labelling with (^{14}C-serotonin and) ^{3}H-adenine

Following their final resuspension washed platelets were labelled with ^{14}C-serotonin as described previously. Prior to this however, platelets were also labelled using ^{3}H-adenine (2'8'-^{3}H-adenine, 33.0 Ci/mmol in 1ml aqueous solution; Dupont. NEN Research Products, MA, U.S.A.). When platelets were pooled in the first washing buffer, 18µl ^{3}H-label (for 126 ml collected blood) was added and platelets were then incubated (with minimal exposure to air) for thirty minutes prior to centrifugation (as described in the platelet washing procedures) and continuation of the usual washing protocol. The uptake efficiency of ^{3}H-adenine over this 30 minute incubation was assessed in six consecutive experiments and found to be 47.4 ± 3.10% (mean±sem). The extent of uptake (although consistent) was somewhat lower than the ~70% uptake over this time period reported by Curwen, Gimbrone & Handin (1980) or Curwen *et al,* (1982) who also used this platelet labelling procedure. Following the second wash and final resuspension of platelets in RB, an aliquot of the suspension was used to determine final total and background dpm. It was found that following labelling, the washing procedures removed residual ^{3}H-adenine such that background levels in the final platelet suspension were only 4.32± 0.85% (mean±sem, n=6) of the final total ^{3}H dpm.

Investigations to assess platelet release of ^{3}H-adenine or its uptake by HUVECs

Controls were included in initial experiments to determine the extent to which stimulated platelets released platelet ^{3}H-adenine and whether HUVECs uptake of adenine occurred to a detectable level. It was recognised that both factors had to be taken into account in order to ensure that ^{3}H-adenine dpm associated with coverslips could be used to accurately quantify platelet association with them (either with or without HUVECs).

Where thrombin was used to initiate platelet ^{14}C-serotonin release, (49.5±0.9%, n=2) the background ^{3}H-adenine dpm did not increase over unstimulated background levels (which were 3.9±0.2% of the total). These assessments were performed at ~3 hours following platelet labelling with ^{3}H-adenine when it was considered that more ^{3}H-adenine may have become associated with the releasable nucleotide pool (Reimers, 1985). This indicated that platelet activation did not result in sufficient release of ^{3}H adenine to invalidate estimates of deposition.

In two further control experiments, HUVEC uptake of ^{3}H-adenine was examined. The association of ^{3}H-adenine (~background ^{3}H-dpm) with thrombin stimulated HUVECs (grown on coverslips) was assessed over 5 minutes and compared to levels of association with unseeded coverslips. In both experiments, more than 98% of the added ^{3}H-adenine dpm were recovered. Also, following standard washing of HUVEC seeded coverslips the ^{3}H dpm actually remaining associated with the coverslips was minimal and represented less than 0.4% of the added ^{3}H dpm. This association of ^{3}H dpm with unseeded coverslips was similar in the absence of HUVECs. These results were consistent with (over 5 minutes) a lack of detectable uptake of ^{3}H-adenine by HUVECs under these experimental conditions.

These results meant that the background ^{3}H-adenine dpm in the supernatant of the control wells (or any other wells) could be used to correct the total ^{3}H-adenine dpm added to the wells such that the specific platelet associated ^{3}H-adenine dpm added to wells could be determined. This value would be representative of platelet associated ^{3}H-adenine irrespective of the presence or absence of HUVECs or stimuli.

Preparation of HUVECs for deposition studies

Thermanox plastic coverslips (Nunc Inc., Naperville IL, U.S.A.) 15mm in diameter were placed in 16 wells (16mm diameter) of 24 well culture plates and were gelatinised prior to seeding. Following typical culturing and subculturing and upon their third passage, HUVECs were seeded into 8 wells of 24 well cluster plate wells as for typical platelet and HUVEC interaction studies and unseeded wells were filled with sterilised water. Initial experiments were performed to determine the effects of the coverslips on platelet activation (serotonin release and thromboxane generation) and on HUVEC prostacyclin generation prior to any platelet deposition experiments. These studies showed that platelet activation responses were not affected by the presence of coverslips but prostacyclin generation from HUVECs seeded into wells containing coverslips was increased (compared to that observed in the absence of coverslips). Since it was possible for HUVECs to grow circumferentially in the 0.5mm gap between the edge of the coverslips and the side of the wells, the damage to these cells upon plate agitation could contribute to the enhanced prostacyclin generation observed in the presence of coverslips. The protocol developed for deposition studies therefore involved removing such peripheral cells at 24 hours following seeding, by shaking the seeded plates (for 5 minutes in under sterile conditions, 5% CO_2 and 37°C, at shaker speed number 4) and then changing the culture medium. This procedure reduced but did not eliminate the enhanced prostacyclin generation associated with the use of coverslips.

Experimental protocol for platelet deposition studies.

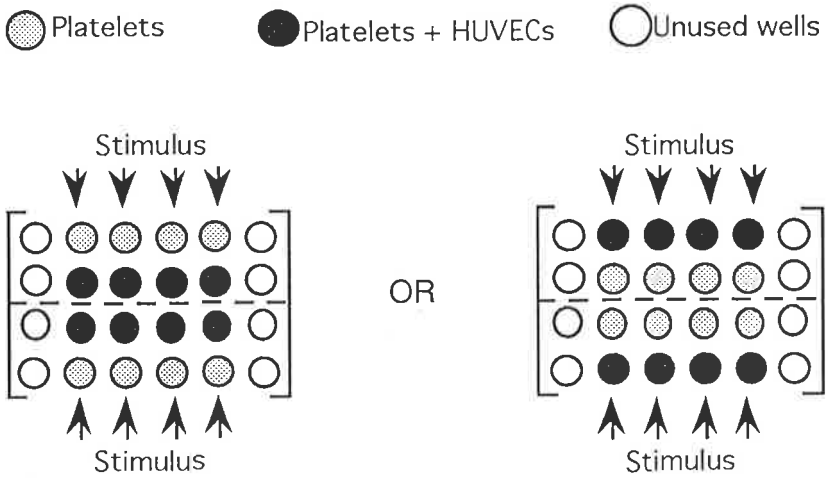
Forty eight hours following seeding, deposition studies were performed according to a similar protocol to that previously described for platelet and HUVEC interaction studies. Wells containing HUVECs grown on coverslips or coverslips alone were washed for 15 minutes with medium 199 and then 5 minutes with RB prior to the addition of platelets (975µl) and the subsequent addition of a stimulus (25µl). Sixteen wells in total were used per 24 well plate; 8 were seeded with HUVECs and 8 contained only coverslips (see Figure 2b, page 99) and all were finally incubated with platelets. A fibrinogen source (PFP: 5µl/ml washed platelets) was added to platelets just prior to their use. Following agitation at shaker speed number 4 (at 37°C

under 5% CO_2 in air) for 5 minutes, platelets were removed for the determination of ^{14}C-serotonin release (as described previously).

Figure 2b below, illustrates the typical use of culture plate wells for experiments used to assess platelet deposition.

Figure 2b

Platelet deposition studies



Following the removal of platelets (and whilst platelets were being centrifuged), coverslips were removed from wells and were then immersed sequentially through 3x100ml beakers of HBSS (37°C). Excess HBSS was removed following each immersion, by blotting the edge of each coverslip on absorbant paper. Following these washes, coverslips were placed in scintillation vials. In initial studies, these coverslips were exposed to 0.05% trypsin-0.02% EDTA for 1 hour prior to the addition of scintillation fluid (similar to the method described by Curwen, Gimbrone & Handin, 1980 or Curwen *et al,* 1982). However in paired studies, it was determined this proteolytic treatment of HUVECs did not alter estimates of the amount of ^{3}H-adenine in association with the coverslips compared to the use of scintillation fluid alone (Ready Value, Beckman). Consequently this proteolytic treatment step was not included in reported experiments. Where samples for scanning electron microscopy were required, following the usual HBSS washes, the coverslips were placed in 2.5% gluteraldehyde fixative in HBSS (see Appendix 1) prior to processing as described under 'Scanning Electron Microscopy'.

Collection and expression of platelet deposition data

The dpm associated with ^{14}C and ^{3}H were measured using a Beckman Liquid Scintillation counter, model LS3801 programmed for processing dual labelled ($^{14}C/^{3}H$) compounds. The separated ^{14}C and ^{3}H dpm allowed the assessment of platelet serotonin release (from the supernatant) and platelet deposition (from coverslips) from a single experimental well.

% platelet serotonin release

The ^{14}C dpm data (derived from the platelet supernatants) was used to calculate % ^{14}C-serotonin release, as described for platelet and HUVEC interaction studies.

% platelet deposition

The total ^{3}H-adenine dpm was obtained from a 100µl aliquot of non-centrifuged platelet suspension, taken directly from the storage syringe and this dpm value was corrected by the mean platelet-free background ^{3}H-adenine dpm, derived from 100µl of the two control (RB) wells. This value (denoted x), was multiplied by 4.75 (to correct for the 475µl volume added to wells) and represented the total platelet associated ^{3}H-adenine dpm added to experimental wells, which could become associated with coverslips or HUVEC seeded coverslips. Platelet deposition was equal to the ^{3}H-adenine dpm associated with the coverslips (±HUVECs) expressed as a % of x.

Scanning electron microscopy

Sample processing

The fixation of samples for scanning electron microscopy was performed following deposition studies by immersing coverslips in 2.5% gluteraldehyde in HBSS (see Appendix 1).

After 1.5-2 hours, samples were transferred into an iso-osmotic washing buffer pH 7.2, with sucrose (see Appendix 1). Coverslips were etched for identification and then cut and placed into dehydration processing baskets. Samples were then incubated at 4°C overnight in fresh washing buffer. Dehydration of samples was not performed by critical point drying procedures, but through the use of Peldri II, a fluorocarbon compound (Ted Pella, Inc. California, U.S.A., purchased through Probing and Structure Electron Microscopy & Probing supplies, Thuringowa Central, Queensland, Australia), used as a sublimation dehydrant.

Drying procedures were undertaken as recommended by the makers of Peldri II, except that an osmium tetroxide post-fixation step was not performed. Sample processing involved the use of a Lynx^{TM}electron microscopy tissue processor. Samples were immersed (in two successive 30 minute incubations at room temperature) in 70%, 90%, 95% and 100% ethanol. These steps were followed by 1 hour immersions in 100% and then 50% ethanol/50% Peldri II with a final immersion at 27°C in 100% Peldri II. The samples were then placed in a desiccator at room temperature overnight, under vacuum (5 x 10^{-2}mbar Hg).

Following sample dehydration procedures samples were mounted on aluminium stubs for coating with carbon and gold palladium. Examination of samples involved the use of Philips 505 scanning electron microscope and photographs were taken using Kodak black and white 100 ASA Tmax film. Dehydration and sample coating procedures were undertaken in the Centre for Electron Microscopy and Microstructure Analysis, University of Adelaide.

Scanning electron microscopy was performed on samples generated from two experiments. The first had to be performed on cells 24 hours following seeding. The second was performed on cells 48 hours following seeding but when the dehydration procedures were delayed for 2 days there was poor morphological preservation of the cells (photographs not included in results).

Treatments with pharmacological agents

Pharmacological agents were used in many platelet and HUVEC interaction studies, and in platelet deposition studies, and were prepared as described in Appendix 2. When added to culture plate wells, these agents were included in the preincubation washes and/or the final incubation step, but details are given in relevant chapters. For platelets, (except for aspirin) pharmacological agents were generally added to platelets just prior to their addition to wells, but again details of how platelet treatments were undertaken are specified in relevant chapters.

Chapter 3

Culture, identification and prostacyclin generation from human umbilical vein endothelial cells (HUVECs)

Introduction

In vitro, cultured HUVECs retain many specific functions known to be expressed by endothelial cells *in vivo* (Thilo-Korner, Heinrich & Lasch, 1983; Stern, Kaiser & Nawroth, 1988; Nachman & Hajjar 1991) including the generation of the platelet inhibitory eicosanoid, prostacyclin. It has also been shown that HUVECs have a greater capacity to produce high levels of prostacyclin relative to other endothelial cell lines (Revtyak, Johnson & Campbell, 1987), making them particularly useful in studies where the effects of this mediator on platelet functions are of interest. Other prostanoids such as PGD_2 and PGE_2 (which may influence platelet functions) are reported to be produced from HUVECs to only a minor extent (Revytak, Johnson & Campbell, 1987; Marcus *et al,* 1980; Ingerman-Wojenski *et al*, 1981). In addition, except for one study (Greismacher *et al,* 1989), most investigators have found cultured HUVECs do not generate the platelet stimulatory eicosanoid thromboxane A_2 whereas other endothelial cell lines (bovine aortic endothelial cells) apparently generate thromboxane as well as prostacyclin (Ingerman-Wojenski *et al,* 1981).

In many studies in which the effects of prostacyclin or factors affecting its production are of interest, the quantification of its generation is essential. HUVECs from primary and subcultured lines have been reported to generate prostacyclin in response to a wide range of physiological stimuli and this generation is frequently monitored by radioimmunoassay of the stable product of prostacyclin hydrolysis, 6-keto-$PGF_{1\alpha}$. *In vivo*, the metabolic disposition of prostacyclin is complex, involving enzymic degradation via 15-hydroxy dehydrogenase, β oxidation and other hydroxylation and oxidative pathways (Sun *et al*, 1979). In contrast, *in vitro* with the use of physiological buffers, prostacyclin undergoes spontaneous and non-enzymic hydrolysis to form 6-keto-$PGF_{1\alpha}$, and analysis of this metabolite can be employed as an index of prostacyclin present in the buffer (Marcus *et al,* 1980).

Prior to using endothelial cells cultured *in vitro*, validation procedures must be instituted to ensure the purity of cell lines. For the purposes of endothelial cell identification *in vitro*, typical growth patterns and morphological characteristics at confluence are frequently described. Importantly however, one of the most commonly reported means of endothelial cell identification involves the staining of factor VIII related antigen. This staining is typically localised in endothelial cell specific organelles, Weibel-Palade bodies, and reflects the distribution of von Willebrand factor (Wagner, Olmsted & Marder, 1982, Reinders *et al,* 1988; Ruggeri & Ware, 1993).

The overall objective of the studies presented in this chapter was to characterise isolated and cultured HUVECs by morphological and immunohistochemical techniques in order to determine that the procedures used in their isolation gave rise to acceptably pure lines. In addition, since ongoing studies would involve the assessment of the platelet inhibitory effects of HUVECs and the role of prostacyclin in such effects, it was necessary to demonstrate that HUVECs could express prostacyclin generation. Consequently, it was desirable to determine specific methodological factors which may affect this generation.

Aims

The specific aims addressed in the following 4 sections of this chapter were:

3a. To confirm that the cells (primary and passaged) possessed typical growth characteristics and morphology during growth and at confluence.

3b. To confirm that cells which morphologically resembled HUVECs also expressed factor VIII related antigenic activity.

3c. To examine technical variables affecting stimulated prostacyclin generation from HUVECs; quantifying prostacyclin generation by RIA of 6-keto-$PGF_{1\alpha}$.

3d. To assess prostacyclin generation from HUVECs in response to a range of stimuli, and determine the relative potencies of these stimuli.

Section 3a

Characterization of HUVECs by morphological criteria during growth and at confluence

Methods

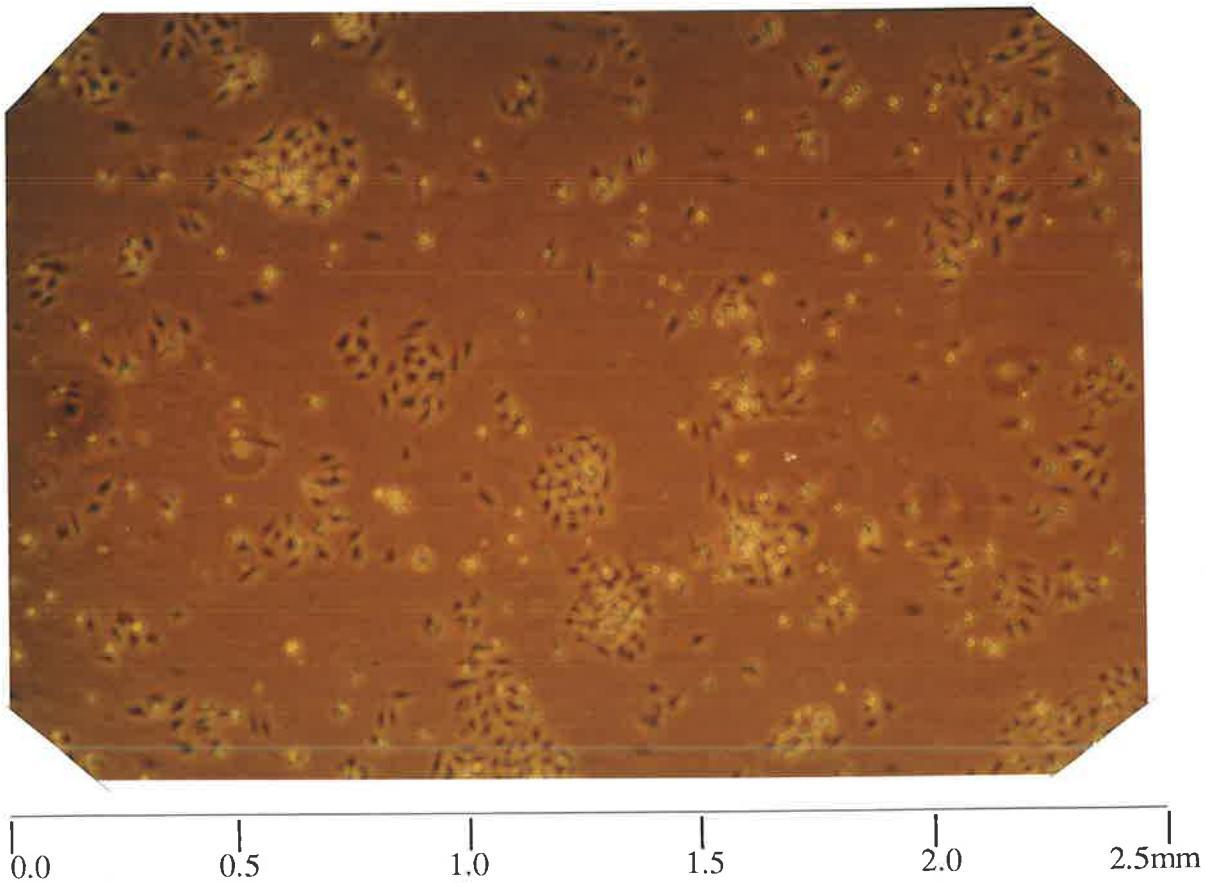
HUVECs were isolated, cultured, subcultured and photographed as described in Chapter 2. Photographs were taken of cells from 2 independent lines including primary (1°) cells at varying stages of growth and cells which had been passaged up to 3 times (i.e. quaternary (4°) cells).

Results

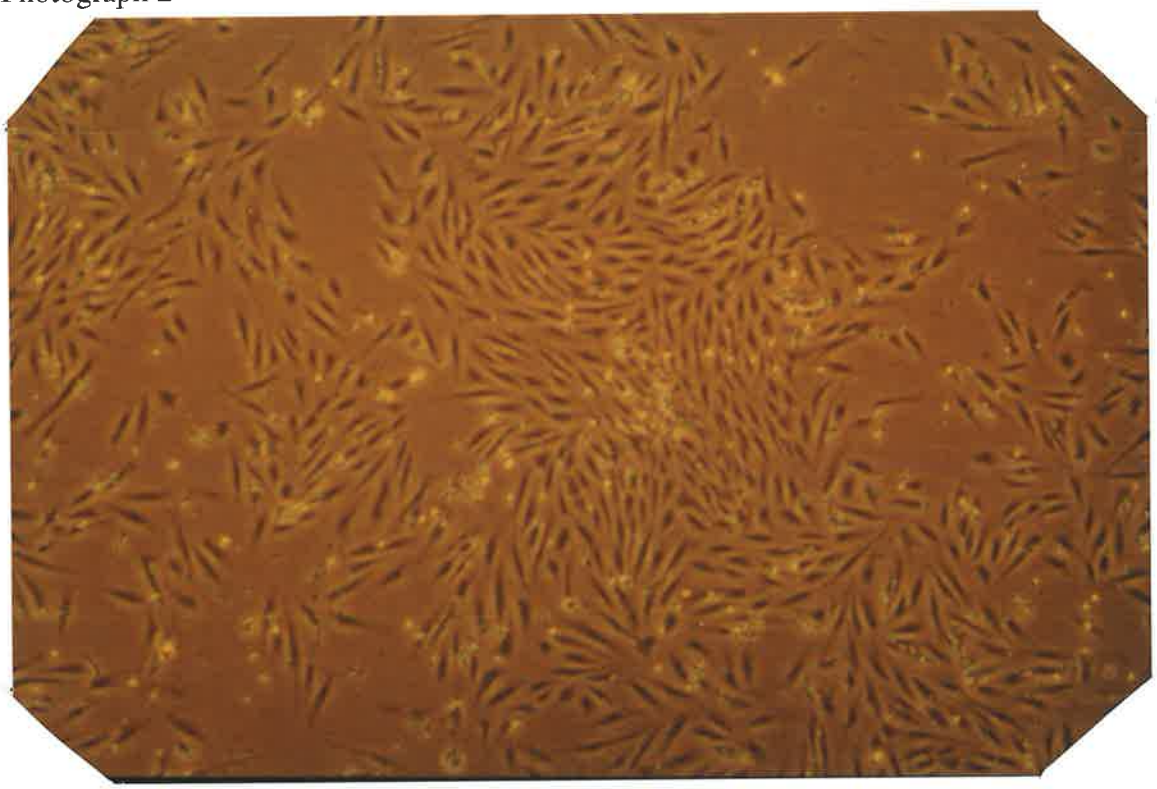
Morphological assessments of primary HUVEC cultures.

Photographs 1, 2, and 3 show the stages of growth of primary (1°) HUVECs at 12 hours, 2 and 4 days respectively following seeding. The original magnification was 40 times (x) and the approximate width of the field of view is indicated. This particular yield (0.9 x 10^6 cells, seeded in ~5ml CCM) was lower than average (1.9 x 10^6 cells, n=5).

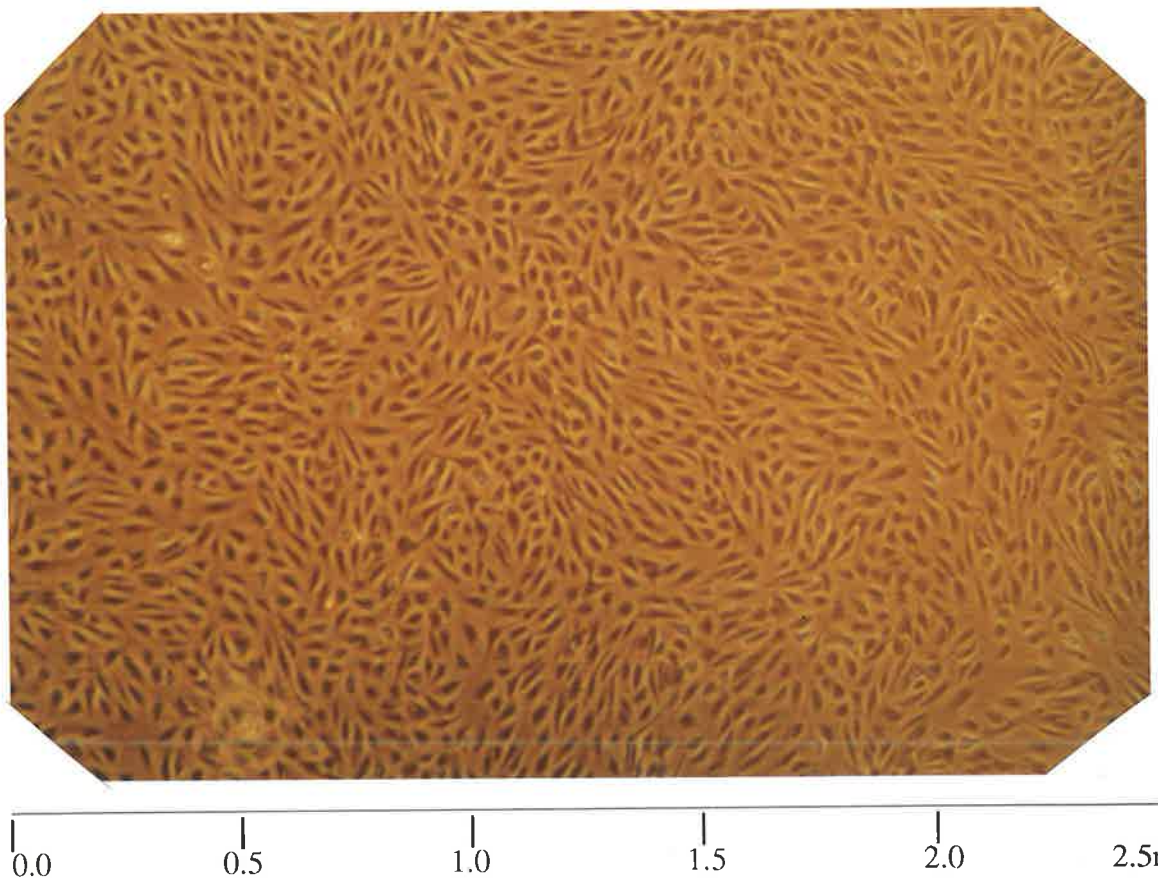
Photograph 1



Photograph 2

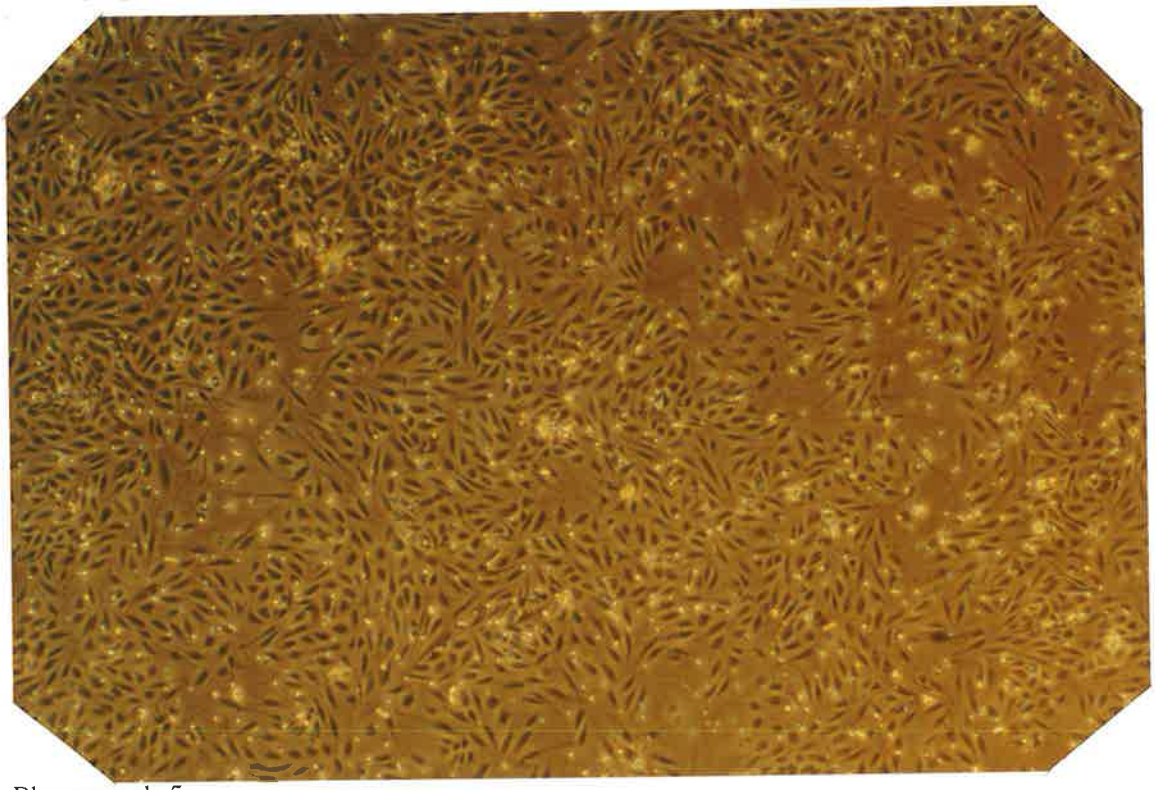


Photograph 3

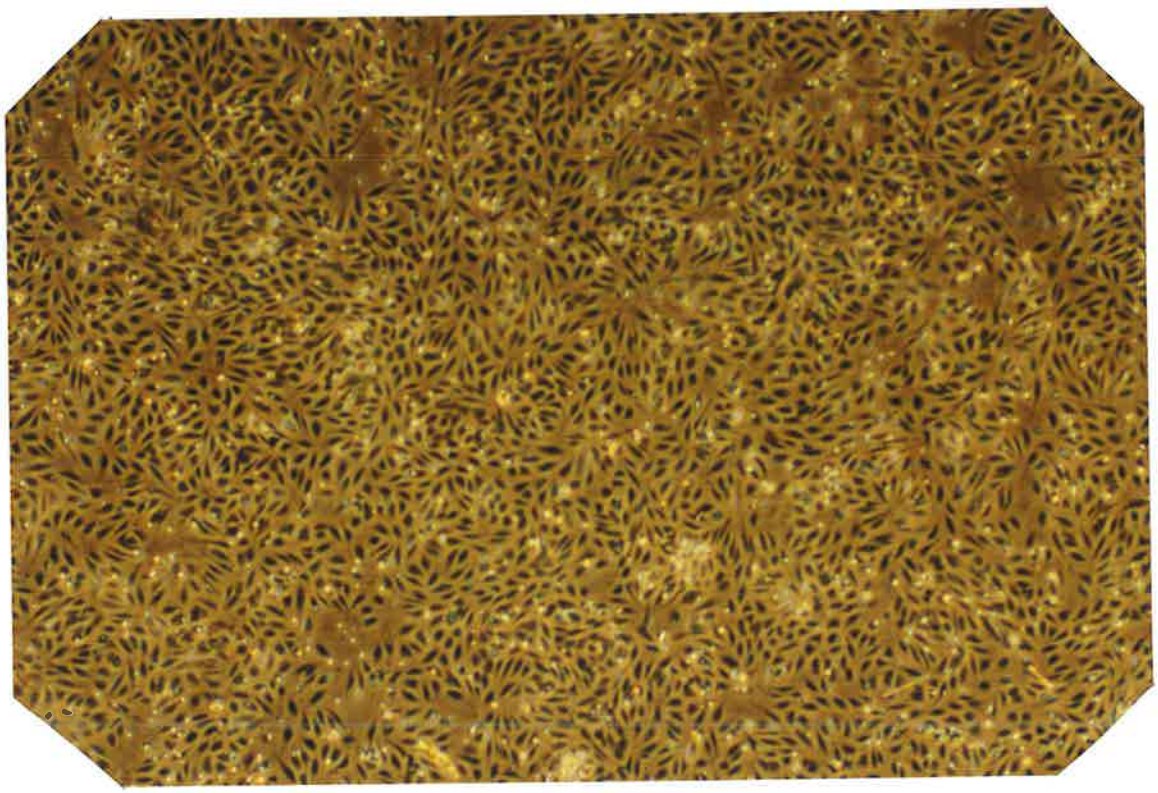


Photographs 4 and 5 show a second 1° line, (yield ~ 2.5 x 10^6 cells) photographed at 2 days and approaching confluence at 3 days (Photograph 5) following seeding. At confluence, cells displayed a 'cobblestone-like' appearance. The original magnification was 40 times (x) and the approximate width of the field of view is indicated.

Photograph 4



Photograph 5



The growth patterns of HUVECs showed that with lower than average primary cell yields (see Photographs 1-3) initial adherence of small clusters of cells was apparent (Photograph 1) and cells began to undergo migratory activity resulting in cell spreading and elongation (Photograph 2). This activity together with mitotic activity continued until a homogeneous cell population was achieved (Photograph 3) and cell to cell contact prohibited further migration. Cells at this stage continued to undergo mitotic activity resulting in increased cell density until optimal confluence was achieved. Lines seeded at densities similar to that of the first line depicted in Photographs 1-3, took up to 6 days from initial seeding to reach confluence.

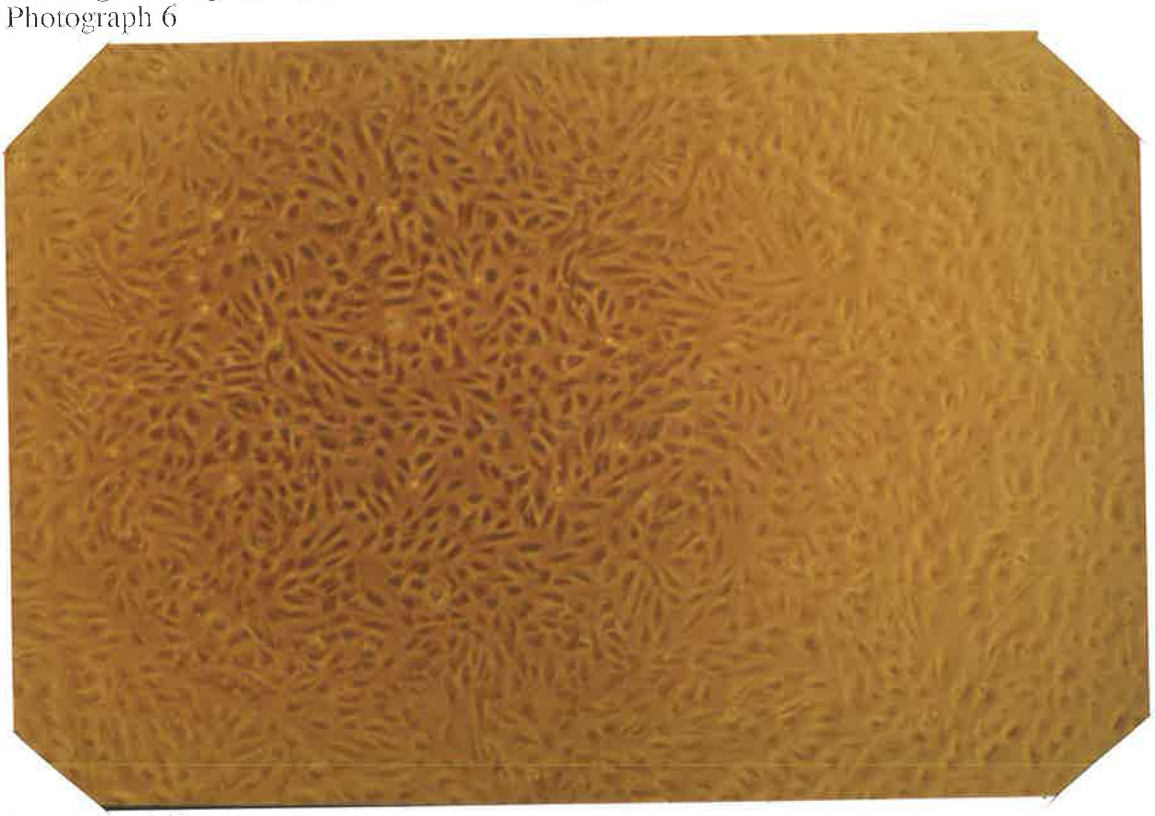
With relatively high initial cell yields (Photographs 4 and 5) following seeding, the adherent cells had to undergo fewer population doublings to reach confluence. Following adherence, due to their initial close association, cells underwent less migratory activity and the population (even during their mitotic phase) demonstrated a 'cobblestone' appearance more clearly than lines where cells were initially seeded at a lower density. Such lines typically reached confluence within 3 to 4 days of seeding.

Further observations

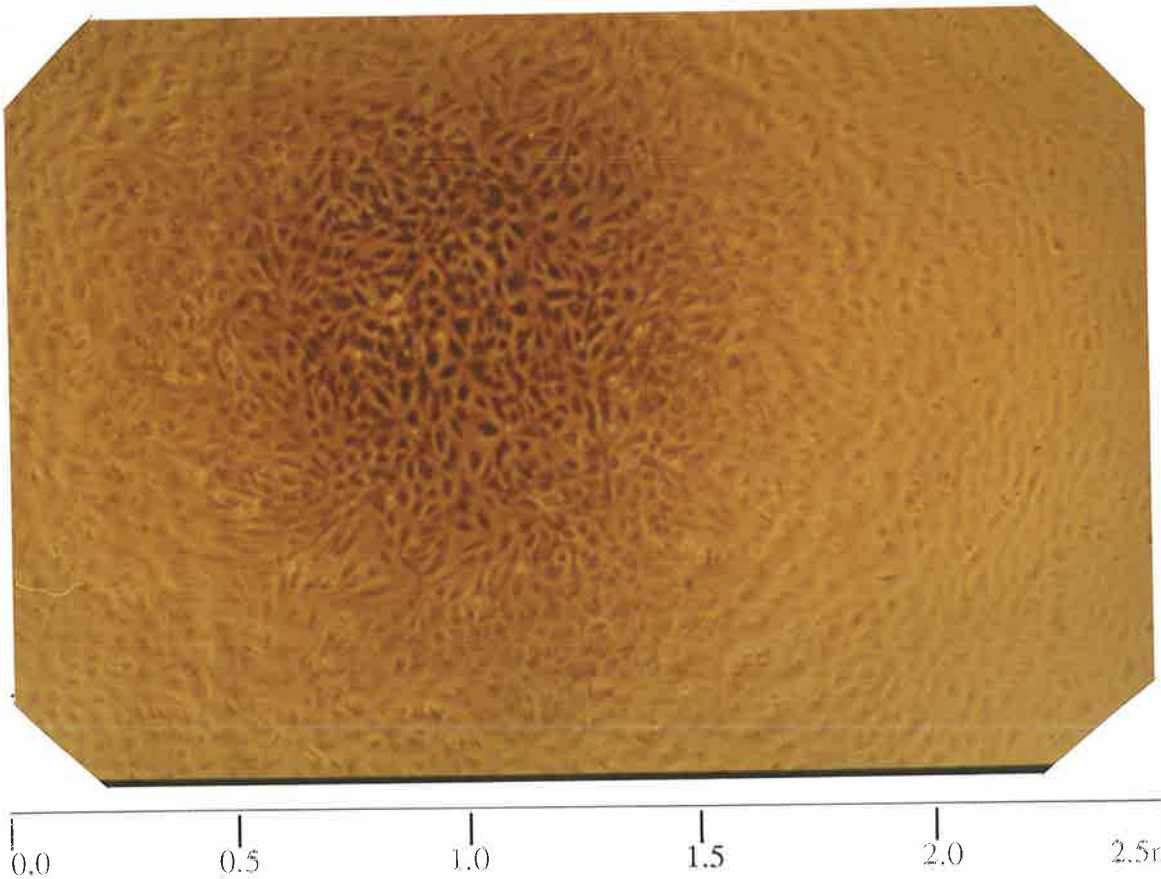
Average cell yields per 20cm umbilical cord were $1.9 \pm 0.34 \times 10^6$ cells (range, $0.5\text{-}3.4 \times 10^6$ cells, n=5 lines). Therefore, on average, cells were seeded into culture flasks (one flask per yield; base surface area of $25cm^2$) at $7.6 \times 10^4 cells/cm^2$. Lower yields (Photograph 1) were generally associated with unusually high erythrocyte contamination of the HUVEC yield indicating that the initial procedures used to flush the vein lumen had been inadequate to completely remove all erythrocytes. Although assessed by trypan blue exclusion in only two lines, isolated 1° cell viability was found to be ~80% in both instances. Primary cell lines which were seeded below 0.4×10^6 cells/$25cm^2$ flask often failed to reach confluence even after 7 days from the time of seeding and frequently contained cells which were stellate and apparently senescent. They also appeared to lose their capacity to replicate, so rarely reached confluence even when given further time in culture. For this reason (from Chapter 5 onwards) if a 1° line failed to reach confluence within 6 days of seeding, the line was discarded.

Morphological assessments of subcultured HUVEC cultures: Photographs 6 and 7 show cells passaged 3 times, seeded into 12 well culture plate wells, and viewed 48 hours following seeding (i.e. quaternary (4°) cells). Cells were seeded at 1.2 and 2.0 x 10^5cells/well respectively. The original magnification was 40 x and the approximate width of the field of view is indicated.

Photograph 6



Photograph 7



Secondary, tertiary and quaternary (4°) cultures (Photographs 6 and 7) displayed similar morphological characteristics to 1° cells, following splitting (1:2) at each passage.

For initial experiments, cells were seeded into 12 well culture plate wells at ~1.2 x 10^5 cells/well. By 48 hours, cells generally reached an apparent level of visual confluence of ~70-80% (Photograph 6). From lines where cell counts were made at 24 and 48 hours following seeding, cell counts (mean±sem, n=3 lines) of 1.5±0.16 x 10^5 cells/well and 1.3±0.13 x 10^5 cells/well were obtained respectively. Assuming a high seeding efficiency, this indicated that negligible mitotic activity occurred for up to 48 hours following seeding.

Occasionally, cell seeding efficiency into wells was exceedingly low and confluence (based visually upon % surface area coverage) failed to reach an acceptable level for experimental purposes (cells had to appear >70% confluent) and these cells were discarded. The variability in the seeding efficiency between lines appeared to be effectively overcome for later experiments (Chapter 5 onwards) following the implementation of two changes to the cell culturing and seeding methods. Firstly, the seeding density into 12 well culture plates (surface area/well, 4cm^2) was increased to 1.8-2.0 x 10^5 cells/well or ~5 x 10^4 cells/cm^2 (Photograph 7). Secondly, the decision was made to discard primary (1°) lines if they failed to reach confluence 6 days following seeding. With these changes, 4° cells at both 24 and 48 hours following seeding consistently appeared to reach visual confluence of > 80%.

Using this seeding density of 1.8-2.0 x 10^5cells/well, the 48 hour cell count (2.04±0.15 x 10^5/well: mean±sem from 10% of seeded wells) was, as in earlier studies, virtually identical to the number of cells seeded (Photograph 7) (n=5 lines or experiments). Where 24 well clusters were used (surface area/well, 2cm^2) the seeding density was adjusted to ~1.0 x 10^5cells/well and again 48 hours following seeding the cell counts (8.2 ±0.41 x 10^4 /well: mean±sem from 10% of seeded wells) were similar to the number of cells initially seeded (n=5 lines or experiments).

Discussion

The results of the morphological assessments of HUVEC growth patterns in 1° and in passaged lines were typical for HUVECs. The cells expressed patterns of adherence, colony development, cell migration and 'cobblestone' appearance at confluence similar to those reported and photographed by other investigators (Gimbrone, Cotran & Folkman, 1974; Jaffe, 1984; Thilo-Korner, Heinrich & Temme, 1983; Bull, 1988). With an average seeding density of primary cells of 7.6 x 10^4cell/cm^2, the presence of 20% serum in the CCM was obviously sufficient to support cell replication even in the absence of specific added growth factors such as bovine hypothalamus derived endothelial cell growth factor. Occasionally such factors have been used by others to enable HUVEC mitotic activity where cells are seeded below 1.25 x 10^4 cells/cm^2 (Maciag, 1984). Loss of cell mitotic activity and eventual loss of cell viability are characteristics typical of HUVECs seeded at insufficient density to replicate in the absence of such externally added growth factors (Maciag *et al,* 1984). This was observed in primary lines which failed to reach confluence within 7 days of seeding and (from Chapter 5) primary lines which failed to reach confluence within 6 days were eliminated.

As shown in Photograph 1, 1° cells initially seeded with a below average density first adhered in small cell clusters, similar to those photographed by Gimbrone, Cotran & Folkman (1974) and Bull, 1988). This primary inoculum of 0.9 x 10^6 cells represented close to the lowest number from which adherent cells could sustain mitotic activity following seeding. Lines derived from an inoculum of fewer than 0.4 x 10^6 cells (1.6 x 10^4 cells/cm^2) tended to fail. This was consistent with findings by Gimbrone, Cotran & Folkman (1974) who found that a minimum inoculum of >0.3 x 10^6 viable primary HUVECs per 25cm^2 or (1.2 x 10^4cells/cm^2) was required to ensure line survival. Similarly, Maciag *et al* (1984) found that where HUVECs were inoculated at less than 1.25 x 10^4 cells/cm^2, growth factors were required to sustain mitotic activity. Gimbrone and colleagues (1974) also observed that in freshly isolated HUVECs high levels of viability of ~90% were attained (similar to the 80% level observed in two lines assessed in these studies) although in their studies only 20-50% of such primary cells actually adhered.

The cell density of HUVECs at confluence in 25cm^2 culture flasks has been described to vary from 2.4 x 10^4 cells/cm^2 up to 1.2 x 10^5 cells/cm^2 and such wide variation has also been observed for other culture vessels (reviewed in 'Endothelial Cells in Culture': Thilo-Korner Heinrich & Temme, 1983). This range of cell densities which has been used to describe confluence probably reflects the differing views with respect to the definition of confluence. Confluence may be accepted to mean visual confluence where 100% of the observed surface area appears to be covered by endothelial cells, even though cells may continue to undergo mitotic activity. More commonly, confluence is accepted to mean that the optimum (contact inhibited) number of cells for a given surface area has been obtained. Despite discrepant reports in the number of cells per unit surface area which has been used to describe confluence, it is generally accepted that HUVEC confluence, where contact inhibits further cell mitotic activity, arises at ~0.8-1 x 10^5cells/cm^2 (Jaffe *et al,* 1973; Gimbrone, Cotran & Folkman, 1974).

When HUVECs were seeded into 12 well culture plates at 1.2 x 10^5 cells/well (4cm^2), they generally achieved >70% visual confluence by 48 hours following seeding (Photograph 6). On the basis of cell counts, and in terms of their optimum density for that surface area, they were little more than 35% confluent. Similarly, following seeding, at the higher density of 1.8-2.0 x 10^5 cells/well, at 48 hours the cells appeared (on visual examination) homogeneous in morphology and showed >80% visual confluence (12 well cluster, Photograph 7). However, according to the cell counts obtained for the well surface area (3.0-5.0 x 10^4 cells/cm^2), at 48 hours cell counts only represented 51-64% of their optimum density. It was therefore evident that at 48 hours, even when cells were seeded at the higher density, the cell monolayer was semi-confluent.

The reason for the discrepancy between levels of visual confluence and confluence in terms of cell density clearly lies in the ability of HUVECs to spread and appear appositional to each other, despite being at sub-optimal cell density for a given surface area. This capacity to appear visually confluent or near confluence at sub-optimal cell densities could certainly explain why such a wide range of cell counts for a given surface area have been reported previously to describe HUVEC confluence (Thilo-Korner Heinrich & Temme, 1983).

Following passaging and final seeding into wells, the cell counts at 48 hours implied high seeding efficiency was achieved (80-100%, assuming the absence of mitotic activity). As the cell counts at 24 and 48 hours were similar, this indicated that mitotic activity was minimal over this period of time. This was consistent with the effects of a lag phase of approximately two days which typically occurs following HUVEC passaging procedures (Jaffe, 1984) prior to cells undergoing mitotic activity.

Occasionally, when cells were finally seeded at 1.2 x 10^5 cells/well (into 12 well culture plates) a very low seeding efficiency was observed (<70% visual confluence at 48 hours following seeding) and cells were discarded. The cause was not determined but interestingly the problem was eliminated when the final seeding density was raised to 1.8-2.0 x 10^5 cells/well. This change was however implemented at the same time as the decision was made to discard 1° cultures which failed to reach confluence by 6 days following seeding. As a consequence, the improvement could have been attributable to either or both of the altered factors.

Section 3b

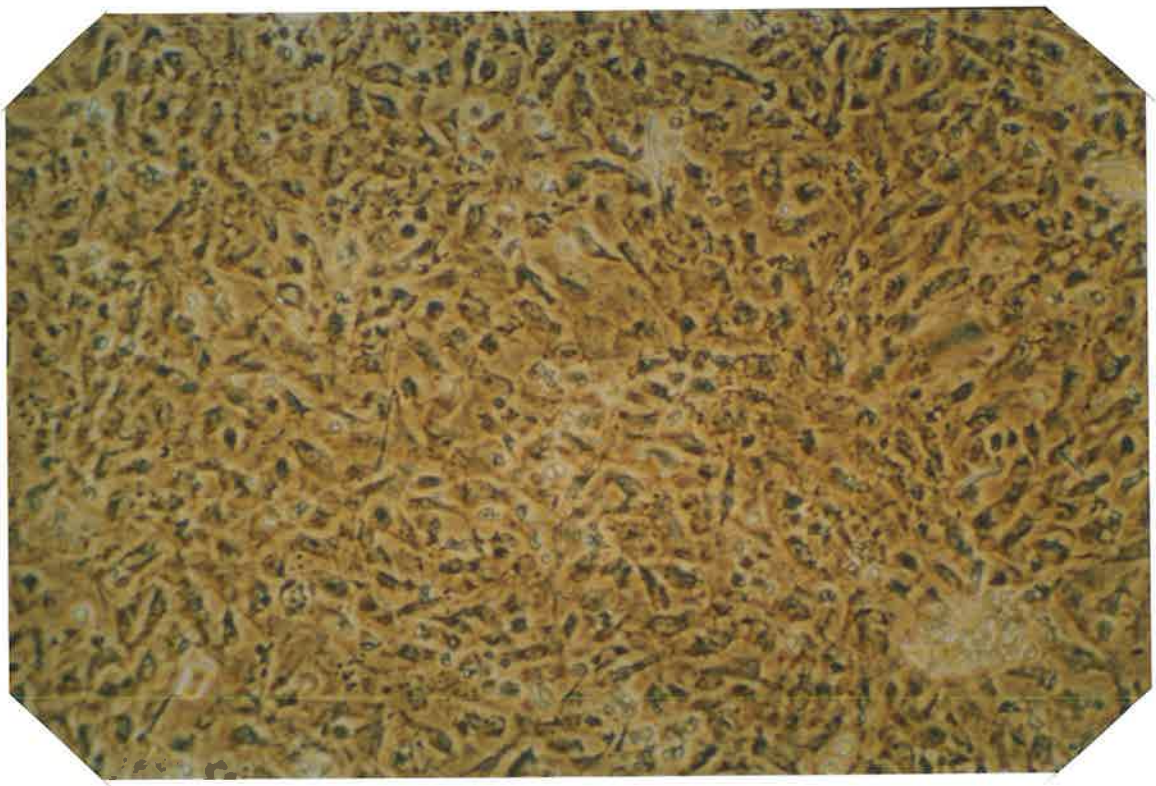
Identification of HUVECs by the immunofluorescent staining of factor VIII related antigen

Methods

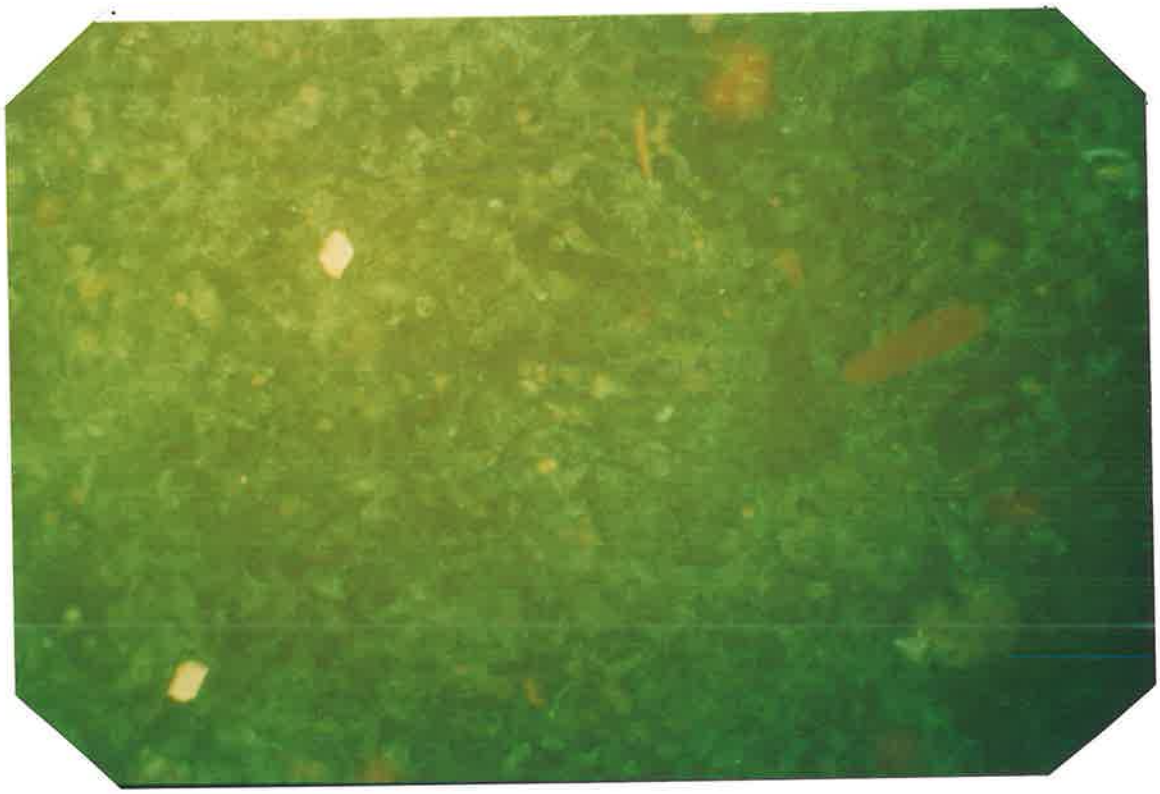
HUVECs which were assessed for factor VIII related antigenic expression were isolated, cultured, subcultured and photographed as described in Chapter 2. HUVECs (from 2 cell lines, passaged 2 and 3 times respectively) were seeded onto gelatinised glass coverslips at 1.2 x 10^5 cells/well. Cells were fixed, stained and photographed 48 hours following seeding.

Results

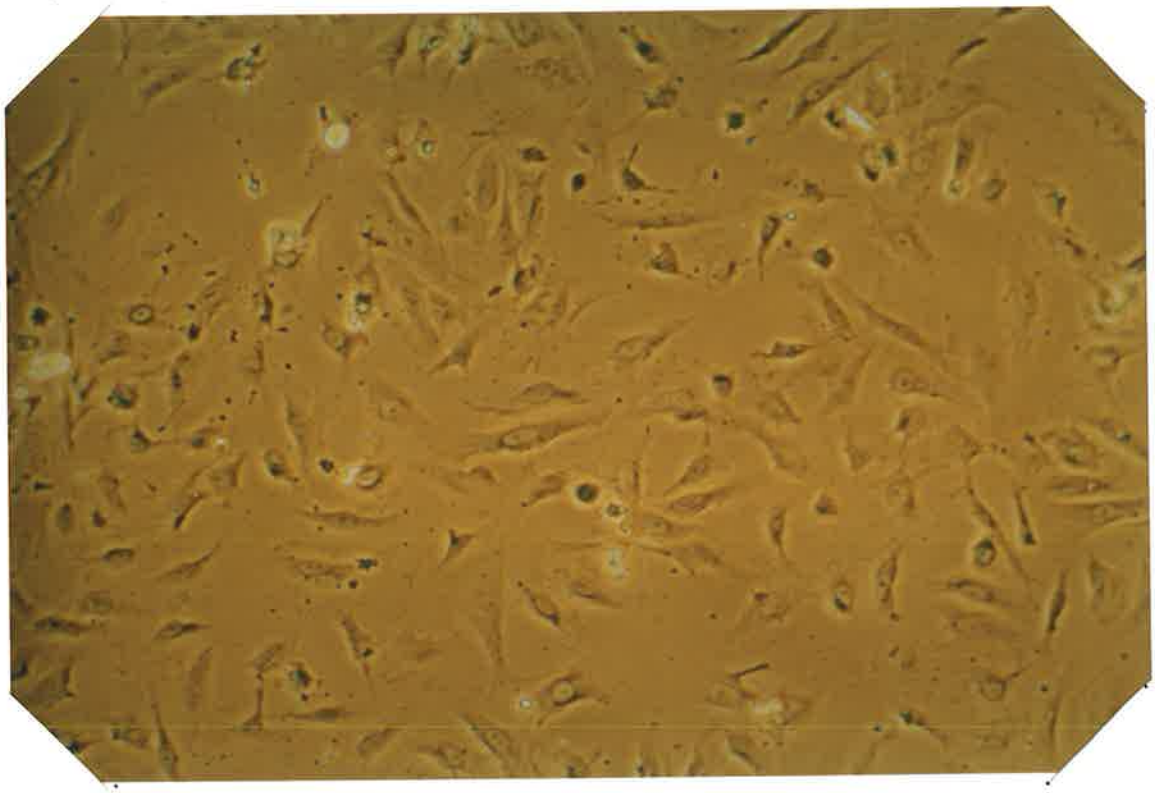
Photograph 8 shows a population of confluent HUVECs (used 48 hours following seeding) which had been passaged twice (i.e. 3° or tertiary cells). The cells were acetone fixed and incubated with fluorescein-conjugated antiserum to detect factor VIII related antigen and were examined by phase contrast or reflected light fluorescence microscopy at an original magnification of 40 x.



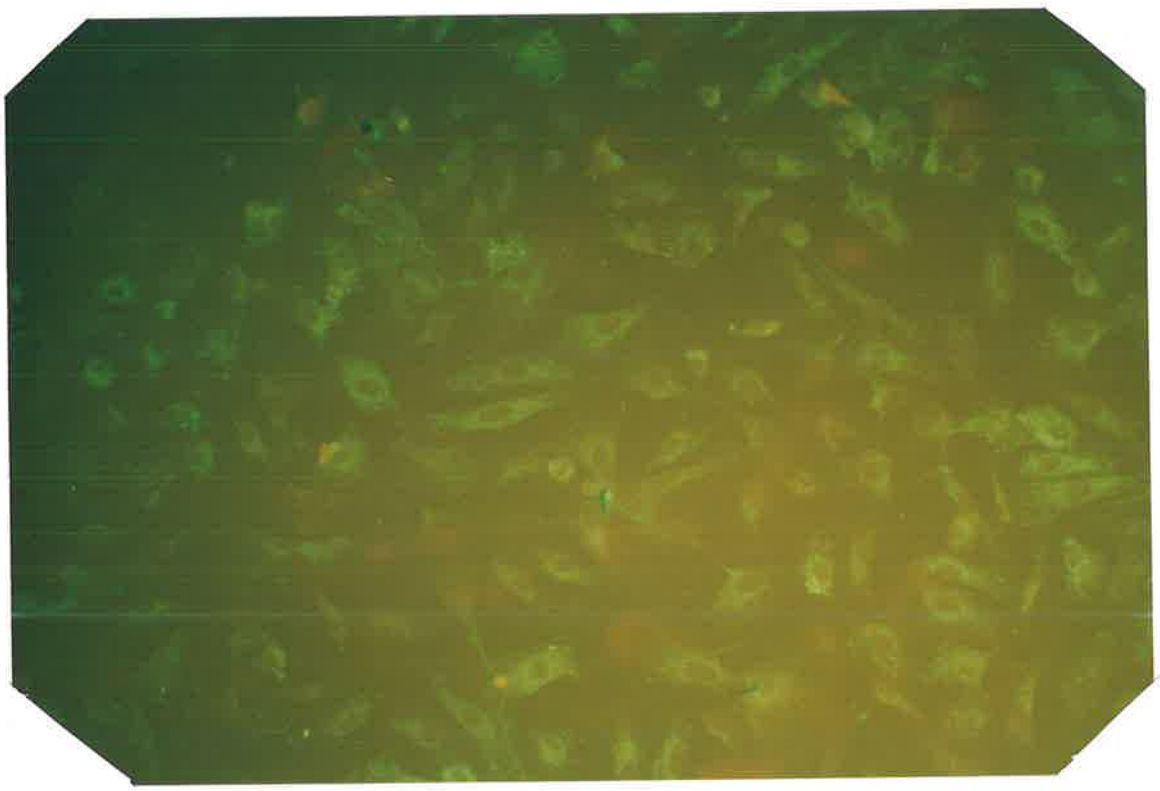
Photograph 9 shows the same population of cells at equivalent magnification, examined under fluorescent light.



Photograph 10 shows a population of non-confluent HUVECs (used 48 hours following seeding) which had been passaged three times (i.e. 4° or quaternary cells). The cells were acetone fixed and incubated with fluorescein-conjugated antiserum to detect factor VIII related antigen and were examined by phase contrast or reflected light florescence microscopy at an original magnification of 100 x.

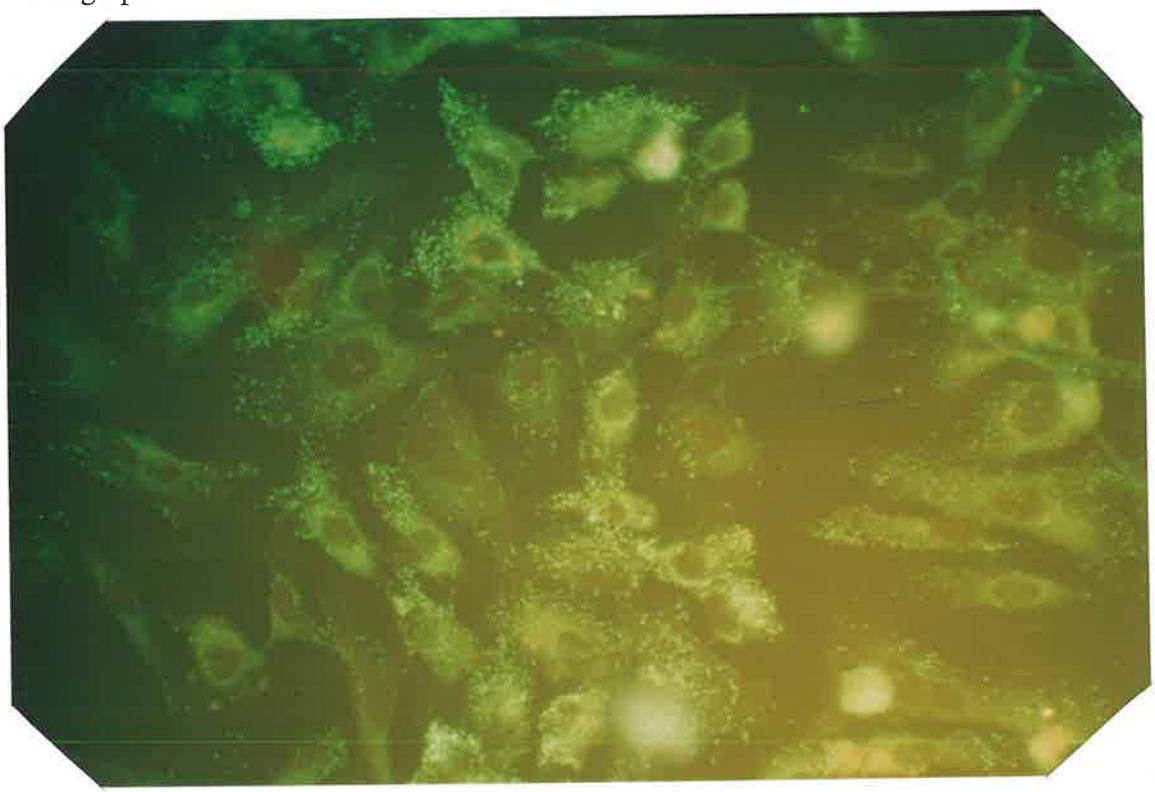


Photograph 11 shows the same cell population examined under fluorescent light at an original magnification of 100x .

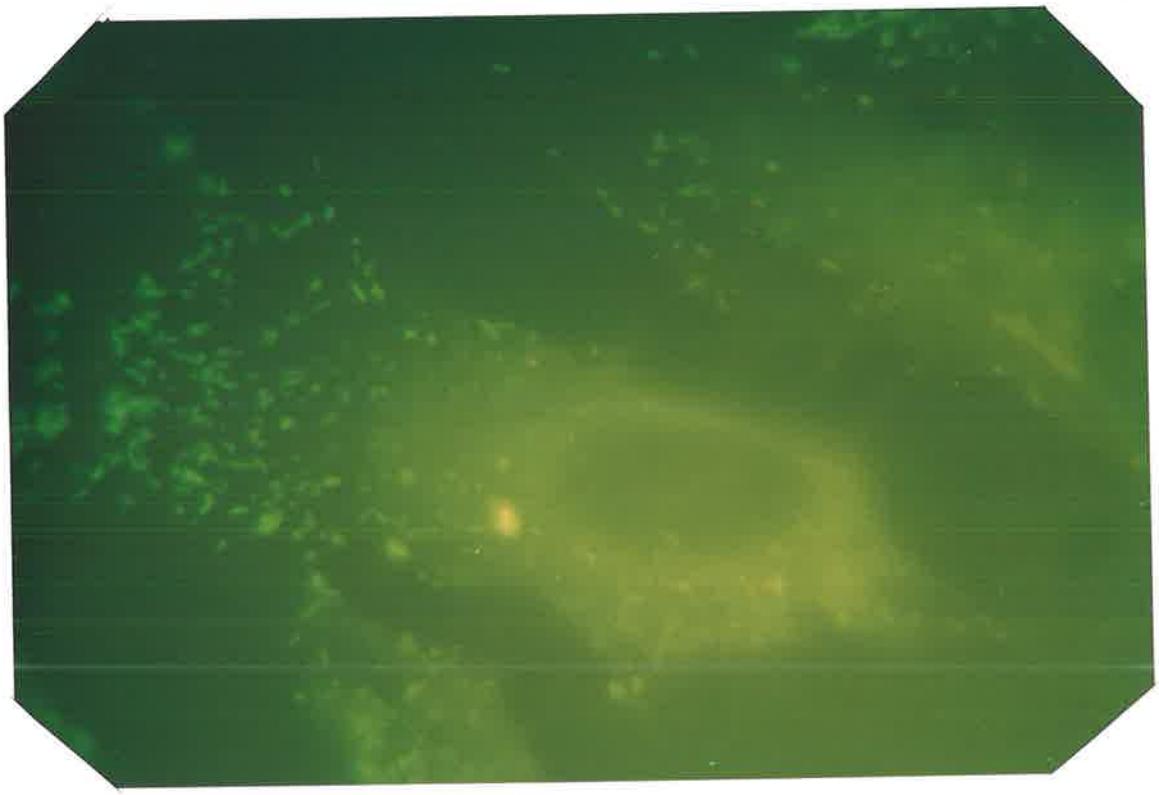


Photographs 12 and 13 show the same cell population (as that used in Photographs 10 and 11) and examined under fluorescent light at an original magnification of 200 x (Photograph 12) and a single cell of the population at magnification of1000 x , under oil immersion (Photograph 13).

Photograph 12



Photograph 13



Discussion

Fixed and stained HUVECs showed clear factor VIII related antigenic staining in cells passaged both 2 and 3 times (Photographs 9, 11, 12 and 13). Factor VIII related antigenic staining appeared to be located in the cellular matrix. There was no evidence of surface localisation as there was no apparent staining delineating the outer edge of the cells (Photograph 13). In addition, no staining was evident in association with the nucleus (Photographs 11, 12 and 13). In areas where cells were more isolated and at higher magnification (Photographs 12 and 13) staining of factor VIII related antigen appeared strongest at the outer perimeter of the nucleus, and dispersed from this area into the cell cytoplasmic matrix where it became localised in distinct vesicle-like points. This pattern of staining was consistent with the expected distribution of von Willebrand factor and its storage in Weibel-Palade bodies. Certainly HUVECs in culture retain such structures and the number of these organelles per cell is reportedly higher in HUVECs than in endothelial cells derived from other species, for example bovine endothelial cells (Wagner, Olmsted & Marder, 1982). The staining, described by Wagner, Olmsted & Marder (1982) and observed in the present studies, was consistent with the cells actively producing von Willebrand factor in the cytoplasm, presumably in association with rough endoplasmic reticulum (hence its strongest association around, but not in the nuclear area) and its dispersion into vesicles in the surrounding cytoplasm. The lack of staining at the cell periphery (Photograph 13), indicated a lack of deposition at the surface of the cells and may have indicated a low degree of HUVEC activation in the cells used for these studies. Certainly, release of von Willebrand factor stores (predominantly into the subendothelium) can accompany endothelial cell activation and occurs concomitantly with a reduction in endothelial cell stores of von Willebrand factor (Reinders *et al,* 1988; Ruggeri & Ware, 1993).

The results obtained in these studies were also consistent with the distribution patterns of factor VIII related antigen described by other similar investigations (Jaffe *et al,* 1973; Jaffe, Hoyer & Nachman, 1973; Booyse *et al,* 1981). These results provided evidence, that cells identified as HUVECs on the basis of their morphology (see section 3a), also expressed typical factor VIII

antigenic expression. They also confirmed the ability of endothelial cells to synthesize and store von Willebrand factor and the capacity of heterologous factor VIII antiserum (raised to factor VIII:vWF complex) to locate its position, although notably, vascular endothelial cells are not a recognised source of factor VIII itself (Zimmerman & Meyer, 1987). All cells which morphologically resembled HUVECs, when examined by this method, displayed clear staining for factor VIII related antigen.

Section 3c

Technical variables affecting prostacyclin generation by HUVECs

Methods (for sections 3c and 3d)

Initial experiments using cultured endothelial cells were performed with cells isolated and cultured using methods as described in Chapter 2. Briefly, cells for these initial studies were cultured using CCM based upon RPMI 1640 and were seeded into gelatinised 12 well culture plates with a seeding density (unless otherwise indicated) of 1.2×10^5 cells/well. Following washing procedures, the HUVECs were incubated (10 minutes) in the presence of a range of stimuli (prepared as described in Appendix 2). Prostacyclin generation was quantified by determining concentrations of 6-keto-$PGF_{1\alpha}$. Cell counts were performed in some but not all studies, therefore 6-keto-$PGF_{1\alpha}$ concentrations are expressed in ng/ml, with no adjustment for cell numbers.

Results

Table 3c : i shows prostacyclin generation (measured as 6-keto-$PGF_{1\alpha}$ in ng/ml) in response to thrombin. Where a range of thrombin concentrations was used the result from one (mid-range) stimulus concentration is shown. The variables examined (see **bold typeface**) were the seeding density, the time at which cells were used (24 or 48 hours) following seeding and differences in prostacyclin generation from the first (W1) and last well (W12) processed of 12 well culture plates. Between 1 and 3 independent lines were used to examine each variable; mean±sems are indicated when more than 1 line was used or in some instances where for a single line, replicates were included. N.q indicates where 6-keto-$PGF_{1\alpha}$ was non-quantifiable.

Supernatant volume (ml)		Time from seeding (hrs)	Passage number 1°,2° etc.	Seeding density (cells/well)	Stimulus	6-keto-$PGF_{1\alpha}$ concentration (ng/ml)
2		24	4	**3.8×10^4**	Thrombin	n.q.
				7.5×10^4	2u/ml	n.q.
				1.5×10^5		0.7 ± 0.08
				3.0×10^5		1.2 ± 0.20
				6.0×10^5		1.7 ± 0.06
				1.2×10^6		4.1 ± 0.32
1		**24**	3,4	1.2×10^5	Thrombin	0.5 ± 0.01
		48			0.05u/ml	2.2 ± 0.20
1	**W1**	48	2,4,5	1.2×10^5	Thrombin	0.7 ± 0.25
	W12				0.02u/ml	0.8 ± 0.28
	W1				Thrombin	3.3 ± 0.42
	W12				2.0u/ml	3.5 ± 0.32

Firstly, it was evident that where seeding densities of 1.5×10^5 cells/well up to 1.2×10^6 cells/well (an 8 fold increase in seeding density) were used, that prostacyclin generation in response to 2u/ml thrombin also increased approximately 8 fold. As the detection of prostacyclin was hampered by the use of a 2ml supernatant in these initial experiments this volume was reduced to 1ml in subsequent studies. Secondly it was apparent that prostacyclin generation responses were greater when the cells had been seeded 48 rather than 24 hours prior to their use. Finally in processing the 12 wells of a culture plate, the mean prostacyclin level in the last well processed was only slightly greater than in the first well, and the difference was insufficient to conclude plate processing affected the level of prostacyclin generation observed.

Table 3c : ii shows prostacyclin generation (measured as 6-keto-$PGF_{1\alpha}$ in ng/ml) in response to thrombin (used at 0.02-0.5u/ml) or arachidonic acid (used at 0.5-10μM). Where a range of concentrations was used the result from one (mid-range) stimulus concentration is shown. Cells were consistently used 48 hours following seeding and a 1ml supernatant volume was used. The effects of three variables on prostacyclin generation responses were examined (see **bold typeface**). These included the use of different supernatant buffers, different passage numbers and passaging itself (before→following passaging). Between 1 and 4 independent lines were used to examine each variable; mean±sems are indicated when more than 1 line was used or in some instances where for a single line, replicates were included.

Supernatant buffer	Passage number 1°,2°etc.	Seeding density cells/well	Stimulus	6-keto-$PGF_{1\alpha}$ concentration (ng/ml)
HBSS	4,4,5,7	1.2×10^5	Thrombin	1.2 ± 0.29
RPMI	↓	↓	0.05u/ml	1.6 ± 0.47
HBSS	↓	↓	Arach. A.	2.2 ± 0.62
RPMI			2.0μM	2.8 ± 0.95
RPMI	**1**	1.2×10^5	Thrombin	2.5
↓	**3**	↓	0.05u/ml	2.5 ± 0.19
↓	**4,4**	↓	↓	0.9 ± 0.12
↓	**5**	↓	↓	3.1
↓	**7**	↓	↓	1.0 ± 0.13
↓	**4,4**	↓	Arach. A.	2.6 ± 0.78
↓	**5**	↓	2.0μM	3.8 ± 0.50
↓	**7**	↓	↓	0.8 ± 0.10
RPMI	**4→**	1.2×10^5	Thrombin	0.8 ± 0.09
↓	**→5**	↓	0.05u/ml	3.1 ± 0.40
↓	**1→**	↓	0.10u/ml	4.8 ± 0.40
↓	**→2**	↓	↓	1.5 ± 0.09
↓	**4→**	↓	Arach A.	1.4 ± 0.15
↓	**→5**	↓	2.0μM	3.8 ± 0.50

Firstly, in the presence of RPMI 1640, responses were on average higher than with HBSS. Secondly when prostacyclin generation responses were compared from lines passaged different numbers of times, variation encompassing approximately 4 fold differences in the prostacyclin generation responses to both thrombin and arachidonic acid were apparent. Finally, there was no strong evidence to suggest that with increasing passage numbers, there was a consistent loss of responsiveness in prostacyclin generation. Although 7° cells produced low prostacyclin generation it was not determined if further passaging may reduce responsiveness further. Also, when 4° cells were examined before and after a single passage, increased responsiveness was seen following passaging. The reverse was observed when a 1° cell line was passaged, highlighting the inconsistent effects of passaging on cell responsiveness to the stimuli used.

Discussion

These initial experiments, although not extensive, identified a number of elements associated with the culture of HUVECs and the methods used to assess their responses which appeared to influence prostacyclin generation from HUVECs. The findings allowed the system to be modified to optimise the detection of HUVEC-derived prostacyclin generation. A reduction in the well supernatant volume (2ml to 1ml) was first instituted such that prostacyclin would be concentrated into a reduced volume, and lower levels of prostacyclin generation could be quantified.

Effects of seeding density on prostacyclin generation

Where the effect of seeding density on prostacyclin generation responses was initially examined it was clear (see Table 3ci) that the magnitude of stimulated responses were related to the number of cells which could respond. The two highest seeding densities (6.0 and 1.2 x 10^5 cells/4cm^{2}well) both provided a visually confluent HUVEC monolayer and would have provided an optimal cell density. Possibly the greater prostacyclin generation from the higher seeding density was caused by the HUVECs being grown in the presence of excess non-adherent HUVECs.

It was observed in these initial studies that a minimum seeding density of 1.5 x 10^5 cells/well was required to obtain detectable prostacyclin generation (when using cells 24 hours after seeding and a 2ml supernatant volume). Modifications to the experimental conditions (including the use of a lower supernatant volume of 1ml and cells at 48 rather than 24 hours following seeding) allowed a lower seeding density of 1.2 x 10^5 cells/well to be used. Under these conditions quantifiable levels of prostacyclin could be obtained in response to thrombin concentrations as low as 0.02u/ml and more wells could be seeded from a given population of HUVECs.

Effects of time following seeding on prostacyclin generation responses

In examining HUVEC responses 48 hours following seeding, the responses were considerably greater (4-5 fold) than when 24 hour cells were used. This may be explained on the basis that the proteolytic harvesting of HUVECs prior to seeding produced stimulation of prostacyclin generation. Indeed it has been shown that trypsin stimulates prostacyclin generation (Weksler *et al,* 1978). Also both mechanical and proteolytic (with trypsin-EDTA) resuspension of cells prior to passaging stimulates prostacyclin generation (Chesterman, Ager & Gordon, 1983; Ager *et al,* 1982). Following this stimulation, the cells may have been relatively refractory to further stimulation at 24 hours following seeding, but may have recovered sufficiently to react more strongly at 48 hours. Certainly upon stimulation of prostacyclin generation (in response to stimuli including thrombin, arachidonic acid and ionophore A23187), endothelial cells have been shown to become refractory to further stimulation for up to 48 hours (Brotherton and Hoak, 1983; Dejana *et al,* 1983), until protein synthesis allows replacement of inactivated cyclooxygenase. Given such findings, cell harvesting and seeding procedures in the present studies probably activated HUVECs. As a result they may have been relatively unreactive to further stimulation at 24 hours compared to 48 hours following seeding, when greater recovery from the effects of harvesting would have occurred .

Effects of time involved in processing wells on prostacyclin generation

The experimental protocol attempted to co-ordinate the processing of culture plates such that the supernatants from the first and last wells would be removed as close as possible to 10 minutes following the addition of a stimulus. The results of studies where processing time was assessed (see Table 3ci) indicated the last or twelfth well processed contained a prostacyclin concentration (for a given stimulus) similar to that found in the the first well. This suggested no systematic error was introduced in prostacyclin generation responses in association with the order in which wells were processed. Despite this evidence, different stimuli or treatments applied to wells were still randomised within plates and between experiments in subsequent studies, to ensure systematic errors could not be introduced.

Effects of differing supernatants on prostacyclin generation

It has been shown that HUVECs, in the presence of a supernatant buffer rocked over their surface, can be stimulated to produce prostacyclin and the extent of this production is affected by the type of buffer to which they are exposed (Brox & Nordoy,1982). In response to specific stimuli, and in the presence of RPMI 1640, prostacyclin generation by HUVECs was shown to be consistently slightly higher than in the presence of HBSS. RPMI 1640 contains a wide range of amino acids, vitamins and other components not present in HBSS. In addition, the buffering capacity of RPMI (containing 20mM Hepes) would have provided more stable pH conditions than HBSS, another potential contributing factor to the differing results. Despite such differences in these buffers, the magnitude of divergence in responses in the presence of each was not great and neither buffer alone stimulated quantifiable prostacyclin generation (see control in Table 3d). This lack of HUVEC stimulation was possibly assisted by a lack of any form of culture plate agitation in these studies, and such agitation was only undertaken when starting platelet and HUVEC interaction studies (see Chapter 4).

Effects of different lines on prostacyclin generation responses

Variable prostacyclin generation responses were observed between different endothelial cell lines (of similar passage number (3-5) and seeded at equivalent density) and such variation has been described previously (Christofinis *et al*, 1979; McIntyre *et al,* 1985; Ingerman-Wojenski & Silver, 1988). Ingerman-Wojenski & Silver (1988) obtained data from seven cell lines, and for primary and subcultured cells noted, for a given passage number, up to seven fold variation in stimulated responses. The cause of such variability was not determined by these authors. In the present studies, (Table 3c:ii,) approximately 4 - fold variation in stimulated prostacyclin generation responses was observed between different lines of similar passage number (e.g.3-5). This variability in responses could have been caused by not examining identical passage numbers, although in subsequent studies (Chapter 4) where 4° cells were used consistently, similar variation in responses was observed. Alternatively, the variability may have been associated with variability in the time between the initial isolation of cells and their use in experiments. Variability may also have been caused by differences in initial yields of primary

cells such that cells from different lines may have undergone variable numbers of population doublings before sufficient cells were available for experimental purposes. Naturally, a relationship existed between these two factors. For example, lines derived from low initial cell yields had to be cultured for longer periods of time and undergo more population doublings to provide (at a given passage number) enough cells for experiments.

Evidence suggests that increased HUVEC population doublings may be linked with a loss of stimulated prostacyclin generation (Ingerman-Wojenski & Silver, 1988). There is also evidence to suggest that the time in culture affects endothelial cells. Tracey & Peach, (1992) provided an example of the loss of mRNA and muscarinic receptor expression in bovine aortic endothelial cells, with an associated loss of such receptor mediated EDRF production. Endothelial cell ectonucleotidase and ACE enzyme activity in porcine aortic endothelial cells has also been seen to decrease with time in culture (Chesterman, Ager & Gordon, 1983). Notably, such changes over time could be seen in primary cells (harvested by both collagenase and mechanical means) such that passaging procedures could not have caused the changes.

Although it seemed likely that the factors mentioned above may explain some of the variability in responses between different lines of similar passage number, overcoming this variability posed some difficulties. Ideally the cells accepted for experimental purposes would be derived from lines where specific primary cell yields were seeded and confluence was reached within a consistent period of time. Following this, all cell lines would have had to be subcultured over a consistent time period prior to their use in experiments. The implementation of such highly stringent culturing conditions would have meant too few lines would have yielded 'acceptable' HUVECs which could be used in experiments.

Effects of passaging on prostacyclin generation responses

On viewing different passage numbers (Table 3c : ii), for passage numbers 1 through to 7, no consistent fall in responsiveness was observed with increasing passage number. In the only two lines assessed before and following passaging (1°- 2°, and 4°- 5°) there was a decrease in prostacyclin generation responses in one case, but an increase in the second, highlighting the lack of consistent effects associated with passaging itself. A number of studies have assessed the effects of passaging upon stimulated prostacyclin generation from HUVECs. Christofinis *et al* (1979) indicated (through the indirect assessment of platelet inhibitory effects) that with up to 47 passages, isolated HUVECs continued to display prostacyclin generation capacities. Others (Ingerman-Wojenski & Silver,1988) have found that HUVECs proteolytically subcultured twice lost most of their capacity to generate prostacyclin in response to thrombin, arachidonic acid and the ionophore A23187. These authors also produced data to indicate that recovery to original levels of responsiveness was observed between subcultures 5 and 12 and they could not explain this recovery. This inconsistency in 'lost' HUVEC responsiveness was certainly in accord with findings in the present studies.

Although proteolytic (trypsin-EDTA) passaging can be linked with reductions in receptor density (Sung *et al,* 1989), the loss of receptors in cultured endothelial cells may also occur with time following cell isolation (Tracey & Peach, 1991). Loss of endothelial cell functions such as surface enzyme activity has also been shown to occur with time, irrespective of differing isolation and passaging techniques (Chesterman, Ager & Gordon, 1983). In the results shown in Table 3c : i, there was no strong evidence to indicate that the proteolytic passaging procedures used produced loss of HUVEC responses. Had this been the case, one may have expected thrombin (ADP and bradykinin) mediated responses to be more susceptible to the effects of passaging than non-receptor mediated responses, such as those in response to arachidonic acid. There was no evidence to suggest this was the case (ADP passage effects not shown, bradykinin responses in 1° cells shown in Table 3d).

Had passage-dependent loss of HUVEC responsiveness been apparent, the use of primary cells, or mechanical harvesting for passaging purposes may have been necessary (Sung *et al,* 1989). Notably however, some investigators have found that even the use of mechanical harvesting techniques for passaging procedures has not been successful in preventing the loss of some endothelial cell functions (Chesterman, Ager & Gordon, 1983).

The conclusions from these studies, although of limited number, was that passaging itself did not produce predictable alterations in stimulated prostacyclin generation responses. There was therefore no particular benefit associated with using only primary cells in future studies. There was in contrast an obvious advantage associated with the use of passaged rather than primary cells in that the numbers of cells available for experimental purposes could be doubled with every passage. To obtain near equivalent cell numbers for subsequent experiments, it was decided to use cells from lines which had consistently been passaged 3 times. In addition, with respect to cell prostacyclin generation responses, a seeding density of 1.2×10^5cells/well and the use of cells at 48 hours following seeding appeared to provide conditions where detectable prostacyclin responses were obtained in a 1ml supernatant volume. These responses (as discussed in the next section) were considered to be sufficient to produce platelet inhibition. Consequently, these parameters were kept constant for the first series of platelet and HUVEC interaction studies (Chapter 4), and were revised following these studies.

Section 3d

Prostacyclin generation by HUVECs in response to various stimuli

Results

Table 3d summarises pooled results from experiments using HUVECs passaged 3-5 times and seeded at 1.2 x 10^5 cells/well. A final supernatant volume of 1ml RPMI 1640 was used and prostacyclin generation (measured as 6-keto-$PGF_{1\alpha}$ in ng/well; mean and sem value) is indicated. Within experiments, duplicates were averaged and reps indicates where more than two replicates were used to determine a mean value. N indicates the number of independent experiments performed (i.e. number of lines).

		Mean	sem	N
Control		n.q.	-	8
Thrombin	0.02u/ml	1.2	0.2	4
	0.05	1.4	0.4	4
	0.10	2.1	0.7	4
	0.20	2.2	0.5	4
	0.50	2.6	0.5	4
	2.0	2.8	0.5	2
Arachidonic acid	2.0µM	0.9	0.2	4
	5.0	2.5	0.5	4
	10	4.8	0.9	4
	20	5.7		1
Adrenaline	1µM	0.2	0.1	4
	5	n.q	-	4
ADP	5µM	0.5		1
	40µM	0.8		1
Histamine	10µM	3.0		1
*PAF	25µM	0.3		1
Bradykinin	0.01µM	n.q		1
	0.05µM	1.8	0.7	4
	1.0	2.0	1.3	4
*1°	0.05	1.0	4reps	1
**1°	0.05	1.5	4reps	1
*1°	0.5	1.6	4reps	1
*1°Thrombin	0.1u/ml	2.5	4reps	1

* Primary cells, ** Primary cells in the presence of 1µM captopril. N.q.=not quantifiable (i.e. <0.16ng/ml).

Discussion

Prostacyclin generation in response to various stimuli (summarised in Table 3d) provided an indication of the relative strengths of several stimuli to elicit prostacyclin generation from HUVECs. The use of a 10 minute final incubation time in these experiments was used to ensure that optimal prostacyclin levels were obtained. It has been shown that HUVECs in a closed system such as the one used in these studies produce the majority of prostacyclin within 2-5 minutes following stimulation (Czervionke *et al,* 1979a) and this was confirmed in subsequent studies (Chapter 4).

The control (where an aliquot of buffer was used as a 'stimulus') was evaluated on the basis that these wells could provide information with respect to the basal level of prostacyclin generation. Preincubation washes were undertaken to remove serum and also served to equilibrate HUVECs with the medium to be used in the final incubation. It was desirable that in the control wells the washing procedures and the supernatant buffer itself should produce minimal HUVEC activation. The results confirmed that HUVEC activation in control wells was minor as no quantifiable prostacyclin generation was observed (< 0.16ng/ml 6-keto-$PGF_{1\alpha}$,).

Arachidonic acid and thrombin produced 6-keto-$PGF_{1\alpha}$ generation responses which were comparable (based on cell counts and stimulus concentrations) to responses obtained by other workers where both primary and passaged HUVECs were used (Hong, 1980; Weksler, Ley & Jaffe, 1978; Dejana *et al,* 1983; Czervionke *et al* 1979a). Arachidonic acid caused the greatest responses through acting as a direct substrate for HUVEC cyclooxygenase whereas thrombin is known to activate endothelial cells through binding (and cleavage) of specific receptors (Venturini & Kaplan, 1992) on endothelial cells. The results indicated that both these stimuli, despite their very different mechanisms of action produced strong (and concentration-dependent) prostacyclin generation responses.

Although assessed on only one occasion, ADP at both 5 and 40μM produced a weak increase in prostacyclin generation over the control value, and even 40μM resulted in the generation of only 0.81ng/ml 6-keto-$PGF_{1\alpha}$. Some investigators have indicated that cultured HUVECs do not generate prostacyclin in response to ADP (Chesterman *et al,* 1986; Weksler, Ley & Jaffe, 1978), whilst others have shown responses (Carter *et al*, 1988). Notably, these latter workers indicated ADP gave a relatively weak (consistently only 30%) response compared with the response to ATP. In addition, adenosine and AMP gave no response. Relative to ATP (1mM), others have shown that many other stimuli, including bradykinin and histamine, produce far stronger responses in HUVECs at lower (micromolar) concentrations (McIntyre *et al,* 1985). Certainly the results shown in table 3d showed other stimuli were more potent than ADP in stimulating prostacyclin generation.

Adrenaline failed to stimulate an increase in prostacyclin generation, consistent with previous findings where HUVECs (Weksler, Ley & Jaffe, 1978) or bovine aortic endothelial cells (Adler *et al*, 1981) were used. This lack of activity of adrenaline was also confirmed in subsequent studies (see Chapter 5). Such results were consistent with possible effects of adrenergic stimuli (seen in bovine endothelial cells) acting on β–adrenergic receptors to increase cellular cAMP, thus opposing rather than stimulating prostacyclin generation (Adler *et al,* 1981).

Responses to bradykinin were obtained in a number of experiments but replicated experiments were only performed using bradykinin at concentrations of 50nM and 1μM. Even for these concentrations the variability in responses was high and no clear dose-related effects were seen. The ability of bradykinin to evoke prostacyclin generation did however show that receptor evoked prostacyclin generation responses could be gained from both primary and passaged cells at bradykinin concentrations of 50nM or more. Others, using primary HUVECs have observed 6-keto-$PGF_{1\alpha}$ generation in response to ~1μM bradykinin ranging from 6.3ng/10^6 up to 23.5ng/10^6 cells (McIntyre *et al,* 1985; Hong, 1980). In the present studies, in response to 1μM bradykinin, passaged HUVECs generated an estimated 16.5ng/10^6 cells. Given the evidence that bradykinin receptors are reduced by up to 40% during proteolytic harvesting

procedures (Sung *et al,* 1989), it was anticipated that responses may have been even greater in non-passaged primary cells but this was not the case. Captopril was also used in the one experiment where primary cells were used (to inhibit bradykinin metabolism by HUVECs) and led to a ~50% increase in 6-keto-$PGF_{1\alpha}$ formed. The response however, remained less than those gained using passaged cells in the absence of captopril, and little emphasis could be placed on the results from this single study. Some investigators have reported that in primary HUVECs bradykinin can provoke comparable responses to thrombin (Hong, 1980). However, the results of the present studies showed that with primary or passaged cells, at the indicated concentrations, thrombin resulted in the formation of more consistent and relatively high levels of prostacyclin compared with bradykinin.

The single (and possibly non-representative) experiments examining the effects of histamine and PAF were performed only out of an interest in receptor mediated responses, rather than to obtain information for ongoing studies. The 10μM concentration of histamine produced a response which equated to 16.7ng of 6-keto-$PGF_{1\alpha}$ per 10^6 cells, and an almost identical response to the same concentration was reported by McIntyre *et al* (1985) from primary HUVECs (16.8ng/10^6). In this system, even in primary HUVECs, no response was obtained in response to PAF, consistent with the findings of others who have also failed to detect a response to PAF (Chesterman *et al,* 1986).

From these preliminary experiments it was apparent that the responses of HUVECs to some stimuli were in accord with those described by some other workers but also differed to some previous reports, either in the magnitude or in the existence of prostacyclin (6-keto-$PGF_{1\alpha}$) generation. For ongoing studies involving platelets, it was considered important to gain responses which represented prostacyclin generation in the nanomolar or platelet inhibitory range (Krishnamurthi, Westwick & Kakkar, 1984; Radomski, Palmer & Moncada, 1987a). Prostacyclin generation of greater than 1nM over the total incubation time would entail generation of 6-keto-$PGF_{1\alpha}$ above 0.38ng/ml (assuming complete conversion of prostacyclin to 6-keto-$PGF_{1\alpha}$).

Such platelet inhibitory concentrations of prostacyclin (>1nM) were evoked from HUVECs, in ascending order of potency, in response to ADP, bradykinin, thrombin and arachidonic acid. The strength of arachidonic acid (a direct substrate of cyclooxygenase) was not unexpected. It was however a non-physiological stimulus, and the use of a physiological stimulus with similar activity in the system was desirable. Thrombin met this criterion and the strong and consistent responses to thrombin indicated it would be a useful stimulus in subsequent studies. In addition, it had the additional desirable characteristic of being able to cause platelet aggregation as well as HUVEC activation. In contrast to thrombin, ADP was only a weak stimulus of prostacyclin generation, but as a recognised stimulus of platelet activation its effects in subsequent studies was of interest.

It was hoped that in subsequent experiments, bradykinin (500nM) could have been used to advantage to enhance the platelet inhibitory activity of HUVECs, mediated by prostacyclin and EDRF (Alheid, Frolich & Forsterman, 1987; Radomski, Palmer & Moncada, 1987 a-d). Unfortunately, in the present studies, bradykinin proved an inconsistent stimulus of prostacyclin generation and was therefore not used in subsequent studies.

Given that later experiments were to involve assessments of platelet and HUVEC interactions, it would also have been of interest in these preliminary studies to ascertain the responses of HUVECs to serotonin and ATP, both of which are released from activated platelets, as well as the effects of a TXA_2/PGH_2 mimetic (e.g. U44619).

Rather than continuing to evaluate HUVEC responses in isolation, subsequent studies were designed to evaluate platelets as well as HUVECs, the experimental factors which affected the responses of both cell types, and the effects of combining them. For these studies, washed platelets and HUVECs were to be examined in 12 well culture plates. As described earlier, HUVECs (passaged 3 times) were to be seeded into wells at 1.2×10^5 cells/well 48 hours prior to use. Platelet and HUVEC responses to stimuli including ADP and thrombin were to be examined with agitation provided by a shaker plate. The effects of different calcium concentrations in the supernatant (1ml) and different incubation times were to be evaluated.

Chapter 4

Factors affecting platelet and HUVEC responses when incubated independently and in combination

Introduction

The experiments described in the previous chapter were performed to confirm that cultured HUVECs presented typical morphological characteristics and antigenic expression. They also confirmed that HUVECs produced the potent platelet inhibitory factor prostacyclin in response to various stimuli. Prior to making assessments of how platelets and HUVECs responded when co-incubated, it was necessary (as in the studies with HUVECs in isolation) to evaluate the properties of platelets in isolation. In particular, it was essential to determine factors which affected isolated platelet responses to a number of stimuli.

For these studies the isolation of platelets from citrate anticoagulated blood involved the use of a washing technique adapted from those previously described (Kinlough-Rathbone *et al,* 1977). Using this technique, it has been found that (without adding $CaCl_2$ to buffers) ionised calcium concentrations of only ~20μM are evident in the final platelet suspension, and platelet responses to some stimuli in such low ionised calcium conditions differ from those observed in the presence of more physiological $CaCl_2$ concentrations (e.g. 2mM; Packham, Kinlough-Rathbone & Mustard, 1987; Packham *et al*, 1989). To investigate such reported phenomena, 3 platelet aggregometry studies were performed (as described in Chapter 2) prior to undertaking the studies described in this chapter. In accord with the previous reports, it was observed that platelet serotonin release in response to the stimulus ADP could only be detected when platelets were prepared in the absence of added (1.2mM) $CaCl_2$ and in the presence of a fibrinogen source. Therefore, in order to obtain detectable responses for this stimulus in subsequent studies with HUVECs, low ionised calcium conditions (and the use of a fibrinogen source) would be required.

It is known that differing extracellular calcium concentrations also affect endothelial cell functions, including the generation of prostacyclin (Brotherton & Hoak, 1982) and EDRF (Adams *et al,* 1989; White & Martin, 1989; Lopez-Jaramillo *et al,* 1990; Busse *et al*, 1993). For this reason, in the studies described in this chapter, the effects of differing $CaCl_2$ concentrations on both platelet and HUVEC responses to stimuli including ADP, collagen and thrombin were examined.

To assess platelet aggregation, it was not possible to measure changes in light transmission in the culture plates used for platelet and HUVEC interaction studies. Therefore platelet serotonin release (and thromboxane generation responses) were used as alternative means of describing platelet activation. It was determined (see Chapter 2) that in the presence of ^{14}C-serotonin, negligible amounts became associated with HUVECs, and these levels were no greater than those associated with plastic. Also over 30 minutes HUVECs did not appear to accumulate ^{14}C-serotonin (even in the presence of a monoamine oxidase inhibitor). Consequently, the measurement of platelet serotonin release could be used to determine platelet activation in both the presence and absence of HUVECs. In order to provide platelet agitation (such as that provided by a stir bar in the aggregometry studies) a titre plate shaker was used. In preliminary studies using platelets alone in culture plates platelet activation responses (serotonin release) over 5 minutes increased with increasing shaker speed. Shaker speed number 4 was found to provide platelet serotonin release responses which were similar to those obtained in the preliminary platelet aggregometry studies and this speed was therefore used in the studies described in this and all subsequent chapters.

In the studies described in Chapter 3 in which HUVEC responses were examined, only a 10 minute time point was examined. Also, in the preliminary platelet aggregometry studies, platelet responses were only examined at a 5 minute time point. It was therefore considered important to evaluate in greater depth how differing incubation times affected platelet and HUVEC responses such that an optimum time point could be selected to evaluate the responses of both these cells in subsequent studies.

Aims

The studies described in this chapter were undertaken to assess the effects of a number of experimental variables which were considered likely to affect platelets (serotonin release and thromboxane generation) and/or HUVECs (prostacyclin generation). Responses to ADP, collagen and thrombin and in an unstimulated control (RB) were to be assessed and the specific aims were to examine:

1. How the responses of platelets and HUVECs were affected by either the absence or presence of 1.2mM $CaCl_2$.

2. How the responses of platelets and HUVECs were affected by different final incubation times of 2, 5 and 15 minutes.

3. How the responses of isolated platelets and HUVECs were affected by the presence of the alternative cell type when the cells were coincubated.

4. Whether the experimental factors examined produced comparable or different effects on platelet serotonin release and thromboxane generation responses.

Methods

For interaction studies, HUVEC isolation, culturing and subculturing was undertaken as in Chapter 2 (CCM was based upon RPMI 1640). Following 3 passages, HUVECs were seeded at 1.2 x 10^5cells/well into 8 wells of 12 well titre plates and were used 48 hours following seeding. Platelets were washed and labelled using ^{14}C-serotonin as described in Chapter 2. Washed platelets were prepared in both the presence and absence of added 1.2mM $CaCl_2$ and a crude fibrinogen source (platelet-free plasma) was added (at 5µl/ml) to washed platelets, just prior to their use.

Interaction study protocol

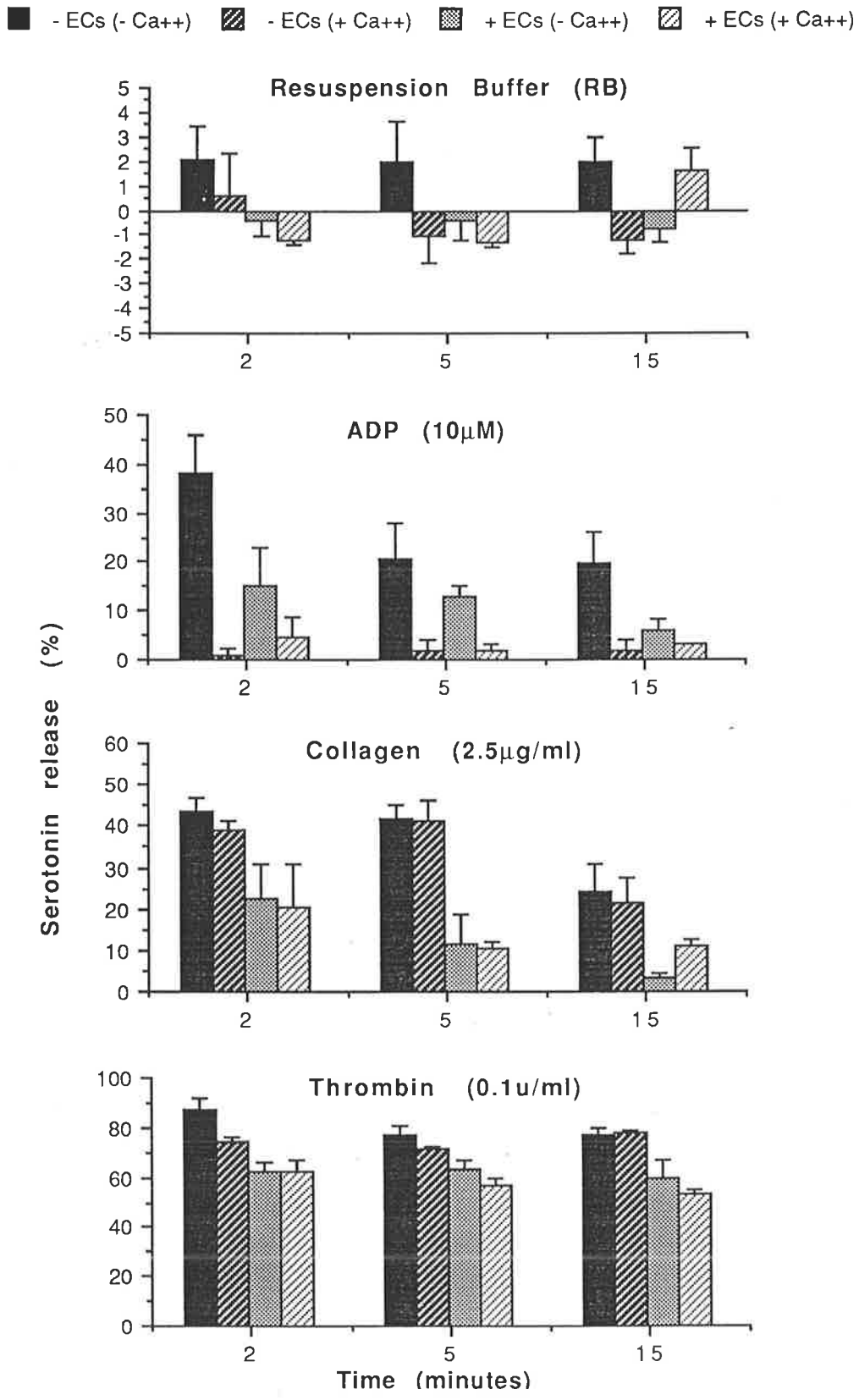
Interaction studies were performed as described in Chapter 2. Briefly, culture plate wells (±HUVECs) were washed twice prior to the addition of platelet or platelet resuspension buffer (RB) (950µl). This was followed by the addition of a stimulus (50µl) or in the control, 50µl of RB, and a final incubation of 2, 5 or 15 minutes. Plate agitation for the final incubation was achieved using a Titertek titre plate shaker (Flow Labs) using a setting of 4. All incubations were performed at 37°C under 5% CO_2 in air. Stimuli assessed included ADP (10µM), collagen (2.5µg/ml), and thrombin (0.1u/ml), and were chosen from a range of concentrations used in pilot interaction studies (data not shown), which resulted in readily detectable platelet or HUVEC responses. The stimuli and treatments were randomised between plates and experiments. However, one final incubation time was used per plate. Following shaking for the specified time, supernatants were immediately removed. Those containing platelets were centrifuged at 8400g for 1 minute and 100µl of the supernatant was used for the determination of % serotonin release. Samples were then stored for RIA assessments of platelet thromboxane generation (measured as TXB_2) and HUVEC prostacyclin generation (measured as 6-keto-$PGF_{1\alpha}$).

Statistical analyses

The unbalanced nature of the studies performed (data were derived from between 2 and 4 experiments) prevented the application of analysis of variance (ANOVA). Comparative statistical analyses were however performed to determine the significance of apparently consistent effects caused by time, the presence of $CaCl_2$ or platelets/HUVECs. Following assessments for heterogeneity or homogeneity of variance, time-dependent effects (between 2 and 15 minutes) were assessed by either Student's paired t test or Wilcoxon's matched-pairs signed-rank test. Multiple paired type comparisons were not undertaken; however for specific unpaired comparisons either Student's unpaired t tests or Mann-Whitney U tests were applied. Significance was accepted at a $p<0.05$ level .

Results

Figure 4a illustrates platelet serotonin release (%) (mean + or - sem; n=2-4) in the RB control and in response to the 3 stimuli indicated. Responses were assessed following 2, 5 and 15 minute incubation times. The presence or absence of HUVECs is shown by ± ECs. The presence (+Ca^{++}) or absence (-Ca^{++}) of $CaCl_2$ (1.2mM) in the supernatant is also shown.



In the RB control, negligible ^{14}C-scrotonin release (mean values < 3%) was obtained. Negative release values were frequently obtained due to the correction factor used to calculate % release. In the absence of HUVECs, the maximal platelet serotonin release was ~40% in response to both collagen and ADP. Thrombin produced relatively high release (~80%) and distinct clumping of platelets was apparent.

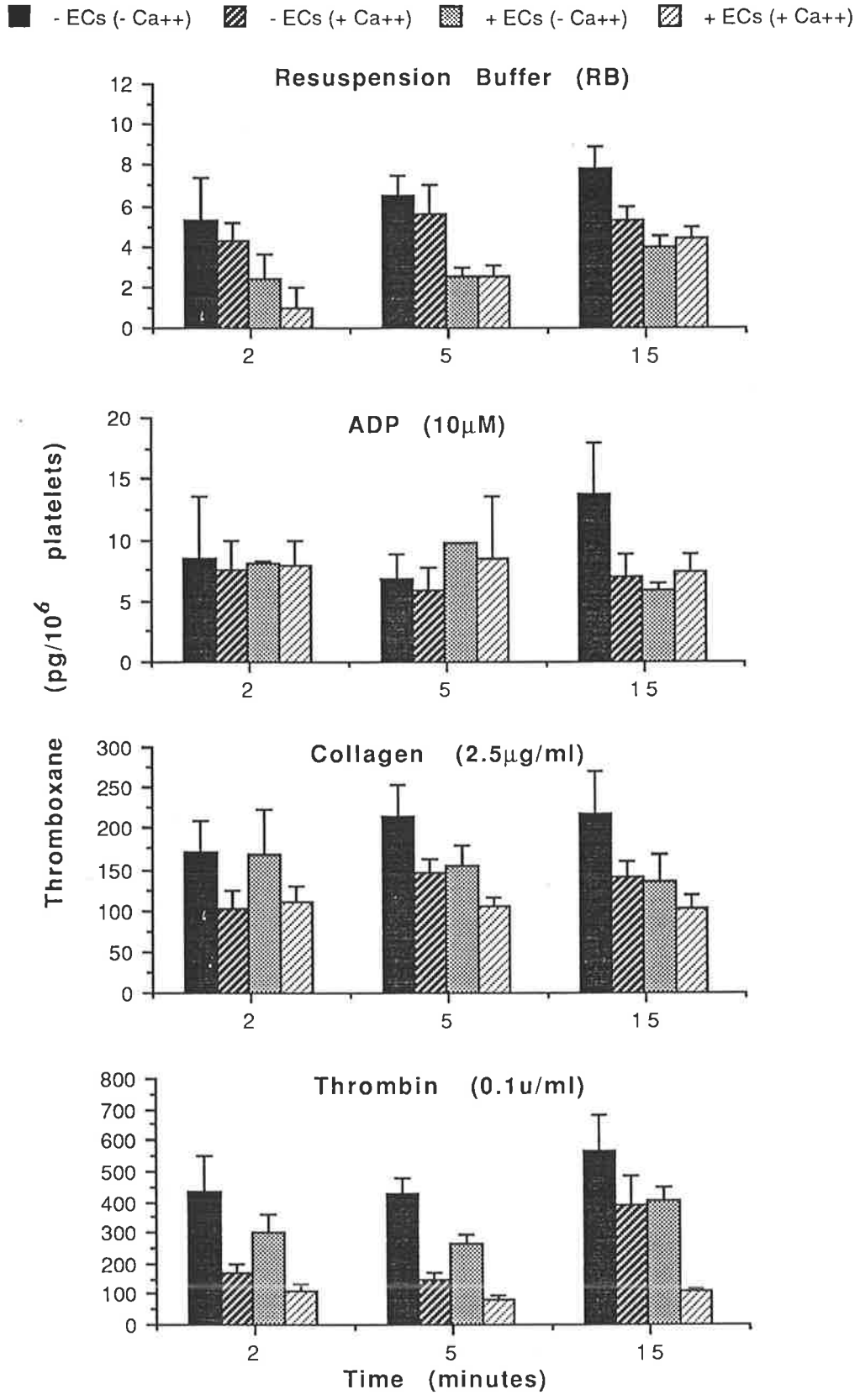
The effects of omitting 1.2mM $CaCl_2$ from, or adding 1.2mM $CaCl_2$ to the buffers used in these studies will be described as effects attributable to the either the absence or presence of $CaCl_2$ respectively. It was assumed however, that where platelets were prepared in $CaCl_2$ free buffers, their final resuspension may still have contained low levels (~20μM) of ionised calcium (Ca^{2+}).

In the presence of $CaCl_2$ negligible serotonin release (mean values < 5%) occurred in response to ADP, whereas up to 40% release was apparent in the absence of $CaCl_2$. Similarly, although not statistically significant, the presence of $CaCl_2$ was also associated with lower serotonin release in the RB control ($p=0.064$). The presence of $CaCl_2$ appeared to produce only minor reductions in serotonin release in response to both collagen ($p=0.386$) and thrombin ($p=0.110$).

Consistent time-dependent effects were not apparent in the RB control or in response to thrombin. In contrast, diminution of serotonin release between 2 and 15 minutes was apparent where collagen ($p=0.002$) and ADP ($p=0.096$) were used as stimuli. For collagen, this time-dependent reduction in serotonin release was particularly evident in the presence of HUVECs.

The presence of HUVECs significantly inhibited serotonin release responses to RB ($p=0.013$) and (in the absence of added $CaCl_2$) ADP ($p=0.019$). HUVECs also significantly inhibited release in response to collagen and thrombin ($p<0.001$ for both). For responses to RB, ADP and collagen, the inhibitory effects of HUVECs were generally in excess of 50% (compared to responses in the absence of HUVECs) but less than ~20% inhibition was evident when thrombin was used as the stimulus.

Figure 4b illustrates platelet thromboxane generation (pg/10^6 platelets) (mean+sem; n=2-4) in the control RB and in response to the 3 stimuli indicated. Responses were assessed at 2, 5 and 15 minutes. The presence or absence of HUVECs is shown by ±ECs. The presence (+Ca^{++}) or absence (-Ca^{++}) of $CaCl_2$ (1.2mM) in the supernatant is also shown.



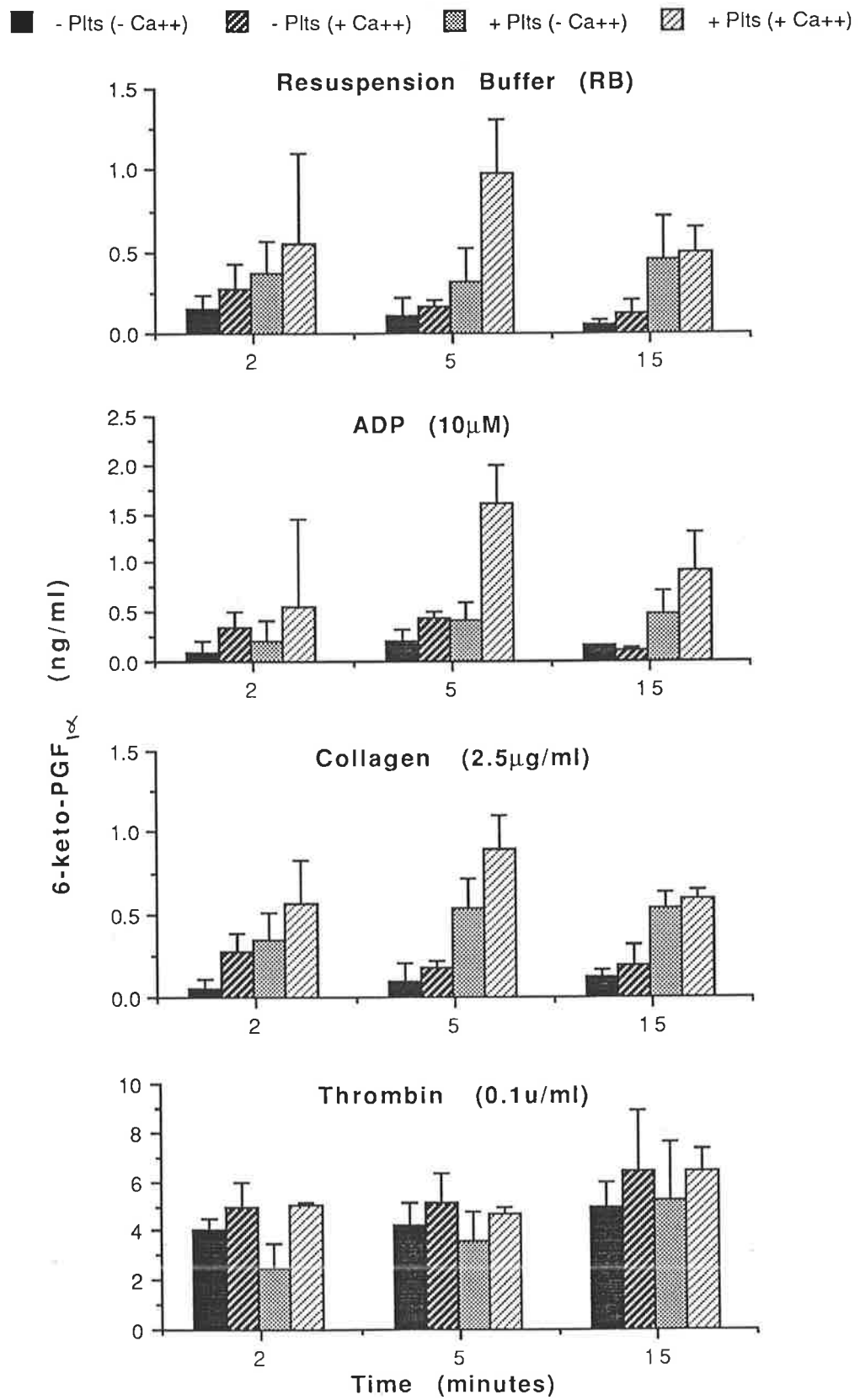
In the RB control, low but consistently detectable thromboxane generation was observed (RIA detection limit equivalent to ~ 0.16 pg/10^6 platelets). Thromboxane generation in response to ADP was similar or marginally greater than seen in the control. In contrast, collagen and thrombin evoked high levels of thromboxane generation approximately ~30 to 60 times that observed in the control (-HUVECs,-$CaCl_2$).

No significant, time-dependent accumulation of thromboxane generation was observed in the control or in response to ADP or collagen. In response to thrombin however, between 2 and 15 minutes, a near significant increase in thromboxane generation was apparent ($p=0.080$). Maximal generation generally occurred by 5 or 15 minutes and it was apparent that the majority of thromboxane generation occurred prior to the 2 minute time point.

The greatest thromboxane generation responses were generally observed in the absence of both $CaCl_2$ and HUVECs. Thromboxane generation responses in the presence of $CaCl_2$ were frequently lower than in the in the absence of $CaCl_2$ and this trend reached significance for the stronger stimuli, collagen ($p=0.001$) and thrombin ($p<0.001$). For these stimuli the presence of $CaCl_2$ was frequently associated with a marked 30-50% reduction in the mean thromboxane generation. In the RB control and in response to ADP, the effects of the presence of $CaCl_2$ on thromboxane generation were less marked and less consistent than for the stronger stimuli. Interestingly for the collagen and thrombin stimulus groups in which the effects of $CaCl_2$ on thromboxane generation were most pronounced, the effects of $CaCl_2$ on serotonin release responses (Figure 4a) were least evident.

The presence of HUVECs provided significant inhibition of thromboxane generation in response to RB ($p<0.001$) and thrombin ($p=0.018$), and near significant inhibition of the response to collagen ($p=0.058$). For ADP, HUVECs failed to inhibit thromboxane generation which contrasted with the pronounced inhibitory effects of HUVECs on serotonin release responses (Figure 4a; in the absence of $CaCl_2$).

Figure 4c illustrates prostacyclin generation by HUVECs measured as 6-keto-$PGF_{1\alpha}$ (ng/ml) (mean+sem; n=2-4) in the control (RB) and in response to the 3 stimuli indicated. Responses were assessed following 2, 5 and 15 minute incubation times. The presence or absence of platelets is shown by ±Plts. The presence ($+Ca^{++}$) or absence ($-Ca^{++}$) of $CaCl_2$ (1.2mM) in the supernatant is also shown.



Prostacyclin generation responses (as measured by 6-keto-$PGF_{1\alpha}$) were similar in magnitude for RB, ADP and collagen. In contrast, thrombin produced relatively strong prostacyclin generation.

Prostacyclin generation by HUVECs did not show consistent time-dependent cumulative effects and the majority of prostacyclin generation occurred by the 2 or 5 minutes time points. Only in response to thrombin was a significant ($p=0.043$) increase in generation apparent between 2 and 15 minutes.

The presence of 1.2mM $CaCl_2$ was associated with a minor but significant enhancement of prostacyclin generation in response to ADP ($p=0.023$), collagen ($p=0.042$) and thrombin ($p=0.013$). This increase failed to reach significance in the control (RB) ($p=0.097$).

The presence of platelets also produced significant enhancement of prostacyclin generation in response to RB ($p=0.002$), ADP ($p=0.005$) and collagen ($p<0.001$). For thrombin, this platelet associated enhancement effect was not observed.

There was evidence which suggested that additive effects may occur between the $CaCl_2$ and platelet associated enhancement of prostacyclin generation in response to RB, ADP and collagen. Indeed it was clear that the highest prostacyclin generation occurred consistently when $CaCl_2$ and platelets were combined.

It was noted that the platelet associated enhancement in prostacyclin generation occurred to a similar extent in the presence of activated platelets (stimulated with collagen or ADP) and in the RB control. Also, activated platelets did not confer time-dependent effects onto prostacyclin generation responses. Where, in response to thrombin, time-dependent effects were observed, these occurred both in the absence and presence of platelets, and therefore did not appear to be platelet-dependent.

Discussion

Effects of experimental factors on platelet responses

From the results illustrated in Figure 4a, the platelet serotonin release responses to ADP were of particular interest. Of the stimuli assessed, the serotonin release responses to ADP were affected to the greatest extent by the ionised $CaCl_2$ concentration present in the platelet resuspension buffer .

It has been reported previously (Packham, Kinlough-Rathbone & Mustard, 1987 and Packham *et al,* 1989), that in the presence of low ionised calcium concentrations (~20μM) and a fibrinogen source, ADP (2-5μM) evokes two phases of aggregation and only the second extensive aggregation phase is associated with thromboxane generation and serotonin release. In contrast, in the presence of millimolar concentrations of ionised calcium, only a single reversible aggregation response is observed and serotonin release does not occur.

The results of the preliminary aggregometry studies (data not shown) were consistent with the findings described above (Packham *et al,* 1989) and in the presence of a fibrinogen source and physiological (1.2mM) calcium concentrations, reversible aggregation responses to ADP were observed with no detectable serotonin release. In contrast, in the absence of $CaCl_2$, extensive aggregation responses and measurable serotonin release were apparent.

Similarly in the studies presented in Figure 4a, serotonin release responses of up to ~40% occurred in response to ADP in the absence of added $CaCl_2$, but in the presence of added 1.2mM $CaCl_2$, less than 5% serotonin release was observed.

It was noted by Packham *et al* (1989) that during extensive aggregation in response to 5μM ADP, thromboxane generation of 22pg/10^6 platelets occurred. In that study, with increasing ionised calcium concentrations there was a reduction in thromboxane generation, coincident with diminished serotonin release. This implied that secondary aggregation and serotonin release in response to ADP may be thromboxane-dependent. Certainly others have reported that

serotonin release to ADP is highly thromboxane-dependent (Best *et al* 1980; Packham, Kinlough-Rathbone & Mustard, 1987). In contrast to their findings, in the present studies (Figure 4b) irrespective of the presence or absence of $CaCl_2$, ADP produced thromboxane generation similar to that observed in the control and only in one instance (15 minutes in the absence of added $CaCl_2$) did thromboxane generation appear elevated ($15pg/10^6$ platelets). No reason for the lack of responsiveness to ADP at 2 and 5 minutes (or relatively great generation at 15 minutes) could be found. Certainly it was anticipated that greater thromboxane generation in response to ADP would have been seen in association with the considerable serotonin release responses observed. In addition, except at 15 minutes, neither HUVECs nor $CaCl_2$ provided detectable inhibition of thromboxane generation whereas such inhibition was observed for the other stimuli. This was another unexpected result which cast doubt on the validity of the thromboxane generation data at the 2 and 5 minute time points.

With respect to other platelet stimuli, Packham *et al* (1989) observed that in response to thrombin (0.05-0.08u/ml), and collagen (0.25µg/ml and 0.4µg/ml), serotonin release (particularly at higher concentrations) was not greatly affected by differing ionised calcium concentrations. Similarly, in the present studies (Figures 4a and 4b) serotonin release in response to thrombin and collagen was not significantly lowered in the presence of 1.2mM $CaCl_2$. Furthermore, again consistent with previous reports (Packham *et al,* 1989), thromboxane generation in response to thrombin was clearly lower in the presence of 1.2mM $CaCl_2$, ($p<0.001$) and a similar trend was observed with collagen ($p=0.001$).

Time-dependent effects on serotonin release responses reached significance or near significance only when ADP or collagen were used as stimuli. For these stimuli, the greatest platelet serotonin release was observed at 2 minutes and reduced release was observed at the subsequent 5 and/or 15 minute time points. The reduction in the response to ADP over time may have occurred because of ADP metabolism, but such effects could not explain the same occurrence observed over time when collagen was used as a stimulus. The most plausible explanation for the time-dependent effects was that serotonin re-uptake (by platelets or HUVECs) from the supernatant buffer was occurring.

As described in Chapter 2, the passaged HUVECs used for experiments, even when exposed over a period of up to 30 minutes, could not accumulate detectable amounts of serotonin (even in the presence of a monoamine oxidase inhibitor). Furthermore, had HUVECs been capable of detectable serotonin uptake, then time-dependent reductions in apparent platelet serotonin release responses should only have been observed in the presence of HUVECs, and not in their absence and this was not evident. In addition such effects should have been seen for all the stimuli used, and was not.

Because HUVECs could not be considered responsible for time-dependent serotonin uptake, the uptake of serotonin by platelets between 2 and 15 minutes offered the only alternative explanation. Certainly, it is recognised that platelet release of serotonin does not prohibit re-uptake, and investigators sometimes use serotonin uptake inhibitors such as imipramine to prevent this occurrence (Packham, Kinlough-Rathbone & Mustard, 1987; Packham *et al,* 1989). The reason behind the detection of such re-uptake in response to collagen and to a lesser extent ADP could have been related to the similar magnitude of the initial (2 minute) serotonin release responses evoked by these stimuli. Such re-uptake may not have been observed in the RB control because of the negligible background release levels, and in the thrombin stimulus group platelet responses were possibly too extensive and irreversible to allow the detection of re-uptake. Although not shown, evidence in favour of serotonin re-uptake by platelets came from the preliminary aggregometry studies where fluoxetine (0.5μM) was used as an uptake inhibitor, and caused an increase in measured serotonin release in response to various stimuli including ADP and collagen.

Time-dependent effects on platelet thromboxane generation were also of interest. No significant time-dependent effects were evident and platelet thromboxane generation responses were generally maximal by 5 minutes following stimulus addition. The absence of consistent time-dependent effects was in keeping with the ability of platelets to undergo a rapid burst of arachidonic acid liberation with subsequent thromboxane generation (complete within ~20 seconds of platelet activation)(Marcus, 1987).

Finally, in the present studies, the effects of co-incubating platelets and HUVECs on platelet responses was a point of some interest. In all stimulus groups, and in the RB control, the presence of HUVECs was associated with significant inhibition of platelet serotonin release, although the extent of such inhibition differed between stimuli. Discrepancies were also evident between the effects of HUVECs on platelet serotonin release and thromboxane generation. That is, although HUVECs inhibited platelet serotonin release significantly in all the stimulus groups, they only produced significant inhibition of platelet thromboxane generation in response to thrombin, and in the RB control.

Relationships between measured parameters of platelet activation

Discrepant effects of the experimental factors (such as the effects of $CaCl_2$ or HUVECs) upon the two measured parameters of platelet activation, thromboxane generation and serotonin release were not unexpected. Indeed one would only have expected these parameters to be affected in a parallel fashion if a close linear relationship existed between thromboxane generation and platelet serotonin release. It is known that such a simple relationship does not exist between these platelet responses, and the dependence of serotonin release on thromboxane generation differs between stimuli and between different stimulus concentrations. For example for ADP and for collagen and thrombin at low concentrations, platelet aggregation and release may depend upon thromboxane generation. In contrast at higher concentrations of collagen and thrombin, platelet aggregation and release may become thromboxane independent (Best *et al,* 1980; Emms and Lewis, 1986; Ambler *et al,* 1985). The complex relationship between these parameters could explain many of the discrepant results observed in the present studies between thromboxane generation and serotonin release responses.

For example the reason behind the considerable effects of $CaCl_2$ and HUVECs on thromboxane generation in response to thrombin (0.1u/ml) and the undetectable or relatively minor effects of these factors on serotonin release may have been attributable to serotonin release in response to this stimulus at 0.1u/ml being essentially thromboxane independent. It was however expected that for collagen a closer relationship between thromboxane generation

and serotonin release would have been apparent. Certainly where low collagen (< 4μg/ml) concentrations are used, serotonin release has been shown to be thromboxane-dependent (Best *et al,* 1980). It was considered likely that the use of HUVECs in these studies added another confounding element to the relationship between the measured parameters of platelet activation. Due to the multiple platelet inhibitory effects of mediators derived from HUVECs, the relationship between thromboxane generation and serotonin release responses may well have differed in the presence or absence of HUVECs. This question concerning the relationship between thromboxane generation and serotonin release was explored further in subsequent chapters (Chapters 5 and 6).

Effects of experimental factors on prostacyclin generation from HUVECs. Possible involvement of prostacyclin in HUVEC-mediated platelet inhibition?

In viewing the prostacyclin generation results (Figure 4c), under these experimental conditions ADP and collagen appeared to be essentially platelet specific stimuli as they were able to evoke platelet serotonin release of ~40% but caused no clear increases in prostacyclin generation responses from HUVECs. Although prostacyclin generation in the RB control, ADP and collagen groups was low, the 6-keto-$PGF_{1\alpha}$ levels (in the presence of platelets) represented prostacyclin concentrations of between 1 and 1.6nM. Nanomolar (>1nM) concentrations of prostacyclin are able to inhibit platelet aggregation (Radomski, Palmer & Moncada, 1987a) and serotonin release (Krishnamurthi, Westwick & Kakkar, 1984) in response to a range of platelet stimuli (including ADP, and low concentrations of collagen and thrombin). Thus, it was considered possible that even in the RB control, prostacyclin generation by HUVECs may have participated in their platelet inhibitory effects. Indeed, significant inhibition of platelet serotonin release by HUVECs was observed even in the RB control, and HUVEC-mediated inhibition of platelet serotonin release responses to ADP and collagen generally exceeded 50%. Thromboxane generation in response to RB and to a lesser extent collagen were also inhibited by the presence of HUVECs. The lack of effect of HUVECs on ADP induced thromboxane generation at 2 and 5 minutes as mentioned earlier could not be explained unless thromboxane generation in the absence of HUVECs was not representative of typical responses.

In contrast to ADP and collagen, thrombin not only stimulated platelet activation but was also a potent stimulus of prostacyclin generation. Despite high prostacyclin generation in response to thrombin (6-keto-$PGF_{1\alpha}$ represented prostacyclin levels of 13-18nM) and significant inhibition of platelet thromboxane generation and serotonin release by HUVECs, the inhibition, particularly of serotonin release was less pronounced (<20%) than for the other stimuli. This was possibly caused by the aggregation response to thrombin (which caused ~80% serotonin release) being less reversible in nature than responses to the other stimuli. Certainly others have demonstrated that in washed platelets stimulated with 0.1u/ml thrombin, prostacyclin concentrations in excess of 150nM are required to inhibit serotonin release responses (Krishnamurthi, Westwick & Kakkar, 1984). Further analyses of associations between HUVEC-mediated inhibition of platelet responses and prostacyclin generation responses were undertaken in subsequent studies (Chapter 5).

The results illustrated in Figure 4c showed that HUVECs in the presence of $CaCl_2$ but in the absence of platelets (conditions most similar to those used in Chapter 3) generally generated more prostacyclin than in the studies reported in Chapter 3. The increased responses in the present studies were possibly attributable to the use of an agitation system, changes made to the preincubation wash times (extended from 10 minutes up to 20 minutes) or the different supernatant buffer used in these studies.

In viewing possible time-dependent effects on prostacyclin generation, it was apparent that in response to all stimuli, the majority of prostacyclin generation occurred within 2 or at the most, 5 minutes of HUVEC stimulation. This was supported by the lack of time-dependent cumulative effects observed between 2 and 15 minutes (significant in only one instance). Former investigators have indicated that in a closed system similar to that used in these studies, maximal prostacyclin generation in response to thrombin occurred within 2 to 5 minutes of initiation (Czervionke *et al*, 1979a). Those results were consistent with the findings in the present study. This may have been attributable to the maximal liberation of arachidonic acid for a given stimulus occurring shortly following HUVEC activation. The results were also consistent with, in response to stimuli such as thrombin (and arachidonic acid), prostacyclin

generation being limited by irreversible inactivation of cyclooxygenase, caused by products formed as a result of its own activity (Dejana *et al,* 1983; Brotherton & Hoak, 1983).

The presence of added 1.2mM $CaCl_2$ was generally associated with higher prostacyclin generation. Previous investigators have indicated that prostacyclin generation from endothelial cells is unaffected by the absence of extracellular $CaCl_2$ (White & Martin, 1989; Carter *et al,* 1988) and have concluded that internal stores of calcium are sufficient to allow PLA_2 activation and maintain prostacyclin generation responses. Others have found that low extracellular calcium can reduce prostacyclin generation responses from endothelial cells (Brotherton & Hoak, 1982) and that in response to stimulation, optimal PLA_2 activity and arachidonic acid release appears to require the influx of external calcium (Buckley *et al,* 1991). The present findings were consistent with the latter study and were in accordance with the concept that physiological concentrations of external calcium are not essential for prostacyclin generation, but that the presence of such concentrations enable endothelial cells to produce greater (possibly optimal) generation of prostacyclin.

Platelet-associated enhancement of prostacyclin generation from HUVECs

The presence of platelets also appeared to enhance prostacyclin generation responses. Notably this effect was significant for the weaker stimuli (e.g to ADP, RB and collagen), which alone (without platelets) stimulated only minor prostacyclin generation. The effect was not apparent when thrombin was used as a stimulus which even in the absence of platelets evoked relatively high levels of prostacyclin generation compared to the other stimuli. Possibly, for thrombin, the lack of further enhancement in the presence of platelets was attributable to a near maximal prostacyclin generation response even in the absence of platelets, such that further enhancement was not possible. In addition, for the weaker stimuli the greatest prostacyclin generation was observed in the presence of both added $CaCl_2$ and platelets, suggesting possible additive effects of these factors.

Two possible reasons behind the platelet associated enhancement in prostacyclin generation were considered. Firstly, the presence of aggregating platelets may have caused HUVEC injury,

and thus have stimulated prostacyclin generation. Certainly others have found a relationship between endothelial cell injury and and prostacyclin generation (Brox & Nordoy, 1982) and such injury has been described in response to thrombin itself as well as collagen or thrombin stimulated platelets (Jorgensen *et al*, 1986; Kishi & Numano, 1989). Such reports typically show that endothelial cell injury increases with the time of exposure of endothelial cells to the source of injury. In the studies described in this chapter, there was no evidence that prostacyclin generation was stimulated between 2 and 15 minutes, except in the thrombin stimulus group, the only group in which platelet-associated enhancement failed to reach significance. Furthermore, if the presence of platelets caused HUVEC injury and prostacyclin generation, then it would be expected that greater platelet-associated enhancement of prostacyclin generation would occur in the presence of activated platelets than in the control, and this was not observed.

A second possible explanation for the platelet-associated enhancement of prostacyclin generation was that products liberated from aggregating platelets (such as endoperoxides, thromboxane etc.) could cause enhancement of prostacyclin generation from HUVECs. If this was the case, then (as for effects of injury to HUVECs) the degree of enhancement in the stimulated groups should have been greater than in the control, and this was not seen.

It was possible that some minor participation of both injury and effects of platelet-derived factors may have been involved in the platelet-associated enhancement of prostacyclin generation observed in these studies. In particular, in the groups in which platelets were activated, the possibility that platelet-derived products may have affected prostacyclin generation from HUVECs could not be excluded. Consequently, the mechanisms of the enhanced prostacyclin generation seen in the presence of stimulated platelets were examined further in subsequent studies (chapters 5 and 6).

Conclusions and aims of future investigations

It was evident that in the absence of 1.2mM $CaCl_2$, prostacyclin generation responses by HUVECs were compromised. This was consistent with optimal arachidonic acid liberation and therefore prostacyclin generation by activated endothelial cells depending upon the influx of

external $CaCl_2$. Because the platelet inhibitory effects of prostacyclin were of interest, it was desirable to optimise its generation, and therefore it was decided that future studies would proceed with the inclusion of 1.2mM $CaCl_2$ in all buffers. In addition, to provide greater prostacyclin levels per well, and possibly achieve more pronounced platelet inhibitory effects of HUVECs in future experiments, the HUVEC seeding density was raised from 1.2 x 10^5cells/well to 1.8-2.0 x 10^5 cells/well (12 well culture plates).

The inclusion of 1.2mM $CaCl_2$ meant that ADP would be unable to stimulate detectable platelet serotonin release. As a consequence, responses to ADP were not examined further in this thesis. Because responses to the other stimuli used did not require the addition of a fibrinogen source, it was omitted in subsequent studies. From the present studies it was apparent that maximal thromboxane generation and prostacyclin generation responses occurred by 5 minutes. There was also, in response to collagen, a significant time-dependent re-uptake of serotonin into platelets. Based upon these results a standard final incubation time of 5 minutes was selected for ongoing studies.

The greatest serotonin release responses were not always observed in the presence of the greatest thromboxane generation. Factors (e.g the presence of $CaCl_2$ or HUVECs) which affected one parameter of platelet activation did not necessarily affect the other in a similar manner. Therefore the relationships between the two measured parameters of platelet activation in this system needed clarification. An analysis of whether prostacyclin generation could be linked to platelet inhibition was also of interest. Finally, there were trends which suggested that in the presence of platelets, prostacyclin generation from HUVECs was enhanced, and whether indices of platelet activation may be associated with this effect required further evaluation.

To examine such relationships in greater depth, it was considered that more information could be obtained from examining platelet and HUVEC responses to a range of concentrations of a given stimulus, rather than a single stimulus concentration. Therefore, platelet and HUVEC responses to a range of collagen and thrombin concentrations were assessed in subsequent studies.

Chapter 5

Effects of single and combined stimuli on platelet and HUVEC responses when incubated independently and in combination

Introduction

The initial investigations of platelet and HUVEC interactions (described in the previous chapter) indicated that a number of factors affected both the platelet and HUVEC responses. These studies involved the use of single stimuli at single concentrations and dose-response effects were not evaluated.

In those studies, it was observed that platelet serotonin release responses and thromboxane generation responses were frequently at variance. It was therefore of interest to examine in greater depth the possible relationships which may exist between these indices of platelet activation. Some assessment of relationships, if apparent, between levels of HUVEC prostacyclin generation and levels of HUVEC-mediated platelet inhibitory effects was also in order. Finally, in the previous chapter, platelet-associated enhancement of prostacyclin generation was observed. The extent to which this phenomenon occurred appeared unrelated to differing levels of platelet activation. It was decided however, in the present studies, to examine further whether any relationship could be seen between indices of platelet activation (serotonin release and thromboxane generation) and the extent of platelet-associated enhancement.

Another area of interest not evaluated in the prior studies was the effect of combined stimuli on both platelets and HUVECs. It was demonstrated in Chapter 3 that adrenaline did not stimulate prostacyclin generation from HUVECs. Adrenaline can however affect platelet activation by enhancing their responses to other stimuli (Keraly *et al,* 1988; Lanza *et al*, 1988; Vanags *et al*, 1992). It has also been reported that the platelet inhibitory effects of prostacyclin may be reduced by adrenaline (Buttrick *et al*, 1985). Such observations have led to speculation that *in vivo*, when catecholamine levels are elevated (during for example myocardial infarction), platelets may become unresponsive to vascular cell-derived prostacyclin. Consequently in the present series of experiments the effects of adrenaline alone and in combination with other stimuli were

examined to assess how this stimulus affected platelets and also whether it influenced HUVEC inhibition of platelet responses.

In the studies presented in this chapter a higher final seeding density of HUVECs was employed compared to that used in the initial studies. Consequently, it was anticipated that prostacyclin generation would be higher and that platelet inhibitory effects of HUVECs may be more pronounced than in earlier studies. In addition, cell counts were performed at the end of each experiment to allow prostacyclin generation to be standardised to these counts and to correct for variance attributable to slight differences in seeding efficiencies between experiments.

Aims

The experiments undertaken in this chapter were performed in order to examine:

1. The concentration-dependent effects of collagen and thrombin on platelet and HUVEC responses when these cells were examined independently and in combination.

2. The effects of combined stimuli on platelet and HUVEC responses, when these cells were examined independently and in combination.

3. Relationships between platelet thromboxane generation and serotonin release responses and those between HUVEC prostacyclin generation responses and the extent of platelet inhibition provided by HUVECs.

4. Relationships between measured parameters of platelet activation and the extent of platelet-associated enhancement of prostacyclin generation.

Methods

HUVECs were isolated, cultured (using CCM based upon medium 199) and subcultured as described in Chapter 2. Platelets were washed and labelled with ^{14}C-serotonin also as described in Chapter 2. All buffers used for their preparation included 1.2mM $CaCl_2$. A fibrinogen source was not used in any of the studies. The HUVEC seeding density/well used for experiments was increased over that used for Chapter 4, such that HUVECs were seeded into wells of 12 well culture clusters using a seeding density of 1.8-2.0 x 10^5cells/well. Plates were used 48 hours following seeding and at the termination of the experiment cells from 10% of the wells were resuspended and counted to determine a representative estimation for cells/well for the experiment and in this series of experiments, this was found to be 1.99±0.29 x 10^5cells/well (mean ±sem, n=5).

Interaction study protocol

For a typical 12 well culture plate, 8 of the 12 wells were seeded with endothelial cells. Following dual washing of all wells, washed platelets or RB were aliquoted into all wells (950µl per well) such that 4 wells contained platelets alone, 4 wells contained endothelial cells and RB and 4 wells contained both platelets and endothelial cells.

Four stimuli could be applied per plate. A stimulus (or paired stimuli) was added (50µl) bringing the total well volume to 1 ml. Plates were then shaken on a Titertek titre plate shaker at setting number 4 for 5 minutes at 37°C under 5% CO_2 in air. Stimuli included RB (as a control), collagen (1 - 10 µg/ml), thrombin (0.02 - 0.1 u/ml), and adrenaline (0.05 or 10µM). Platelet activation was determined by assessments of % ^{14}C-serotonin release and thromboxane A_2 generation, measured as TXB_2 and expressed as pg/10^6 platelets. Endothelial cell prostacyclin generation, measured as 6-keto-$PGF_{1\alpha}$, was expressed as ng/10^6 cells. The inhibitory effects that HUVECs had on activated platelet responses were also assessed.

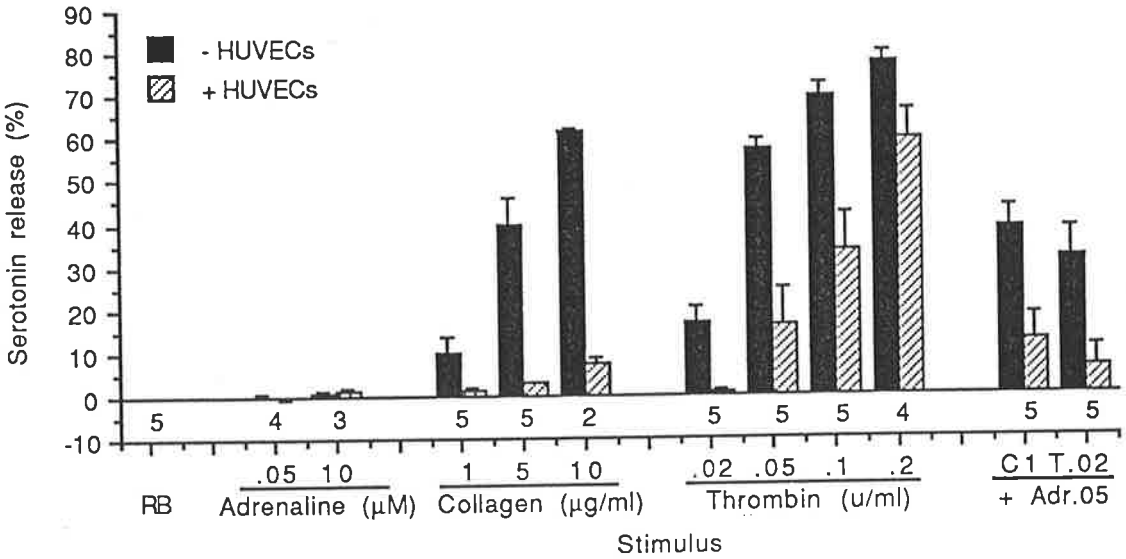
Statistical analyses

As described in Chapter 2, TXB_2 and 6-keto-$PGF_{1\alpha}$ data were log transformed prior to analysis. Data for % platelet serotonin release were left untransformed.

Due to imbalanced data ($n<5$ in some stimulus groups) ANOVA was not applied to the data. To avoid multiple paired comparisons the data from the collagen and thrombin stimulus groups were assessed using unpaired tests to determine the significance of platelet inhibitory effects of HUVECs, or platelet-associated enhancement of prostacyclin generation from HUVECs. Paired comparisons were only made in the RB control and where adrenaline was used alone or in the combined stimulus groups. Depending upon the homogeneity or heterogeneity of the data, parametric or or non-parametric comparisons were made. Unpaired comparisons were made by either Student's unpaired t or Mann-Whitney U tests. Paired comparisons were made by Student's paired t tests. Significance was accepted at a $p<0.05$ level.

For collagen and thrombin dose-response data, a number of correlation analyses were performed to determine how the dependent variables such as serotonin release and thromboxane generation related to stimulus concentrations. Correlation analysis was also performed to determine the significance of relationships between independent variables (Bolton, 1984). For a number of these analyses, data were plotted and lines of best fit were determined (with their associated coefficients of determination) to illustrate the form of any relationship between correlated parameters.

Results: Figure 5a shows % platelet serotonin release (expressed as mean +or - sem) in response to the stimuli or stimulus combinations indicated. RB=resuspension buffer (control), C=collagen, T=thrombin and Adr=adrenaline. The results were from 5 experiments unless otherwise indicated by n numbers below the 0% line.

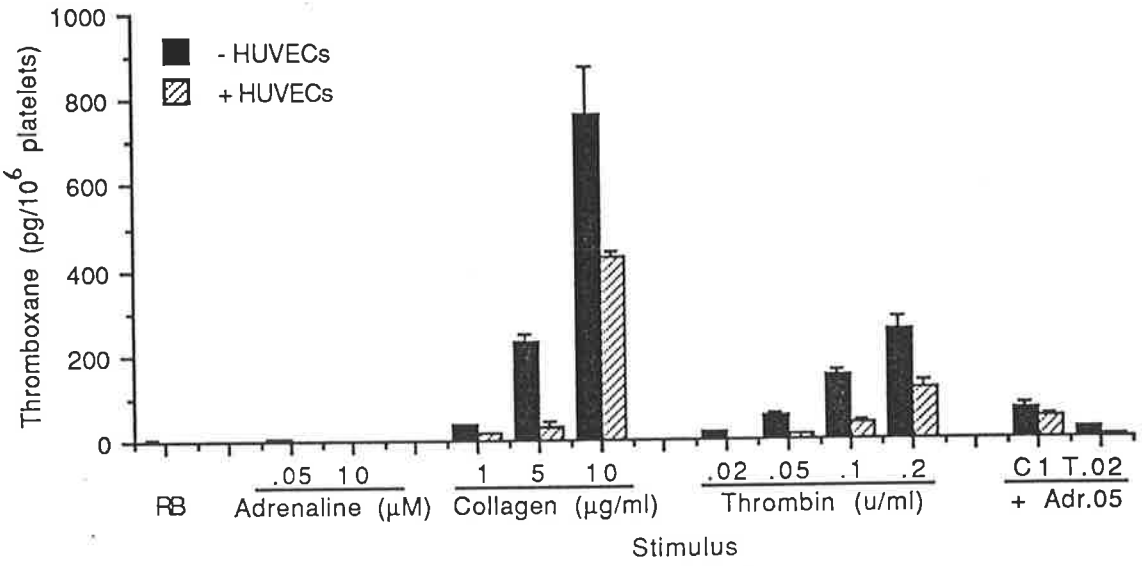


Collagen and thrombin produced concentration-dependent increases in platelet serotonin release reaching (in the absence of HUVECs) maximal levels of 62% and 78% respectively. Such concentration-dependent effects were apparent both in the absence and presence of HUVECs. In contrast to these stimuli, in response to adrenaline at both 0.05 and 10μM and in the RB control, negligible serotonin release (mean values <1.5%) was evident.

HUVECs caused inhibition of platelet serotonin release in all except the 10μM adrenaline stimulus group. Inhibition reached significance in the thrombin and collagen groups ($p<0.001$) (Student's unpaired t tests or Mann-Whitney U test) but in the RB and adrenaline (0.05μM) groups, inhibition of serotonin release was too minor to reach significance ($p>0.05$)(Student's paired t tests). HUVECs also produced significant inhibition of serotonin release in the combined stimulus groups ($p<0.05$; Students paired t tests).

Where collagen or thrombin were combined with adrenaline (0.05μM), adrenaline produced significant ($p<0.05$) enhancement of responses compared to collagen and thrombin alone (± HUVECs; Mann-Whitney U tests).

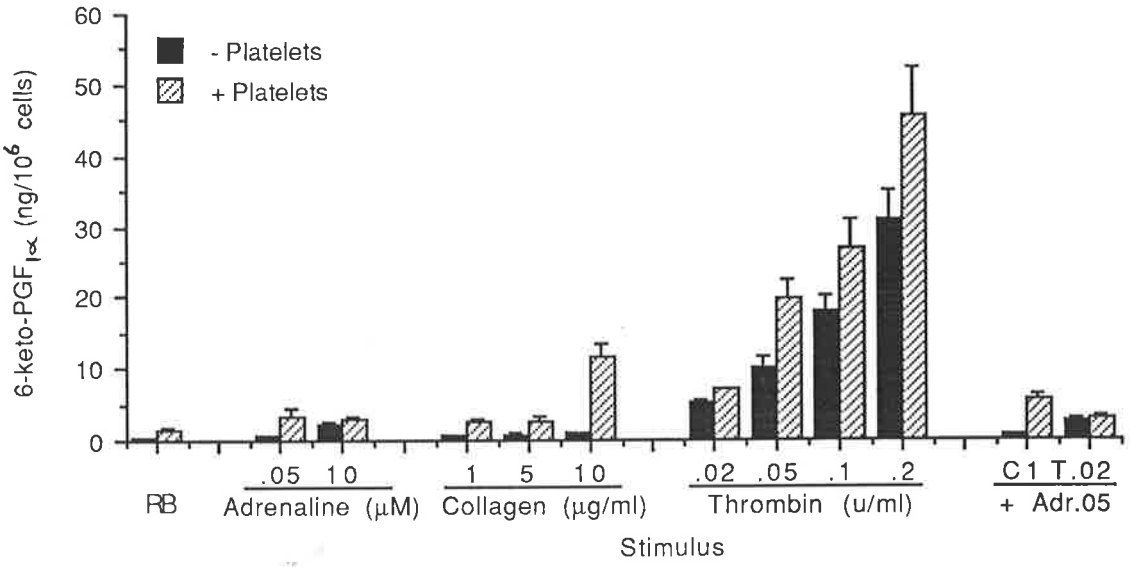
Figure 5b shows platelet thromboxane generation (expressed as gm+gse) in response to the stimuli or stimulus combinations indicated (abbreviations as in Figure 5a) The results were from 5 experiments and the n numbers were as indicated in Figure 5a.



Collagen and thrombin produced concentration-dependent increases in platelet thromboxane generation, in both the absence and presence of HUVECs. The highest thromboxane generation response (in the absence of HUVECs) was far greater for collagen (gm=760pg/10^6 platelets) than thrombin (gm=250pg/10^6 platelets). Thromboxane generation in response to adrenaline (at both 0.05µM and 10µM) was less than 4.0 pg/10^6 platelets and similar to the 3.0 pg/10^6 value in the RB control. HUVECs caused significant inhibition of thromboxane generation in the thrombin ($p<0.002$), collagen ($p<0.05$) and adrenaline ($p<0.05$) stimulus groups (Student's unpaired tests) and also in the RB control (Student's paired t test, $p<0.002$).

Adrenaline (0.05µM) caused significant enhancement in thromboxane generation in response to collagen ($p<0.005$) but not thrombin ($p=0.136$)(Student's unpaired t tests). HUVEC-mediated inhibition of thromboxane generation was significant when thrombin ($p<0.01$) but not collagen was combined with adrenaline (Student's paired t tests).

Figure 5c shows HUVEC prostacyclin generation (measured as 6-keto-$PGF_{1\alpha}$: gm+gse) in response to the stimuli or stimulus combinations indicated (abbreviations as in Figure 5a). The results were from 5 experiments and the n numbers were as indicated in Figure 5a.



In the absence of platelets, thrombin produced concentration-dependent increases in 6-keto-$PGF_{1\alpha}$ generation. In contrast neither collagen nor adrenaline (0.05μM) produced increases in prostacyclin generation above control levels, although a minor increase was observed in response to 10μM adrenaline.

In the presence of platelets consistent enhancement of prostacyclin generation responses was observed which reached significance in the RB ($p<0.05$; Student's paired t test) and collagen stimulus groups ($p<0.002$) and near significance in the thrombin stimulus group ($p=0.069$)(Student's unpaired t tests). Significant enhancement ($p<0.05$) was also evident in the collagen + adrenaline stimulus group (Student's paired t test). In response to collagen, although at 1 and 5μg/ml platelet associated enhancement was not pronounced, at 10μg/ml the extent of enhancement was considerable, reaching generation in excess of that produced by thrombin (0.02u/ml both ±platelets).

In the combined treatments, in response to collagen, and in the presence but not the absence of platelets, adrenaline (0.05μM) caused a significant increase in prostacyclin generation ($p<0.05$). In response to thrombin, adrenaline caused a reduction in prostacyclin generation which reached significance in the presence of platelets ($p=0.011$) (Student's paired t tests).

Correlations : Correlation analyses were performed on the thrombin and collagen induced platelet and HUVEC responses illustrated in Figures 5a-c. More clearly defined relationships were observed in response to thrombin than collagen as more data points were generated for this stimulus (i.e. 4 rather than 3 concentrations were examined).

In response to thrombin, and in the absence of HUVECs, concentration-dependent increases in platelet responses (both serotonin release and thromboxane generation) were evident and are highlighted in the correlation analyses below (Figures 5d and 5e). Clearly, with increases in thrombin concentrations, serotonin release reached a maximal or plateau level. In contrast, thromboxane generation failed to reach a maximal level, even in response to 0.2u/ml thrombin.

Figure 5d

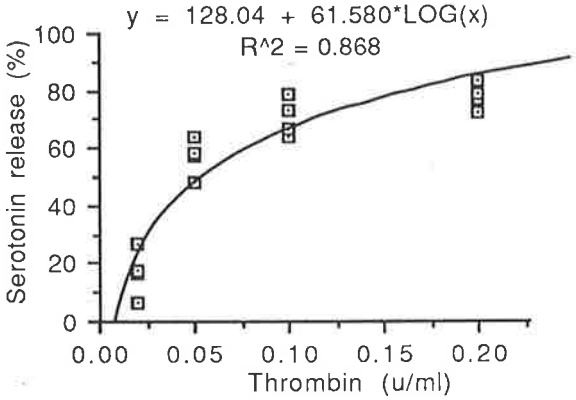
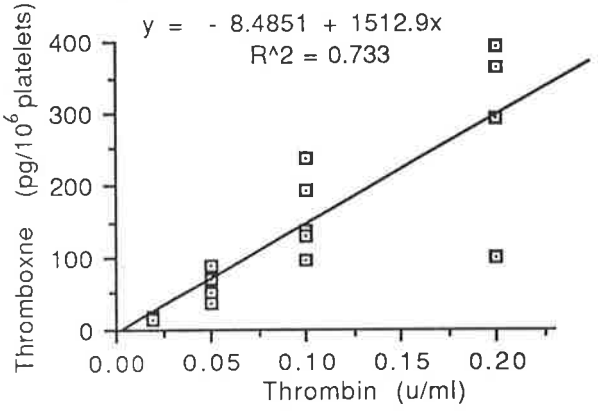


Figure 5e



For collagen, in the absence of HUVECs there was a linear relationship between increasing concentrations and serotonin release and a maximal (plateau) level of release was not observed (R^2=0.797). Over the concentration range examined either a linear (R^2=0.764) or exponential (R^2=0.818) correlation with thromboxane generation produced similar R^2 values. Certainly at 10μg/ml thromboxane generation appeared to begin to increase exponentially but more data points at this concentration would have clarified this trend (graphs not shown).

In the presence of HUVECs, irrespective of the stimulus used, linear increases in serotonin release in response to thrombin, (R^2=0.678) and collagen (R^2= 0.675) were obtained as neither stimulus gave rise to maximal responses. Similarly, thromboxane generation in response to thrombin (R^2=0.583) and collagen (R^2=0.668) also increased linearly with increasing concentrations of these stimuli (graphs not shown).

When the relationship between the variables thromboxane generation and serotonin release was examined, the data illustrated below showed that in the absence of HUVECs, where serotonin release exceeded 50-60%, thromboxane generation appeared to continue to increase exponentially. The figures below show the significant ($p<0.01$) relationships for thrombin (Figure 5f, left) and collagen (Figure 5g, right).

Figure 5f

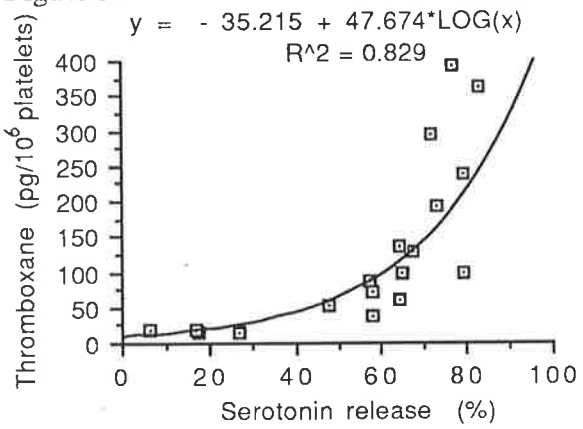
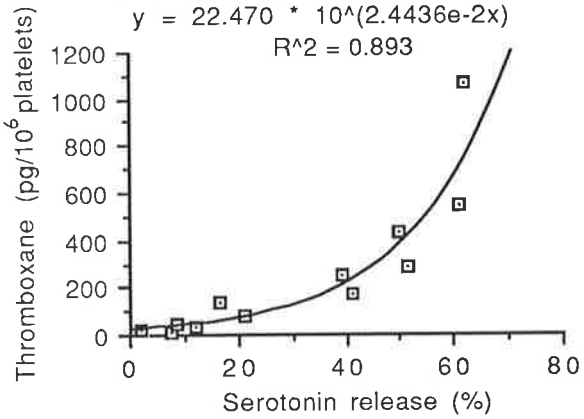


Figure 5g



In the presence of HUVECs the relationship between thromboxane generation and serotonin release remained significant ($p<0.01$) for both thrombin (Figure 5h, left) and collagen (Figure 5i, right). Thromboxane generation in response to thrombin still appeared to increase exponentially where serotonin release exceeded 50-60%. In contrast, for collagen, thromboxane generation increased linearly (rather than exponentially) with increasing serotonin release. Notably in the presence of HUVECs, serotonin release in response to thrombin rarely exceeded 60%, and for collagen, never exceeded 10%.

Figure 5h

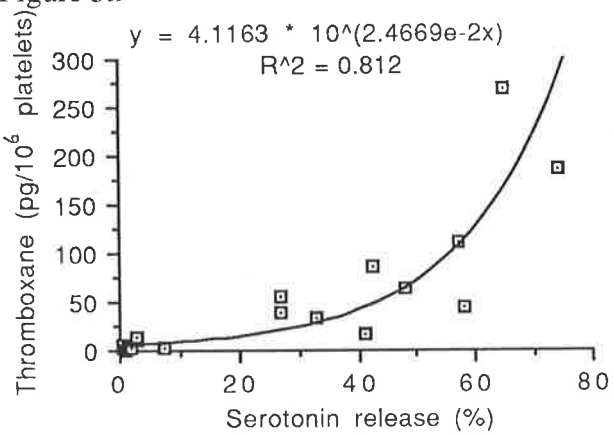
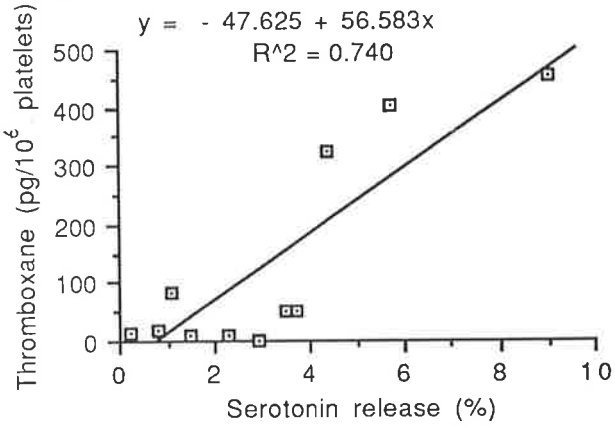


Figure 5i



In response to thrombin, a significant correlation existed between the extent of HUVEC-mediated inhibition of platelet thromboxane generation and serotonin release (R^2=0.631, $p<0.01$). Inhibition of these two indices of platelet activation generally exceeded 20% and above this level they appeared to be inhibited in a near parallel or linearly related manner (Figure 5j, left). Indeed even with a straight line fitted to the data (R^2=0.605) the correlation remained significant ($p<0.01$) (graph not shown).

In contrast, for collagen, irrespective of the level of inhibition of thromboxane generation, HUVECs generally inhibited serotonin release by more than 80%. Thus no correlation existed between the extent of inhibition of thromboxane generation and serotonin release (R^2=0.097, $p>0.05$) (Figure 5k, right).

Figure 5j

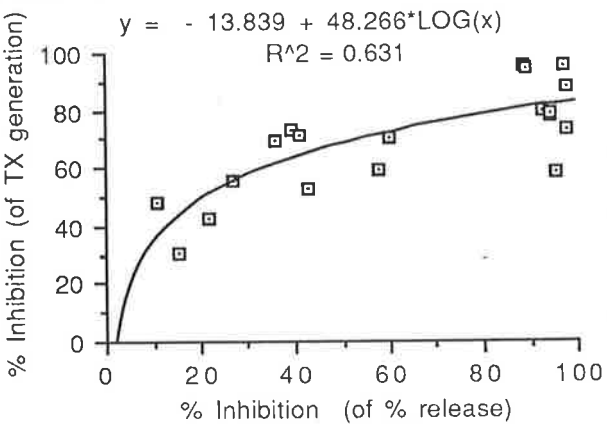
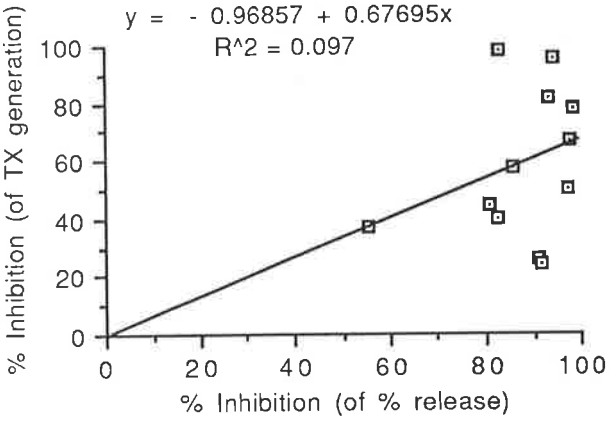


Figure 5k



It was also evident that no significant correlation existed ($p>0.05$) between prostacyclin generation (in the presence of platelets) and the extent of HUVEC-mediated inhibition of platelet responses. This was the case where either thrombin (Figure 5l, left; $R^2=0.113$) or collagen (Figure 5m, right; $R^2=0.001$) were used as stimuli of serotonin release. Similarly, the amount of prostacyclin generated also failed to correlate with HUVEC-mediated inhibition of thromboxane generation where either thrombin or collagen were used as stimuli ($R^2 < 0.037$, $p>0.05$). Graphs not shown.

Figure 5l

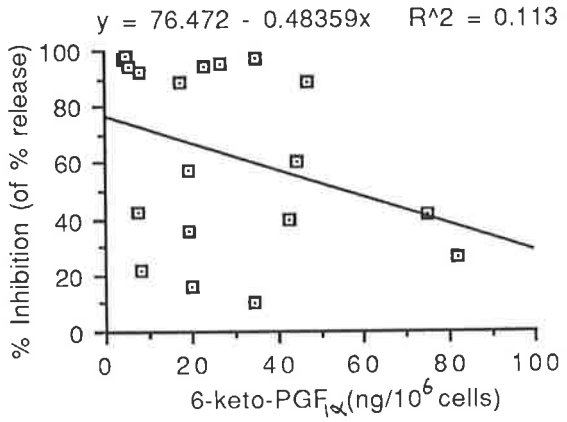
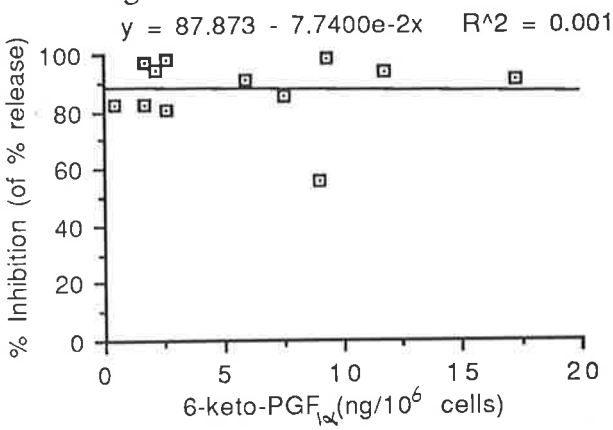


Figure 5m



For thrombin, it was evident that with increasing thrombin concentrations prostacyclin generation increased whereas there was some decrease in the platelet inhibitory effects of HUVECs and these opposing effects would have resulted in the apparent lack of correlation between prostacyclin generation and platelet inhibition observed for this stimulus (Figure 5l). For collagen such trends were less evident and could not explain the lack of correlation between prostacyclin generation and inhibitory effects of HUVECs (Figure 5m). In response to collagen a wider range of prostacyclin generation responses and/or HUVEC inhibitory effects would have assisted in determining whether any correlation could be obtained between these two parameters.

In the present studies, the presence of platelets appeared to be associated with enhancement of prostacyclin generation. Correlation analyses were therefore performed to determine whether in the presence of HUVECs, platelet activation (either serotonin release or thromboxane generation) correlated significantly with the degree of enhancement of prostacyclin generation, described as the prostacyclin generation ratio (+/- platelets). Graphs not shown.

In response to thrombin (and in the presence of HUVECs) no correlation existed between the level of platelet serotonin release (R^2=0.057, $p>0.05$) or thromboxane generation (R^2=0.054, $p>0.05$) and the prostacyclin generation ratio (+/- platelets). Similarly for collagen, no correlation existed between platelet serotonin release in the presence of HUVECs (R^2=0.169, $p>0.05$) and the prostacyclin generation ratio (+/- platelets). A weak but significant linear correlation (R^2=0.481, $0.01<p<0.05$) was however apparent between platelet thromboxane generation in the presence of HUVECs and the prostacyclin generation ratio. In contrast no correlation was seen (R^2=0.132, $p>0.05$) between platelet thromboxane generation in the absence of HUVECs and the prostacyclin generation ratio. Thus the significant relationship between these parameters only existed when platelets and HUVECs were combined.

Discussion

The primary objectives of these studies were to analyse relationships between platelet responses (serotonin release and thromboxane generation) and those between platelet and HUVEC responses, using the data derived from collagen and thrombin concentration-response curves. The present studies were however also used to examine the effects of adrenaline alone and in combination with other stimuli.

Effects of adrenaline on platelet and HUVEC interactions

The possibility existed that in the presence of adrenaline, the platelet inhibitory effects of HUVEC-derived prostacyclin may be reduced (Buttrick *et al,* 1985). Therefore if the platelet inhibitory effects of HUVECs were reduced by adrenaline, further investigations into the mechanism of this effect may be of interest. Although adrenaline alone failed to produce detectable platelet activation, in combination with other stimuli, serotonin release was enhanced in a synergistic manner and thromboxane generation was also increased. Such responses were consistent with those reported previously (Keraly *et al,* 1988; Lanza *et al,* 1988, Vanags *et al,* 1992). Despite such enhanced platelet responses, in the combined stimulus groups, HUVECs still caused pronounced inhibition of platelet serotonin release responses as well as inhibition of thromboxane generation responses (significant for thrombin).

From the prostacyclin generation results, it was evident as in prior studies (Chapter 3) that adrenaline alone was not a strong stimulus of prostacyclin generation and there was certainly no synergistic prostacyclin generation in the combined stimulus situations. Interestingly, although not observed with collagen, with the stronger stimulus thrombin, the additional presence of adrenaline inhibited prostacyclin generation (both ± platelets). This effect may have been associated with the reported (Adler *et al,* 1981) ability of adrenaline to increase endothelial cell cAMP, thus opposing the normal stimulatory effect of thrombin. Irrespective of this effect, and as indicated above, HUVECs still produced significant inhibition of activated platelet responses. Because the presence of adrenaline did not produce any marked reductions in the platelet inhibitory effects of HUVECs, further investigations with this agent were not pursued.

Platelet-associated enhancement of prostacyclin generation from HUVECs

In many instances, and even in the control, endothelial cell prostacyclin generation in the presence of platelets was markedly increased over the responses seen in their absence. This was observed to a greater extent than in earlier studies (Chapter 4) and it was possible that in the present studies, the higher HUVEC numbers per well contributed to the more evident enhancement effects observed. As discussed in Chapter 4 the possible cause may have been linked with HUVEC injury caused by the presence of activated platelets or the liberation of platelet mediators causing stimulation of prostacyclin generation in the presence of platelets. In chapter 4 these arguments were opposed by observations that augmented prostacyclin generation did not differ greatly between stimulus groups. Furthermore, where platelet-associated enhancement was evident, time-dependent augmentation was not observed, such that injury to HUVECs seemed an unlikely cause.

In the present studies, as in Chapter 4, enhancement was not confined to stimuli which caused strong platelet activation. Indeed minor but significant enhancement of prostacyclin generation was even evident in the RB control. This enhancement may have been associated with minor injury effects to HUVECs caused by the presence of unstimulated platelets. In the presence of activated platelets, effects of injury to HUVECs or effects of platelet-derived mediators upon HUVECs should differ with different levels of platelet activation. For this reason, in the collagen

and thrombin stimulus groups, correlation analyses were performed to allow assessments of whether platelet serotonin release or thromboxane generation could be linked with platelet-associated enhancement of prostacyclin generation. These studies indicated that increases in platelet activation, as measured by serotonin release in response to collagen and thrombin, failed to correlate significantly with the extent of platelet-associated augmentation of prostacyclin generation by HUVECs. This implied that the extent of platelet dense granule release did not affect the extent of platelet-associated enhancement of prostacyclin generation.

The possibility that endoperoxide steal or platelet-derived thromboxane was stimulating or augmenting HUVEC prostacyclin generation could not be excluded particularly where collagen was the stimulus assessed. Certainly platelet endoperoxide donation from platelets to HUVECs has been demonstrated by others (Marcus *et al*, 1980; Chesterman *et al*, 1986) and the TXA_2/PGH_2 mimetic, U44619 has been shown to be able to stimulate prostacyclin generation from HUVECs (Kent *et al,* 1993). In the present studies (in the presence of HUVECs) the greatest level of thromboxane generation (and presumably the greatest PGH_2 liberation) occurred in response to collagen (10μg/ml). Collagen (10μg/ml) was also the stimulus which caused the greatest increase in prostacyclin generation in the presence of platelets (11.6 fold that observed in the absence of platelets). In addition, significant correlation (R^2=0.481; $0.01<p<0.05$) was also apparent between platelet thromboxane generation in the presence of HUVECs and the ratio of prostacyclin generation (+/- platelets). This may have lent some credence to a role for endoperoxide steal (from platelets), or possibly direct thromboxane mediated effects in the enhancement of prostacyclin generation from HUVECs for this stimulus.

In contrast to collagen, for thrombin there was no evidence of a correlation between thromboxane generation in the presence of HUVECs and the prostacyclin generation ratio (+/- platelets). Possibly thrombin evoked insufficient liberation of arachidonic acid (and therefore PGH_2 and thromboxane generation) to affect the extent of prostacyclin enhancement significantly. To confirm that, for this stimulus, platelet-derived endoperoxides (or thromboxane generation) were not involved in the platelet-associated enhancement of prostacyclin generation, further studies were required.

In Chapter 6, such studies were performed and specifically assessed whether in response to collagen or thrombin the enhancement of prostacyclin generation in the presence of platelets could be linked to effects of endoperoxide donation (or thromboxane generation) from platelets.

Relationships between measured parameters of platelet activation

The plotted correlation analyses (Figures 5d-m) performed between various experimental parameters emphasised several additional trends within the data. Firstly, the relationship between increasing stimulus concentrations and platelet activation was assessed. Predictably, for increasing stimulus concentrations, increasing serotonin release and thromboxane generation were evident.

Serotonin release in response to thrombin reached a maximum or plateau level (Figure 5d). In contrast, thromboxane generation in response to thrombin increased linearly with increasing concentrations and failed to reach a maximum level (Figure 5e). Serotonin release in response to collagen differed from that of thrombin in that over the concentration range used, maximum release was not apparent. The limited concentration range may however have prevented the detection of this phenomenon. At the highest concentration of collagen (10μg/ml), some evidence of a trend towards an exponential rise in thromboxane generation was observed. This trend was consistent with prior observations that with increasing concentrations of collagen, exponential increases in thromboxane generation become apparent, where such trends are less pronounced for thrombin (Ambler *et al,* 1985).

For various stimuli, platelet aggregation and release responses are frequently enhanced by the formation of thromboxane. It has been demonstrated that in response to stimuli such as low concentrations of collagen, serotonin release follows thromboxane generation and is inhibited where thromboxane generation is inhibited (Ambler *et al,* 1985; Best *et al,* 1980). Consequently, serotonin release responses can be demonstrated to be dependent upon thromboxane generation. For both collagen and thrombin, the degree of thromboxane-dependence is more demonstrable at lower stimulus concentrations, whereas extensive aggregation responses to higher

concentrations can be thromboxane-independent (Best *et al*, 1980; Ambler *et al*, 1985; Emms & Lewis, 1986).

For the studies described in this chapter, no specific assessment of the effects of thromboxane inhibition on serotonin release was made and the thromboxane-dependence of the serotonin release responses to these stimuli could not be determined. However it was evident that for a given submaximal extent of serotonin release for example ~ 60% (Figure 5a) collagen caused thromboxane generation which reached on average 4-5 times that produced by thrombin (Figure 5b). This capacity of collagen (at submaximal aggregatory concentrations) to stimulate greater thromboxane generation than thrombin has been described previously (Ambler *et al*, 1985; Emms & Lewis, 1986). This suggested that serotonin release in response to collagen (at the concentrations examined) may be more thromboxane-dependent than serotonin release in response to thrombin. Certainly it was considered desirable to confirm or refute thromboxane-dependence through specifically inhibiting thromboxane generation, and examining the effects of such inhibition on platelet serotonin release in response to collagen and thrombin, and this objective was approached in the studies described in the next chapter.

Platelet inhibitory effects of HUVECs: Relationships between the inhibition of platelet thromboxane generation and serotonin release

Correlation analyses were also performed to assess platelet responses in the presence of HUVECs. It was evident that in response to increasing stimulus concentrations, in the presence of HUVECs, both serotonin release and thromboxane generation responses were diminished compared to responses seen in the absence of HUVECs. Furthermore, in the presence of HUVECs, platelet responses only increased linearly with increasing stimulus concentrations. That is, in the presence of HUVECs, serotonin release even in response to thrombin did not reach a maximal level, and thromboxane generation, even in response to collagen, did not display a tendency to increase in the exponential manner seen in the absence of HUVECs.

From the correlation analyses, in the absence of HUVECs it was apparent for both stimuli that a significant relationship existed between serotonin release and thromboxane generation (Figures 5f and 5g). A near linear relationship was evident where stimulus concentrations produced less than 50-60% serotonin release. Above this level of serotonin release, thromboxane generation continued to rise, apparently exponentially, due to the relatively minor further increases ~20% in serotonin release. In the presence of HUVECs, the relationship between thromboxane generation and serotonin release remained significant (Figures 5h and 5i). For thrombin, the relationship appeared similar to that seen in the absence of HUVECs, (although serotonin release rarely exceeded 60%). In contrast, for collagen a linear relationship was observed between serotonin release (which never exceeded 10%) and thromboxane generation.

Another observation from Figures 5f-5i was that in response to thrombin, for a given level of thromboxane generation, the corresponding serotonin release was only slightly lower in the presence (Figure 5h) as opposed to the absence of HUVECs (Figure 5f). In contrast, for collagen, for a given level of thromboxane generation (up to 500pg/10^6 platelets) the serotonin release in the presence of HUVECs was always low, that is less than 10% (Figure 5i), compared to that observed in the absence of HUVECs where up to ~60% release occurred (Figure 5g).

Thus it appeared that if platelet serotonin release in response to collagen was thromboxane-dependent, then platelets appeared to be less susceptible to the proaggregatory effects of thromboxane when in the presence of HUVECs. That is, HUVECs altered the relationship between thromboxane generation and serotonin release. Certainly it has been shown that endothelial cell mediators such as prostacyclin can inhibit platelet aggregation responses to arachidonic acid (mediated through the formation of thromboxane A_2) even whilst considerable platelet thromboxane generation remains evident (Fitzpatrick & Gormon, 1979). This apparent change in the relationship between platelet thromboxane generation and serotonin release observed where collagen was the stimulus assisted in explaining why the inhibition of thromboxane generation by HUVECs did not correlate significantly (R^2=0.097, $p>0.05$) with the inhibition of serotonin release for this stimulus (Figure 5k).

In contrast to collagen, for thrombin a significant correlation did exist between inhibition of serotonin release and thromboxane generation by HUVECs (Figure 5j : R^2=0.631; p<0.01). From this correlation, together with those of Figures 5f and 5h, it could be proposed that for thrombin, serotonin release and thromboxane generation were related to each other and thus where thromboxane generation was inhibited by HUVECs so too was serotonin release. An alternative explanation which was also considered, was that thrombin stimulated both platelet thromboxane generation and serotonin release simultaneously but in a mutually independent manner, and HUVECs inhibited both indices of platelet activation in a near parallel but also unrelated fashion. However, from the present studies, whether the mechanism by which HUVECs inhibited serotonin release was thromboxane-dependent or independent remained uncertain. The studies described in the next chapter served to clarify the thromboxane-dependence of serotonin release responses to thrombin, and whether HUVEC inhibition of platelet serotonin release was dependent upon the inhibition of thromboxane.

HUVEC-mediated platelet inhibition; A role for prostacyclin?

Many factors derived from HUVECs could have accounted for their capacity to inhibit platelet activation (both thromboxane generation and serotonin release) and even alter the relationships between thromboxane generation and serotonin release responses. Prostacyclin and EDRF were two such factors.

The ability of prostacyclin to raise platelet cAMP may have reduced platelet reactivity to the stimuli used (i.e. collagen and thrombin) and diminished responsiveness to mediators of feedback amplification of platelet activation, such as thromboxane A_2. Synergism between prostacyclin and EDRF in the inhibition of platelet activation (Macdonald, Read & Dusting 1988: Alheid, Frolich & Forsterman, 1987; Radomski, Palmer & Moncada, 1987b) was also considered of interest, and such effects may have confounded attempts to correlate prostacyclin generation with platelet inhibitory effects. Studies to determine the participation of EDRF in the system were followed in later studies (Chapters 8 and 9).

Certainly, for both thrombin and collagen, prostacyclin generation did not cause concentration-dependent inhibition of platelet activation (Figures 5l and 5m). In addition to factors such as possible synergy between prostacyclin and EDRF, the lack of correlation between prostacyclin generation and the extent of inhibition of platelet activation (particularly of serotonin release) for thrombin could have been explained on the basis of two opposing factors. That is, at high thrombin concentrations, for example 0.2u/ml, although prostacyclin generation was pronounced (estimated to be ~24nM), platelet responses (particularly serotonin release) clearly became irreversible and resistant to inhibition by HUVECs, and possibly the inhibitory effects of prostacyclin. Certainly others have demonstrated that in response to increasing concentrations of thrombin, washed platelet serotonin release and thromboxane generation becomes increasingly resistant to inhibition by prostacyclin (Krishnamurthi, Westwick & Kakkar, 1984). For collagen, over the concentration range examined, even in the presence of platelets, prostacyclin generation was low (compared with thrombin) and this together with the narrow range of inhibitory effects (80-100%) limited the likelihood of detecting (if present) a clear correlation between these parameters. Such evidence against platelet inhibitory effects of prostacyclin raised the obvious question as to how important was prostacyclin generation in platelet inhibition. The studies undertaken in Chapter 6 which involved inhibition of HUVEC prostacyclin generation attempted to address this question.

Summary

These studies demonstrated that platelet activation in response to both collagen and thrombin increased in a concentration-dependent fashion. Moreover, platelet responses to collagen and thrombin could be amplified by 0.05μM adrenaline (which alone produced no response) but such responses were still effectively inhibited by HUVECs.

In response to collagen and thrombin, platelet thromboxane generation and serotonin release increased (both ±HUVECs) in an apparently related manner. For a given serotonin release response, thromboxane generation in response to collagen was consistently greater than that observed in response to thrombin. Although this suggested that serotonin release in response to collagen may be more dependent upon thromboxane generation than release in response to

thrombin, this could not be confirmed without studies involving the specific inhibition of platelet thromboxane generation.

Where thrombin was used as a stimulus, HUVECs diminished both platelet thromboxane generation and serotonin release in a near parallel manner, but for collagen this was not seen. The importance of the inhibition of thromboxane generation by HUVECs in their diminution of platelet serotonin release responses therefore required further investigation. Although HUVECs produced consistent inhibition of platelet activation, it could not be demonstrated that this inhibition was related to the level of prostacyclin generation from HUVECs and evidence that prostacyclin was important in such inhibition remained to be established.

Platelet-associated enhancement of prostacyclin generation was observed where collagen and thrombin were used as stimuli. This did not correlate with platelet serotonin release or thromboxane generation where thrombin was used as a stimulus. For collagen however, a significant correlation between thromboxane generation and the extent of platelet-associated enhancement of prostacyclin generation was seen. It was therefore possible, that platelet-derived endoperoxides may be utilised by HUVECs to generate prostacyclin, or platelet thromboxane A_2 generation may stimulate prostacyclin generation.

The subsequent studies were undertaken with the principle aim of determining whether any component of the platelet-associated enhancement of prostacyclin generation by HUVECs could be attributed to endoperoxide steal (or platelet thromboxane generation). Through these studies many of the other issues or questions outlined above could also be addressed and resolved. With the specific inhibition of thromboxane generation by platelets, it could be determined whether serotonin release in response to collagen or thrombin was thromboxane-dependent. Under these conditions it could also be determined whether thromboxane independent pathways of inhibition of platelet serotonin release by HUVECs could occur. Finally, in the following studies, the inhibition of prostacyclin generation by HUVECs would serve to clarify whether this mediator was important in the platelet inhibitory effects of HUVECs.

Chapter 6

The role of platelet-derived endoperoxides or thromboxane in prostacyclin generation from HUVECs

Introduction

From the results of the previous chapters it was apparent that in the presence of platelets, generation of prostacyclin by HUVECs was enhanced. Where platelets were stimulated, a possible explanation for this effect was that platelet endoperoxides were being transferred from activated platelets to HUVECs, resulting in the increased prostacyclin generation. In a number of previous studies the phenomenon of endoperoxide steal or transfer from platelets to cultured HUVECs has been studied. These former studies have produced variable results which in some instances have confirmed (Marcus *et al,* 1980; Chesterman *et al,* 1986) and in some instances have failed to demonstrate endoperoxide steal (Baenziger, Becherer & Majerus, 1979). On the basis of such prior studies, it has become evident that the platelet to HUVEC ratio, the extent of their interaction with each other and the extent of platelet activation may all influence whether endoperoxide steal occurs. The conditions employed for the present studies (and those of Chapters 4 and 5) were considered unlikely to favour endoperoxide steal (as will be discussed later). In addition, the data accumulated from earlier chapters showed that in response to thrombin, the extent of enhancement of prostacyclin generation seen in the presence of platelets was not associated with the level of platelet activation (as estimated by serotonin release and thromboxane generation). There was however weak evidence that where collagen was used as a stimulus, some association may exist between platelet thromboxane generation and platelet-associated enhancement of HUVEC prostacyclin generation. Thus it was considered of interest to confirm or refute any participation of platelet endoperoxides (or platelet thromboxane generation) in the platelet-associated increases in prostacyclin generation seen in earlier studies.

In many other studies, the inhibition of either platelet or endothelial cell cyclooxygenase with aspirin has been used to prevent either cell type from producing cyclic endoperoxides. Aspirin is particularly favoured as it inhibits cyclooxygenase through irreversible acetylation of the

enzyme (Roth & Siok, 1978). The effects of this inhibition are slowly reversible (associated with new cyclooxygenase synthesis) in nucleated cells but not platelets. Aspirin can be used to inhibit one cell type by this mechanism, and can then be removed such that the other cell type is unaffected in co-incubation studies. Alternative cyclooxygenase inhibitors such as indomethacin have occasionally been used in some investigations into endoperoxide steal (Hornstra, Haddeman & Don, 1979) and have produced reversible effects, which have led to questions by some investigators concerning the validity of initial studies where endoperoxide steal was first hypothesised (Bunting *et al*, 1976).

In the present studies, to evaluate the effects of platelet-derived endoperoxides, two approaches were taken. In the first approach, specific inhibition of HUVEC cyclooxygenase was used and in the second, platelet cyclooxygenase was specifically inhibited. If platelet-derived endoperoxides could be used by HUVECs, then under conditions where HUVEC cyclooxygenase was inhibited, prostacyclin generation should be demonstrable upon their co-incubation with activated platelets. In addition, if during coincubations platelet endoperoxides or thromboxane were participating in the enhancement of prostacyclin generation from HUVECs, then inhibition of platelet cyclooxygenase should prevent the effect.

Another aspect that had been assessed only indirectly in Chapter 5 was the role of prostacyclin in the manifestation of the platelet inhibitory effects of HUVECs. In Chapter 5, no significant correlations could be obtained between the concentration of prostacyclin generated and the platelet inhibitory effects observed. These results raised the question as to whether prostacyclin was involved in platelet inhibition in these studies. Since the studies to be performed in this chapter would require inhibition of HUVEC prostacyclin generation, it was of interest to determine how this would affect platelet inhibition by HUVECs. In addition, the inhibition of platelet thromboxane generation would allow an assessment of the role of this mediator in modulating serotonin release responses of stimulated platelets, a question also raised in Chapter 4 and from the correlation analyses of Chapter 5.

To approach these aims, and include appropriate controls, more experimental wells were required than in former studies. To meet this end, the 12 well system used in Chapter 4 was replaced by a 24 well system. The experimental conditions in the 12 and 24 well system were virtually identical. The only difference was that in the 24 well system, the wells used were half the size. Thus the supernatant and stimulus volumes used per well were halved and half the number of HUVECs were required to seed each well. Similar platelet activation responses and HUVEC prostacyclin generation responses (including platelet-associated enhancement) were expected in the 12 and 24 well systems.

Aims

The aims of this chapter were:

1. To determine the importance of prostacyclin generation from HUVECs in the platelet inhibitory activity of HUVECs by comparing the inhibitory effects of untreated and aspirin pretreated HUVECs on platelet serotonin release and thromboxane generation, in response to collagen and thrombin.

2. To determine the importance of platelet thromboxane generation in serotonin release responses to collagen and thrombin by comparing the release responses in untreated and aspirin pretreated platelets.

3 To determine whether prostacyclin generation could be maintained through the presence of untreated activated platelets when HUVEC cyclooxygenase function was inhibited by pretreatment with aspirin.

4. To determine whether platelet-dependent enhancement of HUVEC prostacyclin generation differed when HUVECs were incubated with aspirin pretreated or untreated platelets.

Methods

All facets of HUVEC isolation, culturing and subculturing were performed as described in Chapter 2. In addition, the preparation of washed (^{14}C-serotonin labelled) platelets was also undertaken as described in Chapter 2 as were the platelet and HUVEC interaction studies.

Interaction study protocol

Briefly, 24 well culture plates were seeded at ~1.0 x 10^5 cells/well 48 hours prior to use. Sixteen wells were used in 24 well plates and these wells (seeded or unseeded) were preincubated for 15 minutes with serum free medium 199, then 5 minutes with platelet RB. For final experimental incubations, 475µl washed platelets or RB were added to wells followed by 25µl of the relevant stimulus. All incubations were performed at 37°C under 5% CO_2 in air, and for the final incubation, plates were shaken at a shaker setting of 4. Platelet activation was determined by assessments of % ^{14}C-serotonin release and thromboxane generation, measured as TXB_2. HUVEC prostacyclin generation was measured as 6-keto-$PGF_{1\alpha}$. Data were expressed as described in Chapter 2.

To address the aims of this chapter, aspirin pretreatment of either platelets or HUVECs was undertaken as described in the following sections.

HUVEC pretreatment with aspirin

For the first series of studies (addressing aims 1 and 3), aspirin pretreatment of HUVECs was undertaken. The consequent inhibition of prostacyclin generation from HUVECs (to non-quantifiable levels in the absence of platelets) was maintained throughout the experimental protocol (~30 minutes per culture plate). The effects of untreated platelets on this inhibited prostacyclin generation were examined. In addition, the effects of aspirin-pretreatment of HUVECs on HUVEC-mediated platelet inhibition were evaluated. When aspirin pretreatment was performed on HUVECs, aspirin (100µM) was incorporated into the first 15 minute medium 199 cell wash. The second 5 minute wash was with RB which contained no aspirin and the final experimental incubation (5 minutes) was also free of aspirin

When aspirin pretreatment of HUVECs was undertaken, parallel treatment of unseeded control wells was also performed. These wells received aspirin pretreatment and subsequent washes in an identical manner to the wells containing HUVECs, and wells were finally incubated with platelets to ensure residual aspirin from washing procedures did not affect platelet responses.

These procedures provided the following treatment combinations in the wells of each plate.

Untreated wells	
Untreated HUVECs	± untreated
Aspirin pretreated wells	platelets
Aspirin pretreated HUVECs	

The stimuli used were collagen (5μg/ml) and thrombin (0.05 and 0.1u/ml). RB was used as the control stimulus to determine that platelets and HUVECs displayed typically low levels of basal activation. The full aspirin pretreatment protocol (above) was not performed on the control. Therefore, the responses are not included in figures 6a-6c, but are referred to where relevant.

Platelet pretreatment with aspirin

In the second series of experiments (addressing aims 2 and 4) the role of platelet-derived endoperoxides or thromboxane in the system was assessed by comparing prostacyclin generation from untreated HUVECs in the presence of platelet-free buffers as well as in the presence of untreated and aspirin-pretreated platelets. Some of the platelets were incubated with 100μM aspirin (for 20 minutes at 37°C) in the first washing step but aspirin was not included in the subsequent washing steps. At the final stage of platelet washing, following platelet count correction but before labelling with ^{14}C-serotonin, the aspirin-pretreated platelet yield was split into two. Half the yield was subjected to a 1000g spin for 15 minutes and the supernatant (or RB derived from aspirin-pretreated platelets) was stored for use as a control to ensure the aspirin had not been carried into the final stage of the platelet preparation. The other half of the yield was labelled with ^{14}C-serotonin and stored for later use.

HUVEC prostacyclin generation was therefore assessed in the presence of platelet RB (not exposed to platelets or aspirin) and platelet free RB (derived from aspirin pre-treated platelets) as well as in the presence of untreated platelets and aspirin-pretreated platelets. Platelet and HUVEC interaction studies were performed in the usual manner.

These pretreatment procedures provided the following combinations in the wells of each plate.

Untreated platelets	
Aspirin pretreated platelets	± untreated
RB (not exposed to platelets)	HUVECs
RB (derived from aspirin-pretreated platelets)	

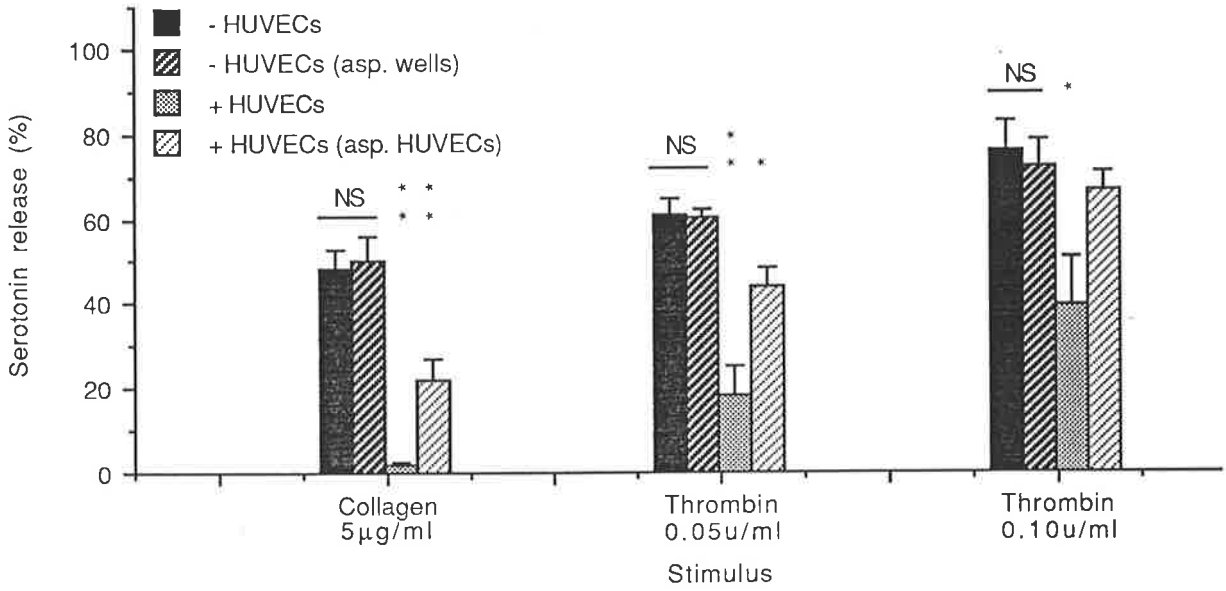
The stimuli used were collagen (5μg/ml), thrombin (0.05 and 0.1u/ml) and RB as a control.

Statistical analyses

The three data parameters of % platelet serotonin release, log transformed platelet thromboxane generation and log transformed HUVEC 6-keto-$PGF_{1\alpha}$ generation were analysed by independent ANOVA. Three-way parametric ANOVA was applied taking into account the variance associated with ± aspirin pretreatments and ± platelets or HUVECs, as well as experimental variation. For the 6-keto-$PGF_{1\alpha}$ data shown in Figure 6c, only a two-way ANOVA was applied taking into account variance attributable to ± aspirin pretreatments and experimental variation. Effects attributable to ± platelet effects were determined using either Student's paired t or Wilcoxon's matched-pairs, signed-rank tests. Following ANOVA, individual sources of variance were assessed using Tukey's honestly significant difference (HSD) test. Significance was described at a $p<0.05$ and $p<0.01$ level.

Results from experiments involving the pretreatment of HUVECs with aspirin (100μM)

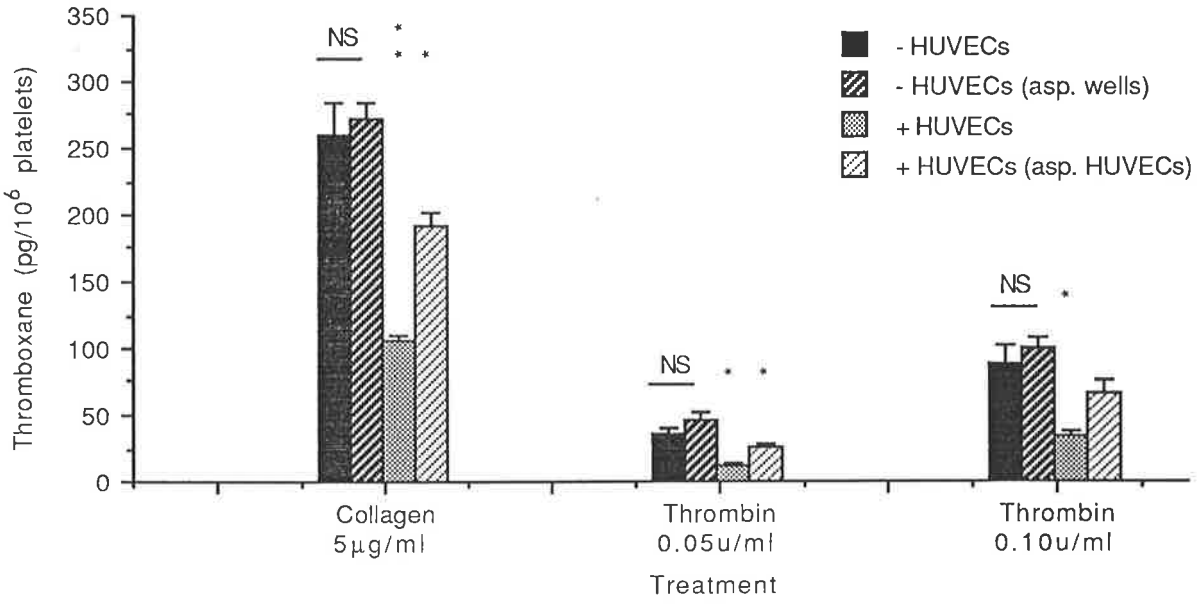
Figure 6a shows platelet serotonin release (mean + sem) from untreated platelets in response to the stimuli indicated in the absence (-) or presence (+) of HUVECs. The 15 minute pretreatment of unseeded wells or HUVECs with 100μM aspirin is also indicated (asp. wells or asp. HUVECs). The results are from 5 independent experiments; thrombin was used in 4 of the five experiments. NS indicates no significant difference between the indicated paired columns. Significant inhibition of platelet serotonin release by HUVECs (paired against the appropriate control) is indicated above the relevant columns at a $p<0.01$ (**) or $p<0.05$ (*) level.



It was evident that the aspirin pretreatment of the wells did not significantly affect the platelet responses to any of the stimuli. This indicated that no carry-over platelet inhibitory effects of aspirin occurred using the protocol described.

The extent of HUVEC-mediated inhibition of platelet serotonin release was reduced when HUVECs were pretreated with aspirin. Despite this effect, aspirin pretreated HUVECs were still able to significantly inhibit platelet serotonin release in response to collagen and thrombin 0.05u/ml but not in response to thrombin 0.10u/ml.

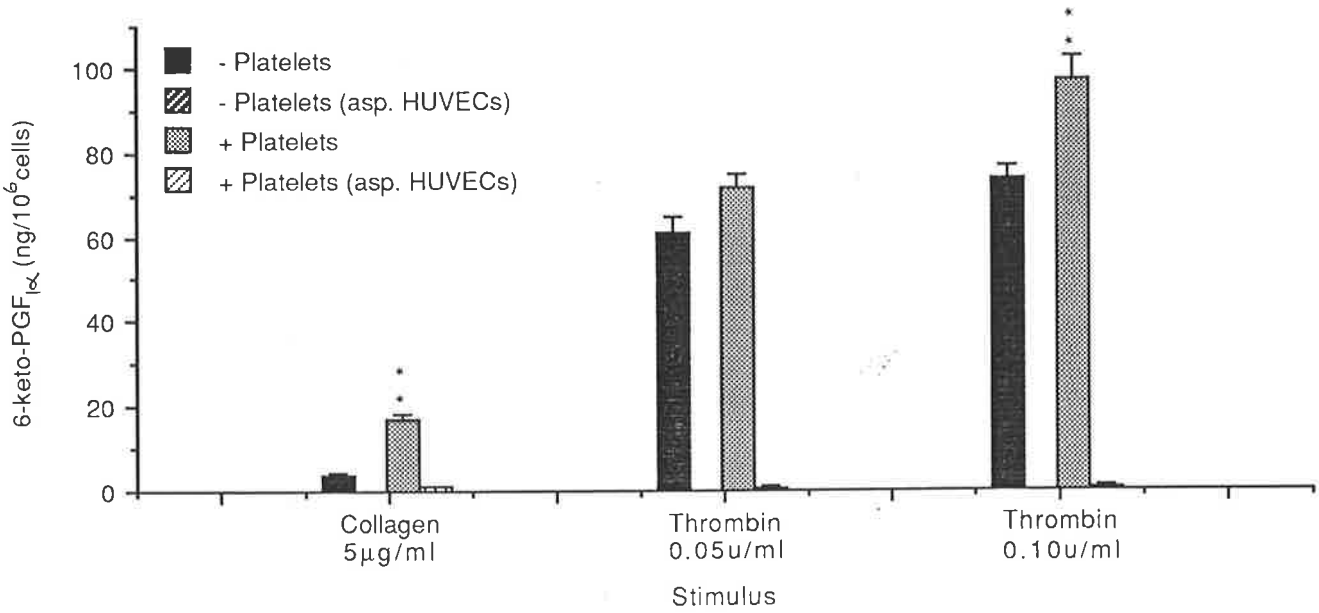
Figure 6b shows thromboxane generation (gm + gse) from untreated platelets in response to the stimuli indicated in the absence (-) or presence (+) of HUVECs, and the effects of aspirin (asp.) pretreatment of wells or HUVECs, as described in Figure 6a. The results are from 5 independent experiments; thrombin was used in 4 of the 5 experiments. NS indicates no significant difference between the paired columns indicated. Significant inhibition of platelet thromboxane generation by HUVECs (paired against appropriate controls) is indicated above the relevant columns at a $p<0.05$ (*) or $p<0.01$ (**) level.



Similarly to the serotonin release results, it was evident that aspirin pretreatment of the wells did not significantly inhibit platelet thromboxane generation for any of the stimulus groups. Indeed in the wells exposed to aspirin, generation (gm) appeared slightly greater (NS) than in the untreated controls, alleviating concerns that carry-over effects of aspirin may have inhibited platelet cyclooxygenase.

In the presence of untreated HUVECs, clear HUVEC-mediated inhibition of thromboxane generation was observed in all stimulus groups ($p<0.01$ or $p<0.05$). When HUVECs were pretreated with aspirin, their platelet inhibitory effects were reduced, displaying a trend similar to that seen in the serotonin release results. Aspirin pretreated HUVECs were still able to significantly inhibit platelet thromboxane generation in response to collagen and thrombin 0.05u/ml but not in response to thrombin 0.10u/ml.

Figure 6c shows prostacyclin generation from untreated or aspirin pretreated HUVECs (asp.) in response to the stimuli indicated. The absence (-) or presence (+) of untreated platelets is also indicated. Prostacyclin generation (measured as 6-keto-$PGF_{1\alpha}$) is expressed as the gm + gse. The results are from 5 independent experiments; thrombin was used in 4 of the 5 experiments. For untreated HUVECs, significant platelet-associated enhancement of HUVEC prostacyclin generation (paired against appropriate controls) is indicated above relevant columns, and where observed consistently reached significance at a $p<0.01$ (**) level.



Untreated HUVECs produced detectable prostacyclin generation in response to all stimuli. Significant platelet-associated enhancement of prostacyclin generation was observed when collagen and thrombin (0.10u/ml) were used as stimuli ($p<0.01$), but not in the 0.05u/ml thrombin stimulus group ($p>0.05$).

RB results were not included in the figure as the complete ± aspirin pretreatment protocol was not applied to this control. This was because it was assumed that platelet activation would be required for platelet-derived endoperoxides to participate in HUVEC prostacyclin generation, and RB (the control) would not provide such activation. In this control however, prostacyclin generation (6-keto-$PGF_{1\alpha}$; $2.8\pm0.55ng/10^6$ cells) in the absence of platelets was similar to that seen where collagen was used as a stimulus. In addition in the presence of platelets there was a significant enhancement of this generation up to $9.0\pm1.65ng/10^6$ cells (Student's paired t test, $p=0.021$).

When HUVECs were pretreated with aspirin, prostacyclin generation (in the absence of platelets) was reduced to non-quantifiable levels for both collagen and thrombin. In contrast, when platelets were present, HUVECs (pretreated with aspirin) could generate low, and just quantifiable amounts of prostacyclin.

For collagen and thrombin (0.05u/ml and 0.10u/ml), in the presence of platelets, aspirin pretreated HUVECs gave rise to 6-keto-$PGF_{1\alpha}$ of 1.10±0.32, 0.93± 0.43 and 0.76± 0.36 ng/10^6 cells respectively. There was no significant difference in these prostacyclin generation responses between the three stimulus groups ($p>0.05$, Student's unpaired t tests). Of these responses, even the greatest response of 1.10ng/10^6 cells was estimated to represent a final prostacyclin concentration of only ~0.6nM. This fell below the level of prostacyclin generation obtained in the untreated RB control which in the absence of platelets represented a final prostacyclin concentration of ~1.5nM).

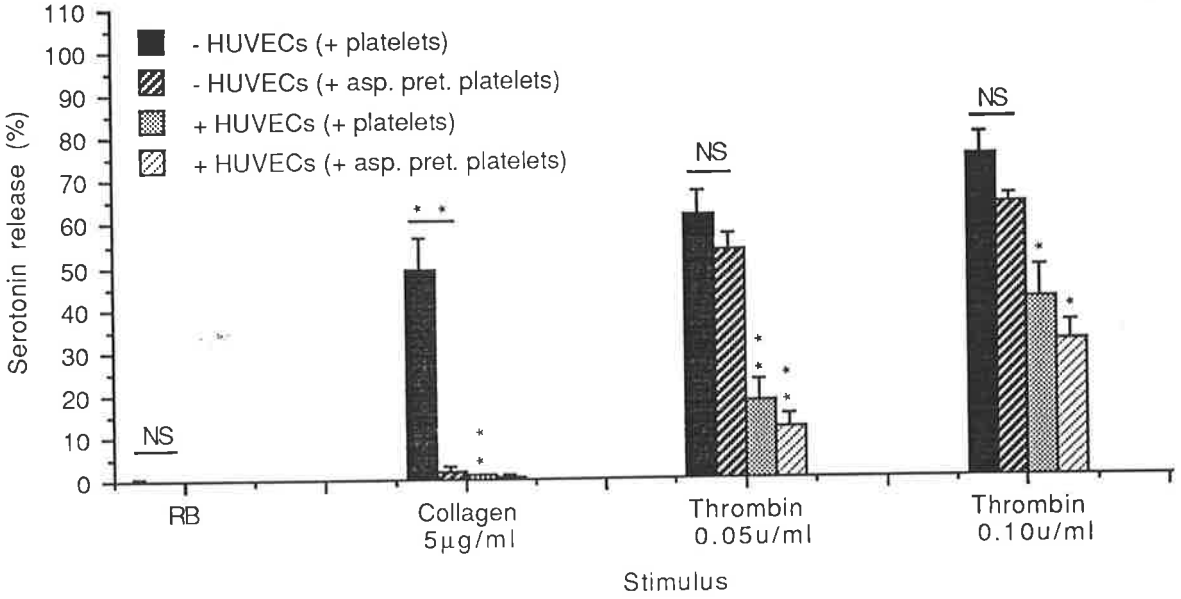
For collagen, thrombin 0.05u/ml and thrombin 0.10u/ml the respective mean prostacyclin generation responses from aspirin pretreated HUVECs in the presence of untreated platelets represented only 6.5%, 1.3%, and 0.8% of the generation from untreated HUVEC controls.

Results from experiments involving the pretreatment of platelets with aspirin (100μM)

A second series of experiments was performed which allowed the evaluation of platelet responses when platelets were pretreated with aspirin but HUVECs were not. In these studies, untreated platelet responses were examined in parallel with those of aspirin pretreated platelets. The inhibitory effects of HUVECs on these responses were also examined.

The platelet thromboxane generation results (not illustrated) showed that untreated platelet thromboxane generation responses to RB, collagen and thrombin were similar to those illustrated in Figure 5b of Chapter 5, and the untreated controls shown in Figure 6b of this chapter. The significant inhibitory effects of HUVECs on these responses were also similar. In these studies, it was also observed that aspirin pretreatment of platelets inhibited platelet thromboxane generation to all stimuli to non-quantifiable levels (both ±HUVECs).

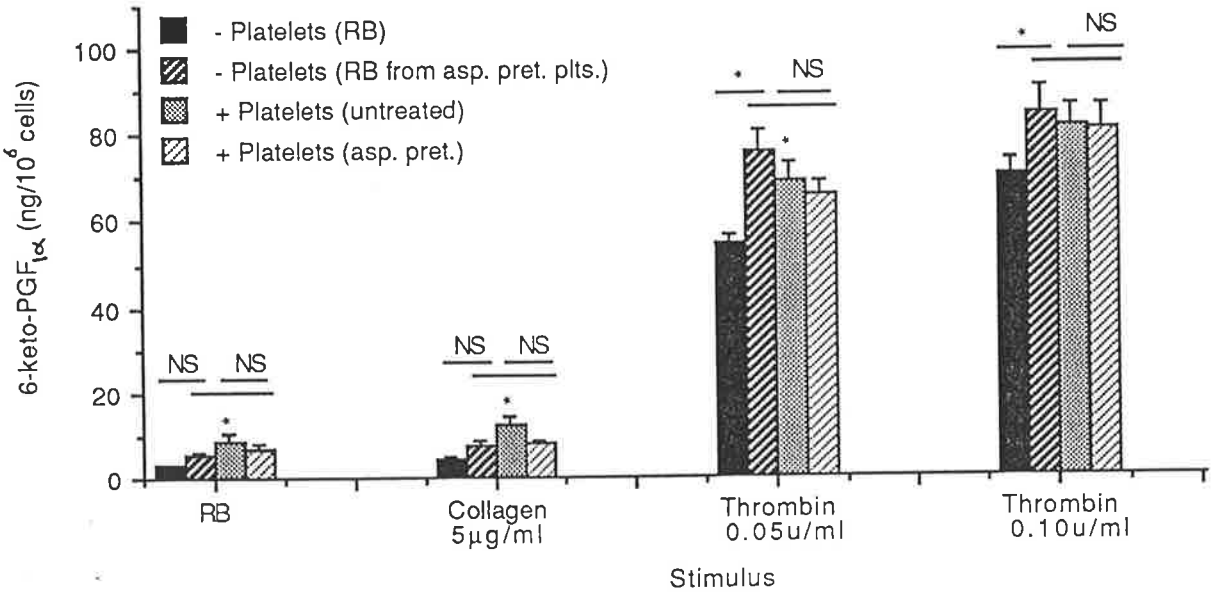
Figure 6d shows platelet serotonin release (mean + sem) from untreated or aspirin pretreated platelets (asp. pret. platelets) in response to the indicated stimuli. The absence (-) or presence (+) of untreated HUVECs is also indicated. Results are from 7 independent experiments; RB and collagen were used in 5 and thrombin (0.1u/ml) in 6 of these. Between the indicated paired columns no significant difference (NS) or significant effects $p<0.01$ (**) are shown. Where the presence of HUVECs caused significant inhibition of serotonin release (paired against appropriate controls) this is indicated above the relevant columns at a $p<0.01$(**) or $p<0.05$ (*) level.



The effects of aspirin on platelets (in the absence of HUVECs) differed for collagen and thrombin. When collagen was used as a stimulus, aspirin pretreatment of platelets and the consequent complete inhibition of thromboxane synthesis was associated with a significant ($p<0.01$) and virtually complete inhibition of serotonin release. In contrast, when thrombin was used as a stimulus, aspirin produced only a minor diminution in platelet serotonin release responses which failed to reach significance.

Irrespective of whether or not the platelets had been pretreated with aspirin, HUVEC-mediated inhibition of serotonin release in response to thrombin was significant. Significant HUVEC-mediated inhibition of serotonin release in response to collagen was also evident, but only when platelet serotonin release had not been inhibited by the aspirin pretreatment. Where platelets were pretreated with aspirin, serotonin release in response to collagen was too low to undergo further significant inhibition by HUVECs.

Figure 6e shows prostacyclin generation from untreated HUVECs in the the absence (-) of platelets but in the presence of RB or RB derived from aspirin pretreated platelets (RB from asp. pret. plts.) or in the presence (+) of either untreated or aspirin pretreated (asp.pret.) platelets. Prostacyclin generation (measured as 6-keto-$PGF_{1\alpha}$) is expressed as the gm + gse. The results are from 7 independent experiments; RB and collagen were used in 5 and thrombin (0.1u/ml) in 6 of these. Between the indicated paired columns no significant difference (NS) or significant effects $p<0.05$ (*) are shown. Where the presence of untreated platelets caused significant enhancement of prostacyclin generation (compared to that seen in the absence of platelets; i.e. RB) this is indicated above the relevant columns at a $p<0.05$ (*) level.



In response to thrombin the platelet-free buffer (derived from aspirin pretreated platelets) caused the greatest significant enhancement in prostacyclin generation. The presence of platelets (aspirin pretreated or untreated) was also associated with increased prostacyclin generation, significant (with untreated platelets) in one instance. There was no significant difference between responses in the presence of untreated and aspirin pretreated platelets or between these responses and that caused by platelet-derived buffer.

For collagen and in the control, the magnitude of the prostacyclin generation response was greatest in the presence of untreated platelets. Such responses were significantly greater than in the absence of platelets. Slightly enhanced prostacyclin generation was also observed in the presence of the RB derived from aspirin pretreated platelets (NS). This response was similar in magnitude and statistically indistinguishable from that observed in the presence of aspirin pretreated platelets.

Discussion

Upon comparing responses in the 24 well system with those obtained in earlier studies using the 12 well system, both systems produced comparable results with respect to platelet responses to RB, collagen and thrombin. However, in the 24 well system prostacyclin generation from HUVECs in response to RB, collagen and thrombin was substantially higher (up to 3 times greater in the absence of platelets) than had been observed previously when using the 12 well system. This was possibly associated with the method of agitation used in this cell interaction system, and different fluid dynamics in the wells of 12 and 24 well plates. No change in the rotation diameter of the titre plate shaker was possible, and halving the rotation diameter (from that used for 12 wells plates) for the 24 well system would have been desirable. In the 24 well system used for the present studies, the increase in prostacyclin generation responses from HUVECs did not affect platelet-associated enhancement of prostacyclin generation where RB and collagen were used as stimuli, but for thrombin the platelet associated enhancement was not as pronounced as in earlier studies. It was considered likely that in response to thrombin, even in the absence of platelets, a near maximal response was being observed, such that the extent to which the presence of platelets could increase prostacyclin generation was limited.

HUVEC-mediated platelet inhibition; a role for prostacyclin?

The results from the first series of experiments (figures 6a to c) produced a number of findings which were useful in determining the importance of HUVEC-derived prostacyclin in the platelet inhibitory effects of HUVECs.

Untreated HUVECs caused clear inhibition of collagen and thrombin stimulated serotonin release (Figure 6a) and thromboxane generation responses (Figure 6b). In contrast when HUVECs were pretreated with aspirin, their inhibitory effects were reduced, or eliminated. With the inhibition of prostacyclin generation, HUVECs still produced significant inhibition of collagen and thrombin (0.05u/ml) stimulated platelet responses although in the latter instance, platelet serotonin release was inhibited to only a minor extent. In contrast, for 0.1u/ml thrombin the aspirin pretreatment of HUVECs prevented them from significantly inhibiting serotonin

release (Figure 6a) or thromboxane generation. These results implied that in response to 0.1u/ml thrombin, the significant inhibition of platelet responses by HUVECs was influenced and indeed dependent upon the generation of HUVEC-derived prostanoids. Because prostacyclin inhibition (Figure 6c) was coincident with this loss of HUVEC inhibitory effects on platelets it was the most likely candidate for such effects. Earlier studies (Chapter 5) had failed to demonstrate that prostacyclin generation correlated with the extent of inhibition of platelet activation by HUVECs, and reasons for this were discussed in the last chapter. The results of the present studies were however consistent with prostacyclin generation playing an important role in HUVEC-mediated inhibition of platelet activation in response to both collagen and thrombin.

Where observed, the residual platelet inhibition seen in the presence of aspirin pretreated HUVECs (Figures 6a and 6b) implied that mediators other than prostacyclin (inhibited more than 94-99%) from HUVECs may be involved in the remaining inhibitory effects of HUVECs. Although aspirin pretreatment did not completely eliminate prostacyclin generation (in the presence of platelets), it was estimated that prostacyclin concentrations under these conditions fell below 1nM. Thus platelet inhibitory effects of residual prostacyclin generation were likely to be minor. Possibly however, prostacyclin even at such low concentrations could have contributed to the significant platelet inhibition retained by HUVECs by acting in combination with other HUVEC-derived mediators. Certainly EDRF has been demonstrated to synergise with prostacyclin in the inhibition of platelet activation (where prostacyclin alone is at sub-inhibitory concentrations) (Radomski, Palmer & Moncada, 1987b; Macdonald, Read & Dusting , 1988; Alheid, Frolich & Forsterman, 1987). The possibility that EDRF was acting in the system was addressed in later studies (Chapters 8 and 9).

Clearly where thrombin 0.10u/ml was used, residual prostacyclin generation (and effects of any other HUVEC-derived mediators) were insufficient to produce significant inhibition of platelet responses. Thus, uninhibited prostacyclin generation from HUVECs was essential in maintaining their significant inhibitory effects against platelet responses to 0.1u/ml thrombin.

Relationships between measured parameters of platelet activation

The second aim of the present studies was to examine the extent of thromboxane-dependence of platelet serotonin release responses. In the absence of HUVECs, when platelets were pretreated with aspirin (i.e. when thromboxane generation was completely inhibited), collagen stimulated serotonin release was also completely inhibited (Figure 6d). This indicated that for the 5μg/ml concentration of collagen used in these studies, serotonin release from platelets was highly dependent upon some quantifiable level of thromboxane generation.

In contrast to collagen, thrombin 0.05u/ml and 0.10u/ml (in the absence of HUVECs) produced platelet serotonin release which was essentially thromboxane-independent. This was apparent as the aspirin pretreatment of platelets failed to cause significant inhibition of serotonin release (Figure 6d).

Mechanisms of platelet inhibitory effects of HUVECs; thromboxane independent (or dependent) inhibition of platelet serotonin release?

As indicated above, it was established that in response to thrombin (but not collagen) serotonin release from platelets could occur even in the absence of platelet thromboxane generation. As a result, the studies depicted in Figure 6d provided evidence that HUVECs could inhibit platelet serotonin release in response to thrombin via thromboxane-independent mechanisms. Where platelet thromboxane generation was eliminated with aspirin, HUVECs continued to produce significant inhibition of platelet serotonin release in response to thrombin. Therefore, this inhibition of serotonin release by HUVECs must have occurred via a thromboxane-independent mechanism. The extent of inhibition of aspirin pretreated platelets by HUVECs was comparable to that seen with untreated platelets, implying the inhibition of thromboxane by HUVECs was not a requirement for their inhibition of serotonin release in response to this stimulus. Certainly prostacyclin (apparently an important inhibitory mediator in this system) can inhibit platelet responses through multiple mechanisms, not necessarily involving the inhibition of thromboxane generation (Fitzpatrick & Gormon, 1979; Hawiger, Steer & Salzman, 1987; Kroll & Schafer, 1989).

In response to collagen, it was established that the inhibition of thromboxane generation with aspirin completely eliminated platelet serotonin release. Because HUVECs inhibited both platelet thromboxane generation and serotonin release to this stimulus, it was possible that the inhibition of thromboxane generation by HUVECs was a causal factor in their inhibition of serotonin release. However, HUVECs inhibited thromboxane generation to a lesser degree than was achieved using aspirin, yet serotonin release was inhibited to a similar extent to that produced by aspirin. It was therefore possible (as discussed in Chapter 5) that in the presence of HUVECs, even when thromboxane A_2 was generated, it was exerting a reduced stimulatory effect upon platelet serotonin release compared to that seen in their absence. That is, the relationship between thromboxane generation and serotonin release in response to collagen differed in the absence and presence of HUVECs. Again, effects of prostacyclin could explain such an effect, as it has been demonstrated that in response to arachidonic acid (a completely thromboxane-dependent platelet stimulus) platelet aggregation can be strongly inhibited by prostacyclin where thromboxane generation is inhibited to only a minor extent (Fitzpatrick & Gormon, 1979).

Mechanisms of platelet-associated enhancement of HUVEC prostacyclin generation: effects of platelet-derived endoperoxides and/or thromboxane A_2 generation

These investigations were predominantly directed towards determining, as indicated in the third and fourth aims, whether platelet-derived metabolites of arachidonic acid could be involved in platelet associated enhancement of HUVEC prostacyclin generation. Although, where thrombin was used as a stimulus, platelet-associated enhancement effects were less pronounced than had been observed than in earlier studies, the present studies were still able to address the source of the enhancement.

Endoperoxide steal, and platelet-associated enhancement of prostacyclin generation

The third aim specifically addressed the question as to whether platelets could provide HUVECs with endoperoxides which could be used by HUVECs to generate prostacyclin. The data in Figure 6c showed that following aspirin pretreatment, HUVECs alone could not generate quantifiable prostacyclin, indicating that the 15 minute pretreatment with 100μM aspirin had been successful in inhibiting such generation, and confirming the findings of previous investigators (Baenziger, Becherer & Majerus, 1979). This inhibition was maintained for the duration of the experimental protocol, confirming the irreversible nature of inhibition with aspirin and lack recovery of HUVECs over this period. For these experiments control wells were used to check that possible carry-over effects from aspirin pretreatment procedures would not lead to platelet inhibition. No such inhibition was observed where wells were exposed to aspirin and it was assumed that in the presence of HUVECs a similar lack of carry-over would be observed.

Although in the absence of platelets prostacyclin generation from HUVECs was eliminated by the aspirin pretreatment, in the presence of platelets prostacyclin generation from aspirin-pretreated HUVECs *was* detectable. However, the concentrations were extremely low and approached the limit of quantification for the assay. It was also apparent that generation of prostacyclin from aspirin pretreated HUVECs in the presence of platelets represented only a negligible (<6.5%) proportion of the prostacyclin which could be generated from untreated HUVECs under the same conditions. Moreover this prostacyclin generation (even at maximal production of 1.10ng/10^6 cells) was not even as high as that observed in the RB control (in the absence of platelets) where generation levels of 2.8ng/10^6 cells was seen. Certainly the level of generation could not account for the platelet-associated enhancement in prostacyclin concentrations noted in the collagen (or other stimulus) groups. Therefore, the results in Figure 6c indicated that where HUVEC cyclooxygenase was inhibited, platelet endoperoxide steal was negligible and could not account for the platelet-associated enhancement of prostacyclin generation seen in untreated controls.

Having established that endoperoxide steal in this cell interaction system was negligible, the reasons for the lack of participation of platelet endoperoxides in HUVEC prostacyclin generation were considered.

Evidence suggests that HUVEC monolayers when incubated with platelets do not provide ideal conditions for demonstrating endoperoxide steal (Marcus *et al,* 1980; Baenziger Becherer & Majerus, 1979), possibly because of insufficient contact between the cells. For example, the extent of endoperoxide steal demonstrated by Marcus *et al* (1980) was greater when HUVEC suspensions and platelets were interacted than when HUVEC monolayers and platelets were examined. In addition, when Chesterman *et al* (1986) successfully demonstrated endoperoxide steal, platelets were not interacted with HUVEC monolayers. Instead, the experimental system optimised platelet contact with HUVECs by perfusing platelets through columns of HUVECs (grown on microcarrier beads).

In the platelet and HUVEC interaction system described in this chapter, the ratio of platelets to HUVECs was 1425:1. On estimates from other studies this ratio represented that which may occur in larger rather than smaller vessels. In capillaries, the ratio of platelets to endothelial cells has been estimated to be ~1:1. and endoperoxide steal has been demonstrated at such ratios (Schafer, Crawford & Gimbrone, 1984). It has been demonstrated that with increasing ratios of platelets to endothelial cells, there is greater platelet-dependent prostacyclin generation from HUVECs (Marcus *et al*, 1980) and from bovine endothelial cells (Schafer, Crawford & Gimbrone, 1984) but clearly even with the high ratio used in these studies, platelet-derived PGH_2 was only utilised by HUVECs to a minor extent. Possibly, the presence of 0.35% bovine serum albumin in the platelet RB reduced the appearance of endoperoxide steal by binding liberated platelet endoperoxides and preventing their cross-cellular transfer (Hechtman *et al,* 1991).

Another reason for the lack of endoperoxide steal in this system may have been related to the low level of platelet activation (and therefore arachidonic acid liberation) in the present studies

compared to earlier studies in this area. Previous investigators have used platelet stimuli including 20μM-50μM arachidonic acid, 30μg/ml collagen, 5u/ml thrombin and even 1μM ionophore A23187 (Marcus *et al,* 1980; Schafer, Crawford & Gimbrone, 1984; Chesterman *et al,* 1986). In the present studies, the stimuli used caused relatively modest platelet activation compared to these previous studies. Had stronger stimuli been used more pronounced endoperoxide steal may have been observed.

Because endoperoxide steal was not apparent to any significant extent under the conditions used for the present studies, the question remained; what was causing the apparent platelet-associated enhancement of prostacyclin generation?

A role for thromboxane A_2 in stimulating prostacyclin generation from HUVECs?

In the second series of experiments (Figures 6d and 6e) the fourth aim of these studies was assessed, that is to determine whether inhibition of platelet cyclooxygenase affected the platelet-associated enhancement of prostacyclin. Although endoperoxides could not account for the enhanced prostacyclin generation in the presence of platelets, this did not exclude the possibility that platelet derived thromboxane may stimulate prostacyclin by a receptor mediated process. Certainly TXA_2/PGH_2 mimetics have been reported to stimulate prostacyclin generation in transformed bovine aortic endothelial cells (Hunt *et al,* 1992; Clesham *et al,* 1992) and HUVECs (Kent *et al,* 1993). The role of platelet-derived endoperoxides and/or platelet-derived thromboxane A_2 in stimulating endothelial cell prostacyclin generation was examined by assessing platelet associated enhancement of prostacyclin generation in the presence of untreated or aspirin pretreated platelets. Because it was evident that endoperoxide steal was negligible in this system, involvement of platelet thromboxane A_2 generation in HUVEC prostacyclin generation would be suggested if aspirin pretreatment of platelets resulted in significant inhibition of prostacyclin generation from HUVECs.

The comparisons of prostacyclin generation from HUVECs incubated with untreated and aspirin-pretreated platelets (Figure 6e) revealed no significant difference between the two cases for any of the stimulus groups. It was concluded therefore that the extent of participation of platelet-derived endoperoxides or the effects of thromboxane A_2 in HUVEC prostacyclin generation responses were not significant.

With collagen, a strong stimulus of thromboxane generation, there was a trend towards diminished prostacyclin generation in the presence of aspirin pretreated platelets (compared to untreated platelets). This suggested that for this stimulus, part (but not a significant component) of the prostacyclin generation from HUVECs in the presence of untreated platelets may be dependent upon cyclooxygenase products derived from platelets. However, somewhat similar trends in the RB control (which stimulated negligible thromboxane generation) lent weight to the argument that enhanced prostacyclin generation in the presence of platelets was not dependent upon the level of platelet thromboxane A_2 generation, as large differences in the prostacyclin generation profiles should have been seen between these stimuli.

Had more clear cut effects been observed, a thromboxane antagonist could have been used to advantage in these experiments to differentiate any effects which were attributable to endoperoxide steal from those attributable to thromboxane A_2.

The effects of platelet-free, platelet-derived buffer

In these studies, platelets were treated with aspirin in only the first stage of washing. For this reason, and because aspirin is unstable in aqueous solutions, it was considered unlikely that any aspirin would carry into the final incubation and inhibit HUVEC responses. However, to provide a control against any possible carry over effects of aspirin, HUVEC responses were assessed in the presence of buffer derived from aspirin pretreated platelets. Certainly, there was no evidence that aspirin in this buffer produced inhibition of HUVEC responses; conversely, as depicted in Figure 6e, in response to thrombin the presence of the platelet-free, platelet-derived buffer caused significant enhancement of prostacyclin generation.

For thrombin, prostacyclin generation in the presence of platelet-derived buffer was comparable to that produced in the presence of platelets. This observation effectively eliminated the possibility that in response to thrombin, stimulated platelets themselves were responsible for causing significant effects upon prostacyclin generation through mechanical damage (Jorgensen *et al,* 1986).

The differences in the trends in prostacyclin generation observed for RB and collagen compared to thrombin were also of interest because for the former stimuli, the greatest prostacyclin generation was still observed in the presence of platelets, not-platelet-derived buffer. Even for collagen and in the RB control however, the platelet-derived buffer did cause increases in prostacyclin generation which although not statistically significant could account for more than 50% of the apparent platelet associated enhancement. The different trends between the stimulus groups seemed to imply that a factor in the platelet-derived buffer may interact with the stimulus. For example the factor may interact with thrombin or could act differently in the presence of thrombin stimulated HUVECs compared with its action in the presence of effectively unstimulated HUVECs (i.e. where RB or collagen were used).

This raised the question: what was the element in the platelet-derived buffer which could be responsible for stimulating HUVEC prostacyclin generation? The evidence suggested it was not of endoperoxide origin. The experimental protocol used suggested it had to be a stable platelet-derived product, as following the preparation of platelets, interaction experiments were performed over a period of approximately 3 hours. The most likely possibility was therefore that a factor was released from platelets during the course of the final resuspension or during the final 15 minute 1000g centrifugation used to prepare the control buffer. The element may have been a protein or a low molecular weight factor, possibly released from platelet granules. The release probably occurred in response to mechanical damage to platelets rather than in response to a specific stimulus, and constituents of alpha and dense granules were considered as possible mediators. The instability of the dense granule factors ADP or ATP made them unlikely candidates and apyrase in the buffer would have eliminated such factors. Serotonin was

a possibility because, although not stable in physiological buffers (in the absence of antioxidants) for extended periods of time, it may have been sufficiently stable over three hours to exert a stimulatory effect on HUVEC prostacyclin generation. In opposition to this argument, it is generally noted that serotonin is a stronger stimulus of EDRF rather than prostacyclin generation from endothelial cells (Vanhoutte, 1990). Released contents of platelet alpha granules may have been involved. The constituents include GMP140, βTG and platelet factor 4 and although βTG has been shown to have no effect on endothelial cell prostacyclin generation (Jaffe, 1984) the effects of the other mediators are not known.

Studies were not performed to characterise the factor responsible for the enhanced prostacyclin generation observed in platelet-derived buffer. Consideration was however given to procedures which could have been used to identify the factor. These included determination of its susceptibility to heat inactivation, whether it was dialisable, and if a protein using molecular weight sieving techniques to determine its molecular weight and assist in its identification.

Summary and conclusions

In the 24 well system used for these studies, HUVEC prostacyclin generation responses were enhanced compared to responses observed previously using a 12 well system. However, platelet responses were similar to those previously observed using the 12 well system.

When prostacyclin generation by HUVECs was inhibited with aspirin (≥93.5%), a reduced platelet inhibitory capacity of HUVECs against platelet responses to both collagen and thrombin was evident. This evidence was consistent with prostacyclin being a mediator of HUVEC inhibition of platelet responses to these stimuli. For collagen and thrombin (0.05u/ml), the remaining inhibitory effects of aspirin pretreated HUVECs were significant. In contrast, the remaining inhibition of platelet responses to thrombin (0.10u/ml) failed to reach significance. Because for collagen and the lower thrombin concentration, aspirin pretreated HUVECs still produced significant platelet inhibitory activity, it was considered likely that HUVEC-derived mediators other than prostacyclin may be involved in platelet inhibition by HUVECs.

At the stimulus concentrations used, serotonin release in response to collagen was dependent upon thromboxane generation whilst thrombin responses were not. HUVECs were clearly capable of inhibiting both platelet thromboxane generation and serotonin release to both stimuli, but for thrombin it was demonstrated that HUVECs could inhibit serotonin release by mechanisms which were independent of their inhibition of thromboxane.

Aspirin pretreatment of HUVECs inhibited prostacyclin generation completely in the absence of platelets and virtually completely even in their presence (inhibited $\geq$ 93.5% compared to untreated levels). These results indicated that irrespective of the stimulus, in this experimental system, endoperoxide steal occurred to a negligible extent.

In response to thrombin, no significant difference in the enhancement of prostacyclin generation was observed between untreated or aspirin pretreated platelets. Thus platelet-derived endoperoxides or thromboxane were not participants in the enhancement. It was evident that although prostacyclin generation by HUVECs was enhanced in the presence of platelets it was enhanced to a similar extent by buffer which had been exposed to platelets. The identity of the platelet-derived factor (present in the buffer) which caused this enhancement was not elucidated.

Where collagen was used as a stimulus, although mean prostacyclin generation in the presence of aspirin pretreated platelets was slightly less than in the presence of untreated platelets, this trend failed to reach significance. Consequently it could not be concluded that platelet-derived thromboxane A_2 generation (or endoperoxide donation) was involved to a significant extent in platelet-associated enhancement of HUVEC prostacyclin generation. Moreover, the similar trends in the RB control argued against a role for these mediators as causal factors in prostacyclin generation from HUVECs.

In conclusion, these studies answered a number of questions raised in Chapter 5. Firstly, they clarified the mechanisms by which HUVECs exerted platelet inhibition in this system, and the results were supportive of an important role for prostacyclin as a mediator of such inhibition. Secondly HUVECs could inhibit platelet serotonin release responses by thromboxane independent mechanisms. This was established by the demonstration of thromboxane-independent serotonin release in response to thrombin. Such mechanisms of inhibition could not be evaluated for collagen because serotonin release in response to this stimulus was demonstrated to be thromboxane-dependent. The association between the moderate inhibition of thromboxane generation by HUVECs and their virtually complete inhibition of serotonin release remained undefined. Finally, the phenomenon of platelet-associated enhancement of prostacyclin generation was addressed. It was established that this effect could not be explained upon the basis of endoperoxide steal, which occurred to a negligible extent in these studies. There was also no significant evidence to suggest that any cyclooxygenase-derived metabolites of platelet arachidonic acid metabolism, for example thromboxane A_2, were involved in the phenomenon.

Further studies to determine the involvement of HUVEC-derived mediators (other than prostacyclin) in the platelet inhibitory effects of HUVECs would have been of interest. However, it was also of interest to examine whether endoperoxide steal (virtually absent in the present studies) *could* be demonstrated in this system. The antithrombotic potential of antiplatelet agents such as thromboxane synthetase inhibitors may involve their effects on endoperoxide steal. Through redirecting platelet-derived endoperoxides from activated platelets towards vascular cells, these agents may cause enhanced prostacyclin generation. The subsequent studies were therefore designed to determine whether in the presence of activated platelets, thromboxane synthetase inhibition could enhance prostacyclin generation in this system. Furthermore, as more recently developed thromboxane synthetase inhibitors also express TXA_2/PGH_2 receptor antagonistic activity, it was decided to compare the effects of a pure thromboxane synthetase inhibitor with those of a similar inhibitor with, in addition, TXA_2/PGH_2 receptor antagonistic activity.

Chapter 7

Effects of thromboxane synthetase inhibition and/or TXA_2/PGH_2 receptor antagonism on platelets and HUVECs independently and in combination

Introduction

In Chapter 1, the use of thromboxane synthetase inhibitors as platelet inhibitory agents was introduced, and the capacity of these agents to inhibit thromboxane-dependent platelet aggregation responses was described. When platelets under the influence of such inhibition are incubated with vascular tissue, the resulting redirection of platelet-derived endoperoxides has been shown to facilitate prostacyclin generation from such tissue (Needleman, Wyche & Raz 1979; Mayeux, Kadowitz & McNamara, 1989; Papp *et al*, 1986; Hechtman *et al,* 1991) and from cultured HUVECs (Baenziger, Becherer & Majerus, 1979; Marcus *et al,* 1980). It is assumed that *in vivo* similar redirection may also occur, particularly during thrombotic events where platelet activation and platelet interactions with the vascular wall occur.

In isolated platelets however, it has been shown that thromboxane synthetase inhibitors demonstrate limited platelet inhibitory efficacy against platelet responses to thromboxane-dependent stimuli such as arachidonic acid (Bertele *et al,* 1984). Because these inhibitors result in redirected eicosanoid generation from activated platelets they cause enhanced generation of PGE_2 and PGD_2 from isolated platelets (De Clerck et al, 1989a; Bertele *et al*, 1984) or during whole blood coagulation (Hoet *et al,* 1990; De Clerck *et al,* 1989b; Watts *et al*, 1991). PGE_2 can potentiate and PGD_2 can reduce platelet aggregation in response to other stimuli (Gray & Hepinstall, 1991; Bertele *et al,* 1984). Consequently the ability of thromboxane synthetase inhibitors to alter the generation of such prostanoids from activated platelets has been considered as one possible explanation for their limited platelet inhibitory efficacy (Bertele *et al,* 1984). Evidence in support of this concept has not however been convincing. For example, in subjects in whom the administration of thromboxane synthetase inhibitors produced poor platelet inhibition *ex vivo*, prostanoid generation profiles (from whole blood coagulation) did not differ from those seen in more responsive subjects (Bertele, *et al* 1984).

A second reason for the limited platelet inhibitory efficacy of thromboxane synthetase inhibitors may be associated with their ability to increase local concentrations of cyclooxygenase derived endoperoxides such as PGH_2. PGH_2 can, like thromboxane A_2 (TXA_2), bind to platelet TXA_2/PGH_2 receptors and cause platelet activation (Jaschonek & Muller, 1988; Fitzpatrick & Gorman, 1979) and may thus oppose the platelet inhibition achieved through inhibiting platelet thromboxane generation.

Recent evidence confirms that in the presence of thromboxane synthetase inhibitors, platelet-derived PGH_2 accumulation may stimulate platelet activation and limit the antiplatelet activity of these agents. The *ex vivo* platelet inhibitory effects of thromboxane synthetase inhibitors have been shown to be inferior to those provided by thromboxane synthetase inhibitors with thromboxane/PGH_2 receptor antagonistic activity (De Clerck *et al,* 1989b). The latter agents are also able to increase bleeding times more than pure thromboxane synthetase inhibitors or cyclooxygenase inhibitors (Hoet *et al,* 1990). *In vitro* evidence also supports the concept that combined thromboxane synthetase inhibition and TXA_2/PGH_2 receptor antagonism provides superior antiplatelet activity compared with either cyclooxygenase inhibition or pure thromboxane synthetase inhibition (Bertele & De Gaetano, 1982; Watts *et al,* 1991; Hoet *et al,* 1990; De Gaetano, Bertele & Cerletti, 1987a,b; Gresele *et al,* 1991)

There have been numerous reports of *in vitro* systems where both platelets and vascular cells have been interacted and where pure thromboxane synthetase inhibitors such as dazoxiben or imidazole have been used. These agents have been included in such studies in an attempt to demonstrate that endoperoxide steal from platelets can result in enhanced prostacyclin generation from vascular cells (Needleman, Wyche & Raz 1979; Mayeux, Kadowitz & McNamara, 1989; Papp *et al*, 1986; Hechtman *et al,* 1991; Baenziger, Becherer & Majerus, 1979, Marcus *et al,* 1980). Surprisingly, with the development of thromboxane synthetase inhibitors which also demonstrate TXA_2/PGH_2 receptor antagonistic activity, studies have consistently addressed the antiplatelet activity of these agents in the absence of vascular tissue.

It has been demonstrated that cultured endothelial cells possess TXA_2/PGH_2 receptors (Hunt *et al,* 1992; Kent *et al,* 1993). Although early reports implied that occupation of such receptors in bovine pulmonary artery endothelial cells could inhibit stimulated prostacyclin generation (Sung, Arleth & Berkowitz, 1989), other reports have found that TXA_2/PGH_2 mimetics (U44619) can stimulate prostacyclin generation from transformed bovine aortic endothelial cells (Hunt *et al,* 1992; Clesham *et al,* 1992) and HUVECs (Kent *et al,* 1993). These effects can be opposed with specific TXA_2/PGH_2 receptor antagonists such as SQ29548 (Kent *et al,* 1993). However, more information regarding the effects of endothelial cell TXA_2/PGH_2 receptor occupation on endothelial cell functions is clearly required.

The evidence to date suggests that in the presence of platelets, prostacyclin generation from endothelial cells may be affected by platelet derived TXA_2 (or in the presence of thromboxane synthetase inhibition, PGH_2) via receptor mediated effects rather than or in addition to endoperoxide steal. Thus, where thromboxane synthetase inhibitors also exert TXA_2/PGH_2 receptor antagonistic activity, their effects upon endothelial cell prostacyclin generation in the presence of platelets may differ from those of pure thromboxane synthetase inhibitors. The studies described in this chapter were undertaken to compare the effects of dazoxiben, a pure thromboxane synthetase inhibitor, with those of R68070, a thromboxane synthetase inhibitor with modest PGH_2/TXA_2 receptor antagonistic properties (Watts *et al,* 1991; De Clerck *et al,* 1989a,b). The effects of SQ29548, a specific PGH_2/TXA_2 receptor antagonist and the effects of a cyclooxygenase inhibitor (aspirin) were also examined. The studies were designed to compare the effects of the agents on platelet and HUVEC responses when these cells were examined alone and in combination. Since limited information was available on the effective concentrations of R68070 and SQ29548, the platelet inhibitory effects of a concentration range of these agents was initially evaluated in two experiments using platelets in the absence of HUVECs.

Aims

The aims addressed in this chapter were:

1. To assess the concentration-dependent inhibitory effects of dazoxiben and R68070 on platelet thromboxane generation in response to collagen (5μg/ml) and thrombin (0.05u/ml) in the absence and presence of HUVECs, and to compare such inhibitory effects with those produced by aspirin (100μM) or SQ29548 (10nM).

2. To consider the relationship between the effects of the four agents described in 1 on platelet thromboxane generation and serotonin release responses.

3. To assess the effects of the agents described in 1 on prostacyclin generation from HUVECs and compare their effects in the absence and presence of platelets.

Methods

Two preliminary investigations were used to used to ascertain the platelet inhibitory concentrations of R68070 (ridogrel), SQ29548 and the effects of their vehicle on isolated platelet responses to collagen and thrombin. These studies were performed (as for typical interaction studies) in 24 well culture plates and assessments of platelet serotonin release and thromboxane generation were evaluated (results not shown). Following these studies, 6 repeated platelet and HUVEC interaction studies were undertaken. For these studies, all facets of cell isolation, culturing and subculturing were undertaken as described in Chapter 2. Washed platelet preparation and labelling with ^{14}C-serotonin was also performed as described in Chapter 2. Twenty four rather than 12 well culture plates were used for all experiments. Only 12 wells were used per plate and HUVECs were seeded into only 8 wells per plate (~1.0 x 10^5 cells/well) 48 hours prior to their use.

Interaction study protocol

The experimental protocol (used to address aims 1-3) was based on that described in Chapter 2. All experimental wells were preincubated with serum free medium 199 for 15 minutes then with platelet RB for 5 minutes. Platelets or RB were then added to wells (475µl) prior to the addition of the stimulus (25µl) and there was a final incubation of 5 minutes during which culture plates were shaken at shaker speed 4. Two platelet stimuli, collagen (5µg/ml) and thrombin (0.05u/ml), were used, together with a control (RB). Platelet serotonin release, thromboxane generation (measured as TXB_2) and HUVEC prostacyclin generation (measured as 6-keto-$PGF_{1\alpha}$) were assessed and data were expressed as described in Chapter 2.

Treatments of HUVECs and platelets

For the two preliminary experiments the platelet inhibitory effects of R68070 (final concentrations:10^{-8}M-10^{-5}M) and SQ29548 (final concentrations:10^{-10}M-10^{-7}M) and their ethanol vehicle were examined. These two inhibitors were diluted as required in 0.9% w/v saline and final concentrations of the vehicle (1/100 into platelets; maximal ethanol, 0.1% v/v) did not affect platelet responses to either collagen or thrombin. SQ29548 produced detectable inhibitory effects only against collagen (not thrombin) stimulated platelet responses and was used in subsequent studies at a final concentration which in these preliminary studies caused maximal effect (10^{-8}M). R68070 produced detectable inhibition of platelet thromboxane generation at 10^{-8}M but produced detectable inhibition of collagen stimulated serotonin release only at concentrations of 10^{-7}M or more and this was the lowest final concentration used in subsequent studies.

For the six subsequent platelet and HUVEC interaction studies, the combined thromboxane synthetase inhibitor and TXA_2/PGH_2 receptor antagonist R68070 (final concentrations: 10^{-7}M to 10^{-5}M) and the pure thromboxane synthetase inhibitor dazoxiben (final concentrations: 10^{-7}M to 10^{-4}M) were used in all experiments. In three experiments, the TXA_2/PGH_2 receptor antagonist SQ29548 was also used, both alone (at 10^{-8}M) and in combination with dazoxiben or R68070. The cyclooxygenase inhibitor aspirin (10^{-4}M) was also used in three experiments.

These platelet inhibitors (except for aspirin) were incorporated in the second 5 minute HUVEC preincubation wash as well as in the final 5 minute experimental incubation using a final dilution factor (into platelets or RB) of 1/100. Aspirin (final concentration of 10^{-4}M) was incubated with platelets, following their final resuspension and labelling with ^{14}C-serotonin. These platelets, and untreated platelets were then stored in separate syringes. Aspirin pretreatment of HUVECs was performed in the first 15 minute preincubation wash. All drugs used were prepared as described in Appendix 2.

Statistical analyses

Except for data shown in Figures 7c and 7e, complete, n=6 data sets, including serotonin release (%) , log transformed TXB_2 and log transformed 6-keto-$PGF_{1\alpha}$ data, were assessed by three-way parametric ANOVA. This allowed the assessment of variance attributable to the treatments, ± platelets or ± HUVECs, and experimental variation. Specific sources of variance were identified by Tukey's HSD test with significance determined at a $p<0.05$ and $p<0.01$ level. For the remaining n=6 data, (Figures 7c and 7e) separate two-way ANOVA were used to assess responses of the cells either in isolation, or when co-incubated. This allowed the assessment of variance attributable to treatments and experimental variation. Specific sources of variance were again identified by Tukey's HSD test. When required the effects associated with combining the cells were assessed by specific Student's paired t or Wilcoxon's matched-pairs, signed-rank tests. When untreated control data (n=6) were compared with data in the SQ29548 or aspirin treatment groups (included in only 3 experiments) comparisons were made (depending upon homogeneity or heterogeneity of variance) using Student's unpaired t tests or Mann-Whitney U tests. In such instances the maximum obtainable significance was at a $p<0.012$ level. Consequently, for consistency, in all figures significant effects are described at either a $p<0.05$ or $p<0.015$ level.

Results

Figures 7a and 7b show the platelet thromboxane generation (gm+gse) in response to collagen 5µg/ml (Figure 7a : upper panel) and thrombin 0.05u/ml (Figure 7b : lower panel) in the absence and presence of HUVECs. The final concentrations of the agents used in these studies are expressed on the horizontal axis as the log of their molarity (log M). These agents included dazoxiben (Daz.), R68070 (R6), SQ29548 (SQ) and aspirin (Asp.). The results are from 6 experiments. Data for SQ and Asp. were obtained from only 3 of the 6 experiments. Significant inhibitory effects of treatments (compared with the appropriate untreated control) are indicated and were consistently observed at a $p<0.015$ (**) level.

Figure 7a

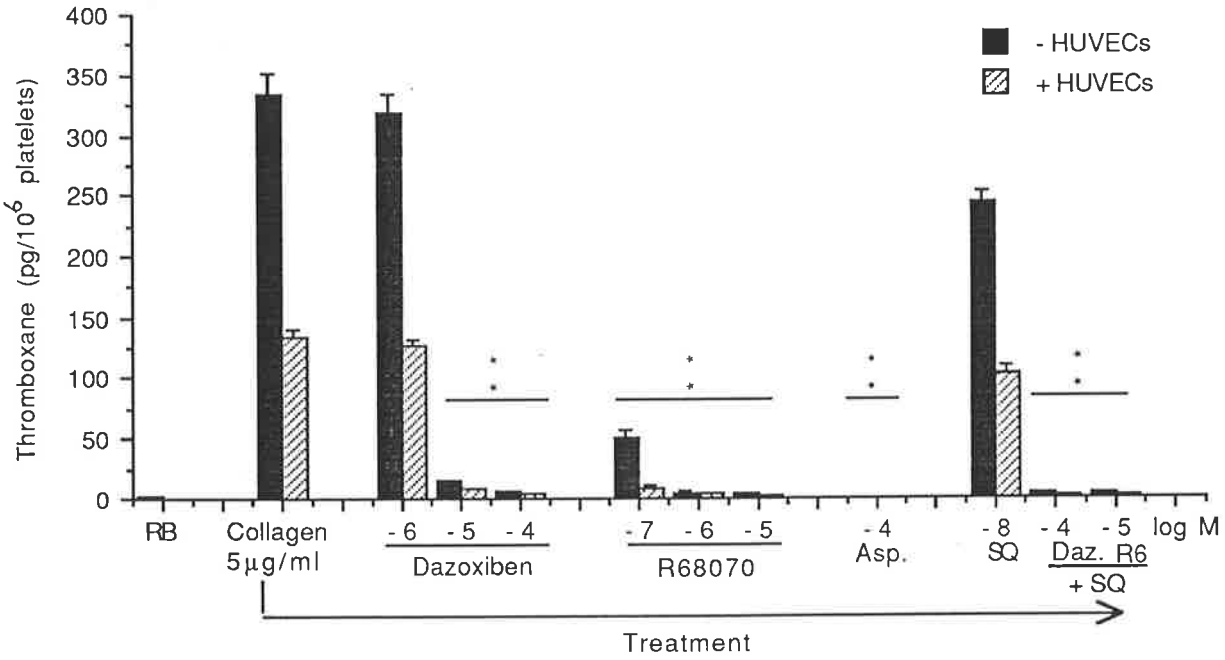
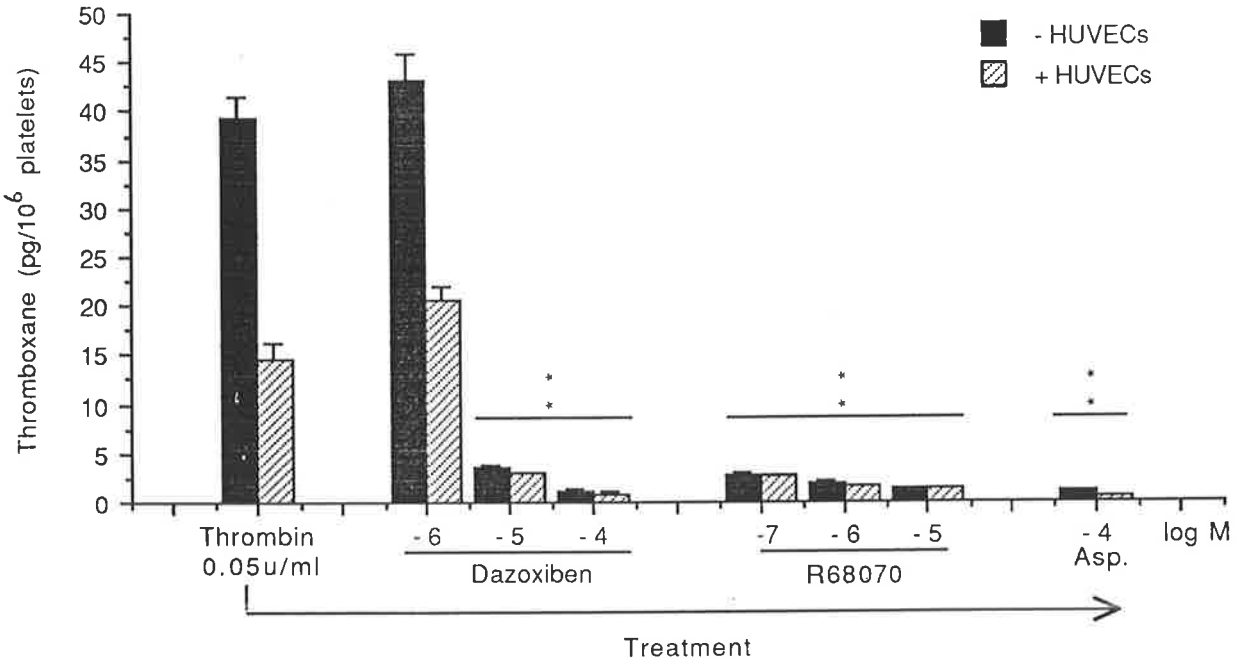


Figure 7b



Figures 7a and 7b show that thromboxane generation in response to both collagen and thrombin could be strongly inhibited by dazoxiben and, as in the preliminary studies, by R68070.

For both stimuli (in the absence or presence of HUVECs), dazoxiben and R68070 produced concentration-dependent inhibition of thromboxane generation (compared to the untreated control). The lowest R68070 concentration examined (10^{-7}M) clearly produced greater inhibition of thromboxane generation than the lowest dazoxiben concentration examined (10^{-6}M), where no inhibition of thromboxane generation was evident.

Although for both stimuli, dazoxiben at 10^{-5}M and R68070 at 10^{-7}M produced highly significant inhibition of thromboxane generation, this failed to reach a maximal level. That is, aspirin caused significantly greater inhibition ($p<0.05$) of thromboxane generation (both ±HUVECs; Student's unpaired t tests).

Dazoxiben 10^{-4}M and R68070 at 10^{-6}M and 10^{-5}M achieved maximal inhibition of platelet thromboxane generation which was statistically indistinguishable ($p>0.05$) from the effect of aspirin 10^{-4}M (Student's unpaired t tests). This was seen irrespective of the stimulus used, and both in the absence and presence of HUVECs.

The presence of HUVECs typically produced inhibitory effects on thromboxane generation responses. The extent of this inhibition by HUVECs was greater than 50% in the absence of treatments. However, where dazoxiben or R68070 provided virtually complete inhibition of thromboxane generation, HUVECs could no longer provide further detectable inhibition.

SQ29548 at 10^{-7}M produced minor but near-significant ($p=0.058$) inhibition of thromboxane generation in response to collagen, in the absence but not the presence ($p=0.087$) of HUVECs.

Figures 7c and 7d show the platelet serotonin release (mean + or - sem) in response to collagen 5µg/ml (Figure 7c : upper panel) and thrombin 0.05u/ml (Figure 7d : lower panel) in the absence and presence of HUVECs. The final concentrations of the agents used in these studies are expressed on the horizontal axis as the log of their molarity. These agents included dazoxiben (Daz.), R68070 (R6), SQ29548 (SQ) and aspirin (Asp.). The results are from 6 experiments. Data for SQ and Asp. were obtained from only 3 of the 6 experiments. Significant inhibitory effects of treatments (against the appropriate untreated control) are indicated at a $p<0.05$ (*) or $p<0.015$ (**) level.

Figure 7c

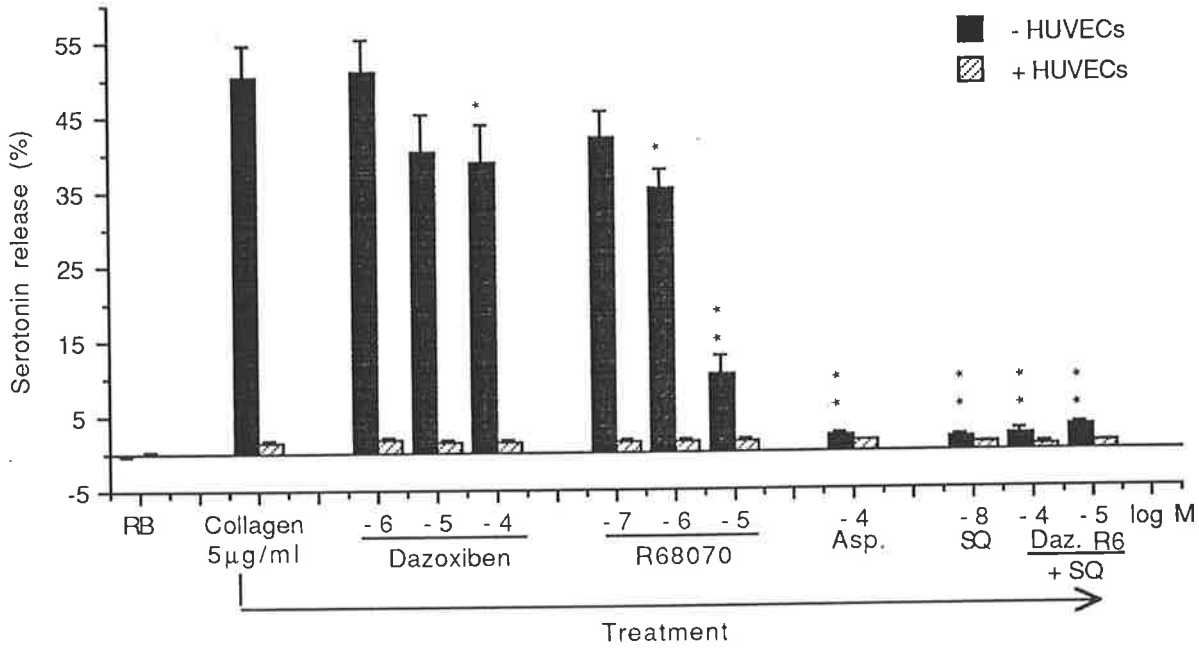


Figure 7d

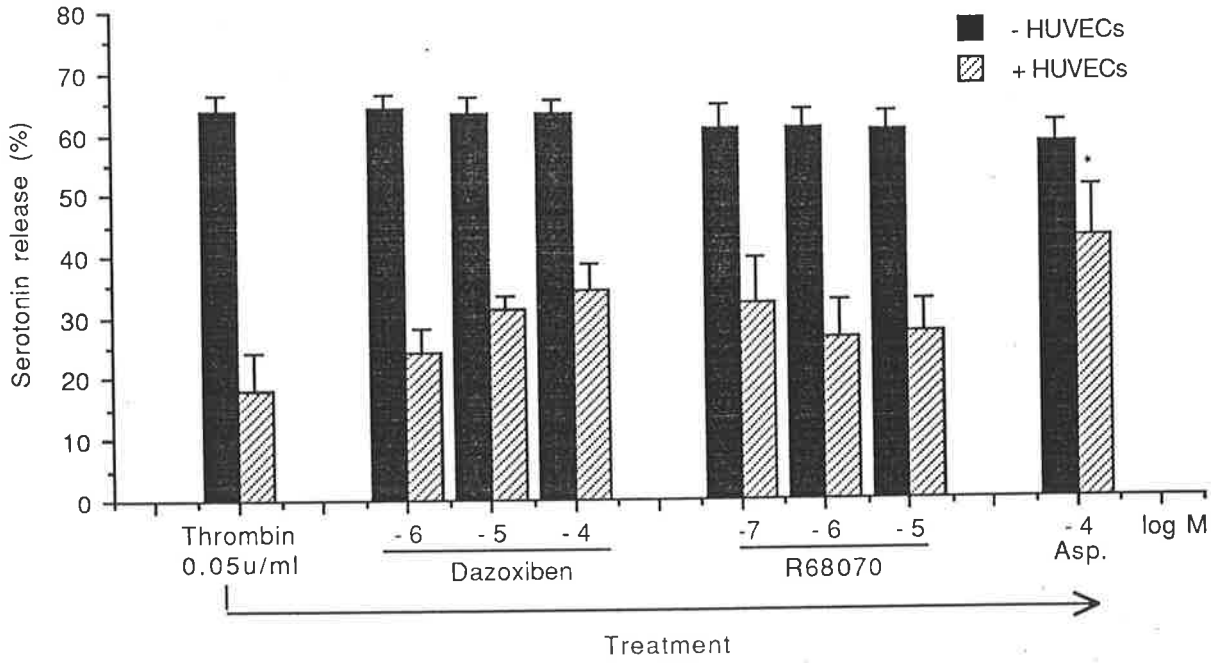


Figure 7c shows that in the absence of HUVECs, collagen stimulated serotonin release appeared to be inhibited in ascending order of potency by dazoxiben, R68070, aspirin and SQ29548. In the presence of HUVECs serotonin release responses were consistently reduced to a mean of < 2 % and the treatments produced no significant effects.

Upon closer examination of the platelet responses to collagen, in the absence of HUVECs, it was shown in Figure 7a that the inhibition of thromboxane generation produced by 10^{-4}M dazoxiben, 10^{-6}M and 10^{-5}M R68070 was comparable to that produced by aspirin. However, as shown in Figure 7c, at these concentrations, neither dazoxiben nor R68070 produced inhibition of serotonin release to a level comparable to that achieved by aspirin (Mann-Whitney U tests, $p< 0.05$). For 10^{-4}M dazoxiben and 10^{-6}M R68070, mean serotonin release of 39% and 35% respectively, was still evident. For 10^{-5}M R68070, 10% serotonin release remained evident, whereas aspirin inhibited release to only 2%.

When collagen stimulated serotonin release was still apparent in the presence of R68070 (10^{-5} M) and dazoxiben (10^{-4}M) alone, the presence of the SQ29548 in combination with these agents produced further significant inhibition ($p<0.05$ and $p<0.015$ respectively; Mann-Whitney U tests). SQ29548 at 10^{-8}M alone, and in combination with R68070 and dazoxiben produced maximal inhibition of the collagen induced serotonin release which was statistically indistinguishable from that caused by aspirin ($p> 0.05$).

Figure 7d shows that in the absence and presence of HUVECs, thrombin stimulated serotonin release was not statistically significantly affected by any of the treatments with the exception of aspirin, which reduced the extent of platelet inhibition typically exerted by HUVECs such that in the presence of HUVECs, significantly more serotonin release was seen compared to the untreated control. This level of serotonin release was not statistically different from that seen with the R68070 10^{-7}M or dazoxiben 10^{-4} M treatments ($p>0.05$, Student's unpaired t tests) both of which appeared to cause a modest reduction in the typical platelet inhibitory effects of HUVECs but unlike aspirin, this did not reach statistical significance.

Figures 7e and 7f show HUVEC prostacyclin generation (measured as 6-keto-$PGF_{1\alpha}$; described as gm+ gse) in response to collagen 5µg/ml (Figure 7e : upper panel) and thrombin 0.05u/ml (Figure 7f : lower panel) in the absence and presence of platelets. The final concentrations of the agents used in these studies are expressed on the horizontal axis as the log of their molarity. These agents included dazoxiben (Daz.), R68070 (R6), SQ29548 (SQ) and aspirin (Asp.). The results are from 6 experiments. Data for SQ and Asp. were obtained from only 3 of the 6 experiments. Significant inhibitory or enhancement effects of treatments (against the appropriate untreated control) are indicated at a $p<0.05$ (*) or $p<0.015$ (**) level.

Figure 7e

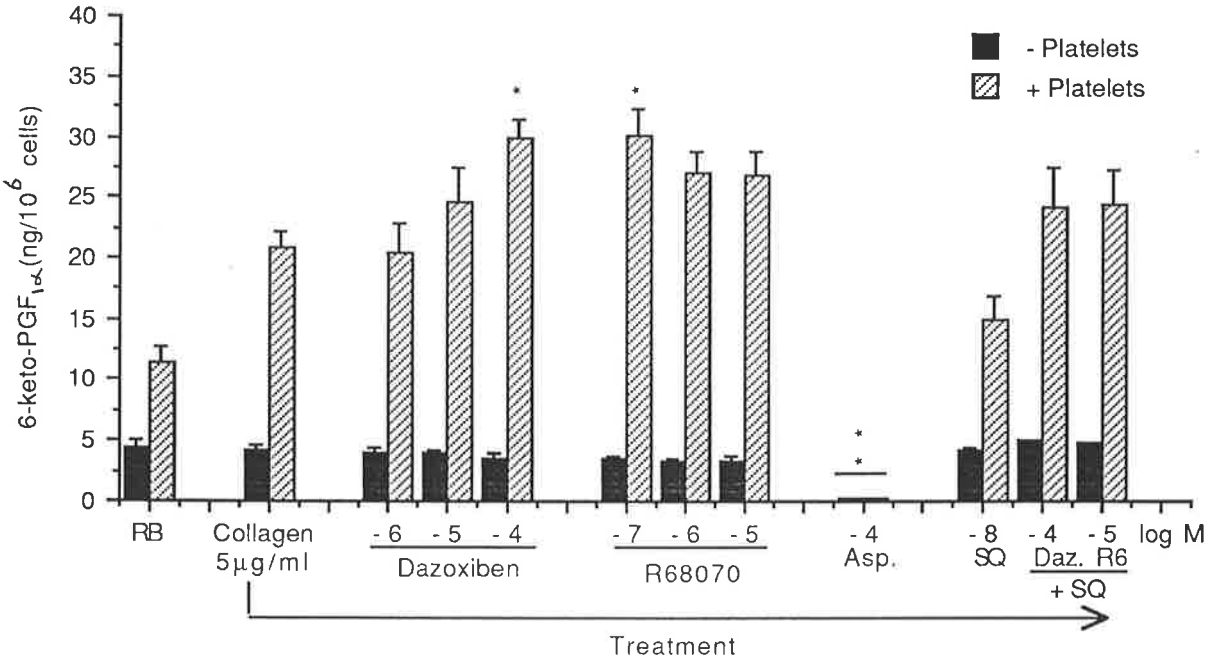
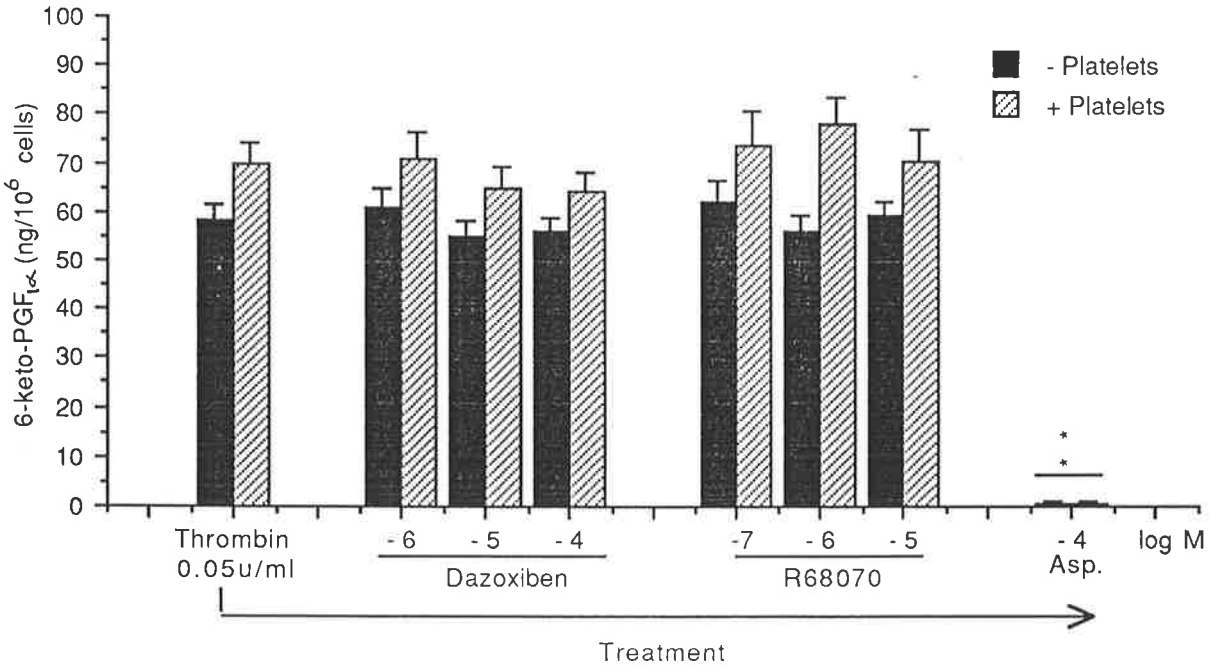


Figure 7f



Figures 7e and 7f show that in the absence of platelets, only one significant treatment effect was observed : 10^{-4}M aspirin significantly inhibited the generation of prostacyclin in response to collagen and thrombin to levels below those seen in the RB control.

In the untreated controls, significant ($p<0.05$; not shown) platelet-associated enhancement of prostacyclin generation was observed for collagen and RB but this effect did not reach significance for thrombin (Student's paired t tests).

For collagen, in the presence of platelets, a concentration-dependent enhancement in the prostacyclin generation was observed in the presence of dazoxiben over the concentration range 10^{-6}M to 10^{-4}M, significant at the latter concentration. This increase coincided with the concentration-dependent inhibition of thromboxane generation produced by dazoxiben over the same concentration range (Figure 7a). R68070 also produced enhancement of prostacyclin generation at concentrations of 10^{-7}M to 10^{-5}M. However, in contrast to dazoxiben, the maximal and only significant increase was achieved at the lowest concentration used (10^{-7}M). The maximum effects of R68070 and dazoxiben on prostacyclin generation were virtually identical in magnitude. For thrombin, neither R68070 nor dazoxiben produced any significant effects on thrombin responses (±platelets).

In response to collagen, SQ29548 at 10^{-8}M both alone and in the combined treatments produced no significant effects on prostacyclin generation in the absence of platelets. In the presence of platelets however, compared to the untreated control, SQ29548 appeared to inhibit prostacyclin generation. Similarly in the combined treatments, compared to dazoxiben and R68070 alone, SQ29548 appeared to reduced prostacyclin generation. Neither of these effects reached statistical significance, but SQ29548 did prevent dazoxiben from causing significant enhancement of prostacyclin generation over that seen in the control. In the combined treatments, although mean prostacyclin generation in the presence of platelets was lower than seen with dazoxiben or R68070 alone, it remained significantly greater ($p<0.015$, Student's paired t tests) than in the presence of SQ29548 alone. In addition, prostacyclin generation was virtually identical in the two combination treatments.

Table 7a (below) shows measurements of apparent 6-keto-$PGF_{1\alpha}$ generation from platelets in the absence of HUVECs. The results are expressed as ng/10^6 cells only for comparative purposes with 6-keto-$PGF_{1\alpha}$ data already presented. Mean values were derived from 3 experiments. N.q. indicates where concentrations were below the assay quantification limit.

	6-keto-$PGF_{1\alpha}$ (ng/10^6 cells)						
	-	Dazoxiben (M)			R68070 (M)		
Treatment [ ]		10^{-6}	10^{-5}	10^{-4}	10^{-7}	10^{-6}	10^{-5}
Collagen 5µg/ml	n.q	8.4	14.2	14.4	9.40.	11.0	6.0
Thrombin 0.05u/ml	n.q.	-	-	1.4	-	-	1.1

These data, although not derived from all 6 studies, showed that in the presence of the indicated concentrations of R68070 and dazoxiben, platelets appeared to be able to generate prostacyclin, measured as 6-keto-$PGF_{1\alpha}$. This was not evident in the untreated controls. This apparent prostacyclin generation from isolated platelets was probably attributable to cross-reactivity with other platelet-derived prostanoids (produced in the presence of thromboxane synthetase inhibition), and could not be distinguished from actual HUVEC generation of prostacyclin in wells where platelets and HUVECs were combined. This platelet-dependent error observed in the absence of HUVECs could not be used to correct 6-keto-$PGF_{1\alpha}$ values when platelets were combined with HUVECs, as platelet activation (and therefore prostanoid generation) would occur to a different extent when the cells were combined. As a consequence, it not possible to accurately determine the extent of HUVEC generation of prostacyclin in these studies, where either R68070 or dazoxiben were used as treatments.

Discussion

Effects of R68070, dazoxiben and SQ29548 on isolated platelet responses.

From the studies depicted in Figures 7a and 7b (and the preliminary studies; not shown) in the absence of HUVECs the IC_{50} of R68070 against thromboxane generation was similar to or slightly lower than that described by others (Watts *et al*, 1991; De Clerck *et al*,1989a). Dazoxiben inhibited thromboxane generation with an IC_{50} value of between 10^{-6}M and 10^{-5}M, somewhat higher than the IC_{50} of 5.4 x10^{-7}M suggested in washed platelets by De Clerck *et al* (1989a).

SQ29548 was shown in the preliminary studies not to affect thrombin stimulated responses. In response to collagen however, although it did not significantly affect platelet thromboxane generation, it inhibited collagen stimulated platelet serotonin release (see Figure 7c) with an IC_{50} of $<10^{-8}$M. This was consistent with the 5 x 10^{-9} M value suggested by Ogletree *et al* (1985) against collagen (10μg/ml) induced platelet aggregation in PRP. These effects of SQ29548 confirmed that in isolated platelets, thrombin stimulated platelet responses were thromboxane independent. In contrast, in response to collagen, platelet serotonin release was completely dependent upon the positive feedback effects of thromboxane A_2; while thromboxane generation itself was not significantly affected by such feedback effects.

As shown in Figures 7a-d, in the absence of HUVECs, platelet serotonin release was reduced by the inhibition of thromboxane generation and/or the antagonism of TXA_2/PGH_2 receptors where collagen (5μg/ml) but not thrombin (0.05u/ml) was used as a stimulus. For both thromboxane synthetase inhibitors, significant inhibition of collagen stimulated serotonin release only occurred when thromboxane generation was inhibited by more than 50%. However, unlike R68070 which could inhibit serotonin release by up to 80%, dazoxiben even with complete inhibition of thromboxane generation, only produced minor (22%) inhibition of serotonin release and an IC_{50} value could not be obtained (Figure 7c).

The differing platelet responses to collagen (in the absence of HUVECs) in the presence of the the inhibitors dazoxiben and R68070 were particularly interesting. Assuming that aspirin, dazoxiben at 10^{-4}M and R68070 at 10^{-6}M and 10^{-5}M all produced equivalent and maximal inhibition of platelet thromboxane generation, then the differences in the serotonin release responses had to be caused by differences in the mechanisms of action of the different agents. With 10^{-6}M R68070 or 10^{-4}M dazoxiben, less than 50% inhibition of serotonin release was observed. The maintained platelet activation and serotonin release responses in these cases could be attributed to accumulation of PGH_2, a known stimulus of platelet activation (Jaschonek & Muller, 1988; Fitzpatrick & Gormon, 1979), or to enhanced generation of platelet prostanoids.

At the highest concentration of R68070 (10^{-5}M), greater (but still incomplete) inhibition of serotonin release was seen compared with that observed at 10^{-6}M. If inhibition of thromboxane generation was already maximal at 10^{-6}M, then the greater inhibitory efficacy of R68070 at the higher (10^{-5}M) concentration was probably due to the TXA_2/PGH_2 receptor antagonistic effects opposing the platelet stimulatory effects of PGH_2. Such receptor antagonism by R68070 at $>10^{-5}$M has been demonstrated by others in PRP (De Clerck *et al,* 1989a) or whole blood (Watts, *et al* 1991) and because of reduced protein binding in washed platelets, these effects may have occurred in the present studies at even lower concentrations.

Aspirin caused significantly greater inhibition of serotonin release in response to collagen than either dazoxiben or R68070 at their highest concentrations. This was consistent with the ability of aspirin, but not dazoxiben or R68070, to block the generation of all cyclooxygenase products including endoperoxides such as PGH_2. It also implied that with 10^{-5}M R68070, complete antagonism of PGH_2 effects on platelets was not achieved.

The production of PGH_2 and its stimulatory effect on platelet serotonin release (in response to collagen and with thromboxane synthetase inhibition) was supported by the effects of SQ29548 alone and in combination with dazoxiben and R68070. SQ29548 10^{-8}M produced only minor effects on thromboxane generation, but was able to completely inhibit serotonin release through effectively blocking the effects of thromboxane A_2 (or PGH_2) on platelets. Moreover, in the presence of 10^{-4}M dazoxiben or 10^{-5}M R68070 together with SQ29548, serotonin release was reduced to an extent equivalent to that achieved by aspirin (and SQ29548 alone). Consequently, given the mechanism of action of SQ29548, there was a strong case for the involvement of accumulated platelet-derived endoperoxides in the serotonin release seen in the presence of complete thromboxane synthetase inhibition by both 10^{-4}M dazoxiben and 10^{-6}M and 10^{-5}M R68070. Where 10^{-5}M R68070 was used, incomplete platelet TXA_2/PGH_2 receptor antagonism was confirmed by the ability of SQ29548 in combination with 10^{-5}M R68070 to provide further significant inhibition of collagen stimulated serotonin release. This capacity of SQ29548 to completely eliminate the remaining serotonin release seen in the presence of thromboxane

synthetase inhibition also argued against a role of prostanoids such as PGE_2 in stimulating such release, as SQ29548 could not have inhibited PGE_2 mediated platelet activation.

Effects of R68070, dazoxiben and SQ29548 on HUVEC-mediated platelet inhibition

It was desirable to determine how the treatments affected the typical extent of inhibition by HUVECs; that is, whether the inhibitory effects of HUVECs were potentiated (or reduced) by any of the treatments. Unfortunately, the data from these studies displayed two trends which hampered the acquisition of this information. Firstly, the inhibitors used frequently caused maximal or near maximal inhibition of platelet thromboxane generation. Under these conditions, HUVECs could no longer cause further inhibition and differences in the effects of the treatments in the absence and presence of HUVECs were not detectable. The use of more inhibitor concentrations between 10^{-6}M and 10^{-5}M for dazoxiben, and concentrations between 10^{-8}Mand 10^{-7}M for R68070 may have provided incomplete inhibition of thromboxane generation such that the effects of HUVECs together with these agents could be examined. Notably, dazoxiben at 10^{-7}M failed to inhibit thromboxane generation either in the absence or presence of HUVECs. Thus in this instance it appeared the presence of HUVECs produced no alteration in the ability of dazoxiben to inhibit platelet thromboxane generation.

Another limitation imposed upon the conclusions which could be drawn from the present studies was due to the ability of HUVECs to completely inhibit platelet serotonin release in response to collagen (Figure 7c). Indeed in the presence of HUVECs, no significant effects of the various treatments could be discerned. Lowering the HUVEC seeding density even below the density of ~1.0×10^5/well (for 24 well plates) may have been useful in the present studies to reduce the efficiency of HUVEC inhibitory effects on platelet activation.

Only where thrombin was used as a stimulus (where HUVECs were unable to completely inhibit serotonin release) could differing effects of the various treatments in the presence of HUVECs be assessed (Figure 7d). In response to this stimulus (in the absence of HUVECs) serotonin release was clearly thromboxane independent. However it was observed that platelet

responses in the presence of HUVECs were affected to a minor extent by dazoxiben, R68070, and significantly by aspirin.

Both R68070 (particularly at 10^{-4}M) and dazoxiben (particularly at 10^{-7}M) appeared to exert modest impairment of the platelet inhibitory effects of HUVECs against thrombin stimulated serotonin release (Figure 7d) although these trends failed to reach statistical significance. Aspirin also compromised the platelet inhibitory effects of HUVECs and this effect did reach significance.

For aspirin, the cause of this effect could have been attributed to the inhibition of prostacyclin generation by HUVECs. This argument could not however be used to explain the effects of dazoxiben or R68070 as neither agent significantly affected prostacyclin generation (Figure 7f). The trends observed in the serotonin release responses in the presence of HUVECs and R68070 or dazoxiben, were consistent with possible proaggregatory effects of endoperoxide (e.g. PGH_2) accumulation, which with higher concentrations of R68070 (but not dazoxiben) would have been diminished by TXA_2/PGH_2 antagonism. Importantly however, if PGH_2 accumulation did enhance thrombin stimulated serotonin release in the presence of HUVECs, it was certainly not evident in their absence. Because SQ29548 was not examined where thrombin was used as a stimulus, the possible role of PGH_2 on platelet responses in the presence of HUVECs could not be confirmed.

Effects of platelets, dazoxiben, R68070 and SQ29548 on prostacyclin generation from HUVECs

The agents used in these studies have been reported to exert non-specific effects at high concentrations. Watts *et al* (1991), reported that inhibition of cyclooxygenase by R68070 may occur at 1×10^{-4} M, or 10 fold the highest concentration used in the present studies. Similarly, SQ29548 has been reported to inhibit enzymes involved in thromboxane generation and prostacyclin generation but only at very high concentrations, above 1×10^{-3}M (Ogletree *et al*, 1985). Finally, dazoxiben has been reported to cause cyclooxygenase inhibition in bovine aortic

endothelial cells at concentrations above 1 x 10^{-4}M (Martin *et al,* 1983), which was the highest concentration used in the present studies. Although evaluation of the specificity of the effects of these agents on platelets was not possible, in the presence of HUVECs any non-specific effects on HUVEC prostacyclin generation could be assessed. It was found that at the concentrations of the agents used in these studies, neither R68070, SQ29548, nor dazoxiben (in the absence of platelets) produced any significant effects on HUVEC prostacyclin generation (Figures 7e and 7f).

The prostacyclin generation results (Figures 7e and 7f) showed the typical platelet-associated enhancement of such generation in the control and where collagen was used as a stimulus. A relatively modest increase in generation was observed where thrombin was used as a stimulus. As proposed in the previous chapter, the magnitude of platelet-associated enhancement of prostacyclin generation appeared to be determined by the initial level of HUVEC activation seen in their absence and possibly a maximal level of generation which could be obtained. In the present studies, as in those of Chapter 6, in response to thrombin 0.05u/ml HUVECs produced high levels of prostacyclin (6-keto-PGF1α >60 ng/10^6cells) in the absence of platelets and additional generation associated with the presence of platelets appeared to be limited. In response to thrombin, and in the presence of platelets (as shown in Figure 7f) inhibition of thromboxane synthetase did not increase generation further. This suggests that thrombin may not have provided sufficient platelet endoperoxide liberation to enhance prostacyclin generation. Alternatively, maximal generation was obtained in the presence of untreated platelets and further generation in the presence of thromboxane synthetase inhibition was not possible.

In contrast, in response to thrombin, for collagen, thromboxane synthetase inhibition with either dazoxiben and R68070 caused apparent enhancement of prostacyclin generation over and above the typical enhancement associated with the presence of platelets. The maximal enhancement in generation did not even reach a 2 fold level. These results contrast with previous studies which have found that in the presence of platelets stimulated with ionophore A23187 or thrombin (5u/ml), thromboxane synthetase inhibition enhanced prostacyclin generation from aspirin

treated HUVECs by ~10 fold (Marcus *et al,* 1980). Others using arachidonic acid as a stimulus have reported a 2 fold enhancement (Baenziger, Becherer & Majerus, 1979). In addition, in the present studies, the apparent enhancement in prostacyclin generation, particularly for collagen, may in part have been an artefact caused by non-specificity in the 6-keto-$PGF_{1\alpha}$ radioimmunoassay.

It has been shown that in isolated platelets, in response to arachidonic acid, ~22% of total platelet arachidonic acid metabolism (via cyclooxygenase) results in the formation of thromboxane, while only 5% is metabolised to PGE_2, PGD_2 and $PGF_{2\alpha}$ (Bertele *et al,* 1984; De Clerck *et al*, 1989a) and none to prostacyclin (Marcus *et al*, 1980). In the presence of complete thromboxane synthetase inhibition by dazoxiben 4 x 10^{-5}M, it has been shown that 20% of arachidonic acid metabolism via cyclooxygenase results in the formation of PGE_2 with ~4 and 8% metabolised to $PGF_{2\alpha}$ and PGD_2 respectively (Bertele *et al,* 1984). Similar alterations by R68070 in the metabolism of arachidonic acid by washed platelets has also been observed (De Clerck *et al,* 1989a). In the present studies, given that high levels of platelet PGE_2 may be generated from platelets in the presence of thromboxane synthetase inhibition, the cross-reactivity of the 6-keto-$PGF1_{\alpha}$ antiserum for such prostanoids (typically produced at low levels) had to be reconsidered. The manufacturers reported that the 6-keto-$PGF_{1\alpha}$ antiserum used for RIAs in the present studies displayed cross-reactivity with PGE_2 of only 0.6% (there was also 2.2% cross reactivity with $PGF_{2\alpha}$ and 0.1% with PGD_2). Unfortunately, this cross reactivity was clearly sufficient reduce the validity of measurements made for 6-keto-$PGF_{1\alpha}$ in samples derived from collagen stimulated platelets in the presence of thromboxane synthetase inhibition.

As shown in Table 7a, in the untreated collagen and thrombin control, there was no evidence of prostacyclin generation from untreated platelets, consistent with the findings of preliminary studies using this RIA (Chapter 2). In contrast, in the presence of dazoxiben and R68070, platelets appeared to generate detectable concentrations of prostacyclin. When thrombin was used as a stimulus, this apparent platelet-associated prostacyclin generation represented only a minor proportion of total concentrations (<2% error) observed in the presence of HUVECs, and measurements of prostacyclin may therefore have estimated prostacyclin generation with

reasonable accuracy. For collagen however, in the presence of thromboxane synthetase inhibitors, up to 50% of prostacyclin generated in the presence of HUVECs could have been attributable to apparent prostacyclin generation from platelets. In addition, the error observed where isolated platelets were examined could not be used to correct the data in the presence of HUVECs due to the possible inhibitory effects of HUVECs on platelet arachidonic acid metabolism, making the two situations incomparable.

This artefact caused by cross-reactivity meant that in the presence of thromboxane synthetase inhibition and collagen stimulated platelets, prostacyclin measurements had to be considered, at least in part, as a reflection of prostanoid concentrations derived from platelets. Such cross-reactivity was obviously not a concern in treatments where thromboxane synthetase inhibition was not undertaken (e.g treatments with SQ29548 or aspirin). In the TXB_2 assay problems due to cross reactivity (of <0.1% with possible platelet-derived prostanoids) were not evident as it was observed that the thromboxane synthetase inhibitors used could reduce thromboxane generation to levels equivalent to those of aspirin (Figures 7a and 7b). This could not have been achieved had cross-reactivity been evident. Furthermore, others encountering inaccuracies in their 6-keto-$PGF_{1\alpha}$ RIAs in serum samples have not found similar problems with respect to TXB_2 RIAs (Pedersen, Watson & FitzGerald, 1983).

Due to these effects of cross-reactivity, no definite conclusions could be drawn regarding HUVEC- derived prostacyclin generation where thromboxane synthetase inhibitors were used. However, where collagen was used as a stimulus, the observation that in the presence of platelets the apparent prostacyclin generation (or prostanoid generation) profiles differed for dazoxiben and R68070 was of interest.

In platelet and HUVEC coincubations, where collagen was used as a stimulus, dazoxiben caused concentration-dependent enhancement of apparent prostacyclin generation (significant at 10^{-4}M) but R68070 achieved its greatest enhancement at the lowest concentration used (10^{-7}M). The cause of the lesser generation seen with higher concentrations of R68070 (but not dazoxiben) may have been associated with increasing TXA_2/PGH_2 receptor antagonism by

R68070 at such concentrations. Consistent with this suggestion, in the presence of thromboxane synthetase inhibition the addition of SQ29548 also reduced apparent prostacyclin generation, supporting the hypothesis that platelet-derived PGH_2 may stimulate prostanoid generation from platelets and/or prostacyclin generation from HUVECs. In this combined treatment situation, where TXA_2/PGH_2 receptor antagonism was complete, the effects of dazoxiben or R68070 became indistinguishable.

Two possible explanations were proposed to explain why TXA_2/PGH_2 receptor antagonism appeared to compromise the typical prostanoid and/or prostacyclin generation seen in the presence of platelets (and HUVECs) during thromboxane synthetase inhibition. Firstly, if part of the enhancement was considered to be caused by platelet-derived prostanoids, then the minor reductions seen with TXA_2/PGH_2 receptor antagonism could be explained by the blockade of feedback effects of PGH_2 on platelets, thereby reducing their activation. This could have reduced prostanoid liberation and the size of the artefact caused by such prostanoids. Because complete receptor antagonism with SQ29548 (10^{-8}M) alone did not produce marked diminution of platelet thromboxane generation (and presumably prostanoid liberation) in response to collagen (Figure 7a) an alternative explanation was favoured. Part of the enhancement could have been caused by enhanced generation of prostacyclin from HUVECs, and although endoperoxide steal may have contributed to this, PGH_2 may also stimulated generation through acting as an agonist on HUVEC TXA_2/PGH_2 receptors. If this was the case, then the blockade of HUVEC TXA_2/PGH_2 receptors (by R68070 at higher concentrations, or SQ29548) could have been responsible for reducing prostacyclin generation from HUVECs.

In further studies, of a similar nature (using collagen stimulated platelets but a more specific method for quantifying prostacyclin), closer examination of the differing effects of these two thromboxane synthetase inhibitors on HUVEC prostacyclin generation would have been of interest. From these, the relative extent of endoperoxide steal or receptor mediated effects of platelet-derived PGH_2 on HUVEC prostacyclin could be determined. If *in vivo,* in the presence of thromboxane synthetase inhibitors, elevated platelet-derived PGH_2 can result in a physiologically relevant extent of receptor mediated prostacyclin generation, then agents such as

R68070 may at higher concentrations compromise this potentially important pathway of prostacyclin generation.

In the present studies (due to the problem of RIA cross-reactivity) no conclusions could be made as to whether thromboxane synthetase inhibition actually enhanced endoperoxide steal and thereby HUVEC prostacyclin generation. However, in the combined treatments, apparent prostacyclin generation remained significantly greater than prostacyclin generation in the presence of SQ29548 alone. This difference had to be caused either by endoperoxide steal causing enhancement of prostacyclin generation from HUVECs, and/or by the effects of RIA cross-reactivity.

In the absence of thromboxane synthetase inhibition, and in the presence of collagen stimulated platelets (but not in their absence), SQ29548 caused a non-significant reduction of HUVEC prostacyclin generation. This effect of SQ29548, if confirmed, would be consistent with it blocking thromboxane A_2 receptor mediated prostacyclin generation from HUVECs, and part of the typical platelet-associated enhancement phenomenon would thus be attributable to the effects of platelet-derived thromboxane A_2. Although others have shown that TXA_2/PGH_2 mimetics can stimulate prostacyclin generation from HUVECs (Kent *et al*, 1993) and other endothelial cell lines (Hunt *et al*, 1992; Clesham *et al,* 1992), no studies have provided evidence that authentic thromboxane A_2 can produce similar effects. Had a larger number of experiments incorporated the SQ29548 treatment, then significant receptor mediated effects of platelet-derived thromboxane A_2 upon HUVEC prostacyclin generation may have been observed.

The possibility that thromboxane A_2 may also be involved in the enhancement of prostacyclin generation observed in the presence of collagen stimulated platelets was not followed further. However, in ongoing studies the use of U46619 (a thromboxane mimetic) could have confirmed or refuted the ability of TXA_2/PGH_2 receptor occupation to mediate prostacyclin generation from HUVECs. In subsequent studies, the use of higher collagen concentrations could also enhance the effects of platelet-derived thromboxane A_2 on HUVECs. If thromboxane A_2 could

stimulate prostacyclin generation, then under these conditions, statistically significant decreases in such generation may be observed in the presence of SQ29548.

Summary and conclusions.

The results from these studies showed that, at equivalent concentrations, R68070 was a more potent inhibitor of thromboxane synthetase than dazoxiben. In addition, at 10^{-5}M, R68070 appeared to cause partial inhibition of platelet TXA_2/PGH_2 receptors. This effect resulted in the ability of R68070 to display superior inhibition of collagen stimulated platelet serotonin release when compared to dazoxiben. In response to thrombin, irrespective of the agents used, even the complete inhibition of thromboxane generation failed to affect serotonin release. These observations confirmed the thromboxane-dependent nature of platelet serotonin release in response to collagen, but not thrombin.

The results also produced substantial evidence that with the complete inhibition of thromboxane synthetase, accumulated platelet PGH_2 production was sufficient to maintain substantial serotonin release in response to collagen. There was no evidence to suggest that with maximal inhibition of thromboxane generation, R68070 or dazoxiben could enhance the platelet inhibitory effects of HUVECs. For collagen, the efficacy of HUVECs in inhibiting serotonin release was maximal and unchanged by any treatments. For thrombin, the inhibitory effects of HUVECs on platelet serotonin release were reduced slightly by R68070 at low (10^{-7}M) and by dazoxiben at high (10^{-4}M) concentrations, but these effects failed to reach significance.

TXA_2/PGH_2 receptor antagonism produced slight but non-significant inhibition of HUVEC prostacyclin generation in the presence of collagen stimulated platelets. Although this effect failed to reach significance in these experiments, it would be consistent with the notion that platelet-derived thromboxane A_2 generation in response to this stimulus contributes in part, through HUVEC receptor occupation, to the typical platelet-associated enhancement in HUVEC prostacyclin generation.

In response to collagen (5μg/ml) and in the presence of complete thromboxane synthetase inhibition, platelet prostanoid generation was clearly sufficient to invalidate (through the effects

of cross-reactivity) 6-keto-$PGF_{1\alpha}$ assessments. This meant that where platelets and HUVECs were combined, specific assessments of HUVEC-derived prostacyclin generation could not be made. However, under these conditions platelet-derived PGH_2 generation appeared, either through endoperoxide steal or receptor mediated effects of PGH_2, to be involved in enhanced prostanoid generation. This may have included enhanced prostacyclin generation from HUVECs.

In response to thrombin (0.05u/ml) and in the presence of complete thromboxane synthetase inhibition, platelets evidently generated prostanoids to an extent which (through effects of cross-reactivity) would have produced only minor errors in 6-keto-$PGF_{1\alpha}$ assessments. This meant that where platelets and HUVECs were combined, prostacyclin generation from HUVECs could be determined with reasonable accuracy. Under these conditions, neither agent enhanced apparent prostacyclin generation from HUVECs, suggesting that platelet-derived endoperoxide generation was insufficient to affect HUVEC prostacyclin generation.

The limitations in the RIA used for these studies meant that if further investigations into the effects of thromboxane synthetase inhibitors were to be undertaken, alternative, more specific methods to quantify 6-keto-$PGF_{1\alpha}$, (e.g. gas chromatography-mass spectrometry) would be required. Such studies were not pursued as greater emphasis was placed on further assessment of the HUVEC-derived mediators (other than prostacyclin) which were responsible for HUVEC-mediated inhibition of activated platelet responses in this experimental system.

In earlier studies, it had been demonstrated that HUVEC mediated inhibition of platelets was diminished with the inhibition of prostacyclin generation. It had however been shown in some instances that, where virtually all prostacyclin generation was eliminated, HUVECs retained an inhibitory effect on platelet activation (Chapter 6). It was speculated that EDRF may be involved in this phenomenon. It was therefore decided to examine whether in subsequent interaction studies any platelet inhibitory effects of EDRF could be detected in the complete absence of prostacyclin generation by HUVECs.

Chapter 8

Effects of methylene blue and haemoglobin on platelets, HUVECs, and HUVEC-mediated inhibition of platelet activation

Introduction

Vascular endothelial cells produce a number of substances which are important in the prevention of thrombotic events (Vane *et al,* 1990). Two such substances are prostacyclin (Moncada, 1982; Weksler & Jaffe, 1986) and endothelium-derived relaxing factor (EDRF) or nitric oxide (NO) (Palmer, Ferrige & Moncada, 1987; Ignarro, *et al.* 1988; Gillespie & Sheng, 1988; Ignarro, 1989). As described in the general introduction, both prostacyclin (through stimulating cAMP generation) and EDRF (through stimulating cGMP generation) can inhibit platelet aggregation, cause vasorelaxation and when acting together have been reported to produce synergistic inhibition of platelet aggregation (Radomski, Palmer & Moncada, 1987b; Alheid, Frolich & Forsterman, 1987; Macdonald, Read & Dusting, 1988). EDRF can also inhibit platelet adhesion (Radomski, Palmer & Moncada, 1987c,d; Venturini, Del Vecchio& Kaplan, 1989).

In the studies described in Chapter 6, it was proposed that HUVEC-derived mediators other than prostacyclin may be responsible for some of the platelet inhibitory effects of HUVECs. In this chapter it was of interest to examine the effects of EDRF upon interactions between platelets and HUVECs. The possibility of detecting the effects of EDRF in this experimental system was considered to be favoured by the use of washed platelets, as platelet inhibitory effects of EDRF are reportedly 2-3 times greater in washed platelets than in PRP (Radomski, Palmer & Moncada, 1987a). However, given that the system was closed (rather than perfused) possible oxidant accumulation may reduce the half-life of EDRF and therefore hamper the detection of its effects.

Methods have been developed to measure the formation of EDRF (reviewed recently by Archer, 1993). However, the presence of EDRF can be also be inferred through examining how, in a system where EDRF is proposed to exert detectable effects, those effects are affected by pharmacological agents which influence the formation, activity and elimination of EDRF.

In many *in vitro* studies, pharmacological agents have also been used to differentiate between the platelet inhibitory effects of mediators such as prostacyclin and EDRF derived from isolated vascular tissue or endothelial cells. Aspirin or indomethacin have frequently been used to inhibit endothelial cell cyclooxygenase and thus the generation of prostacyclin. To inhibit or eliminate the effects of EDRF, methylene blue (to inhibit soluble guanylate cyclase) or haemoglobin (to scavenge extracellular EDRF) have often been used (Alheid, Frolich & Forsterman, 1987; Alheid, Reichwehr & Forsterman,1989; Radomski, Palmer & Moncada 1987a-d; Gillespie & Sheng, 1988; Martin *et al.*, 1985; Salvemini *et al*, 1990; Macdonald, Read & Dusting, 1988).

In the studies to be undertaken in this chapter, in order to evaluate the effects of EDRF alone, it was necessary to eliminate prostacyclin generation by HUVECs because of reported synergy between EDRF and prostacyclin (Radomski, Palmer & Moncada, 1987b; Alheid, Frolich & Forsterman, 1987; Macdonald, Read & Dusting, 1988). This could be achieved by using aspirin to block the production of prostacyclin from HUVECs. However, to be certain that prostacyclin generation was completely eliminated, dual aspirin treatment of platelets and HUVECs was undertaken to ensure no endoperoxide steal could occur.

Aims

The aims of the studies described in this chapter were:

1. To assess whether in response to collagen and thrombin, haemoglobin or methylene blue (used to inhibit the effects of EDRF) affected the typical functions of platelets (i.e. serotonin release or thromboxane generation) or HUVECs (i.e. prostacyclin generation).

2. To examine the effects of these agents on the ability of HUVECs to inhibit platelet responses to thrombin and collagen.

3. To determine in the absence of prostacyclin, the effects of haemoglobin on the ability of HUVECs to inhibit platelet serotonin release in response to thrombin.

Methods

HUVECs were isolated, cultured and subcultured as described in Chapter 2. Washed platelets were prepared and labelled with ^{14}C-serotonin also as described in Chapter 2.

Interaction study protocol

Twelve well culture plates were used and eight wells per plate were seeded (at 1.8-2.0 x 10^5 cells/well) with HUVECs 48 hours prior to use. For interaction studies, all wells (±HUVECs) were washed for 20 minutes with M199 (serum free), then for 5 minutes with platelet RB. Platelets or RB was then added to wells (950µl) prior to stimulus addition (50µl). The stimuli used in these studies were collagen (5.0µg/ml) or thrombin (0.05u/ml) and RB was used in the unstimulated control. Following the addition of the stimulus, final incubations of 5 minutes were performed using a shaker speed of 4. Platelet serotonin release, thromboxane generation (measured as TXB_2) and HUVEC prostacyclin generation (measured as 6-keto-$PGF_{1\alpha}$) were assessed and the data were expressed as described in Chapter 2.

Treatments of platelets and HUVECs

Inhibitors included aspirin, methylene blue and haemoglobin (i.e. oxyhaemoglobin) and were prepared as described in Appendix 2. The stability of haemoglobin for the duration of experiments was confirmed by spectrophotometric scan (450-650nm).

Where the complete inhibition of prostacyclin generation was required, both platelets and HUVECs were incubated with aspirin (final concentration 50µM). Aspirin was added to platelets just prior to their transfer into the storage syringe and HUVECs were pretreated with aspirin during the first 20 minute preincubation wash with medium 199.

Where required, methylene blue was incubated (final concentration 1 or 10 μM) with HUVECs during both the first 20 minute preincubation with medium 199, and the second 5 minute preincubation with RB. It was also included at equivalent concentrations in the final 5 minute incubation with platelets or RB. Where required, haemoglobin was incubated (1 or 10 μM) with HUVECs during the second 5 minute preincubation with RB and was also included at equivalent concentrations in the final 5 minute incubation with platelets or RB .

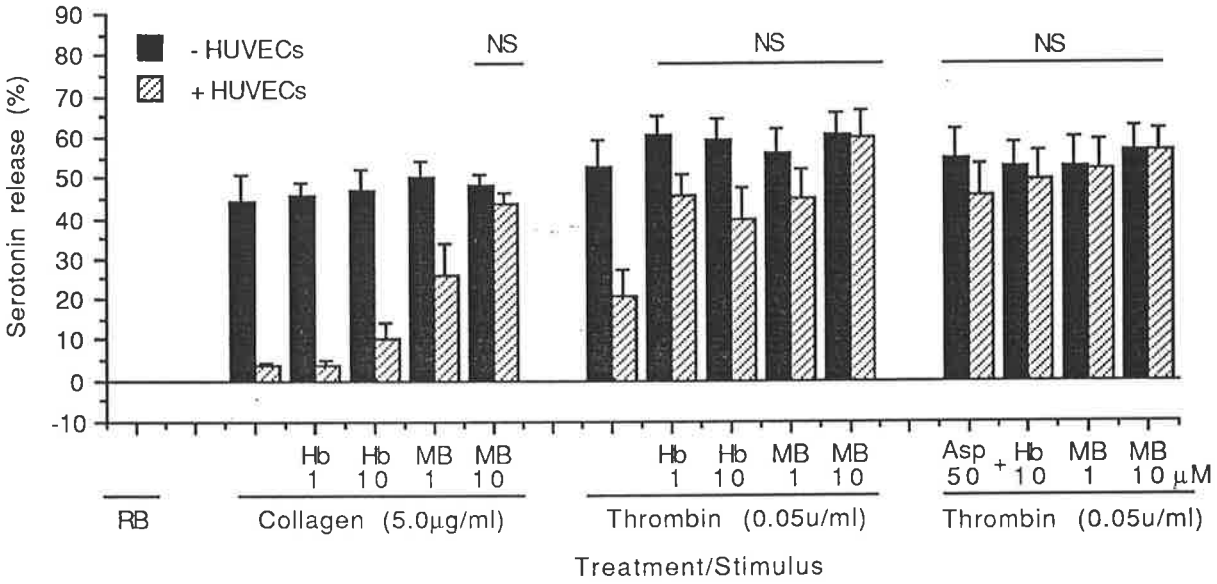
Combined treatments of aspirin with either methylene blue or haemoglobin were also made as required.

Statistical analyses

Data within the collagen (n=5) and thrombin (n=6) stimulus groups were analysed separately. For platelet % serotonin release, log transformed TXB_2 or log transformed 6-keto-$PGF_{1\alpha}$ data, parametric three-way ANOVA was used to assess variance attributable to different treatments, the absence or presence of HUVECs (or platelets), and the experimental variation. Following ANOVA specific sources of variance were assessed using Tukey's HSD tests.

Results

Figure 8a shows platelet serotonin release in response to RB (control), collagen (5μg/ml) or thrombin (0.05u/ml) in the absence or presence of HUVECs. Platelets and HUVECs were treated with haemoglobin (Hb) or methylene blue (MB) or with 50μM aspirin (Asp.) either alone or in the combinations indicated. None of the treatments caused significant effects upon typical platelet responses in the absence of HUVECs. HUVECs caused significant ($p<0.01$ or $p<0.05$) inhibition of platelet serotonin release responses except where indicated by NS. Data where thrombin was used as a stimulus were generated from 6 experiments and collagen was used as a stimulus in 5 of these.



In the untreated controls platelet serotonin release in response to both collagen and thrombin was significantly inhibited in the presence of HUVECs ($p<0.01$).

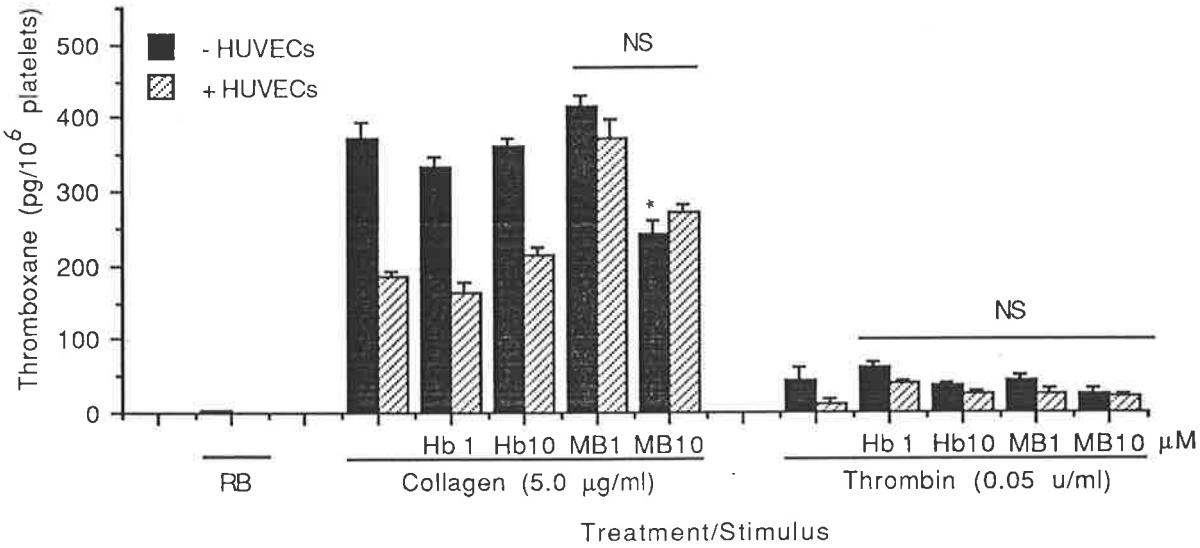
In the absence of HUVECs neither methylene blue nor haemoglobin significantly affected platelet release responses to either collagen or thrombin. In addition, aspirin alone did not significantly inhibit platelet serotonin release in response to thrombin.

Irrespective of the stimulus, methylene blue at 1μM and 10μM reduced the typical inhibitory effects of HUVECs on platelet release responses. This effect was particularly evident at 10μM which abolished the ability of HUVECs to inhibit platelet serotonin release in response to either stimulus.

Similarly, haemoglobin at 1μM and 10μM also reduced the typical inhibitory effects of HUVECs. Haemoglobin appeared less potent than methylene blue particularly when collagen was the stimulus, and even in the presence of 10μM haemoglobin HUVECs were still able to produce significant inhibition of serotonin release ($p<0.01$). Where thrombin was used as a stimulus however, in both the presence of 1μM and 10μM haemoglobin, HUVECs were unable to significantly inhibit platelet serotonin release.

Following aspirin treatment (of both platelets and HUVECs), HUVECs failed to produce significant inhibition of platelet serotonin release in response to thrombin. Consequently, no specific effects attributable to the additional treatment with either haemoglobin or methylene blue could be determined.

Figure 8b shows platelet thromboxane generation in response to RB (control), collagen (5μg/ml) or thrombin (0.05u/ml) in the absence or presence of HUVECs. When aspirin was used as a treatment platelet thromboxane generation was not quantifiable and the results are not shown. Platelets and HUVECs were treated with haemoglobin (Hb) or methylene blue (MB) as indicated. * shows where, in the absence of HUVECs the treatment caused significant ($p<0.05$) effects on platelet responses compared to the untreated controls. HUVECs caused significant ($p<0.05$) inhibition of platelet thromboxane generation responses except where indicated by NS. Data where thrombin was used as a stimulus were generated from 6 experiments and collagen was used as a stimulus in 5 of these.

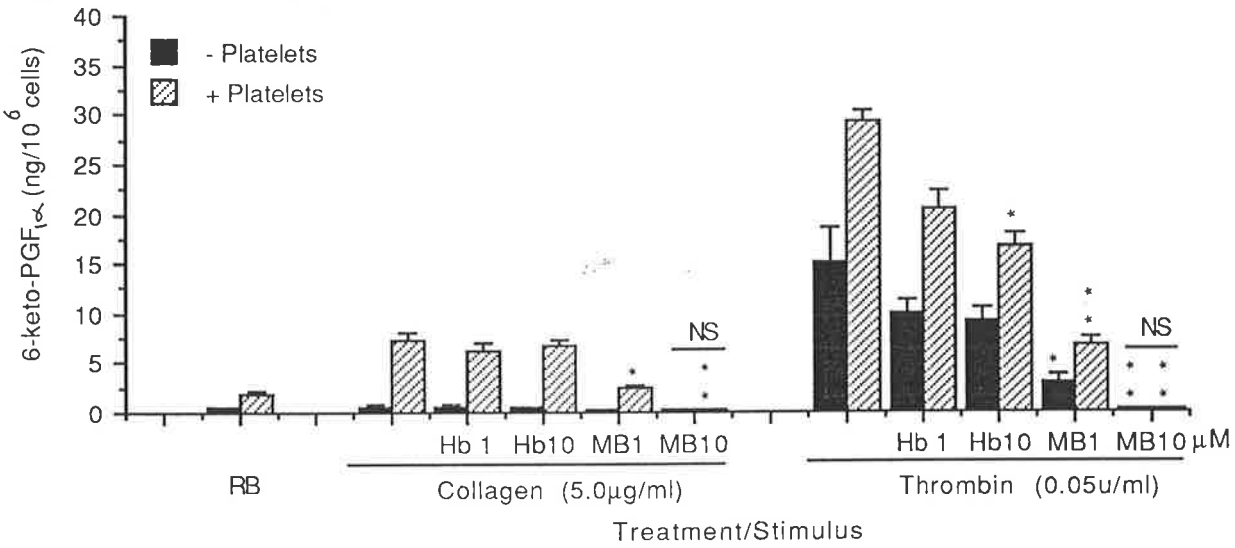


In the untreated controls platelet thromboxane generation in response to both collagen and thrombin was significantly inhibited in the presence of HUVECs ($p<0.05$).

In the absence of HUVECs, the only significant effect of the treatments on platelet thromboxane generation was that 10μM methylene blue caused significant inhibition of platelet thromboxane generation in response to collagen. In the presented studies, platelets were only exposed to methylene blue for 5 minutes. However, in two separate studies, platelet responses were examined after 5 or 25 minute exposure to 10μM methylene blue. Greater mean inhibition of thromboxane generation was achieved with the longer preincubation time, but responses to collagen (62% inhibition) and thrombin (47% inhibition) were not completely inhibited.

In the presence of 1μM and 10μM methylene blue the typical inhibitory effects of HUVECs on stimulated platelet thromboxane generation were abolished, irrespective of the stimulus used. Haemoglobin (1μM and 10μM) also prevented significant HUVEC-mediated inhibition of platelet thromboxane generation, but only where thrombin (not collagen) was the stimulus used.

Figure 8c shows HUVEC prostacyclin generation (measured as 6-keto-$PGF_{1\alpha}$) in response to RB (control), collagen (5μg/ml) or thrombin (0.05u/ml) in the absence and presence of platelets. When aspirin was used as a treatment HUVEC prostacyclin generation was not quantifiable and the results are not shown. Platelets and HUVECs were treated with haemoglobin (Hb) or methylene blue (MB) as indicated. Compared to the untreated controls, * ($p<0.05$) or **($p<0.01$) show where the treatments caused significant inhibition of prostacyclin generation (either in the absence or presence of platelets). The presence of platelets was associated with significant ($p<0.05$ or $p<0.01$) enhancement of prostacyclin generation from HUVECs except where indicated by NS. Data where thrombin was used as a stimulus were generated from 6 experiments and collagen was used as a stimulus in 5 of these.



In the absence of platelets and in response to collagen, prostacyclin generation was too low to permit the determination of significant inhibition by the treatments but in response to thrombin, significant concentration-dependent inhibition of prostacyclin generation by methylene blue was evident. In the presence of platelets (where prostacyclin generation was greater), concentration-dependent inhibition of prostacyclin generation by methylene blue was evident for both stimuli.

In the absence of platelets, haemoglobin was without significant effects on HUVEC prostacyclin generation but in the presence of platelets, at 10μM, it produced significant inhibition of prostacyclin generation in response to thrombin. This effect was not seen in later studies (Chapter 9) and was therefore viewed as questionable.

In the untreated controls the presence of platelets caused significant enhancement of prostacyclin generation in response to collagen ($p<0.01$), thrombin ($p<0.05$) and in the control ($p<0.05$; Student's paired t test). Haemoglobin (and 1μM methylene blue) did not prevent platelet-associated increases in HUVEC prostacyclin generation, but 10μM methylene blue (which like aspirin, completely inhibited prostacyclin generation) abolished such enhancement.

Discussion

The ability of EDRF and prostacyclin generated from isolated endothelial cells to inhibit platelet aggregation has been demonstrated by a number of groups (including Radomski, Palmer & Moncada, 1987a,b; Alheid, Frolich & Forsterman, 1987; Alheid, Reichwehr & Forsterman, 1989 and Macdonald, Read & Dusting, 1988). In the present studies, it was considered important to identify whether both these mediators were contributing to the platelet inhibitory effects of HUVECs. Haemoglobin and methylene blue were used to inhibit the effects of EDRF and aspirin was used to inhibit the effects of prostacyclin. The experimental protocol allowed direct comparisons between the effects of these agents to be assessed and any differences in such effects to be determined.

Aspirin was used in the present studies where it was important to eliminate the potential synergistic effects of prostacyclin and EDRF in their inhibition of platelet aggregation. Although in this system it had already been demonstrated that endoperoxide donation from platelets to HUVECs only occurred to a minor extent, it was necessary to ensure absolutely no prostacyclin generation could occur. Thus, where aspirin was used, both HUVECs and platelets were treated. Following treatment with aspirin, thrombin was the only stimulus used because it could produce a substantial platelet release response in the absence of platelet thromboxane generation.

Inhibition of prostacyclin generation and or EDRF; associated effects on HUVEC-mediated platelet inhibition

Following the aspirin treatment and the complete inhibition of prostacyclin generation, HUVECs lost virtually all their capacity to inhibit platelet serotonin release in response to thrombin. This was consistent with prostacyclin generation from HUVECs being important in the inhibition of platelet responses to thrombin, but meant no further reductions in platelet inhibitory effects of HUVECs could be detected. Therefore whether methylene blue or haemoglobin could reduce the platelet inhibitory effects of HUVECs could not be determined under these conditions.

In the absence of the aspirin treatment, it was considered that the effects of eliminating EDRF alone could be examined, using either haemoglobin or methylene blue. It was observed however that methylene blue at 10μM caused a number of non-specific effects and could eliminate prostacyclin generation from HUVECs (both ± platelets).

Where collagen was used as a stimulus, this effect was used to advantage to compare the effects of methylene blue with those of haemoglobin (which did not affect prostacyclin generation in this stimulus group). In response to collagen, haemoglobin alone only reduced HUVEC inhibition of platelet responses to a minor extent. This implied that the inhibition of EDRF effects alone did not strongly compromise the inhibitory activity of HUVECs over collagen stimulated platelet responses. Possibly, the retained significant inhibitory effects of HUVECs was associated with their retained generation of prostacyclin. Methylene blue (10μM), because of its non-specific effects, eliminated both prostacyclin generation and the effects of EDRF. Under these conditions, the typical platelet inhibitory effects of HUVECs against collagen stimulated platelet responses were completely eliminated. This was consistent with the importance of prostacyclin (but not necessarily EDRF), in the inhibition of collagen stimulated platelet responses .

Where thrombin was used as a stimulus, the effects of haemoglobin provided evidence for a role for EDRF in the inhibition of thrombin stimulated platelet responses by HUVECs. Haemoglobin 1μM and 10μM (which had produced only minor effects on HUVEC inhibition of collagen stimulated platelet responses) prevented HUVECs from exerting significant inhibition of platelet responses. This meant that the effects of haemoglobin (which in the present studies produced only modest inhibition of prostacyclin generation) were similar to those of aspirin, which produced complete inhibition of prostacyclin generation from HUVECs. Therefore (in contrast to collagen) it appeared that for thrombin, HUVEC mediated inhibition of platelet responses could be eliminated with the inhibition of prostacyclin generation or the effects of EDRF. These results were consistent with both EDRF and prostacyclin being important mediators in the inhibition of platelet responses to thrombin.

Where both prostacyclin and EDRF effects were inhibited, either in the combination treatments with aspirin or with 10μM methylene blue, there was no evidence of HUVEC-mediated inhibition of stimulated platelet responses. Thus prostacyclin and EDRF appeared to completely account for the typical inhibitory effects of HUVECs against both thrombin and collagen stimulated platelet responses.

Non-specific effects of the agents used

Methylene blue and haemoglobin are frequently used *in vitro* as specific inhibitors of the effects of EDRF (Alheid, Frolich & Forsterman, 1987; Alheid, Reichwehr & Forsterman,1989; Radomski, Palmer & Moncada 1987a-d; Gillespie & Sheng, 1988; Martin *et al.*, 1985; Salvemini *et al*, 1990; Macdonald, Read & Dusting, 1988). Methylene blue has been reported to inhibit the effects of EDRF through oxidative inhibition of intracellular soluble guanylate cyclase (Gruetter *et al*, 1980; Gruetter, Kadowitz & Ignarro, 1980; Martin *et al*, 1985). This effect prevents EDRF from activating this enzyme and stimulating increases in cellular cGMP. Haemoglobin reportedly inhibits the effects of EDRF by scavenging extracellular EDRF, as it binds EDRF (or NO) with greater affinity than O_2 (Gibson & Roughton, 1957). It may also promote oxidative inactivation of EDRF (Marshall & Kontos, 1990; Ignarro, 1993).

Clearly in the present studies, the effects of these agents were not confined to the effects described above. In particular, the inhibitory effects of methylene blue upon prostacyclin generation in these experiments resulted (in the absence of aspirin treatments) in haemoglobin and methylene blue exerting differing effects upon HUVEC mediated inhibition of platelet activation. It was of interest to consider the mechanism of the inhibitory effect of methylene blue upon prostacyclin generation.

If in the present studies methylene blue was specifically inhibiting guanylate cyclase in platelets and HUVECs, then in HUVECs this inhibition could prevent EDRF from causing feedback elevation of cGMP (Boulanger *et al*, 1990). In cultured endothelial cells, cGMP analogues (e.g 8-bromo-cGMP) either appear to down regulate prostacyclin generation (Kobayashi *et al*,

1990) or have no effect on basal or stimulated prostacyclin generation (Martin, Drazan & Newby, 1989). Thus, with the specific inhibition of guanylate cyclase one would not have expected there to be any inhibition of prostacyclin generation. Non-specific effects of methylene blue must therefore have caused the decrease in prostacyclin generation observed in these studies.

A number of studies have provided evidence that methylene blue may inhibit the effects of EDRF through mechanisms other than through the inhibition of guanylate cyclase. It has been reported that methylene blue can directly inhibit (probably as a result of oxidation at the haem group) purified nitric oxide synthetase (Mayer, Brunner & Schmidt, 1993). Thus the enzyme inhibitory effects of this agent now appear to be non-specific. In addition, in studies by Wolin *et al* (1990) and Marczin, Ryan & Catravas (1992b) it has been demonstrated that methylene blue, through stimulating the generation of superoxides, may cause direct inactivation of EDRF. Such generation of oxidants in this experimental system could have been responsible for the non-specific inhibitory effects of this agent upon prostacyclin generation.

Enzymes responsible for prostacyclin generation are susceptible to oxidative inhibition (Marshall & Kontos, 1990; Moncada, Palmer & Gryglewski, 1986; Czervionke *et al,* 1979a; Brotherton & Hoak, 1983; Dejana *et al,* 1983; Vane, Gryglewski & Botting, 1987). In the present studies, the mechanism by which methylene blue inhibited prostacyclin generation seemed likely to involve oxidative inactivation of the enzymes responsible for its synthesis. To date there appears to be only one other report (Martin, Drazan & Newby, 1989) in which methylene blue has been shown to inhibit prostacyclin generation from endothelial cells. In this study methylene blue (but not haemoglobin) inhibited basal and bradykinin stimulated prostacyclin generation from pig aortic endothelial cells with an IC_{50} concentration of 0.5μM. Although in the present studies the lowest concentration used was 1μM the results indicated an IC_{50} value would fall below this concentration. The mechanism by which methylene blue exerted its inhibition was not investigated by Martin's group. However, as in the present studies, the system they used was closed, rather than perfused, so oxidant accumulation may have been involved in the effects observed.

Similarly, oxidative inhibition of platelet enzymes may also explain the inhibition by 10μM methylene blue of platelet thromboxane generation. However, even following a total 25 minute exposure of platelets to 10μM methylene blue, platelet thromboxane generation was not completely inhibited. It was therefore apparent that HUVEC prostacyclin generation was more susceptible to inhibition than was platelet thromboxane generation. It is tempting to speculate that if cyclooxygenase in platelets and HUVECs was equally affected by methylene blue in these studies, then the differing effects of this agent on thromboxane and prostacyclin generation may reflect a greater susceptibility of HUVEC prostacyclin synthetase to the inhibitory effects of methylene blue compared with platelet thromboxane synthetase. Prostacyclin synthetase is apparently more susceptible to inactivation by free radicals than is thromboxane synthetase (Salvemini & Botting, 1993) and if the non-specific effects of methylene blue were due to oxidant effects, then this difference may have explained why prostacyclin generation from HUVECs was inhibited more than platelet thromboxane generation. Although not evaluated in the present studies, the effects of methylene blue upon platelet thromboxane and HUVEC prostacyclin generation in response to PGH_2 would have been of considerable interest. Such an experiment would have allowed the determination of how methylene blue specifically affected HUVEC prostacyclin synthetase or platelet thromboxane synthetase.

Unlike methylene blue, haemoglobin only demonstrated a modest capacity to inhibit prostacyclin generation, and only where thrombin was used as a stimulus. In subsequent experiments in which haemoglobin was used (Chapter 9), this inhibitory effect of haemoglobin could not be reproduced and so the inhibitory effect in the present studies seemed questionable, and its cause was unknown.

Because of the non-specific effects of methylene blue demonstrated in the present studies, and reported by others, it was not used subsequently. Indeed as suggested by Martin, Drazan & Newby (1989), it would be advisable in any experimental system where methylene blue is utilised to consider the potential non-specific effects of this agent.

If in experiments of the nature described in this chapter, methylene blue is to be used to inhibit platelet guanylate cyclase, then possibly the specificity of its effects may be improved by taking the approach used by Alheid and co-workers (1987; 1989). They pretreated platelets with methylene blue but did not include it in the final incubation. This group investigated the platelet inhibitory effects of HUVEC coated microcarriers (stimulated with bradykinin) on washed platelet aggregation in response to thrombin and in contrast to the present studies, haemoglobin and methylene blue produced similar effects. Furthermore, neither agent prevented HUVEC-mediated inhibition of thrombin stimulated platelet responses whereas indomethacin eliminated such inhibition. Thus in their experimental system it was evident that prostacyclin but not EDRF was important in the inhibitory effects of HUVECs against thrombin stimulated platelet responses.

Summary and conclusions

In conclusion, although both methylene blue and haemoglobin are frequently used to inhibit the effects of EDRF in experimental systems, the results from the present studies showed that haemoglobin produced more specific effects than methylene blue.

The experiments described in this chapter further clarified the HUVEC-derived mediators which were involved in the platelet inhibitory effects of HUVEC in this experimental system and there was considerable evidence that both EDRF and prostacyclin exerted platelet inhibitory effects.

Where collagen was used as a stimulus, significant HUVEC-mediated inhibition of platelet responses was not affected by 10μM haemoglobin, but was eliminated by 10μM methylene blue. This was consistent with the proposition that HUVEC-derived prostacyclin (but not necessarily EDRF) is an important mediator in the inhibition of such platelet responses.

Where thrombin was used as a stimulus, significant HUVEC-mediated inhibition of platelet responses were eliminated by haemoglobin (1μM and 10μM), methylene blue (1 and 10μM) and aspirin (50μM). This was consistent with both EDRF and prostacyclin being important mediators in the inhibition of such platelet responses.

Irrespective of the stimulus, the results obtained from these studies indicated that with the inhibition of both prostacyclin generation and the effects of EDRF, platelet inhibition by HUVECs was eliminated. Both these mediators therefore appeared to account for all the typical platelet inhibitory effects of HUVEC in this system.

In the present studies, haemoglobin exposed a role for EDRF in the platelet inhibitory effects of HUVECs against thrombin stimulated platelet activation. It was desirable however, to obtain confirmation that HUVEC-derived EDRF could cause inhibition of thrombin stimulated platelets. To approach this objective it was decided to assess whether the platelet inhibitory effects of HUVECs in the absence of prostacyclin generation could be enhanced in the presence of superoxide dismutase, and whether such effects could be reversed using haemoglobin or a specific inhibitor of EDRF generation N^{ω}-nitro-L-arginine (an L-arginine analogue). Another point of interest was whether the platelet inhibitory effects of HUVECs were specific to endothelial cells. Therefore it was decided to examine in subsequent studies whether non-endothelial cell lines possessed platelet inhibitory effects, and if observed, to determine the role of EDRF or any cyclooxygenase-derived products (e.g. prostacyclin or other prostanoids) in such inhibitory effects.

Chapter 9

The role of EDRF in the platelet inhibitory effects of HUVECs, L and FL cells

Introduction

The studies in the previous chapter provided some supportive evidence that a non-prostanoid factor, namely EDRF, may be responsible for part of the platelet-inhibitory effects of HUVECs. It was desirable without actually attempting to quantify EDRF, to obtain more evidence in support of its participation in the platelet inhibitory effects of HUVECs in this system. To assess this, two other pharmacological agents were introduced into the studies. These were superoxide dismutase and the L-arginine analogue N^{ω}-nitro-L-arginine (LNNA).

EDRF is susceptible to inactivation by molecular oxygen and stronger oxidative species such as lipid peroxides, superoxide anions, and hydroxyl radicals (Salvemini & Botting, 1993). *In vivo,* the latter species ($\cdot OH$), formed from superoxide and hydrogen peroxide by the iron-catalysed Haber Weiss reaction is considered particularly important in limiting the activity of EDRF, whilst *in vitro* the effects of superoxide anions ($\cdot O_2^-$) appear to be of greater importance (Marshall & Kontos, 1990). Superoxide dismutase dismutates superoxide anions to hydrogen peroxide, which is subsequently inactivated by catalase. *In vitro* the addition of exogenous superoxide dismutase to experimental systems can protect EDRF against inactivation and thus increase the biological effects associated with its formation. Indeed superoxide dismutase has been used in a number of experimental systems to enhance the platelet inhibitory effects of EDRF derived from vascular tissue or endothelial cells (Furlong *et al,* 1987; Salvemini, De Nucci & Vane, 1991; Durante *et al,* 1992; Broekman, Eiroa & Marcus, 1991). For these reasons, the studies described in this chapter were performed to determine whether in the absence of prostacyclin generation, superoxide dismutase could enhance the antiplatelet effects of HUVECs through enhancing the effects of EDRF. Superoxides generated during isolated platelet aggregation may also influence the extent of such aggregation (Salvemini & Botting, 1993). Therefore it was also of interest to examine the effects of superoxide dismutase on platelet responses in isolation.

If superoxide dismutase could enhance the platelet inhibitory effects of HUVECs, confirmation that the mediator responsible was EDRF required specific inhibition of its effects or generation. Therefore, haemoglobin was used to bind extracellular EDRF, or the L-arginine analogue LNNA was used to inhibit EDRF synthesis.

L-arginine analogues have been used in many experimental systems to inhibit EDRF generation. These analogues possess a substitution at the N-guanadino terminal of the peptide and one of the most widely used analogues is N^G-monomethyl-L-arginine (LNMMA). Most evidence suggests that these agents inhibit generation by acting as competitive inhibitors of nitric oxide synthetase (Moncada, Palmer & Higgs,1991), although some reportedly also inhibit L-arginine uptake by endothelial cells (Bogle *et al*, 1992). On an equimolar basis, N^{ω}-nitro-L-arginine (LNNA) displays superior inhibition of EDRF generation compared to LNMMA both *in vitro* (Bogle *et al*, 1992; Ishii *et al*, 1990; Moore *et al,* 1990; Rees *et al,* 1990; Vargas *et al,* 1991) and *in vivo* (Vargas *et al,* 1991). One reason for the superior inhibitory effects of LNNA may lie in the capacity of this agent, but not its methyl ester, to resist metabolism and the formation of L-arginine, which could oppose its inhibitory activity (Hecker *et al,* 1990b; Theimermann *et al,* 1991). Because LNNA has been used successfully to eliminate the platelet inhibitory effects of endothelial cell-derived EDRF (Broekman *et al*, 1991; Durante *et al,* 1992) it was anticipated that similar effects would be observed in the studies of this chapter.

A second question which had come into consideration from the previous studies was how specific were the platelet inhibitory effects of endothelial cells. Vascular cells other than endothelial cells can generate prostacyclin, albeit less efficiently than endothelial cells (Smith, 1986). In addition, a wide range of cells other than vascular cells can generate EDRF, including neuronal and phagocytic cells (Moncada, Palmer and Higgs, 1991). It was therefore of interest to determine whether non-endothelial cells could produce detectable inhibition of platelet activation in this system, and if evident, the extent of such inhibition compared to HUVECs, and the mediators involved. Two transformed cell lines were examined, neither of which possess recognised endothelial cell properties. These alternative lines were L cells which express fibroblast-like functions and FL cells which express epithelial cell-like functions.

Aims

The aims addressed in this chapter were to determine:

1. Whether in the absence of prostacyclin generation by HUVECs, the platelet inhibitory effects of HUVECs could be enhanced by superoxide dismutase and, if evident, whether such effects could be reversed using haemoglobin or LNNA.

2. Whether either FL or L cells could present platelet inhibitory effects, and whether these effects could be changed through the inhibition of prostanoid generation or by haemoglobin.

Methods

HUVEC isolation, culturing and subculturing and the culturing of transformed FL and L cell lines were performed as described in Chapter 2. Twenty four rather than 12 well culture cluster plates were used; however for experiments only 8 wells per plate were seeded and in total only 12 wells per plate were used. HUVECs were used 48 hours following their final seeding (~1.0 x 10^5 cells/well) and FL and L cells were used 24 hours following their final seeding (~5 x 10^5 cells/well). The preparation of washed platelets and labelling with ^{14}C-serotonin were performed as described in Chapter 2.

Interaction study protocol

All experimental wells underwent an initial 20 minute preincubation wash with serum free medium 199 (15 minutes for FL and L cells) followed by a 10 minute preincubation with RB (5 minutes for FL and L cells). The final 5 minute incubation with RB or platelets (475µl) was then performed following the addition of a stimulus (25µl) and plates were shaken at shaker speed number 4. All incubations were made at 37°C under 5% CO_2 in air, and the stimuli used were collagen (5.0µg/ml), thrombin (0.05u/ml) or RB (in the control). Platelet serotonin release, thromboxane generation (measured as TXB_2) and HUVEC or FL/L cell prostacyclin generation (measured as 6-keto-$PGF_{1\alpha}$) were assessed. For aim 1, data were expressed as described in Chapter 2. For aim 2, the % inhibition of platelet responses by the various cell lines was determined prior to the calculation of mean and sem values.

Treatments (platelet and HUVEC studies)

Where treatment with aspirin was required, aspirin (final concentration 50μM) was included in the first 20 minute incubation with HUVECs. It was also added to platelets following completion of washing and ^{14}C-serotonin labelling procedures and prior to their storage in a separate syringe to that used for untreated platelets.

Haemoglobin was prepared as described previously. Where required it was incorporated in the final 5 minute incubation with RB or platelets (final concentration 10μM). Similarly superoxide dismutase was also incorporated in the final 5 minute incubation (final concentration 30u/ml).

LNNA (500μM) was only used in 3 of the 4 experiments performed, and was incorporated in both the first washing step (extended for all experimental wells to 20 minutes) and in the second wash (extended to 10 minutes). It was also included in the final 5 minute incubation.

Treatments (platelet and FL/L cell interaction studies)

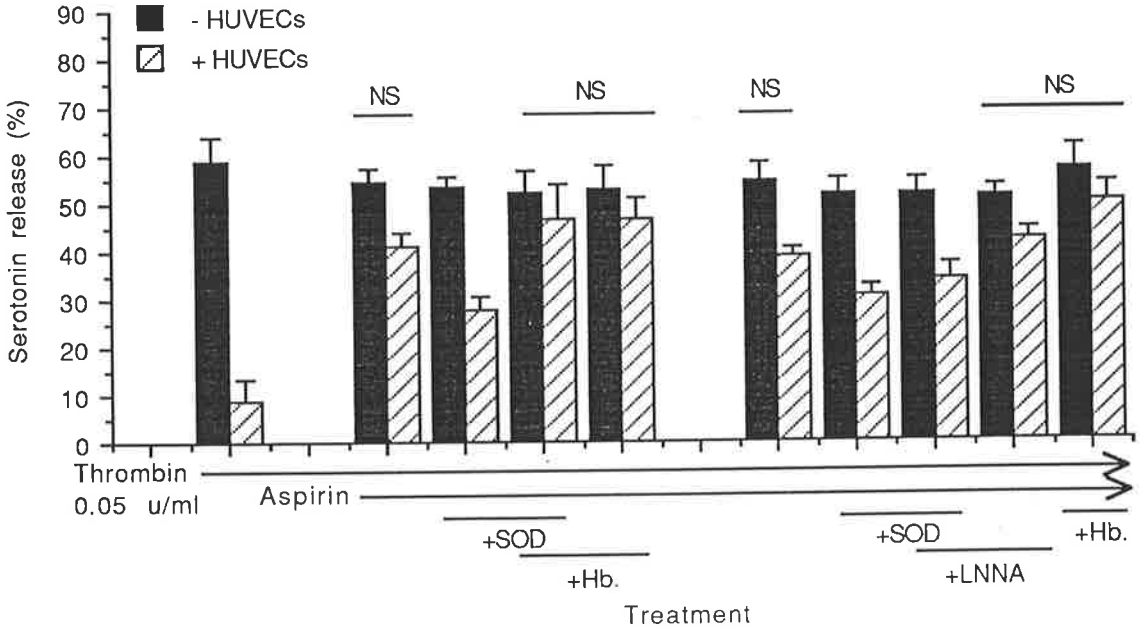
Where required, FL or L cells but not platelets were pretreated with aspirin (100μM). This was included in the first 15 minute preincubation wash with FL or L cells and as a control against carry over effects, in the paired unseeded wells (to which platelets were later added). Where required haemoglobin was incorporated in the final incubation at a final concentration of 10μM.

Statistical analyses

In the first series of experiments with HUVECs (n=4), aspirin treatments completely inhibited thromboxane and prostacyclin generation, thus statistical analyses were performed only on platelet serotonin release responses. Three-way ANOVA was applied to the data (n=4) to assess variance attributable to the various treatments, the presence or absence of HUVECs and the experimental variation. LNNA was only used in 3 experiments and a separate ANOVA was performed on n=3 data. The significance of differences between appropriately paired data were assessed using Tukey's HSD test. In the second series of experiments, FL/L cell data were not balanced and treatment effects were determined by using Student's unpaired t tests or Mann-Whitney U tests with p values adjusted using Bonferroni's correction for multiple comparisons (Miller, 1984). Significance was accepted at a $p<0.05$ level.

Results from platelet and HUVEC interaction studies

Figure 9a shows platelet serotonin release responses (mean+sem) to thrombin (0.05 u/ml) in the absence and presence of HUVECs. Treatments included LNNA (500μM), superoxide dismutase (SOD; 30u/ml) and haemoglobin (Hb; 10μM). Aspirin (50μM) was used to inhibit both platelet and HUVEC cyclooxygenase. The data were obtained from 4 experiments; however the right hand block of 10 columns was derived from only 3 of these. Similar results were obtained using thrombin 0.10u/ml (data not shown). NS shows where HUVECs failed to significantly ($p>0.05$) inhibit platelet serotonin release.



Following aspirin treatment of platelets and HUVECs, HUVECs failed to exert significant inhibition of platelet serotonin release in response to thrombin.

The inhibitory effects of aspirin treated HUVECs were enhanced in the presence of 30u/ml superoxide dismutase such that significant inhibition of platelet responses was evident

The effect of haemoglobin alone was similar to that of haemoglobin in the presence of superoxide dismutase and no platelet inhibition by aspirin treated HUVECs was evident. That is, haemoglobin (10μM) completely reversed the effects of superoxide dismutase and reduced the platelet inhibitory effects of aspirin treated HUVECs to a non-significant level. These results were consistent with superoxide dismutase enhancing EDRF-mediated platelet inhibition by HUVECs, an effect completely reversed by the ability of haemoglobin to eliminate EDRF.

When the platelet inhibitory effects of aspirin treated HUVECs were increased to significance by SOD, the additional presence of 500μM LNNA was not effective in reducing such platelet inhibitory effects. This meant that in the presence of superoxide dismutase and LNNA, inhibition of serotonin release by aspirin treated HUVECs remained significant. This suggested that LNNA may not have been completely effective in inhibiting the formation of EDRF and was consistent with superoxide dismutase continuing to enhance the effects of HUVEC-derived EDRF.

In addition to the results shown in Figure 9a, control experiments (with duplicates) were performed to assess whether any of the agents alone (i.e. in the absence of aspirin treatment) affected typical platelet serotonin release, thromboxane generation or HUVEC prostacyclin generation in response to thrombin. Neither superoxide dismutase nor haemoglobin (n=2 and 3 respectively) produced any detectable alteration of typical responses of platelets and HUVECs in isolation. However, LNNA at 500μM (but not at 100μM) appeared to inhibit prostacyclin generation by HUVECs. For example, in response to thrombin and in the absence of platelets, 6-keto-$PGF_{1\alpha}$ generation was reduced from 33.6±8.1 to 12.5±3.3 ng/10^6 cells. A similar reduction from 50.4±3.4 to 25±2.5ng/10^6 cells was also evident in the presence of platelets (n=2). In the studies shown in Figure 9a where 500μM LNNA was used, prostacyclin generation had already been inhibited using aspirin. Consequently this non-specific effect of LNNA should not have influenced the results obtained.

Results from FL and L cell studies

Table 9a (below) shows the % inhibition (mean ± sem) of platelet serotonin release and thromboxane generation responses caused by the presence of FL or L cells. Responses from platelets in response to collagen (5µg/ml) and thrombin (0.05u/ml) were examined. Pretreatments of the cells (not platelets) with 100µM aspirin (Asp.) and/or treatments using 10µM haemoglobin (Hb.) are indicated. Numbers in brackets () indicate where n values differ from those indicated at the top of the table. * indicates where treatments presented statistically significant ($p<0.05$) effects (on the % inhibition) compared to the untreated controls.

Table 9a

		% Inhibition of platelet serotonin release			
		Control (n=5)	Aspirin (n=2)	Hb. (n=3)	Asp. +Hb. (n=2)
HUVECs	Collagen	**92** ±2.5(2)			
FLcells		**55** ±14.1	**40** ±27.4	**-2.8*** ±6.7	**-6.9*** ±5.5.
HUVECs	Thrombin	**68** ±10.5(2)			
FLcells		**11** ±3.9	**8.4** ±3.3	**-2.2*** ±2.5	**-3.7*** ±2.6
		% Inhibition of platelet thromboxane generation			
HUVECs	Collagen	**56** 30.0(2)			
FLcells		**9.6** ±6.5	**-0.9** ±2.6	**9.4** ±5.9	**-10.3** ±11.1
HUVECs	Thrombin	**51** ±0.0(2)			
FLcells		**29** ±9.1	**25** ±3.0	**-1.4*** ±4.7	**-6.5*** ±1.1
		% Inhibition of platelet serotonin release			
HUVECs	Collagen	**93** ±3.0(3)			
L cells		**37** ±13.5	**35** ±33.1	**-0.7*** ±4.2(4)	**5.3** ±1.5
HUVECs	Thrombin	**77** ±10.3(3)			
L cells		**10** ±2.0(4)	**2.3** ±4.2	**5.1** ±3.1(4)	**3.8*** ±1.5(4)

Table 9a shows the extent of inhibition of stimulated platelet responses (in cell free wells) caused by the presence of FL or L cells. When these experiments were performed in parallel with experiments using HUVECs, the data from the HUVEC studies were utilised to enable a comparison between the effects of the HUVECs and the alternative cell lines. The studies performed included appropriate controls (i.e. RB wells: data not shown), but platelet thromboxane generation (measured as TXB_2) was quantified only in the studies where FL cells were used.

In preliminary studies, it was found that neither cell line appeared to produce quantifiable thromboxane (measured as TXB_2). In addition, the cells failed to generate quantifiable prostacyclin as determined by measurement of 6-keto-$PGF_{1\alpha}$ in either the absence or presence of platelets. Aspirin pretreatment was however used to prevent any possible prostanoid formation from these cells if it could occur. ^{14}C-serotonin accumulation by cell lines was also assessed over a 5 minute incubation time, and over this time, no greater association of ^{14}C-serotonin in the presence of cells (compared to plastic) was evident.

Serotonin release in response to collagen was inhibited by HUVECs on average by ~90%, whereas L and FL cells produced only 36.7% and 54.5% inhibition respectively. Inhibition by HUVECs of thrombin stimulated serotonin release was on average ~70%, whereas L and FL cells produced only 10.3% and 10.4% inhibition respectively. Similarly, compared to HUVECs, FL cells were less effective in inhibiting either collagen or thrombin stimulated platelet thromboxane generation. These results indicate that the platelet inhibitory effects of HUVECs were superior to those of either alternative cell line.

Pretreatment of FL and L cells with aspirin (n=2) had negligible effects on platelet inhibition (in 3 of 6 instances) or reduced the platelet inhibitory effects of these cells, but such reductions failed to reach significance.

Haemoglobin (10μM) produced no detectable alteration in typical platelet responses. However, the mean inhibitory effects of both L and FL cells against either platelet serotonin release or thromboxane generation were reduced by haemoglobin and became consistently less than 10%. This was reflected by haemoglobin significantly reducing the platelet inhibitory effects of these cells in 4 of 6 instances. In the two remaining cases, inhibition in the control was already less than 11%, and this may have explained why haemoglobin could not produce a significant effect.

Similarly in the combined aspirin and haemoglobin treatments when compared to the control, the platelet inhibitory effects of both L and FL cells were again significantly reduced in 4 of 6 instances. In three of these cases, significance was consistent with the effects being attributable to haemoglobin alone and haemoglobin (not aspirin) was considered largely responsible for significant effects seen in the combined treatments.

Discussion

Effects of aspirin, superoxide dismutase, haemoglobin and LNNA on HUVEC-mediated platelet inhibition

As in the studies of Chapter 8, where aspirin treatment of both platelets and HUVECs was used, thrombin proved a useful stimulus, able to cause platelet serotonin release in the absence of platelet thromboxane generation. Similarly, as in chapter 8, aspirin treated HUVECs failed to produce significant inhibition of platelet responses. This meant that if EDRF was being generated from HUVECs, the level of such generation was insufficient to maintain significant inhibition of thrombin stimulated platelet serotonin release. In the absence of prostacyclin, although platelet inhibitory effects of HUVECs were not significant, haemoglobin produced a further minor reduction of HUVEC-mediated inhibition such that in the presence of HUVECs and haemoglobin, mean platelet serotonin release in response thrombin was almost identical to that observed in unseeded wells.

Consistent with the hypothesis that aspirin treated HUVECs could produce a platelet inhibitory mediator susceptible to inactivation by superoxides, the presence of superoxide dismutase resulted in enhanced platelet inhibition by aspirin treated HUVECs. This effect of superoxide

dismutase meant that aspirin treated HUVECs were rendered capable of producing significant inhibition of platelet serotonin release in response to thrombin. This capacity of superoxide dismutase to enhance the platelet inhibitory effects of aspirin or indomethacin treated endothelial cells has been reported by others. Salvemini, De Nucci & Vane (1991) showed that the platelet inhibitory effects of perfusates generated from indomethacin treated porcine aortic endothelial cells on platelet responses to ADP, collagen and thrombin were enhanced by 20u/ml superoxide dismutase. Similarly, Durante *et al,* 1992 showed that the inhibition of aggregation of aspirin pretreated platelets (stimulated with thrombin 0.1u/ml) by aspirin pretreated suspensions of bovine aortic endothelial cells or HUVECs was potentiated in the presence of superoxide dismutase 60u/ml.

In the present studies, the effects of superoxide dismutase were completely reversed in the presence of haemoglobin. This confirmed that in the presence of superoxide dismutase, the mediator responsible for significant platelet inhibitory effects of aspirin treated HUVECs was EDRF. Others have also shown that the effects of superoxide dismutase are directly opposed or overcome by the effects of haemoglobin. Broekman, Eiroa & Marcus (1991), in a system where HUVECs were partitioned from washed platelets (both treated with aspirin), showed that endothelial cells inhibited aggregation and serotonin release in response to thrombin 0.3u/ml. This inhibition was enhanced with 15u/ml superoxide dismutase and reduced by haemoglobin. Furlong *et al* (1987), again using washed platelets, also demonstrated that platelet aggregation in response to U46619 was inhibited by EDRF generation from vascular rabbit aortic strips (stimulated with acetylcholine); this effect was enhanced using 60u/ml superoxide dismutase and reversed using haemoglobin. In this latter study, as in the present studies, 10μM haemoglobin was able to completely eliminate the effects of superoxide dismutase.

Where LNNA was used as a specific inhibitor of nitric oxide synthetase, the results from the present studies failed to provide further evidence that HUVEC-derived EDRF was participating in the system. In the presence of aspirin-treated HUVECs, platelet serotonin release in response to thrombin and in the presence of LNNA appeared similar to that seen in the presence of haemoglobin. In both instances, these responses were similar to those observed in unseeded

wells. Thus in the absence of superoxide dismutase the effects of haemoglobin and LNNA appeared similar. However, where LNNA was used in combination with superoxide dismutase, the effects of LNNA and haemoglobin differed. In the presence of superoxide dismutase and LNNA, aspirin-treated HUVECs were still able to exert significant platelet inhibitory effects whereas in the presence of haemoglobin HUVECs failed to exert such inhibition. Superoxide dismutase could have enhanced the effects of EDRF in the present studies only through increasing the half-life of free EDRF upon its liberation from HUVECs. Since such enhancement by superoxide dismutase occurred in the presence of LNNA but not haemoglobin, it appeared that LNNA did not completely block the generation of EDRF, but haemoglobin did completely bind and eliminate extracellular EDRF. An unlikely alternative explanation was that if LNNA was effective in the inhibition of EDRF, then another platelet inhibitory factor was present in platelet and HUVEC coincubations. The activity of this factor must have been enhanced by superoxide dismutase but inhibited by haemoglobin and, other than EDRF, no specific factors fitting this description have been documented. Thus the results suggested that LNNA may not have completely inhibited EDRF generation. Therefore the conditions under which it was used were considered.

Factors affecting the efficacy of LNNA

The HUVECs used in these studies were cultured in medium 199 which contained 70mg/L or 332μM L-arginine and during incubations with LNNA this agent would have had to compete with intracellular L-arginine to exert inhibitory effects. In cultured endothelial cells intracellular concentrations of L-arginine have been shown to vary according to the culturing conditions (Hecker *et al*, 1990a; Mitchell, Hecker & Vane, 1990). Bovine endothelial cells are reported to maintain internal L-arginine concentrations at ~122μM (Mitchell, Hecker & Vane, 1990) and it was considered likely that similar concentrations were maintained in the HUVECs used in the present studies.

Where endothelial cells have been cultured in the presence of L-arginine, a number of groups have used L-arginine analogues successfully to inhibit endothelial cell EDRF generation (or associated increases in cGMP). Inhibition has been attained using 20-30 minute incubations in physiological salt solutions with LNMMA (10μM) in porcine aortic endothelial cells (Boulanger *et al,* 1990) and HUVECs (White *et al,* 1993), and with LNNA (IC_{50} 230nM) and LNMMA (IC_{50} 16μM) in bovine aortic endothelial cells (Ishii *et al,* 1990). In addition, in studies (similar to those described in this chapter), where HUVECs (or bovine aortic endothelial cells) and platelets were treated with aspirin, the inhibition of thrombin stimulated platelet aggregation has been examined. In these studies, it was shown that EDRF-mediated effects were inhibited by 1mM LNMMA or 500μM LNNA where endothelial cells were treated for only 3 to 5 minute incubations (Broekman, Eiroa & Marcus, 1991) or by 100μM LNNA where 60 minutes pretreatments were used (Durante *et al,* 1992).

Given this evidence, in the studies described in this chapter in which a 30 minute preincubation period with 500μM was used (the last 10 minutes in L-arginine free buffer), LNNA should have exerted some significant effects on the EDRF-mediated platelet inhibitory effects of HUVECs. However, the results implied that the inhibitory effects of 500μM LNNA upon EDRF generation were incomplete.

Measures which may have been used to improve the efficacy of LNNA were of interest. Had the preincubation protocol, which included a 20 minute incubation with medium 199 (containing L-arginine) been replaced by an incubation with L-arginine free RB, greater effects of LNNA may have been observed. Extending the incubation time of HUVECs with LNNA to more than 30 minutes prior to their use may also have produced greater effects from LNNA. In addition, possibly during one preincubation wash, a stimulus such as bradykinin or histamine could have been used to activate nitric oxide synthetase, which would have used and reduced intracellular L-arginine and allowed more effective blockade of nitric oxide synthetase by LNNA. Growing HUVECs in L-arginine free media in order to lower endothelial cell L-arginine content could reduce L-arginine competition with LNNA and also improve its efficacy.

Possible non-specific effects of LNNA and further investigations

Because the role of EDRF was investigated in the absence of HUVEC or platelet prostanoid generation (i.e. following cyclooxygenase inhibition), the effects of the agents used on such generation was of little consequence in the presented studies. It was noted however, in control studies (n=2 or 3) that in the absence of cyclooxygenase inhibition, neither superoxide dismutase nor haemoglobin altered typical platelet or HUVEC responses. Although in earlier studies (Chapter 8) haemoglobin had been shown to cause minor diminution of prostacyclin generation from HUVECs, this was not evident in these subsequent studies. LNNA at 500μM, although not affecting platelet thromboxane generation, did appear to inhibit HUVEC prostacyclin generation. More experiments would have been required to test whether this possible non-specific effect of LNNA could be reproduced, and if observed, examine the possible mechanisms underlying this activity.

In addition, in subsequent studies, some assessment in this system of the effects of LNNA over a range of concentrations, with similar assessments of alternative nitric oxide synthetase inhibitors such as LNMMA or L-nitro methyl ester (L-NAME) would be of interest. Studies involving comparisons between the effects of such agents with those of haemoglobin in order to examine in greater depth any differing effects of these agents could also be undertaken. Finally, quantitative assessments of actual EDRF generation or HUVEC cGMP concentrations would also be informative to determine the efficacy of these agents and to evaluate technical factors which may alter their effects.

Conclusions

The studies depicted in Figure 9a confirmed that with the inhibition of HUVEC prostacyclin generation, the inhibitory effects of HUVECs on thrombin stimulated platelet serotonin release were reduced to such an extent that HUVECs could no longer exert significant inhibition of platelet responses. Under these conditions, superoxide dismutase enhanced the platelet inhibitory effects of HUVECs such that significant platelet inhibitory effects were evident. This was consistent with HUVECs producing a platelet inhibitory mediator (such as EDRF) which was susceptible to inactivation by superoxides. The significant level of platelet inhibition in the

presence of superoxide dismutase was eliminated by haemoglobin but not LNNA. This effect of haemoglobin was consistent with HUVEC-derived EDRF being the mediator involved in the significant platelet inhibitory effects of aspirin treated HUVECs observed in the presence of superoxide dismutase. Unfortunately, the effects of haemoglobin could not be reproduced by LNNA as the effects of superoxide dismutase were not reversed by LNNA. It was considered that LNNA may not have been exerting complete inhibition of EDRF generation in these studies, and further studies to assess the effects of this agent were required. Finally, these studies provided some further evidence (adding to that gained in Chapter 8) that HUVECs in this system could produce the platelet inhibitory mediator EDRF. However, in the absence of prostacyclin generation from HUVECs, EDRF generation by HUVECs was insufficient alone (in the absence of superoxide dismutase) to produce significant inhibition of platelet responses to thrombin.

L and FL cell studies

The studies using L and FL cells were performed to determine the specificity of the platelet inhibitory effects of HUVECs. As shown in Table 9a, in the presence of these cells, platelet inhibitory effects were detected (up to 54% inhibition) although neither line was able to generate quantifiable concentrations of prostacyclin in either the absence or presence of platelets. The aspirin pretreatment of cells (but not platelets) was undertaken to assess whether other prostanoids may be responsible for the platelet inhibitory effects of L and FL cells. In three instances, the untreated controls inhibited platelet responses by 29% up to 54%. In these cases, following aspirin pretreatments, the inhibitory effects of the cells consistently remained at or above 25%. In contrast haemoglobin (or haemoglobin and aspirin) consistently (and significantly) reduced the inhibitory effects of these cells to less than 6%. From these results it was inferred that for both cell lines, where they produced pronounced platelet inhibitory effects, an EDRF-like mediator appeared to account for the majority of their platelet inhibitory activity. A role for prostanoids in the platelet inhibitory effects of these lines was less evident and prostacyclin was clearly not involved. Where untreated cells failed to produce platelet inhibition

of more than 11% the treatments used in these studies were uninformative in exposing the mediators which may be responsible for such inhibition.

L and FL cells were used to represent fibroblast-like and epithelial cell-like responses respectively. It was not known whether in the L and FL cell lines used for these experiments their expression of constitutive nitric oxide synthetase (or lack of expression of the inducible enzyme) was similar to that of HUVECs. The degree of inhibition of platelet responses exerted by L and FL cells resembled or was slightly higher than the inhibition obtained in the studies with HUVECs following inhibition of prostacyclin generation (see Figure 9a). This implied that if an EDRF-like mediator was responsible for the platelet inhibitory effects of these cell lines, the cells were generating a similar or slightly greater amount of the mediator than HUVECs.

Conclusions

Although L and FL cells produced less pronounced platelet inhibitory effects than HUVECs, it was evident that these cells were not devoid of such activity. These cells did not generate prostacyclin, and when they produced more pronounced platelet inhibitory effects, an EDRF-like mediator appeared to be involved in causing this inhibition. Further studies would be required to confirm that EDRF was the primary mediator of the platelet inhibitory effects of L and FL cells, and to define any involvement of cyclooxygenase-derived products or other mediators from these cells in such effects. Finally, from these studies, it was evident that the manipulation of EDRF should not be considered to be a specific manipulation of endothelial cell function. Agents directed to affect the platelet inhibitory effects of endothelial cell-derived EDRF are likely to affect a wide variety of vascular cells and their effects on platelets.

Chapter 10
The regulation of platelet deposition by HUVECs

Introduction

Platelets do not normally adhere to endothelial cells. However, following vascular trauma, endothelial cell damage or detachment results in platelet adherence to subendothelial cell matrix proteins and deeper vascular tissue. Many groups have developed and adapted *in vitro* methods to assess factors which influence platelet adherence in the presence of monolayers of cultured endothelial cells (Radomski, Palmer & Moncada, 1987c,d; Curwen Gimbrone & Handin, 1980; Czervionke, Hoak & Fry, 1978; Kaplan *et al,* 1989; Venturini *et al,* 1989) or subendothelial cell matrix proteins (Buchanan *et al,* 1987; Radomski, Palmer & Moncada, 1987d; Sixma *et al,* 1991; De Graaf *et al,* 1992; Sakariessen & Baumgartner, 1989; Eldor *et al,* 1989; Alevriadou *et al,* 1993). The purpose of the studies described in this chapter was to evaluate whether platelet adhesion to HUVECs or their substrate occurred in the present system and to evaluate how mediators such as EDRF and prostacyclin may affect such adherence.

The studies performed in Chapters 8 and 9 showed that HUVECs generated a factor which presented characteristics typical of EDRF. It has been reported that EDRF can inhibit platelet adhesion to fibrillar collagen and can also inhibit the adherence of thrombin stimulated platelets to bovine aortic endothelial cells and their extracellular matrix (Radomski, Palmer & Moncada, 1987c,d) and to sheep aortic endothelial cells (Venturini, Del Vecchio & Kaplan, 1989). Moreover, *in vitro* under conditions of flow, the blockade of EDRF generation from cultured HUVECs or porcine aortic endothelial cells (by L-arginine analogues) has been shown to result in increased platelet adherence to adjacent de-endothelialised surfaces (De Graaf *et al,* 1992). Similarly, the inhibition of EDRF generation *in vivo* has been shown to result in increased platelet adherence to rabbit arteries, when such inhibition is combined with endothelial cell injury (Herbaczynska-Cedro, Lembowicz & Pytel 1991). Surface properties of endothelial cells, and other proposed endothelial cell-derived mediators (e.g. 13:HODE; Buchanan *et al,* 1985) are also reported to be involved in the typical ability of endothelial cells to resist platelet adhesion. It it is clear however, that generation of EDRF by endothelial cells is also important in

this capacity. Indeed, given the short half life of EDRF, it is possible that the greatest effects of this mediator on platelets may be exerted at the endothelial cell surface.

Compared with EDRF, prostacyclin is generally accepted to be a more potent inhibitor of platelet aggregation than adhesion. Indeed it appears to inhibit platelet adhesion only at high concentrations or under conditions of high shear. Under such circumstances, through inhibiting platelet GPIIb/IIIa exposure, it can impair platelet binding to fibronectin and fibrinogen and more importantly vWF, and thus impair platelet adhesion (De Groot & Sixma, 1990). In non-perfused *in vitro* studies prostacyclin does not inhibit platelet adhesion to substrates such as collagen (Lapetina *et al,* 1986; Krishnamurthi & Kakkar, 1985; Radomski, Palmer & Moncada, 1987d). Furthermore, exogenous prostacyclin does not affect thrombin stimulated platelet adherence to bovine endothelial cell extracellular matrix proteins (Radomski, Palmer & Moncada, 1987d) or basal adherence to HUVECs (Czervionke, Fry & Hoak, 1978; Czervionke *et al,* 1979b). Occasionally, where exogenous prostacyclin has appeared to reduce stimulated platelet adherence in the presence of endothelial cells (Czervionke, Fry & Hoak, 1978), it has been proposed that this effect may have been caused by prostacyclin reducing platelet aggregation and deposition (that is the adherence of pre-formed platelet aggregates), rather than specifically affecting platelet adherence. Furthermore, the inhibition of prostacyclin generation with concentrations of aspirin of 100μM or less fails to affect basal (Czervionke, Hoak & Fry, 1978; Czervionke *et al* 1979b; Curwen, Gimbrone & Handin, 1980; Radomski, Palmer & Moncada, 1987c) or thrombin stimulated (Radomski, Palmer & Moncada, 1987c,d) platelet adherence in the presence of cultured endothelial cells.

Where aspirin has been used to inhibit prostacyclin generation, it has resulted in increased platelet adherence to endothelial cells only when used at concentrations of 500μM or more (Czervionke, Fry & Hoak, 1978; Czervionke *et al,* 1979; Kaplan *et al*, 1989; Curwen, Gimbrone & Handin, 1980) and non-specific effects (other than the inhibition of prostacyclin generation) upon endothelial cell surface properties have been suggested. In the present studies, to avoid any non-specific effects of aspirin, a 50μM concentration of aspirin was used.

In the present platelet and HUVEC interaction system, the HUVEC monolayer was not completely confluent. Activated platelets could therefore adhere either to HUVECs or to their underlying plastic substrate and any associated proteins secreted by HUVECs.

In assessing adherence in the present studies, comparisons were made between platelet adherence to unseeded control coverslips and HUVEC seeded coverslips. The effects of different stimuli and treatments on typical platelet reactivity towards the unseeded substrate provided an important control. This control allowed the determination of HUVEC independent factors which may alter the reactivity of platelets towards the HUVEC substrate. The effects of seeding coverslips with HUVECs could therefore be assessed and compared to the control. As a result, HUVEC-specific reductions or increases in platelet adherence to coverslips could be determined. The study protocol also utilised haemoglobin and/or aspirin to determine whether HUVEC-specific functions were related to EDRF or prostacyclin formation.

The technique used in these studies was intended to quantify the adherence of single platelets or a monolayer of platelets, rather than platelet aggregates. It was necessary therefore to examine scanning electron micrographs to determine whether monolayer or platelet aggregate adherence was evident. If the method quantified deposition rather than the adherence of single platelets or a monolayer of platelets, then effects of platelet activation (which may affect the size of adherent platelet aggregates) could be falsely construed as effects on adherence.

Because platelet adherence is not dependent upon platelet aggregation, it was desirable to monitor adherence independently of aggregation. Irrespective of whether this was achieved, it was of interest to determine how closely platelet adherence or deposition was linked with measures of platelet activation.

Aims

The aims of the studies described in this chapter were to determine:

1. The nature and level of platelet adherence (or deposition) to coverslips in response to RB, collagen or thrombin.

2. Whether the responses assessed in aim 1 were affected by:
   a. Different extents of platelet activation as assessed by serotonin release.
   b. The presence of HUVECs.
   c. The presence of haemoglobin.
   d. Aspirin treatment of platelets (and HUVECs) where the stimulus used was thrombin.

Methods

Platelets were washed and labelled with ^{3}H-adenine and ^{14}C-serotonin as described in Chapter 2. This dual labelling procedure meant that samples could not be assessed by RIA to quantify the generation of platelet thromboxane or HUVEC prostacyclin. HUVECs were isolated, cultured and subcultured as described in chapter 2. Following their third passage HUVECs were seeded into 8 wells (containing pregelatinised Thermanox plastic coverslips, 16mm diameter) of 24 well culture cluster plates at 0.9-1.0 x 10^5 cells per well. Eight additional wells also contained coverslips. Twenty four hours following seeding, plates were shaken on a titre plate shaker for 5 minutes to reduce the number of HUVECs at the outer perimeter of the wells (i.e. those adherent to the base of wells, not coverslips) and the CCM was changed. The plates were then used for experiments 24 hours following shaking, that is 48 hours following seeding with HUVECs. For each plate a total of 16 wells containing coverslips were used.

Interaction study protocol

All experimental wells were washed for 15 minutes with serum free medium 199, then for 5 minutes with platelet free RB. For the final incubation, a fibrinogen source was added to washed platelets just prior to their use (5µl PFP/ml platelets). The platelets were then aliquoted (475µl) into wells prior to the addition of a stimulus (25µl) and a 5 minute incubation with agitation at

shaker speed number 4. All incubations were performed at 37°C under 5% CO_2 in air. The stimuli used included collagen (5μg/ml), thrombin (0.05u/ml) and RB (in the unstimulated control). Platelet serotonin release responses (%) and platelet adherence or deposition (as a % of total platelets added to wells) to coverslips or HUVEC seeded coverslips were calculated as described in chapter 2.

Scanning electron microscopy

Scanning electron micrographs of coverslips from two experiments were obtained as described in Chapter 2. The photographs shown were from an experiment which (due to constraints on time) had to be performed using HUVECs 24 rather than 48 hour following seeding, and the normal 24 hour shaking procedure had to be omitted.

Treatments

Where required, aspirin (50μM final concentration) was included in the first (15 minute) preincubation with HUVECs and platelets were treated with aspirin following the preparation and ^{14}C-serotonin labelling of washed platelets. Haemoglobin was prepared and used as described previously and was included in the final 5 minute incubation at a final concentration of 10μM.

Statistical analyses

For data obtained where thrombin was used as a stimulus, three-way parametric ANOVA was applied to complete (n=5) platelet serotonin release (%) and adherence (or deposition; %) data . Variance attributable to the presence of HUVECs, the effects of treatments and the effects of experimental variation were evaluated. Specific sources of variance were determined using Tukey's HSD tests. For the platelet responses to RB and collagen (n=3 or 9), specific effects attributable to HUVECs or haemoglobin were determined by either Student's paired t tests or Wilcoxon's matched-pairs signed-rank tests. Significance was accepted at a $p< 0.05$ level.

Results

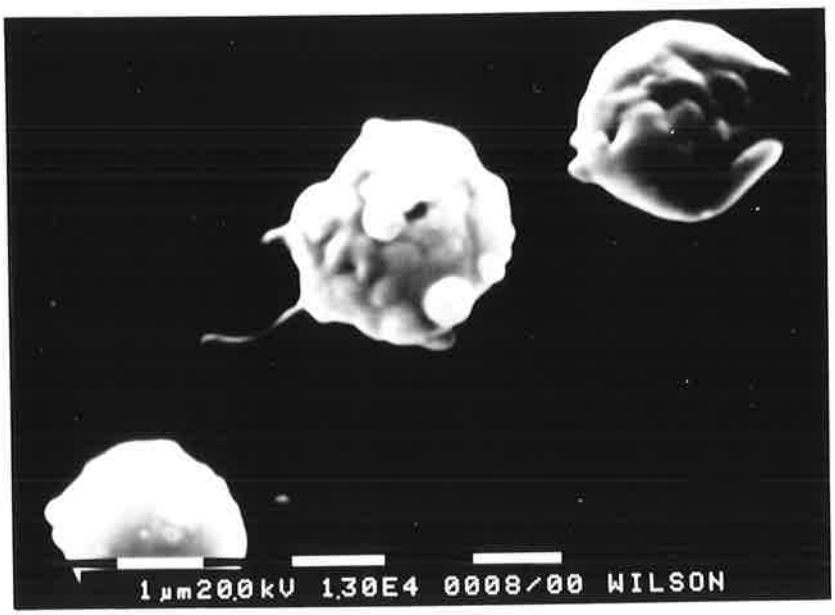
Scanning electron microscopy

Scanning electron microscopy was performed on coverslips prepared following platelet and HUVEC interaction studies. These studies were used to determine the morphological appearance of adherent platelets and to determine whether following the final incubation, the triple washing procedure was successful in the removal of any platelet aggregates. They were also performed to assess the distribution of adherent platelets in the absence and presence of HUVEC monolayers.

Photographs 10a-10cii show platelet deposition to coverslips in the absence of HUVECs following the addition of either RB (Photograph 10a), thrombin, 0.05u/ml (Photographs 10bi and bii) or collagen, 5µg/ml (Photographs 10ci and 10cii).

Photograph 10a shows at high magnification (1.3 x 10^4 times) the adherence of 3 platelets to the surface of a coverslip observed in response to RB (i.e. control adherence). The bars at the base of the photograph illustrate 1µm lengths.

Photograph 10a



Photographs 10bi and 10bii show at a magnification of of 2.5 x 10^3 and 3.7 x 10^3 respectively the adherence of single and aggregated platelets to the surface of a coverslip, following stimulation with thrombin (0.05u/ml). The bars at the base of the photographs illustrate 10µm lengths.

Photograph 10bi

Photograph 10bii

Photographs 10ci and 10cii show at a magnification of 6.2×10^2 and 2.5×10^3 respectively the adherence of single platelets and platelet aggregates to the surface of a coverslip, following stimulation with collagen (5µg/ml) The bars at the base of the photographs illustrate 100µm and 10µm lengths for Photographs 10ci and 10cii respectively.

Photograph 10ci

Photograph 10cii

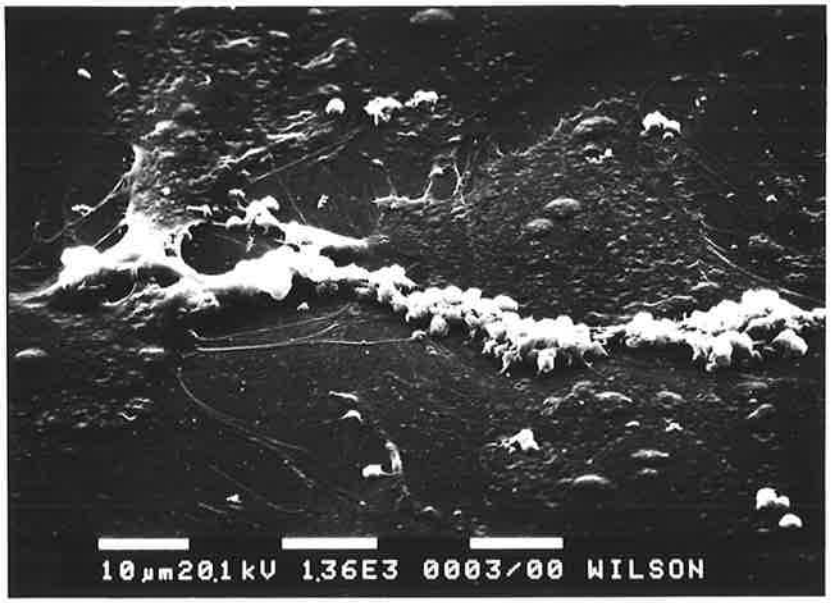
Platelets in the RB control (Photograph 10a) adhered to coverslips sparsely as either single platelets or small aggregates. Generally single adherent platelets exhibited only minor platelet activation as minimal extension of cytoplasmic pseudopodia was evident. In contrast, in response to collagen, platelets tended to adhere as larger aggregates, the numbers of which were greater than in the control. Increased adherence of single platelets was also apparent and adherent platelets exhibited increased activation (compared to the control), with clear pseudopodal extensions and spreading onto the plastic coverslip (Photographs 10ci and 10 cii). Similarly, where thrombin was used as a stimulus, frequent large platelet aggregates as well as single platelets were observed to be adherent to the coverslips (Photograph 10bi). Again, pseudopodal extensions and spreading onto the plastic was also observed (Photograph 10bii). In response to collagen, there was evidence of fibrous links between groups of platelets (Photograph 10cii). These may have been associated with the inclusion of fibrinogen in these studies, or may have been polymerised collagen fibrils. These structures were also in evidence when thrombin was used as a stimulus (Photograph 10bi), and fibrinogen or fibrin deposition seemed the likely source.

Photographs 10d-10f show platelet deposition in response to RB, thrombin (0.05u/ml) and collagen (5μg/ml) in the presence of HUVECs.

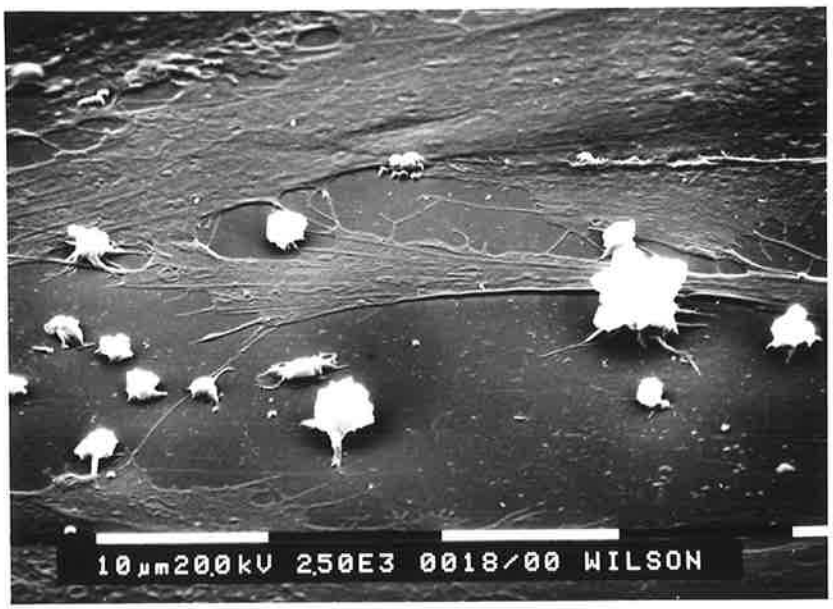
Photograph 10d shows the adherence of single platelets or small platelet aggregates in the presence of HUVECs in response to RB (i.e. control adherence) at a magnification of 1.5 x 10^2. The bars at the base of the photograph illustrate 100μm lengths.

Photograph 10ei shows platelet adherence in the presence of HUVECs in response to thrombin (0.05u/ml) at a magnification of 1.7 x 10^2. The bars at the base of the photograph illustrate 100μm lengths.

Photograph 10eii shows platelet adherence in the presence of HUVECs in response to thrombin (0.05u/ml) at a magnification of 1.3 x 10^3. The bars at the base of the photograph illustrate 10µm lengths.



Photograph 10f shows platelet adherence in the presence of HUVECs in response to collagen (5µg/ml) at a magnification of 2.5 x 10^3. The bars at the base of the photographs illustrate 10µm lengths.

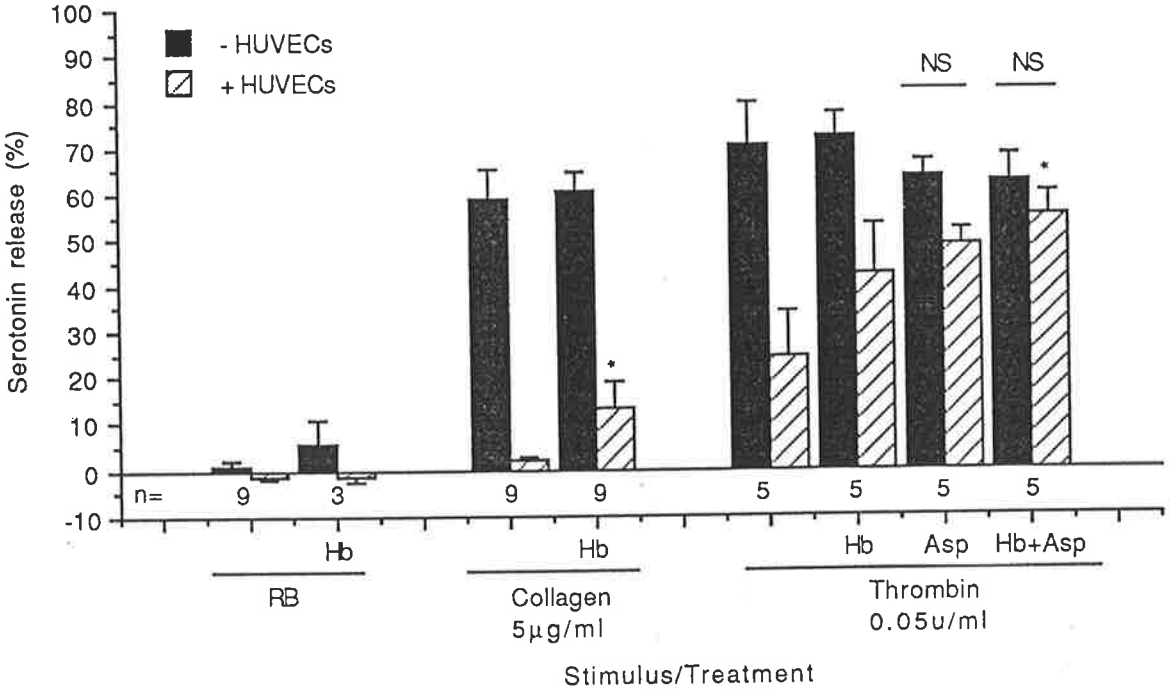


It was apparent that in the RB control in the presence of HUVECs, as in their absence, only sparse single or small platelet aggregates adhered (Photograph 10d). Adherence appeared to occur principally in gaps between or at junctions between HUVECs rather than to the surface of HUVECs. In response to thrombin (Photographs 10ei and 10eii), a far greater number of platelets was observed to adhere and large aggregates were evident. These generally adhered between HUVECs but there was also some evidence of platelet adherence to HUVECs themselves (Photograph 10eii). In response to thrombin, the deposition of platelet aggregates between HUVECs rather than the adherence of single platelets is also clearly shown in Photograph 10eii. Notably at this magnification the lack of confluence of HUVECs is also depicted and although these studies were performed 24 hours following HUVEC seeding (rather than the usual 48 hours) as stated in Chapter 3, the typical 48 hour HUVEC monolayer was also found to be only semi-confluent. In response to collagen (5μg/ml) platelet adherence in the presence of HUVECs also appeared to occur to a greater extent than observed in the control, but most adherence took the form of single platelets or small aggregates and extensive aggregates as observed with thrombin were infrequent (Photograph 10f). Where collagen or thrombin were used as stimuli, neither aspirin nor haemoglobin treatments evoked any visually detectable alteration in the distribution or nature of platelet adherence compared to the untreated controls (photographs not shown).

During the preparation of samples for scanning electron micrograph studies, it was noted that adherence of platelet aggregates to the underside of coverslips could be observed.

The results from the scanning electron micrograph studies showed that following the washing procedures, particularly where thrombin was used as a stimulus, platelet aggregates as well as single platelets remained associated with coverslips (seeded or unseeded). Therefore the term platelet deposition rather than adherence was used to describe the results.

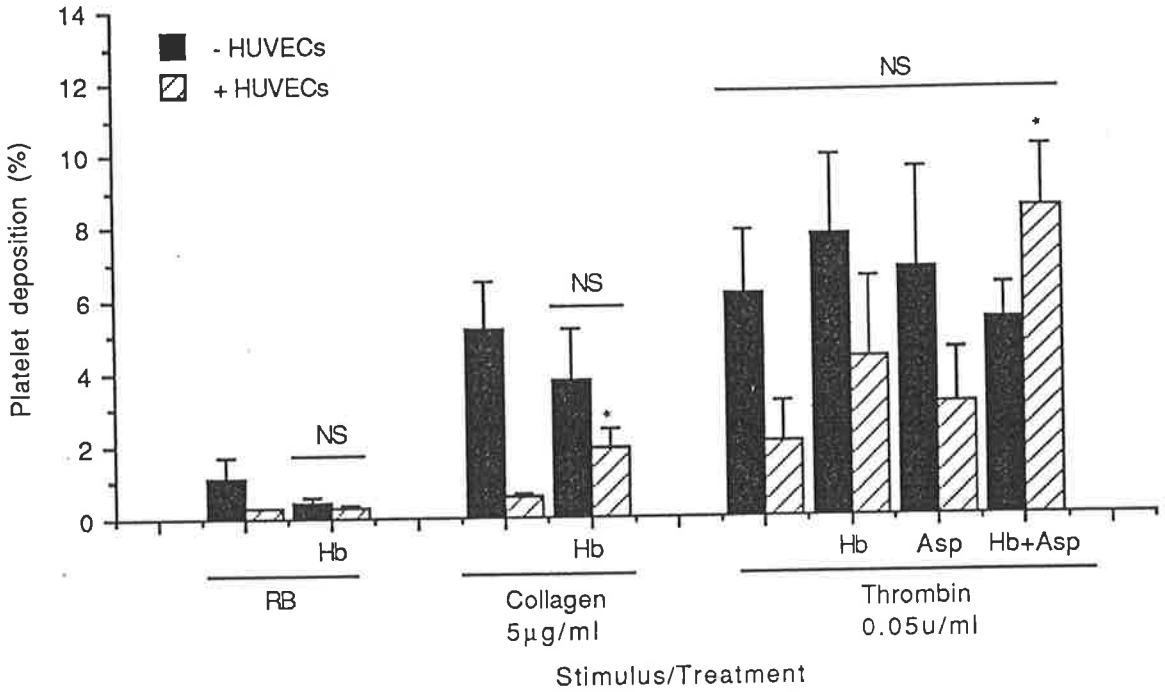
Figure 10a shows platelet serotonin release (mean + or - sem) in response to the specified stimuli in the absence or presence of HUVECs. The data were derived from 9 experiments but some stimuli or treatments were not included in all experiments, and the relevant n numbers are indicated. NS indicates cases where HUVECs failed to produce significant inhibition of the platelet responses ($p>0.05$). * indicates where the treatments, haemoglobin (Hb) and/or aspirin (Asp) produced significant ($p<0.05$) changes in the responses compared with the untreated controls.



In the absence of HUVECs none of the treatments (irrespective of the stimulus used) produced significant effects on platelet serotonin release. Although not a significant effect, in the RB control serotonin release was slightly increased by 10µM haemoglobin in the absence of HUVECs (but not in their presence).

In the presence of HUVECs the treatment with haemoglobin was associated with increased serotonin release in response to collagen and thrombin, but this only reached significance where collagen was used. Where thrombin was used as a stimulus, the aspirin treatment and combined aspirin and haemoglobin treatment were also associated with increases in serotonin release but this effect only reached significance in the combined treatment. Although such results showed that these treatments compromised the platelet inhibitory activity of HUVECs, only where aspirin or aspirin with haemoglobin were used were the inhibitory effects of HUVECs (against thrombin stimulated serotonin release) reduced to a non-significant level.

Figure 10b shows platelet deposition (mean + sem) in response to the specified stimuli in the absence or presence of HUVECs. The data were derived from 9 experiments but the relevant n numbers (3-9) are as indicated in Figure 10a. NS indicate cases where HUVECs failed to produce significant inhibition (or enhancement) of the platelet deposition ($p>0.05$). * indicates where the treatments, haemoglobin (Hb) and/or aspirin (Asp) produced significant ($p<0.05$) changes in the responses compared with the untreated controls.



In the absence of HUVECs, none of the treatments (irrespective of the stimulus) produced significant effects on platelet deposition. In the presence of HUVECs, deposition in response to collagen was significantly enhanced by haemoglobin. In addition, deposition in response to thrombin was significantly enhanced by the combined haemoglobin and aspirin treatment, and the effects of the independent treatments appeared additive.

In the RB control, the presence of haemoglobin prevented HUVECs from inhibiting platelet deposition significantly. This was clearly attributable to the capacity of haemoglobin to reduce platelet adherence to coverslips in the absence of HUVECs rather than to haemoglobin causing increased deposition in the presence of HUVECs.

In contrast, for collagen, haemoglobin caused a significant increase in platelet deposition in the presence of HUVECs and this prevented them from inhibiting deposition significantly.

Where thrombin was used as a stimulus no effects (of inhibition or enhancement) associated with the presence of HUVECs reached significance.

Discussion

The nature of platelet adherence to coverslips (±HUVECs)

The first aim of the studies undertaken in this chapter was to evaluate the nature of platelet adherence to coverslips (±HUVECs) and whether the quantified data represented single or monolayer platelet adherence or platelet adherence together with some level of aggregation (i.e. deposition).

In the unstimulated RB control (both ±HUVECs) single platelets or small platelet aggregates adhered to coverslips, and in the presence of HUVECs there was no evidence of platelets binding to HUVECs themselves. Where collagen or thrombin was used as a stimulus, in the absence of HUVECs large dense platelet aggregates remained adherent to coverslips. Even in the presence of HUVECs, in response to collagen, some small platelet aggregates remained bound to coverslips between HUVECs (Photograph 10f). Similarly, where thrombin was used as a stimulus, in the presence of HUVECs large platelet aggregates remained bound, predominantly between the HUVECs, although occasionally binding of platelets to their surface was also observed (Photograph 10eii).

These scanning electron micrographs showed that the term platelet adherence was inappropriate to describe the measurements of platelet association with coverslips (±HUVECs), particularly where collagen or thrombin were used as stimuli. Therefore the term platelet deposition was applied to these data to represent platelet interactions with the substrates. This observation meant that the results had to be interpreted with the consideration that percentage deposition values would be affected not only by changes in platelet adherence, but also by changes in the extent of platelet activation and aggregation.

Other investigators have reported measurements of platelet adherence using methods similar to those used in the present studies. It was of interest to consider whether their data actually represented single or monolayer platelet adherence or, as in these studies, platelet deposition.

Czervionke, Hoak & Fry (1978) reported assessments of platelet adherence in the presence of HUVEC monolayers. This group assessed the adherence of washed ^{51}Cr labelled-platelets (shaken for 30 minutes) to HUVEC monolayers grown in wells. Following two washing steps to remove non-adherent platelets they solubilised the monolayer and assessed ^{51}Cr remaining associated with it. From the radioactivity remaining in the wells the number of platelets which remained adherent was determined and expressed as a percentage of those added. From scanning electron micrographs, this group found thrombin stimulated platelets adhered as aggregates, not as a monolayer such that, as in the present studies, they actually quantified platelet deposition rather than specific monolayer adherence.

More recently, Kaplan *et al* (1989) used a similar approach (also used by Venturini *et al,* 1989 and more recently by Tloti *et al*, 1991) and assessed the adherence of washed ^{111}In-labelled platelets to pulmonary artery endothelial cell monolayers following co-incubations for 60 minutes (static or shaken). Following incubations, non-adherent platelets were removed with 3 washes of the cell monolayer with buffer. They then solubilised the cells and the percentage of ^{111}In-labelled platelets remaining adherent was calculated. From scanning electron micrographs, this group (Kaplan *et al,* 1989) found that in the unstimulated control, single platelet adherence predominated. In contrast, when platelets and endothelial cells were co-incubated with thrombin (2u/ml) platelets adhered as dense aggregates, similar to those seen in the present study. Thus once again as in the present studies, when platelets were stimulated with thrombin, platelet deposition rather than adherence was quantified. Indeed they acknowledged that in the presence of thrombin, part of their data may have been associated with the deposition of pre-formed platelet aggregates.

Using washed platelets, Radomski, Palmer & Moncada reported the adherence of unstimulated (1987c) or thrombin (3u/ml) stimulated platelets (1987c,d) in the presence of monolayers of bovine aortic endothelial cells. They followed a similar protocol to that of Kaplan's group but co-incubated ^{111}In-labelled platelets with endothelial cells for only 5 minutes and used only two post-incubation wash steps before examining platelet adherence. They also reported adherence,

rather than deposition, but did not give any scanning electron microscopy results to allow confirmation that some level of platelet aggregation was not contributing to the adherence estimates in their studies.

Finally, another method (used by Curwen, Gimbrone & Handin in 1980; Gimbrone & Buchanan, 1981 and adapted by Buchanan *et al*, 1987) was reported by Curwen *et al* in 1982. This method involved coincubating HUVECs grown on coverslips with ^{3}H adenine labelled platelets. Following 15-60 minutes of coincubation, coverslips underwent up to 6 washes in HBSS. ^{3}H-adenine labelled platelets which remained associated with coverslips were determined and reported as cpm. At each washing step, coverslips were transferred through the liquid air interface 3 times before blotting and moving to the next washing step. Following 3-4 such washes, optimum platelet removal was achieved and platelet aggregates were not found in association with coverslips, confirmed by scanning electron microscopy. Similar levels of adherence were reported using either PRP or washed platelets. In these studies this more rigorous post-incubation washing procedure evidently allowed this group to obtain data on single platelet (or monolayer) adherence. The success of this group in measuring monolayer platelet adherence was probably also assisted by their examination of the adherence of unstimulated rather than activated, aggregating platelets.

In summary, the method used in the present studies was unsuccessful in assessing specific monolayer adherence of platelets. Importantly, it would appear that many similar reported methods which allude to the quantification of platelet adherence should more accurately express their data as deposition. Few authors specifically quantify adherence without some level of platelet aggregation contributing to their results. In the present studies platelet deposition was evident, particularly where stimuli were used. Possible improvements in the method were considered which could increase its specificity in quantifying platelet adherence rather than deposition. A more rigorous washing procedure (such as that employed by Curwen *et al* (1982)) following coincubations may have removed platelet aggregates. It was however considered unlikely that dense platelet aggregates formed in response to thrombin would be removed by simply increasing post-incubation washes. Possibly the use of a final wash through

a weak EDTA solution may have effected some dispersion of such aggregates. Alternatively, the fibrinogen source used in these studies possibly assisted in the stabilisation of thrombin and collagen induced platelet aggregates. Omitting this fibrinogen source may have assisted in allowing the dispersion of platelet aggregates during post-incubation washes.

Another improvement to the method employed was also considered. In the present studies, the binding of platelets between HUVECs may in part have been supported by HUVEC-derived extracellular proteins. Plastic coverslips alone were used as a control to compare with HUVEC seeded coverslips, but unfortunately such blank coverslips failed to act as a control for platelet interactions with HUVEC-derived extracellular matrix proteins. Certainly in subsequent studies the use of cellulose acetate stripping (Buchanan *et al,* 1987) of HUVEC monolayers to provide an 'extracellular matrix' control would overcome this limitation in the present studies.

Finally, it was noted that platelet adherence to coverslips occurred not only to the apical side, but also to their underside. This may have been a contributing factor to background levels of platelet deposition and the considerable amount of variation observed in the deposition data (see Figure 10b). For future studies, the use of wells or HUVEC seeded wells without coverslips (used by a number of groups) would have avoided this problem. This, together with the omission of a fibrinogen source and a rigorous post-incubation washing procedure (before a solubilisation step to quantify remaining adherent platelets) may have improved the specificity of the technique for adherence rather than deposition and may also have reduced the variability of the data acquired. Certainly the variance in the deposition data was unsatisfactory, as even the presence of HUVECs could not, where thrombin was used as a stimulus, demonstrate significant inhibition of platelet deposition.

Relationship between platelet activation and deposition; any effects attributable to adherence?

Because platelet deposition reflected not only levels of platelet adherence but also levels of platelet activation (i.e. the deposition of pre-formed platelet aggregates) then assessments of platelet activation and deposition in the present studies were considered likely to be related. Certainly, in the data described in the present studies, in the absence of HUVECs, the greatest

platelet deposition was observed in the groups where platelet activation was most prominent. In addition, the effects of HUVECs upon platelet responses highlighted a consistent relationship between serotonin release and deposition. In response to thrombin, HUVECs reduced both serotonin release and platelet deposition to an identical extent, that is 66% inhibition. Similarly, where collagen was the stimulus, HUVECs reduced platelet serotonin release by 96%, and platelet deposition by a similar amount (89%). Thus, there appeared (in the absence of treatments) to be a strong association between the level of platelet activation as measured by release of serotonin and the level of platelet deposition.

As a result of this evident relationship, the information which could be gained regarding platelet adherence was limited. That is, specific effects on platelet adherence could not be determined as platelet deposition reflected platelet activation. Only where the treatments used caused differing effects upon serotonin release and platelet deposition was it considered that the effects of such treatments on platelet adherence may be exposed.

It was noted in the RB control, that in the absence of HUVECs haemoglobin produced a minor (and non-significant) increase in mean serotonin release. In contrast it produced a modest (also non-significant) reduction in mean platelet deposition to coverslips. The reduced deposition of platelets to coverslips (although serotonin release increased) possibly reflected the adsorption of haemoglobin to the surface of coverslips and a reduction in their surface reactivity (Adams, 1985b) towards platelets.

Where collagen was the stimulus, the presence of haemoglobin resulted in increased serotonin release and deposition and because these trends were similar for both parameters, no specific effects of haemoglobin upon platelet adherence could be proposed.

In contrast, where thrombin was the stimulus, more than a single treatment was used and some divergence in the effects of these treatments was observed. In the presence of HUVECs, the aspirin treatment resulted in greater mean serotonin release than did haemoglobin. A reverse trend was observed in the platelet deposition data. Although not convincing evidence, these

opposing trends in the presence of HUVECs were consistent with a greater role for EDRF than prostacyclin in the inhibition of thrombin stimulated adherence, consistent with other reports that EDRF can inhibit thrombin induced platelet adherence in the presence of endothelial cells (Radomski, Palmer & Moncada, 1987c,d; Venturini, Del Vecchio & Kaplan, 1989).

The other clear discrepancy in the serotonin release and deposition results occurred in response to thrombin in the combined treatment group. In this treatment group as in earlier studies, the inhibition of both prostacyclin generation and EDRF effects resulted in HUVECs losing their ability to inhibit platelet serotonin release. However, in the presence of HUVECs, mean serotonin release did not exceed that observed in the absence of HUVECs, although it was significantly greater than in the untreated control. In the deposition results, in the presence of HUVECs this combined treatment also caused a significant increase in deposition (compared to the control). However, in contrast to the serotonin release results, the mean deposition was greater (although not significantly so) in the presence of HUVECs than in their absence. Based on this trend in the results, it appeared that under these conditions, platelet reactivity towards the substrate (HUVECs, their extracellular matrix and the coverslip surface) may be greater than in the absence of HUVECs. One possible explanation was that under these conditions, the surface properties of HUVECs themselves or their extracellular matrix proteins were altered such that they actually facilitated platelet adherence, an effect which could not be reproduced over plastic. Unfortunately the location of deposited platelets could not be determined as electron micrographs were not taken for this combined treatment. This increased deposition could have been caused by the elimination of both prostacyclin and EDRF. However, other effects of aspirin or haemoglobin upon the surface properties of HUVECs (or their extracellular matrix proteins) could not be excluded.

HUVEC-mediated platelet inhibition: further support for roles of prostacyclin and EDRF

Finally, although these studies did not provide clear answers regarding the participation of EDRF or prostacyclin upon platelet adherence, they did provide further evidence regarding their involvement in the inhibition of serotonin release from stimulated platelets.

Where thrombin was used as a stimulus, the effects observed in the combined treatment group confirmed that both EDRF and prostacyclin accounted for all the platelet inhibitory effects of HUVECs against platelet serotonin release. In the aspirin treatment group, it was evident that with the elimination of prostacyclin generation alone, as in earlier studies (Chapters 8 and 9), HUVECs could not maintain significant inhibition of platelet serotonin release, indicating EDRF generation alone was insufficient to maintain significant inhibition. In the haemoglobin treatment group, HUVEC inhibition of thrombin stimulated platelet serotonin release was reduced but still significant. Thus even in the absence of EDRF effects, HUVECs retained significant inhibition of platelet responses, presumably associated with prostacyclin generation. This differed slightly from the studies reported in Chapter 8 where, in the presence of haemoglobin, significant inhibition of platelet responses was lost. Possibly in the present studies, the reason for this effect may have been associated with greater prostacyclin generation in response to thrombin compared with that seen in Chapter 8. This was considered likely as a 24 well (rather than a 12 well) system was used in the present studies. In addition, the presence of coverslips appeared to result in increased prostacyclin generation responses (see Chapter 2). In summary, the results from the present studies were consistent with HUVEC-derived prostacyclin alone, or EDRF together with prostacyclin, mediating significant platelet inhibitory effects of HUVECs against thrombin stimulated serotonin release, while EDRF alone was unable to exert such inhibition.

Where collagen was used as a stimulus, as in earlier studies, haemoglobin caused a minor but significant increase in serotonin release in the presence of HUVECs. This effect however was not sufficient to prevent HUVECs from significantly inhibiting serotonin release. Thus these results were again consistent with prostacyclin alone being able to support the majority of HUVEC-mediated inhibition of platelet serotonin release in response to collagen.

Although not assessed previously, the effects of haemoglobin in the RB control were assessed in this study. In the absence of HUVECs, haemoglobin produced a minor (and non-significant) increase in mean serotonin release, possibly attributable to diminished autoregulation by

platelet-derived EDRF (Radomski Palmer & Moncada, 1990). In the presence of HUVECs however, platelet serotonin release (and deposition) was not affected by haemoglobin. Thus the the low level of platelet activation (and deposition) in the control could be maintained in the absence of EDRF effects, possibly through the effects of low levels of prostacyclin generation.

Conclusions

The findings from these studies showed that the method used did not assess single or monolayer platelet adherence, but single and aggregated platelet adherence (i.e. deposition), particularly where thrombin was used as a stimulus. Given this finding, the results could not be used to determine specific effects on platelet adherence caused by aspirin or haemoglobin. In addition, the high level of variability in the deposition results meant that improvements to the method had to be considered. With an improved method, further studies could be used to assess some of the proposed effects of prostacyclin and/or EDRF inhibition on platelet adherence. These include whether the inhibition of prostacyclin is less important than that of EDRF in determining the ability of HUVECs to resist thrombin-stimulated platelet adherence, and whether the inhibition of both prostacyclin and EDRF can result in the promotion of platelet adherence in the presence of HUVECs.

These studies provided confirmatory evidence that HUVEC-derived EDRF and prostacyclin were involved in HUVEC-mediated platelet inhibition. Consistent with earlier studies, with the inhibition of prostacyclin generation, significant HUVEC-mediated inhibition of platelet serotonin release in response to thrombin was eliminated. In the presence of HUVECs, and with the inhibition of EDRF effects using haemoglobin, serotonin release in response to collagen and thrombin increased. However, HUVECs retained significant and prominent inhibition of platelet serotonin release in response to collagen and (in contrast to earlier studies; Chapter 8) modest but significant inhibition of such responses to thrombin. Such inhibitory effects of HUVECs in the presence of haemoglobin presumably involved their retained capacity to generate prostacyclin.

Chapter 11

General Discussion

The system described in this thesis was developed in order to assess platelet and endothelial cell interactions *in vitro*. Unlike many *in vitro* systems which assess platelet and endothelial cell interactions, the present system allowed direct and controlled comparisons to be made between the responses of isolated cells (both platelets and endothelial cells) and the cells in combination, such that effects of each cell type on the other could be specifically evaluated within a single experiment. The method also allowed the examination of multiple stimuli and/or treatment effects simultaneously. The end-points of platelet (serotonin release and thromboxane generation) and endothelial cell activation (prostacyclin generation) were assessed. In addition, through the inhibition of prostacyclin or EDRF generation or through the inhibition of EDRF effects, the involvement of these mediators in the platelet inhibitory effects of endothelial cells was also evaluated.

To use the system described to its maximum advantage in subsequent studies, the findings of the studies described in this thesis were important. Under the experimental conditions employed, the use of a range of pharmacological agents explored the mediators and mechanisms which regulated platelet responses and platelet and endothelial cell interactions. Thus in future studies, aims could be addressed which would utilise its strengths and rational adaptations to the system could be considered where it appeared to present limitations.

Factors affecting platelet and HUVEC responses

The results of initial studies described in this thesis (Chapter 4) highlighted how both platelet and HUVEC functions were affected by experimental factors such as ionised calcium levels. It was determined that in order for ADP to be used as a platelet stimulus in this system (and produce detectable responses) a low Ca^{2+} concentration in the medium was required. However, in the presence of low Ca^{2+} it was evident that HUVEC prostacyclin generation was compromised, and therefore this condition was not employed in further studies, and the use of ADP was discontinued.

The majority of studies described in this thesis evaluated the stimuli, collagen and thrombin. Using these stimuli the relationships between the parameters of platelet serotonin release and thromboxane generation were examined. Furthermore, the mechanisms of HUVEC-mediated inhibition of each parameter of platelet activation, and the HUVEC-derived mediators involved in such inhibition were assessed. The effects of these stimuli alone and of platelets activated by these stimuli upon HUVEC prostacyclin generation were also examined.

Relationships between measured parameters of platelet activation (±HUVECs)

For collagen (5μg/ml), in the absence of HUVECs, platelets displayed clear thromboxane-dependent serotonin release (Chapters 6 and 7). When thromboxane synthetase inhibition was used to block thromboxane generation, it was found that platelet-derived PGH_2 could support platelet serotonin release in response to this stimulus (Chapter 7). For collagen, there was no correlation between the extent to which platelet serotonin release and thromboxane generation were inhibited by HUVECs (Chapter 5). In response to 5μg/ml collagen, platelet serotonin release typically underwent near complete inhibition in the presence of HUVECs, whereas thromboxane generation was inhibited to a lesser extent (Chapters 6-8). It was considered that such results probably reflected the ability of HUVEC-derived mediators to alter the relationship between platelet thromboxane generation and serotonin release. That is, in the presence of HUVECs, platelets were less susceptible to activation by thromboxane A_2 and this was reflected by their negligible release of serotonin.

For thrombin (0.05u/ml and 0.10u/ml), in the absence of HUVECs, serotonin release responses were clearly thromboxane-independent (Chapters 6-10). In the absence of platelet thromboxane generation, HUVECs produced significant inhibition of serotonin release, such that thromboxane-independent mechanisms of this inhibition were demonstrable. The extent of HUVEC-mediated inhibition of serotonin release in the absence of thromboxane generation was similar to that seen in its presence (Chapter 6). Thus it appeared that for this stimulus, the inhibition of serotonin release by HUVECs was not necessarily associated with their inhibition of thromboxane generation.

Mediators involved in HUVEC-mediated platelet inhibition

In considering HUVEC-derived mediators which could have provided platelet inhibition in this system, the effects of prostacyclin and EDRF were evaluated. When the effects of both prostacyclin and EDRF were eliminated, no significant platelet inhibition by HUVECs was evident (Chapters 8-10). Thus it was apparent that in this cell interaction system, both EDRF and prostacyclin could account for all the platelet inhibitory effects of HUVECs against collagen and thrombin stimulated platelet responses.

For collagen, where the effects of EDRF were inhibited with haemoglobin (Chapters 8 and 10), HUVEC-mediated inhibition of platelet responses was reduced to a only minor extent. In contrast, with the virtually complete inhibition of HUVEC prostacyclin generation (Chapter 6), HUVEC-mediated inhibition of platelet responses was reduced considerably. Thus it appeared that for HUVECs to produce typical inhibition of collagen stimulated platelet responses in this system both prostacyclin and EDRF were required, but prostacyclin alone could maintain the majority of such inhibition, and EDRF played a less prominent role.

For platelet responses to thrombin, the platelet inhibitory effects of HUVECs were generally reduced to a non-significant level with either the inhibition of prostacyclin generation or the inhibition of EDRF effects (Chapters 6-10). However, in the absence of prostacyclin generation, the platelet inhibitory effects of HUVECs could be increased to significance by using superoxide dismutase to enhance the effects of EDRF (Chapter 9), highlighting the influence of this mediator upon platelet responses to thrombin. As thrombin but not collagen can stimulate prostacyclin and EDRF generation from HUVECs, this capacity may render the role of EDRF more influential in the inhibitory effects of HUVECs against platelet responses to this stimulus. Certainly in order for HUVECs to achieve typical inhibitory effects, both mediators were required, and in contrast to collagen, generally, neither prostacyclin nor EDRF alone could produce significant platelet inhibitory effects.

Effects of platelets on prostacyclin generation from HUVECs

In this experimental system, it was evident that the presence of platelets was consistently associated with enhanced prostacyclin generation from HUVECs. Some enhancement was observed even in the unstimulated control indicating that even the action of shaking unstimulated platelets over HUVECs appeared able to elicit some enhancement, possibly due to modest effects of injury to HUVECs. In the presence of collagen or thrombin stimulated platelets, the possibility existed that platelet aggregation or platelet-derived mediators could be involved in enhancing prostacyclin generation from HUVECs. There was no evidence that the extent of platelet serotonin release and the extent of enhancement were associated (Chapter 5). In addition, for both collagen and thrombin, the effects of endoperoxide steal (which was negligible) could not account for the phenomenon. Furthermore, no significant effects were obtained to indicate a role for thromboxane A_2 generation from platelets in the phenomenon (Chapters 6 and 7). The trends which suggested that platelet thromboxane A_2 generated in response to collagen (but not thrombin) may act upon HUVEC receptors to stimulate prostacyclin generation failed to reach significance (Chapters 6 and 7). There was evidence that some undetermined platelet-derived factor could be involved in the enhancement phenomenon, and where thrombin was used as a stimulus, this mediator could account for all the enhancement associated with the presence of thrombin stimulated platelets. Further studies (as described in Chapter 6) to identify this factor were not pursued.

Applications for this in vitro method of assessing platelet and HUVEC interactions and adaptations to extend its applications

The information summarised above clarified many (although not all) of the mechanisms and mediators which were involved in regulating platelet and HUVEC responses (alone and in combination) where collagen and thrombin were used as stimuli. The system could thus be used with confidence in ongoing studies (using either collagen and/or thrombin as stimuli) to examine the effects of a wide range of pharmacological agents on platelet and HUVEC interactions. For some experimental aims the present system may present limitations, but minor modifications could be used to overcome many of these.

The use of collagen (5µg/ml), in subsequent experiments, would require due consideration of the fact that platelet serotonin release in response to this stimulus was thromboxane A_2 (or PGH_2) dependent and would therefore be affected strongly by any agents which altered thromboxane generation and/or effects. HUVECs could clearly produce virtually complete inhibition of serotonin release in response to this stimulus. Consequently, collagen would be best used for examining the effects of agents which reduced the inhibitory effects of HUVECs. It was apparent that pharmacological agents, endogenous mediators, or even other cell types which inhibited prostacyclin generation or its effects would produce marked compromise of HUVEC inhibitory effects, whereas those which inhibited EDRF generation or its effects would produce less pronounced effects.

If agents were to be used which may enhance the platelet inhibitory effects of HUVECs, a higher collagen concentration or a lower HUVEC seeding density may be advisable to reduce the typical level of inhibition of platelet responses and make enhanced inhibition detectable. Again, agents which enhanced prostacyclin generation or effects would be expected to produce more marked effects than those which enhanced EDRF generation or effects. The use of higher collagen concentrations could also facilitate the assessment of any effects of platelet-derived thromboxane A_2 upon HUVEC prostacyclin generation in this system.

Thrombin (0.05u/ml), unlike collagen, could cause thromboxane-independent serotonin release from platelets. If platelet thromboxane generation was inhibited (for example by cyclooxygenase inhibition), this stimulus could still be used to produce pronounced serotonin release and thromboxane-independent inhibitory effects of HUVECs could be examined. Any pharmacological agents, endogenous mediators, or other cell types which affected either prostacyclin generation or EDRF generation or effects in this system would be likely to exert pronounced effects on HUVEC inhibition of platelet responses. Effects of either enhancing or reducing the inhibitory effects of HUVECs should be demonstrable using the thrombin concentration used in the present studies. For example, agents such as phosphodiesterase inhibitors could produce enhanced inhibitory effects of HUVECs through both prostacyclin and EDRF mediated mechanisms.

The conditions employed in the present system clearly did not favour the demonstration of endoperoxide steal, and this phenomenon occurred to only a minor extent in the presence of collagen and thrombin stimulated platelets. For this phenomenon to be demonstrated in this system, the results of Chapter 7 suggest that platelets (and HUVECs) should be incubated in the presence of a thromboxane synthetase inhibitor, that collagen (5μg/ml) rather than thrombin (0.05u/ml) should be used as a platelet stimulus, and that a highly specific means of measuring prostacyclin generation should be employed. In addition, to obtain more pronounced effects of endoperoxide steal, higher collagen concentrations could be used and these may also (though reducing typical HUVEC inhibitory effects) enable the detection of any enhanced platelet inhibitory effects of HUVEC-derived prostacyclin. Clearly even with thromboxane synthetase inhibition, demonstration of endoperoxide steal in the presence of thrombin stimulated platelets would require thrombin concentrations of greater than 0.1u/ml. At such concentrations, it would also have to be considered (as described in Chapters 5 and 6) that platelet responses may not be readily reduced by the inhibitory factors derived from HUVECs.

EDRF and prostacyclin generation appeared to account for all the platelet inhibitory effects of HUVECs against collagen and thrombin stimulated platelet responses. There was no evidence that any other HUVEC-derived products, for example endothelial cell lipoxygenase products (such as 13: HODE; Buchanan & Brister, 1993), participated in their platelet inhibitory effects. Consequently, it was clear that this method would not be useful in studying any minor influence of such mediators in platelet and HUVEC interactions.

To extend the applications of this system, it could be adapted to examine platelet adherence and how HUVEC-derived mediators affected such adherence. The studies described in Chapter 10 investigated such an adaptation but the method employed lacked specificity in the assessment of platelet adherence and actually quantified platelet deposition. However, with the modifications to the method suggested in that chapter, the role of mediators such as prostacyclin and EDRF on platelet adherence in response to collagen and thrombin could be easily clarified in this system.

Both prostacyclin and EDRF appeared to be important mediators in this system, but the present system only evaluated the effects of pharmacological agents or platelets upon HUVEC prostacyclin generation. To further develop this system, another parameter of HUVEC activation could be also be assessed, namely, EDRF generation from HUVECs. The quantification of EDRF (either directly or through indirect determinations of intracellular cGMP levels) would allow the examination of the effects of pharmacological agents or platelets on HUVEC-derived EDRF generation in this system, information which was not obtained in the studies presented in this thesis.

Comparison of this method with others to assess platelet and endothelial cell interactions

The method was developed to assess platelet and endothelial cell interactions *in vitro*, but in contrast to many systems it was constructed such that both platelet effects upon endothelial cells and endothelial cell effects on platelets could be examined under a single set of experimental conditions. In terms of the physiological relevance of the system, two major disadvantages were apparent. Firstly it was closed rather than perfused which meant that accumulation of platelet and HUVEC-derived products occurred. This presented the advantage that prostacyclin and thromboxane generation could be easily quantified, even when generated at low levels. However, it also meant that the accumulation of such products and others, such as oxidants (which would not occur *in vivo*) may have influenced the cell responses assessed, or compromised the effects of mediators such as EDRF. Certainly (Chapter 8) the non-specific effects of methylene blue in the inhibition of prostacyclin generation may have been attributable to the effects of oxidants generated from this agent, and such effects may have been avoided in a flowing system. Secondly, it must be considered that, as in any system in which cultured endothelial cells are employed, endothelial cell responses and responsiveness may be influenced as a result of isolation procedures and culturing conditions. Consequently, their responses may not necessarily reflect those of endothelial cells *in vivo*, while HUVECs may not necessarily reflect characteristics of endothelial cells from other vascular regions.

The method did include a number of more physiologically relevant features which are not included in many other methods used to assess platelet and endothelial cell interactions *in vitro*. For example, the experimental conditions used (in the majority of these studies) included the presence of physiological concentrations of Ca^{2+}, rather than the low Ca^{2+} of citrate anticoagulated platelet rich plasma (as used by some investigative groups). As demonstrated in initial studies (Chapter 4) the presence of physiological Ca^{2+} concentrations allowed HUVECs to generate higher levels of prostacyclin, and possibly also favoured the generation of EDRF, the platelet inhibitory effects of which were examined in Chapters 8 to 10. A disadvantage of these conditions was that platelet responses to ADP, which could only produce detectable serotonin release under low Ca^{2+} conditions, could not be examined in this system.

To achieve more physiological interactions between platelets and HUVECs, this system was based upon the use of a semi-confluent HUVEC monolayer, rather than (as used by some other investigators) resuspended HUVECs or HUVEC coated microcarriers. The ratio of platelets to HUVECs of ~1400:1 was also physiological, but was considered to represent a platelet to endothelial cell ratio which may occur in larger blood vessels rather than smaller vessels or capillaries. For interaction studies, physiological stimuli were used and the concentrations chosen for use in the majority of studies produced detectable platelet serotonin release (and thromboxane generation) but not maximal responses. This ensured the platelet responses were highly sensitive to the inhibitory effects of HUVECs. To achieve platelet agitation and their interaction with HUVECs in this system, a shaker plate was used. This technique avoided possible damage to cells which may occur in stirred systems used by other investigators. Continuous monitoring of platelet responses (using light transmission aggregometry) was not possible in this system. However, the assessment of two parameters of platelet activation was undertaken, and the two forms of assessment produced considerable information with respect to the mechanisms (which differed for collagen and thrombin) by which platelets responded to the stimuli employed.

Conclusion

The system described in this thesis provided a number of findings which illuminated how both platelet and HUVEC-derived mediators could influence each others' activity. As discussed, many results from these studies provide a basis for other investigations to clarify further the mechanisms and mediators involved in the interactions in this system. The method could also be used or adapted if necessary to address a wider range of questions pertaining to pharmacological agents or endogenous factors which may affect platelet and HUVEC interactions. Certainly, it is recognised that *in vitro* systems such as the one developed in this thesis are essential for initial investigations into the effects of new pharmacological agents which are designed to alter interactions between platelets and endothelial cells. Moreover, where the effects on the isolated cells as well as combined cells can be examined in parallel (as in this thesis), the information gained can add to an appreciation of the complex nature of possible cell interactions which may occur *in vivo*.

Appendix 1

Physiological buffers and other solutions

Unless otherwise stated, all solutions were prepared from UNIVAR analytical grade reagents obtained from AJAX chemicals Pty Ltd.Auburn, NSW, Australia or BDH chemicals, Australia Pty. Ltd. Kilsyth Victoria, Australia.

Solutions were prepared in water, purified through reverse osmosis followed by filtration through a Milli-Q Ultra-pure water purification system, or in glass distilled/deionised water. Solutions were stored at 4°C.

Concentrated (10 x) Hanks balanced salt solution (HBSS)

	g/100ml
NaCl	8.0
KCl	0.4
$MgSO_4\ 7H_2O$	0.2
$Na_2HPO_4\ 7H_2O$	0.09
KH_2PO_4	0.06
$CaCl_2\ 6H_2O$	0.28

HBSS

Two hundred millilitre (200ml) batches of HBSS were prepared by adding 20ml concentrated (i.e.10 x) HBSS to 150ml water prior to the addition of 0.2g d-glucose and sufficient $NaHCO_3$ (5.6%, w/v) to bring the pH to 7.35-7.4 (approximately 1.8ml). The volume was brought to 200ml prior to sterilisation by filtration using 0.22μm filters. HBSS was stored at 4°C prior to use.

Phosphate buffered saline (PBS)

(HUVEC identification studies)

pH adjusted to 7.2

	g/L
NaCl	8.0
Na_2HPO_4	1.15
KCl	0.2
KH_2PO_4	0.2

Phosphate buffered saline (PBS)

(Scanning electron microscopy studies)

pH adjusted to 7.2

	g/L
NaCl	8.5
Na_2HPO_4	0.7
KH_2PO_4	0.16

Post-fixation washing buffer

(Scanning electron microscopy studies)

To prepare the post-fixation washing buffer used in electron microscopy studies, 8g sucrose was dissolved in 200ml PBS (above) prior to use.

Normal (0.9% w/v) saline

150mM/L

	g/L
NaCl	9.0

Hepes-Tyrode's buffer

pH adjusted to 7.4

290 mOs/L

	g/L	
NaCl	8.0	
$MgCl_2$ $6H_2O$	0.2	
$NaHCO_3$	1.0	
KCl	0.2	
NaH_2PO_4	0.05	
Hepes	0.9	Sigma (3.78mM)

Platelet washing buffer (WB) and resuspension buffers (RB)

The platelet washing and resuspension buffers originally described by Mustard *et al,* 1972 were based upon a Tyrode's buffer. In order to improve the buffering capacity the Hepes-Tyrode's buffer described above was used as the basis for the platelet washing and resuspension buffers.

Washing and resuspension buffers (generally prepared in 100ml batches)were prepared on the day of use by supplementing Hepes-Tyrode's buffer with 3.5g/L fatty acid free bovine serum albumin (Sigma,St Louis, MA, U.S.A.) and 0.1g/L d-glucose as described by Timmons & Hawiger, 1978. $CaCl_2$ (from a 1M stock solution) was also added to produce a final concentration of 1.2mM. The only time this addition was not made was for the studies described in Chapter 4. The following additions and pH adjustments were then made to prepare the washing and resuspension buffers which were brought to 37°C prior to use.

	pH	Additives
Washing buffer one (20ml)	6.5	Heparin 50u/ml (Delta West, Pty Ltd.,Bently, W. Aust.)
		Apyrase 3.5µl/ml
Washing buffer two (20ml)	6.5	Apyrase 3.5µl/ml
Resuspension buffer	7.4	Apyrase 0.35µl/ml

Acid-citrate-dextrose anticoagulant

(Baenziger & Majerus, 1974)

pH 4.5

450mOsm/L

7ml anticoagulant was used per 42ml blood collected.

Final pH of anticoagulated blood, ~6.5

	g/100ml
Trisodium citrate	2.5
Citric acid	1.5
D-glucose	2.0

Apyrase

(Molnar & Lorand, 1961)

Apyrase was prepared from potatoes according to the cited method with the exception that the final material was dialysed against 150mM sodium chloride and aliquots were stored at -70°C. Apyrase 0.35µl /1ml buffer allowed aggregation followed by disaggregation of washed platelets in response to 5µM ADP and has been termed 'working strength' by Kinlough-Rathbone *et al*, 1983. The amount added (0.35µl/ml buffer) was determined by calibration of the activity of the specific batch of apyrase used. Buffers containing apyrase were used at 37°C, thus optimising apyrase activity.

Fibrinogen source

A fibrinogen source was prepared on the day of use. Citrate anticoagulated blood was centrifuged at 8400g for 5 minutes. The plasma supernatant was transferred to Eppendorf tubes prior to further centrifugation at 8400g for 2 minutes. The resultant platelet free plasma (PFP) supernatant was removed and stored in fresh Eppendorf tubes at room temperature.When washed platelets were left in the presence of this fibrinogen source for more than 1 hour at 37°C, they spontaneously aggregated and a fibrin clot formed; consequently it was added to washed platelets (generally at 5µl/ml) just prior to the use of platelets.

Medium 199

Medium 199 (modified, with Earle's salts, glutamine and 20mM Hepes without sodium bicarbonate, Flow Laboratories Irvine, Scotland, U.K) was prepared initially in 500ml batches. 7.34g medium 199 was dissolved in water then filtered using positive pressure (under 5% CO_2:95% air) through 0.22µm GS filters (Millipore,Bedford , MA, USA) prior to storage in 250ml sterile glass containers. The medium was stored at 4°C for two weeks (to allow time for possible contamination to become apparent) prior to transferring aliquots (of 36.5ml) into sterile 50ml plastic containers (Techno-plas, St. Mary's, South Australia). The medium continued to be stored at 4°C until required for preparation of 50ml batches of CCM, prepared by the addition of the supplements described under culturing conditions in Chapter 2. For later studies, the medium was prepared in smaller 200ml batches (2.93g/200ml) and sterilised by syringe filtration using 0.22µm syringe filters (Millipore) directly in 36.5ml aliquots into sterile 50ml containers, also stored at 4°C.

0.1% Collagenase

Collagenase was prepared in 30 - 40ml batches. Collagenase (142u/mg CLS 1, Worthington Biochem. Corp., Freehold, New Jersey, U.S.A) was dissolved in freshly prepared 1 x HBSS at 1mg/ml over a period of 20 minutes. Collagenase was then filter sterilised in ~10ml aliquots by syringe filtration using 0.22µm filter syringe filters (Millipore) and aliquots were stored at -20°C until thawed and warmed to 37°C prior to use.

1.0% Gelatin

Gelatin was prepared in 20-30ml batches. Gelatin (Eastman Kodak Co. Rochester, New York, U.S.A) was dissolved in 0.9% w/v saline in a covered beaker whilst stirred and heated to ensure complete dissolution. Prior to cooling, gelatin was filter sterilised in ~5ml aliquots by syringe filtration using 0.22µm filters (Millipore) and aliquots were stored at 4°C until warmed to 37°C prior to use.

POPOP-PPO scintillation fluid

For aqueous samples (100µl), 400µl soluene was used to solubilise samples prior to the addition of 8ml POPOP-PPO scintillation fluid and scintillation counting.

POPOP-PPO scintillation fluid (2.5L) was prepared by dissolving 12.5g 2,5 dipehyloxazole and 0.75g 1,4 di (-2-4 methyl-5phenyloxazoyl) benzene in 495ml toluene and upon near dissolution adding whilst mixing another 2L toluene, then 5ml glacial acetic acid to achieve a final volume of 2.5L scintillation fluid

Appendix 2

Treatments and stimuli

Stimuli

Platelet and or HUVEC stimuli were prepared fresh from stock solutions for each experiment and were kept on ice during the course of experiments

Collagen

2.5mg/ml acid soluble collagen in 3% (v/v) glacial acetic acid was prepared according to the methods of Cazanave *et al,* 1973 and was diluted as required in 0.9% w/v saline. The highest final concentration used with washed platelets was 10µg/ml and at this concentration the presence of glacial acetic acid did not affect platelet pH or responsiveness. Loss of activity sometimes associated with resuspending collagen multiple times during use was avoided by preparing fresh dilutions from the stock solution hourly or as required. Gentle resuspension of collagen by tube inversion was carried out just prior to use.

ADP

ADP sodium salt (Sigma Chemical Co., St. Louis, MO, U.S.A)

10mM/L ADP was prepared in water, and diluted in unmodified Hepes-Tyrode's buffer to 1mM prior to being aliquoted and stored at -70°C. For use, aliquots were thawed and dilutions were made as required, also using unmodified Hepes-Tyrode's buffer. Fresh ADP was prepared half hourly as required.

Thrombin

(Bovine derived α-thrombin; Hoechst-Behring, Rueil-Malmaison, France)

30 international units of thrombin was prepared on the day of use in 3ml platelet resusupension buffer (RB) and diluted as required in the same buffer.

Arachidonic acid

(Bio/Data Corp.;Horsham, PA, U.S.A.) Arachidonic acid was dissolved in 0.5ml water to provide a 16.5mM stock solution which was further diluted using platelet RB.

Adrenaline

(Astra, Chemicals Pty. Ltd., North Ryde, N.S.W., Australia)

Adrenaline tartrate stock solution (550μM) was dissolved in platelet RB just prior to use to provide a 200μM stock which was further diluted in platelet RB as required.

Bradykinin

Acetate salt (Sigma)

Bradykinin (500μM) was prepared in water and stored in aliquots at -20°C. Aliquots were thawed and diluted in platelet RB just prior to use to prevent degradation. Comparisons of responses of freshly prepared solutions and stored solutions indicated they possessed similar activity.

Histamine

Dichloride salt (Sigma)

Histamine (200μM) was prepared in platelet RB just prior to use.

PAF

C16,C18 mixture (Sigma)

PAF was dried down under nitrogen and reconstituted in 0.9% w/v saline containing 0.25% fatty-acid free bovine serum albumin.

Treatments

For each experiment, platelet and or HUVEC treatment solutions were freshly prepared from thawed or freshly prepared stock solutions and were kept on ice during the course of experiments.

Fluoxetine

(a gift from Professor I.S. De La Lande)

Fluoxetine (2mM) was prepared in water on the day of use and diluted in 0.9% w/v saline to 25μM prior to addition using a 1/50 dilution into washed platelets, giving a final concentration of 0.5μM.

Nialamide

(a gift from Professor I.S. De La Lande)

Nialamide was dissolved in 0.1 N HCl to give 6mg/ml solution, and further diluted in 0.9% w/v saline to provide a 2mg/ml solution. This was then added at a 1/100 dilution to give a final concentration of 20μg/ml (70μM).

Captopril

(Bristol-Myers Squibb Pharmaceuticals Pty. Ltd)

Captopril (100μM) was prepared in water and for experiments was added at a 1/100 dilution to give a final concentration of 1μM.

Superoxide Dismutase (SOD)

(bovine liver; Sigma-Aldrich Corp. Milwaukee, WI, U.S.A.)

Superoxide dismutase (10,000u/ml) was prepared just prior to use in 0.9% w/v saline and diluted as required.

Aspirin (acetyl salicylic acid)

(Sigma)

Aspirin was dissolved in 0.9% w/v saline to prepare a 5mM solution over a period of 15 minutes at room temperature using rotary mixing and sonication. Aliquots of the stock solution were frozen at -20°C and were used within 1 week, immediately after thawing. Similar preparation in water indicated this method of preparation and these storage conditions were associated with minimal hydrolysis to salicylic acid. This was confirmed by high pressure liquid chromatography using the method described by Siebert & Bochner (1987). 5mM aspirin was diluted 1/100 or 1/50 to give final concentrations of 50 or 100μM respectively

Oxyhaemoglobin (or haemoglobin; Hb.)

(Sigma: bovine methaemoglobin /oxyhaemoglobin mixture)

1mM haemoglobin was prepared by sodium dithionite reduction of the methaemoglobin/oxyhaemoglobin stock according to methods described by Martin *et al* (1985). Complete conversion to oxyhaemoglobin was confirmed by spectrophotometric scan from 450-650nm. Aliquots of the stocks were stored at -20°C and used within 14 days. Haemoglobin stability during storage and for the duration of experiments was confirmed by spectrophotometric scan. 1mM haemoglobin was diluted to give final concentrations of 1 or 10μM

Nω-nitro-L-arginine (LNNA)

(Sigma)

A 15mM stock solution was prepared in 0.9% (w/v) saline and, to improve solubility, stored at 37°C. For use, this stock was diluted 1/30 to provide final concentrations of 500μM.

Dazoxiben (Daz)

Dazoxiben or UK-37,248-01 (HCl salt; Pfizer, Sandwich, Kent, England) mw 268.7

Provided by Dr. H.Wooller, Pfizer Australia, Pfizer Pty. Ltd. Australia, West Ryde NSW, Australia.

Dazoxiben was usually prepared at 10^{-2}M in 0.9% w/v saline and this stock solution was serially diluted using 10 fold dilutions in 0.9% w/v saline. For experimental purposes, a final dilution factor of 1/100 was used which meant the highest final dazoxiben concentration was 10^{-4}M. At this concentration no effects on pH (~7.4 in the incubates) were observed.

R 68070 (R6)

R 68070 or Ridogrel (Janssen Pharmaceutica Beerse, Belgium), mw 366.34

Provided by Dr F. De Clerck., Janssen Research Foundation, Beerse, Belgium.

A 10^{-2}M stock solution was prepared in ethanol and stored at -70°C. On the day of use an aliquot of the stock solution was serially diluted using 10 fold dilutions in 0.9% w/v saline. For experimental purposes, the highest stock solution concentration used was 10^{-3}M. The further dilution factor of 1/100 in experiments meant the highest final R68070 concentration was 10^{-5}M, associated with a 0.1% ethanol concentration. This concentration of ethanol produced no detectable effects on platelet or HUVEC function.

Squibb-29548 (SQ)

SQ-29548 (Bristol-Myers Squibb Pharmaceuticals Pty. Ltd). fw 387.48

Provided by R. Turner, Noble Park, Victoria, Australia

A 10^{-3}M stock solution was prepared in ethanol and stored at -70°C. On the day of use an aliquot of the stock solution was serially diluted using 10 fold dilutions in 0.9% w/v saline. For experimental purposes, the highest stock solution concentration used was 10^{-5}M . The further dilution factor of 1/100 in experiments meant the highest final SQ-29548 concentration was 10^{-7}M, associated with a 0.01% ethanol concentration. This concentration of ethanol produced no detectable effects on platelet or HUVEC function.

Appendix 3

Materials for RIAs

Unless otherwise stated, all solutions were prepared from UNIVAR analytical grade reagents obtained from AJAX chemicals Pty Ltd.Auburn, NSW, Australia or BDH chemicals, Australia Pty. Ltd. Kilsyth Victoria, Australia.

Solutions were prepared in glass distilled/deionised water. Solutions were stored at 4°C.

0.05M Phosphate buffered saline (PBS)

(with 0.01% w/v sodium azide)

pH 7.5

	g/L		Volume
Solution1			
$NaH_2PO_4.H_2O$	6.9	)	~160ml (Solution1)
NaN_3	0.1	)	
			+
Solution 2			
$Na_2HPO_4.2H_2O$	8.9	)	~840ml (Solution 2)
NaN_3	0.1	)	
			~1000ml (0.05M PBS)

0.05M PBS with 0.01% sodium azide was prepared by mixing solutions 1 and 2 described above in the approximate proportions indicated in order to achieve a final pH of 7.5.

0.01M Phosphate buffered saline (PBS)

pH 7.5

Prepared by dilution of the 0.05M PBS described above 1/5 in water

Tris buffered saline (TBS)

pH 7.3

	g/L
NaCl	9
TrisHCl	1.57
Na azide	0.5

Prepared in 800ml water prior to pH adjustment (with NaOH) to pH 7.3 and making volume up to 1 L.

Gelatin tris buffered saline (GTBS)

+ 0.05% sodium azide

pH 7.3

50 or 100ml GTBS were prepared on the day of use by supplementing TBS with gelatin (Sigma; St Louis, MO, U.S.A.) at 0.1g/100ml and warming the buffer to ~50°C to gain complete dissolution of gelatin. The GTBS was then cooled to room temperature prior to use.

Dextran-coated charcoal suspension

prepared in 0.01 M PBS pH 7.5

	g/L
Acid washed charcoal	5
Dextran	5

Freshly prepared dextran-coated charcoal, at 4°C was used to achieve precipitation of unbound antiserum following overnight incubation of RIA assay tubes at 4°C. Preparation involved mixing activated acid washed charcoal (Sigma; St Louis, MO, U.S.A.) and dextran T40 (Pharmacia; Uppsala, Sweden) prior to dissolving the resultant dextran-coated charcoal in 0.01M PBS (at 4°C). The suspension (0.3ml / tube) was added to assay tubes just prior to centrifugation. Batches of 40ml were sufficient for each assay.

Thromboxane B_2 (TXB_2) standards

Stock solutions of TXB_2 were prepared by dissolving 1mg TXB_2 (Cayman Chemicals, Denver, Colorado, U.S.A.) in 100µl ethanol prior to the addition of 900µl of 1.56mM Na_2CO_3 solution and making the volume up to 100ml with water to give a final ratio of Na_2CO_3 : TXB_2 of 0.5M:1.0M and a final TXB_2 concentration of 10µg/ml. A 1/100 dilution of this primary stock gave a secondary stock of 100ng/ml from which working stocks were prepared every 6-9 months or as required. Working stocks were prepared by dilution of the secondary stock in GTBS to provide 5ng/ml and 0.5ng/ml solutions. All stock solutions were aliquoted and stored at -70°C. For RIAs the working stocks were thawed and diluted to give the standard curve concentrations indicated in Chapter 2.

Thromboxane B_2 antiserum

Antiserum for use in TXB_2 RIAs was kindly provided by Dr. M. James (Department of Rheumatology, Royal Adelaide Hospital, Adelaide, South Australia). Stock antiserum was stored at -70°C and when required was thawed and diluted in GTBS such that on addition of 100µl/tube final specific TXB_2 binding levels of 50% were achieved. Although antiserum batches of 50ml were prepared every 4-5 assays, all these batches for all assays were derived from the same initial batch of antiserum, and between assay variability in specific binding levels was consequently minor.

^{3}H-Thromboxane B_2

^{3}H-thromboxane B_2 (Amersham Int., Buckinghamshire U.K.; 180Ci-210 Ci/mMol in ethanol) was stored at -20°C and was diluted 1/1000 in GTBS on the day of use to provide approximately 10-12mls labelled solution. This volume was sufficient to add 100µl/assay tube and provided final total cpm values of ~8000-10,000.

6-keto-$PGF_{1\alpha}$ standards

Stock solutions of 6-keto-$PGF_{1\alpha}$ were prepared by dissolving 1mg 6-keto-$PGF_{1\alpha}$ (from Advanced Magnetics Inc., Cambridge, MA. U.S.A.) in 100µl ethanol prior to the addition of 900µl of 100µM Na_2CO_3 solution. The solution was made up to 100ml in TBS to give a stock solution of 10µg/ml. This primary stock was further diluted 1/100 in GTBS to give a secondary stock solution of 100ng/ml from which working stocks were prepared every 6 months or as required. Working stocks were prepared by dilution of the secondary stock in GTBS to provide 5.0ng/ml and 0.5ng/ml solutions. All stock solutions were aliquoted and stored at -70°C. For RIAs the working stocks were thawed and diluted to give the standard curve concentrations indicated in Chapter 2.

6-keto-$PGF_{1\alpha}$ antiserum

Antiserum for use in 6-keto-$PGF_{1\alpha}$ RIAs was purchased from Advanced Magnetics Inc. (Cambridge, MA, U.S.A.; formerly Seragen Inc., Boston, MA.,USA). Vials of antiserum were stored at 4°C and when required vial contents were dissolved in 40ml GTBS such that on addition of 100µl/tube final specific 6-keto-$PGF_{1\alpha}$ binding levels of 30% were achieved. Although antiserum batches of 40ml were prepared every 3-4 assays, the batches for all assays were derived from the same lot number of antiserum, and between assay variability in specific binding levels was consequently minor.

^{3}H-6-keto-$PGF_{1\alpha}$

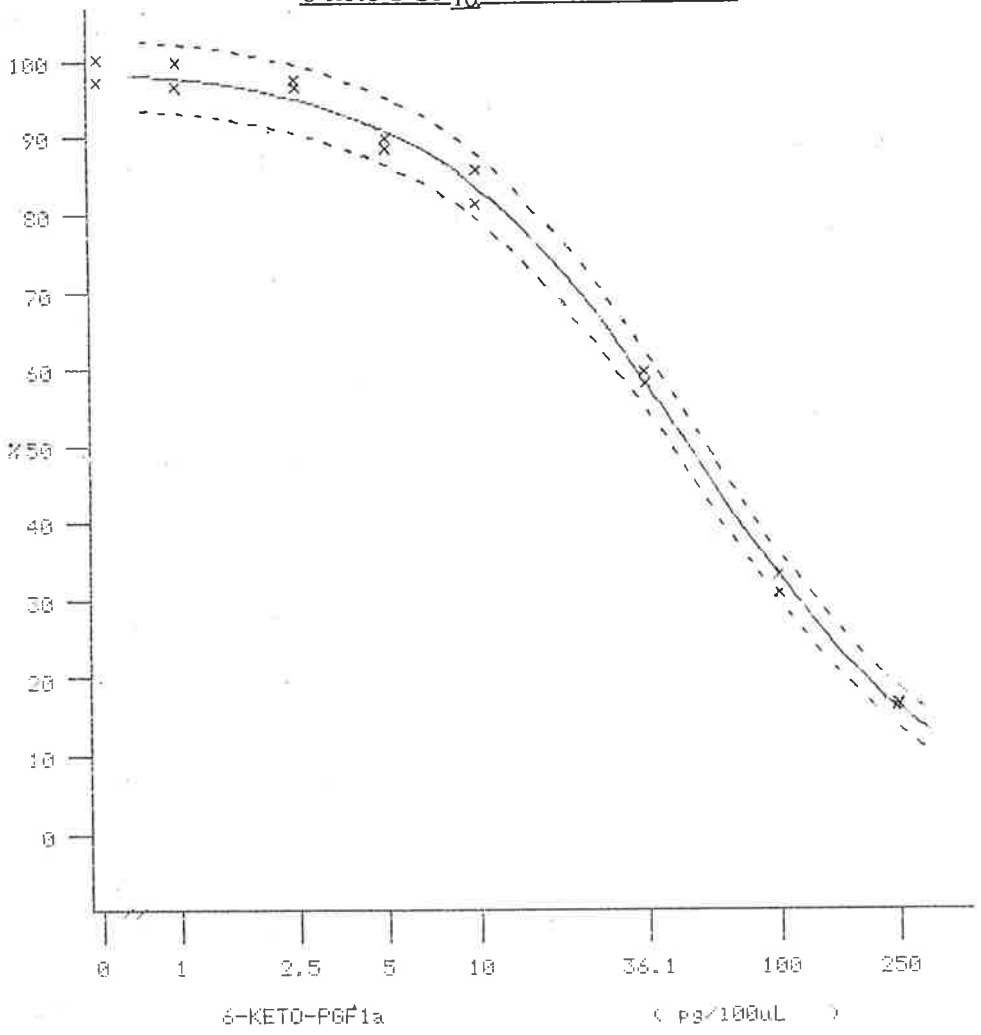
^{3}H-6-keto-$PGF_{1\alpha}$ (Amersham Int.; 150Ci-180Ci/mMol in 250µl acetonitrile:H_20, 9:1) was stored at -20°C and was diluted 1/1000 into GTBS on the day of use to provide approximately 10-12mls of labelled solution.This volume was sufficient to add 100µl/assay tube and provided total bound cpm values of ~8000-10,000.

Appendix 4

Representative standard curves and results from validation of RIAs

The figures below illustrate representative standard curves for the 6-keto-$PGF_{1\alpha}$ and TXB_2 RIAs respectively, performed according to the described in protocols in Chapter 2. The abbreviations used to define parameters of the standard curves are the same as those defined in the methods section (Chapter 2).

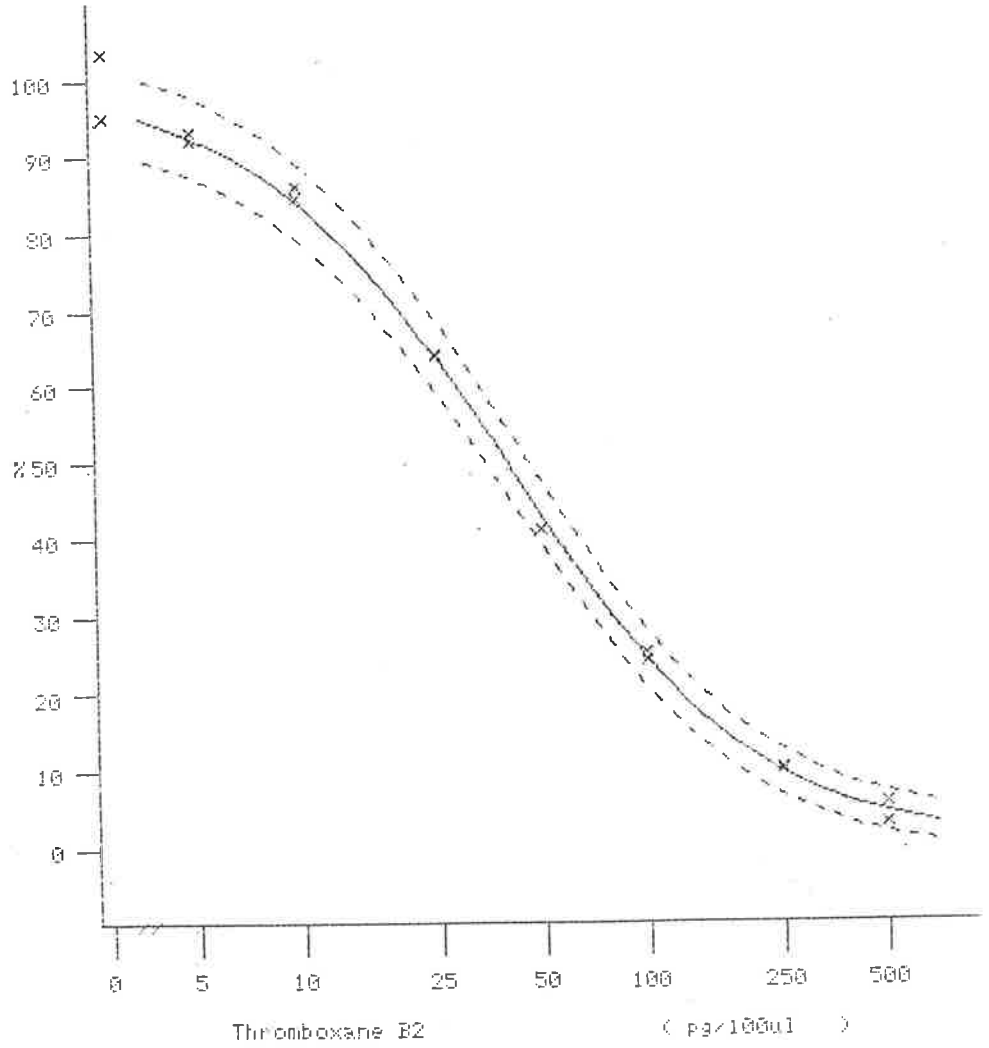
6-keto-$PGF_{1\alpha}$ RIA standard curve



Curve fit by four parameter logistic function

Estimated counts-zero conc.	= 3802 cpm	Total counts (mean)	= 9177 cpm
Slope of logit/log plot	= 1.027	NSB (mean)	= 315 cpm (3.4%)
ED_{50}	= 51.58 %		
Counts for infinite conc.	= 321 cpm		
Minimum detectable conc.	= 2.64 pg/100µl		

TXB_2 RIA standard curve



Curve fit by four parameter logistic function

Estimated counts-zero conc.	= 4739 cpm	Total counts (mean)	= 8013 cpm
Slope of logit/log plot	= 1.217	NSB (mean)	= 431 cpm (5.4%)
ED_{50}	= 39.73 %		
Counts for infinite conc.	= 326 cpm		
Minimum detectable conc.	=3.70pg/100µl		

The table below illustrates RIA parameters calculated from 10 independent 6-keto-$PGF_{1\alpha}$ assays and abbreviations used are the same as those indicated in the methods section, Chapter 2

RIA parameter	Mean	(SD)	Range
ED_{50}	42.56	(3.32)	36.91-46.66
Slope	1.003	(0.08)	0.959-1.075
Blank (%)	31.72	(3.11)	26.71-35.50
NSB (%)	4.76	(1.18)	2.92-7.13
MDC	2.66	(0.29)	1.62-3.87

The table below illustrates RIA parameters calculated from 10 independent TXB_2 assays and abbreviations used are the same as those indicated in the methods section, Chapter 2

RIA Parameter	Mean	(SD)	Range
ED_{50}	37.78	(3.19)	35.24-45.32
Slope	1.16	(0.06)	1.11-1.25
Blank (%)	55.59	(2.51)	50.84-57.95
NSB(%)	3.45	(1.01)	2.51-5.52
MDC	2.36	(0.88)	1.02-3.75

Accuracy, precision and reproducibility

The table below indicates the results from 13 independent 6-keto-$PGF_{1\alpha}$ or TXB_2 assays performed over a period of 9 months. The intended or nominal concentrations are indicated as well as the mean, standard deviation (SD) and coefficient of variation (CV) for their calculated (or actual) values. The MDC represents the calculated minimal detectable concentration for the assays. Given that all samples were assayed using a minimal dilution of 1/2, the actual minimal detectable concentration for samples was twice the MDC calculated for the assay: ~5.44pg/100µl for the 6-keto-$PGF_{1\alpha}$ assay and ~ 4.88pg/100µl for the TXB_2 assay. However, as the lowest standard for the TXB_2 assay was 5pg/100µl, this was allocated as the lowest detectable concentration.

6-keto-$PGF1_\alpha$ (pg/100µl)

Nominal concentration	Mean calc. concentration	Range	SD	CV
MDC	2.72	1.58 - 4.23	0.74	27.1
1	1.02	0.23 -1.20	0.59	58.1
2.5	2.39	1.40 - 3.34	0.56	23.3
5	5.10	4.15 - 6.45	0.72	14.1
10	9.97	9.12 -11.6	1.02	10.2
36.1	36.1	32.2 - 38.6	1.92	5.3
100	101	87.9 - 108	5.81	5.8
250	250	241 - 285	12.51	5.0

TXB_2 (pg/100µl)

Nominal concentration	Mean calc. concentration	Range	SD	CV
MDC	2.44	1.14 - 3.75	0.91	37.3
5	5.07	4.34 - 5.79	0.50	9.9
10	9.80	8.68 -10.61	0.62	6.4
25	24.7	23.3 - 25.7	0.99	4.0
50	50.9	48.2 - 53.0	1.74	3.4
100	100	96.5 - 105	2.75	2.7
250	246	217 - 266	12.53	5.1
500	509	448 - 592	36.03	7.1

Within assay accuracy and reproducibility

Within assay reproducibility was assessed within a single 6-keto-$PGF_{1\alpha}$ assay using QC 1. The concentration of this QC was evaluated 6 times within one assay where dilution of the QC included a pair of 1/2, 1/5 and 1/10 dilution factors. The differing dilutions were used to confirm that dilution factors had no effect on calculated the 6-keto-$PGF_{1\alpha}$ concentrations. The mean undiluted value (corrected by the dilution factors) for the analyses of the QC was found to be 146.2 pg/100µl (CV 8.45%) The results indicated that dilution factors did not influence accuracy, and the within assay reproducibility gained was considered satisfactory.

Within assay reproducibility for the 6-keto-$PGF_{1\alpha}$ assay was also assessed at nominal final concentrations of 1, 10 and 100 pg/100µl. Four concentration determinations on each sample resulted in calculated concentrations and CVs ( ) of 0.8 (12.9%), 9.0(9.2%) and 100.0 (8.2%) pg/100µl respectively.

Within assay reproducibility for the TXB_2 assay was assessed at nominal final concentrations of 25, 100 and 250 pg/100µl. Four concentration determinations on each sample resulted in calculated concentrations and CVs ( ) of 23.5 (5.5%), 94.0(5.7%) and 237.5(9.7%) pg/100µl respectively.

QCs: reproducibility and stability assessments

6-keto-$PGF_{1\alpha}$

The mean final concentration of QC 1 (initially introduced as a'low' QC) following the first 10 assays and using a 1/20 dilution, was found to be 7.69pg/100µl. (CV 13.2%) The stability of QC 1 was assessed and following ~3 years, from the last 5 assays performed a mean QC 1 concentration of 7.36pg/100µl CV (6.2%) was obtained indicating an apparent 4% fall in the mean QC value.

When QC 2 was introduced 6 months after QC1 following 5 assays and following a 1/2 dilution a mean final concentration of 139 pg/100µl (C.V.12.8%) was obtained The stability of QC 2 was assessed over 2.5 years. From the last 5 assays performed a mean QC 2 concentration was 147pg/µl (CV 7.0%) indicating an apparent increase in the QC value probably attributable to slight inaccuracy in the first assessment but certainly no detectable instability in this QC over 2.5 years was detectable.

TXB_2

The TXB_2 assay had been previously used to evaluate TXB_2 in platelet-rich plasma and a formerly established QC was used in the TXB_2 assay prior to the pooling of experimental samples to produce new QCs 1 and 2. The mean final concentration of QC 1 (initially introduced as a 'low' QC) following the first 10 assays and using a 1/20 dilution was found to be 24.3pg/100µl (CV 10.2%) The stability of QC 1 was assessed and following ~2.5 years, from the last 5 assays performed a mean QC 1 concentration of 23.8pg/100µl CV (7.6%) was obtained indicating no signfiicant cahnge in the mean QC value.

When QC 2 was introduced soon after QC 1 following 10 assays and using a 1/20 dilution a mean final concentration of 443pg/100µl (C.V.10.1%) was obtained. The stability of QC 2 was assessed over ~2.5 years, and from the last 5 assays performed a mean QC 2 concentration of 441pg/µl (CV 12.4.%) was obtained indicating no detectable instability in this QC.

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ERRATA

Original line
Corrected line

Page 7, line 6.
Receptor occupation causes the hydrolysis of guanosine diphosphate (GDP) from the ...
Receptor occupation causes the displacement of guanosine diphosphate (GDP) from the...

Page 81, line 14.
Following a 10 minute incubation at room temperature, the supernatant was ...
Following a 10 minute incubation at room temperature, the supernatant solution was...

Regarding all prior and subsequent references to ' the supernatant', or ' the ...supernatant', insert 'liquid', 'solution', or 'buffer' (as appropriate) following the term 'supernatant'.

Page 82, line 4
... detectable thromboxane generation (as assessed by RIA for TXB_2).
... detectable thromboxane (as assessed by RIA for TXB_2).

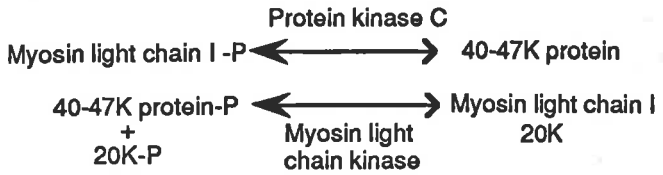
Page 96, line 4
...found in association with coverslips alone or coverslips which had been seeded with...
...found in association with coverslips alone or with coverslips which had been seeded with...

Page 127, line 3
The use of a 10 minute incubation in these experiments was used to...
The 10 minute final incubation time in these experiments was used to...

Page 217, line 20
In earlier studies, it had been demonstrated that HUVEC mediated inhibition of platelets was...
In earlier studies, it had been demonstrated that HUVEC-mediated inhibition of platelets was....

Page 11, Figure 1a

Original



Corrected