



IMPLICATIONS OF NATRIURETIC PEPTIDES AND ENDOTHELIN-1 RELEASE DURING MYOCARDIAL ISCHAEMIA.

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TABLE OF CONTENTS

CHAPTER 1 - INTRODUCTION-----1

1.1. Myocardial ischaemia: Clinical forms, variations in severity and/or duration, and common treatments.

1.1.1. Tachycardia induced myocardial ischaemia with fixed stenoses in epicardial coronary arteries.

1.1.2. Tachycardia independent myocardial ischaemia with subtotal coronary flow reduction.

1.1.3. Incipient necrosis with occlusion of one of the epicardial coronary arteries.

1.1.4. Transient myocardial ischaemia with percutaneous transluminal coronary angioplasty (PTCA).

1.1.5. Sequelae of ischaemia/reperfusion: biochemical and haemodynamic.

1.2. Physiology and Pathophysiology of Natriuretic Peptides

1.2.1. Structure, synthesis, and release of natriuretic peptides

1.2.2. Natriuretic peptide receptors

1.2.3. Physiological actions of natriuretic peptides

1.2.4. Effect of myocardial ischaemia on release of natriuretic peptides

1.2.5. Available techniques for manipulating natriuretic peptides

1.3. Physiology and Pathophysiology of Endothelins

1.3.1. Structure, synthesis, and release of endothelins

1.3.2. Endothelin receptors

1.3.3. Physiological actions of Endothelins

1.3.4. Effect of myocardial ischaemia on release of endothelins

1.3.5. Available techniques for manipulating endothelins

1.4. Interactions between Natriuretic Peptides and Endothelins

1.4.1. Receptor based interactions

1.4.2. Physiological antagonism

1.5. Effects of anti-ischaemic therapy on release of Natriuretic Peptides and Endothelins

1.6. Plan of Research

CHAPTER 2 - MATERIALS AND METHODS-----55

2.1. Animal Studies

2.1.1. Materials and Animals

2.1.2. The Isolated Perfused Rat Heart Preparation

2.1.3. Experimental Protocols

2.1.4. Cardiac Parameters

2.1.5. Radioimmunoassay of atrial natriuretic peptide

2.1.6. Radioimmunoassay of brain natriuretic peptide

2.1.7. Radioimmunoassay of endothelin-1

2.2. Human Studies

2.2.1. Patients undergoing percutaneous transluminal coronary angioplasty

2.2.2. Patients involved in investigation of glyceryl trinitrate-atrial natriuretic peptide-endothelin-1 interaction.

2.2.3. Blood collection

2.2.4. Plasma atrial natriuretic peptide radioimmunoassay

2.2.5. Plasma endothelin-1 radioimmunoassay

2.2.6. Plasma cyclic 3':5'-guanosine monophosphate radioimmunoassay

2.3. Data Analysis

CHAPTER 3 - DETERMINANTS OF ATRIAL NATRIURETIC PEPTIDE/ENDOTHELIN-1 RELEASE DURING ISCHAEMIA--

-----83

3.1. Introduction

3.2. Method

3.3. Results

3.3.1. Extent of Coronary Flow Reduction.

3.3.2. Determinants of atrial natriuretic peptide release

3.3.2.1. Perfusion flow reduction

3.3.2.2. Role of Bradycardia

3.3.3. Brain natriuretic peptide release

3.3.4. Endothelin-1 release

3.3.5. Correlation with cardiac function

3.4. Discussion

3.5. Summary

CHAPTER 4 - MECHANISMS OF ISCHAEMIA ASSOCIATED ATRIAL NATRIURETIC PEPTIDE RELEASE-----107

4.1. Introduction

4.2. Method

4.3. Results

4.3.1. Role of Nitric Oxide on Ischaemia-induced Atrial Natriuretic
Peptide Release

4.3.2. Role of Endothelins on Ischaemia-induced Atrial Natriuretic
Peptide release

4.3.2.1. Effect of exogenous Endothelin-1 on ischaemia-induced
Atrial Natriuretic Peptide release

4.3.2.1.1. Exogenous Endothelin-1 on Atrial Natriuretic
Peptide release

4.3.2.1.2. Exogenous Endothelin-1 on perfusion flow rate

4.3.2.1.3. Exogenous Endothelin-1 on cardiac function

4.3.2.2. Effect of ET receptor antagonists on ischaemia-induced
Atrial Natriuretic Peptide release

4.4. Discussion

4.5. Summary

CHAPTER 5 - EFFECTS OF ENDOTHELIN ANTAGONISTS ON CORONARY FLOW, ENDOTHELIN-1 RELEASE AND SYSTOLIC FUNCTION-----130

5.1. Introduction

5.2. Methods

5.3. Results

5.3.1. Effects of BQ-123 and bosentan on coronary perfusion flow rate

5.3.2. Effects of BQ-123 and bosentan on endothelin-1 release

5.3.3. Effects of BQ-123 and bosentan on cardiac function

5.4. Discussion

5.5. Summary

CHAPTER 6 - EFFECTS OF ENDOTHELIN ANTAGONISTS ON HYPOXIA-INDUCED ATRIAL NATRIURETIC PEPTIDE RELEASE FROM ISOLATED PERFUSED RAT HEARTS-----155

6.1. Introduction

6.2. Method

6.3. Results

6.3.1. Effects of hypoxia on Atrial Natriuretic Peptide, Brain Natriuretic Peptide and Endothelin-1 release.

6.3.1.1. Effect of hypoxia on Atrial Natriuretic Peptide release.

6.3.1.2. Effect of hypoxia on Brain Natriuretic Peptide release.

6.3.1.3. Effect of hypoxia on Endothelin-1 release.

6.3.2. Changes in cardiac performance and coronary flow.

6.3.3. Effect of BQ-123 and bosentan on Atrial Natriuretic Peptide and Endothelin-1 release during hypoxia/re-oxygenation.

6.3.3.1. Effect of BQ-123 and bosentan on Atrial Natriuretic Peptide release.

6.3.3.2. Effect of BQ-123 and bosentan on Endothelin-1 release.

6.3.4. Effect of BQ-123 and bosentan on hypoxia-induced changes in coronary flow and heart performance.

6.4. Discussion

6.5. Summary

**CHAPTER 7 - TRANSIENT MYOCARDIAL ISCHAEMIA IN
MAN: EFFECT ON ENDOTHELIN-1 AND ATRIAL
NATRIURETIC PEPTIDE RELEASE-----177**

7.1. Introduction

7.2. Method

7.3. Results

7.3.1. Patient characteristics and haemodynamic parameters

7.3.2. Effects of percutaneous transluminal coronary angioplasty on
plasma Atrial Natriuretic Peptide concentrations

7.3.3. Effects of percutaneous transluminal coronary angioplasty on
plasma Endothelin-1 concentrations

7.4. Discussion

7.5. Summary

**CHAPTER 8 - INTERACTIONS BETWEEN ANTI-ISCHAEMIC
THERAPY WITH ORGANIC NITRATES, HAEMODYNAMIC
EFFECTS AND RELEASE OF ENDOTHELIN-1/ATRIAL
NATRIURETIC PEPTIDE-----191**

8.1. Introduction

8.2. Method

8.3. Results

8.3.1. Effect of acute glyceryl trinitrate infusion on plasma Atrial Natriuretic Peptide and Endothelin-1 concentrations.

8.3.1.1. Patient characteristics

8.3.1.2. Haemodynamic responses to 10 min iv glyceryl trinitrate

8.3.1.3. Effects of glyceryl trinitrate on plasma Atrial Natriuretic Peptide and cyclic 3':5'-guanosine monophosphate levels

8.3.1.4. Effects of glyceryl trinitrate on plasma Endothelin-1 levels

8.3.2. Effects of chronic nitrate therapy on plasma Atrial Natriuretic Peptide and Endothelin-1 concentrations

8.3.2.1. Patient characteristics

8.3.2.2. Comparisons of haemodynamic parameters between acute and chronic nitrate administration.

8.3.2.3. Comparisons of plasma Atrial Natriuretic Peptide concentrations between acute and chronic nitrate administration.

8.3.2.4. Comparisons of plasma Endothelin-1 concentrations between acute and chronic nitrate administration.

8.4. Discussion

8.5. Summary

CHAPTER 9 - CONCLUSIONS AND FUTURE DIRECTIONS---**-----215****9.1. Conclusions****9.2. Future directions****BIBLIOGRAPHY-----222****APPENDIX-----280**

Abstract

The majority of (*in vitro*) studies were performed in the Langendorff-perfused, isolated rat heart, utilizing a paradigm in which atrial distension was prevented. The release of natriuretic peptides (ANP, BNP) and endothelin-1 (ET-1), along with cardiac function was monitored during periods of transient ischaemia or hypoxia. Additional studies were performed in patients undergoing cardiac catheterization.

A. *In vitro* studies (the Langendorff-perfused rat hearts)

1). Ischaemia-induced ANP release: determinants.

Coronary flow reduction greater than 50% increased ANP, but not BNP or ET-1 release. Right atrial pacing at a rate of 10% over spontaneous heart rate did not affect basal or ischaemia-induced ANP release. Ischaemia-induced bradycardia had no effect on ANP release.

2). Mechanisms of ischaemia/hypoxia-induced ANP release.

The nitric oxide synthase inhibitor L-NAME (10^{-4} M), and the endothelin receptor antagonists BQ-123 (10^{-6} M) and bosentan (10^{-5} M) did not affect basal or ischaemia-induced ANP release. Exogenous ET-1 (5×10^{-10} M) perfusion induced a delayed increase in ANP release. L-NAME perfusion did not alter ET-1 release rate. Hypoxia (5% O₂) induced an 8-fold increase in ANP release rate, but did not affect BNP or ET-1 release. Bosentan attenuated hypoxia-induced ANP release. Thus, ET mediates, in part, hypoxia but not low flow ischaemia-induced ANP release.

3). ET-1 regulation of local vasomotion and myocardial contraction during ischaemia or hypoxia.

Exogenous ET-1 caused significant perfusion flow reduction, but did not significantly affect cardiac force of contraction. BQ-123 decreased coronary flow rate in the non-ischaemic heart. In ischaemic heart, BQ-123 abolished the post-ischaemic increase in

perfusion flow rate. Neither BQ-123 nor bosentan induced significant variation in force of contraction in non-ischaemic hearts. Bosentan significantly accentuated and BQ-123 tended to ($P=0.06$) accentuate the ischaemia-induced impairment in force of contraction. Bosentan also impaired the recovery of systolic function during reperfusion. Both BQ-123 and bosentan perfusion significantly increased ET-1 efflux rate. This effect was abolished during ischaemia for BQ-123, but not for bosentan. During hypoxia, heart rate and force of contraction were reduced, while coronary flow rate increased. Bosentan tended to increase ET-1 release ($P=0.051$) and decrease cardiac force of contraction ($P=0.059$) during hypoxic periods. BQ-123 abolished the hypoxia-induced increase in perfusion flow rate.

B. In vivo studies (human)

1). Effects of percutaneous transluminal coronary angioplasty (PTCA)-induced ischaemia on ET-1 release into systemic circulation in man.

Plasma ANP and ET-1 concentrations, measured prior and subsequent to the initial intra-coronary balloon inflation in patients undergoing elective PTCA, did not change in femoral and pulmonary arteries (FA, PA).

2). Interactions between anti-ischaemic therapy with Glyceryl trinitrate (GTN), haemodynamic effects and ET-1/ANP release.

Intravenous GTN infusion (10 $\mu\text{g}/\text{min}$) significantly decreased pulmonary capillary wedge and systolic arterial pressure. Plasma ANP concentrations in FA, FV and PA and ANP gradient across the femoral vascular bed (FA-FV) decreased following GTN infusion. ET-1 concentrations in FA and PA plasma did not change significantly in the presence of GTN. However, plasma ET-1 concentration gradient across the femoral vascular bed (FV-FA) increased.

Declaration

This thesis contains no material that has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge and belief, contains no material previously written by any other person except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Date: 23 / 9 / 98

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Y Zhang, JR Oliver and JD Horowitz. Endothelin B receptor-mediated vasoconstriction induced by endothelin A receptor antagonist. *Cardiovas. Res.* 1998; (in press).

Presentations at international conferences

CL Zhang, I Stafford, **Y Zhang** and JD Horowitz. Release of 3'5' cyclic guanosine monophosphate across the femoral vascular bed in man during acute and chronic nitrate therapy. *Circulation Suppl.* 88(2), I-91, 1993. Presented at the 66th Annual Scientific Section of American Heart Association, November 1993.

Y Zhang, JR Oliver, JD Horowitz. Atrial natriuretic peptide and endothelin-1 release during myocardial ischaemia and reperfusion of isolated perfused rat hearts. Presented at the 6th International Congress on Cardiovascular Pharmacotherapy, February 1996.

Y Zhang, JR Oliver, JD Horowitz. The role of endothelin in maintaining cardiac systolic function during acute myocardial ischaemia. *Eur. Heart J.* 1997; 18 (abstr. Suppl.): 583. Presented at the 19th Congress of the European Society of Cardiology, August 1997.

Y Zhang, JR Oliver, JD Horowitz. Control of endothelin release within the heart: vasomotor and inotropic implications during ischaemia/reperfusion. *JACC.* 1998; 31(suppl): 134A. Presented at the 47th Annual Scientific session of American College of Cardiology, March 1998.

CHAPTER 1

INTRODUCTION

1.1. Myocardial ischaemia: Clinical forms, variations in severity and/or duration, and common treatments.

Ischaemic heart disease is a common cause of death in most Western countries. In 1993, approximately 44% of all deaths were due to cardiovascular disease, 56% of them caused by acute myocardial infarction. Improved understanding of the pathophysiology of the various forms of ischaemic heart disease would seem to be a prerequisite for improved therapeutic options.

1.1.1. Myocardial ischaemia

By definition, hypoxia is a state of reduced oxygen supply to tissue despite adequate perfusion; and ischaemia is a condition accompanied by inadequate removal of metabolites consequent to reduced perfusion (Fozzard *et al.* 1986). Neither ischaemia nor hypoxia can be defined in absolute terms, since the blood flow and quantity of oxygen required to support myocardium varies under different conditions. In human subjects, blood flow of 60-90 ml/min/per 100 gm of myocardium is generally required under basal physiological conditions. However, when cardiac mechanical activity and metabolic requirements are markedly reduced, under conditions such as hypothermia or using β -adrenoceptor blockers, myocardial viability may be maintained by perfusion at much lower rates (10-20 ml/min/100g) (Braunwald 1988).

The importance of defining ischaemia in relative rather than absolute terms is underscored by the use of a variety of stress tests to detect or assess the severity of coronary artery disease. During relative ischaemia, an imbalance between myocardial oxygen demands

and supply occurs as a consequence of reduced supply. Whether the ischaemia manifests as anginal discomfort, deviation of the ST-segment on the electrocardiogram (ECG), relative diminution of accumulation of ^{201}Tl or other radionuclides in myocardial “perfusion images”, regional wall motion disorders, or diminution of ejection fraction detected by gated blood pool scans, the underlying principle is the same, that is the disparity in the balance between oxygen supply and demand.

Angina pectoris is a discomfort in the chest or adjacent areas, caused by myocardial ischaemia and is associated with a disturbance of myocardial function but without myocardial necrosis (Matthews & Julian 1985). Although chest discomfort is usually the predominant symptom in chronic (stable), unstable, or variant angina and acute myocardial infarction, myocardial ischaemia can present as cardiac arrhythmia, congestive heart failure, or be asymptomatic (“silent” ischaemia).

1.1.2. Tachycardia-induced myocardial ischaemia with fixed stenoses in epicardial coronary arteries.

In patients with *stable angina*, myocardial perfusion becomes regionally inadequate during stress (Camici *et al.* 1986), although it may be adequate under conditions of rest. Stress can be induced by spontaneous or experimental tachycardia (eg. thyrotoxicosis, exercise test or dobutamine test), and/or by increased myocardial contractility/reducing oxygen and energy supply. The principal form of tachycardia-induced stress is an increase in myocardial oxygen consumption secondary to increased heart rate and an increase in myocardial contractility because of the Treppe effect (Ricci *et al.* 1979). Associated with this increase in myocardial oxygen consumption is a reflex coronary vasodilatation with an increase in myocardial blood flow (Hoimberg & Varnauskas 1971). However, in patients who had a major fixed stenosis in epicardial coronary

arteries, the regional coronary blood flow was limited by the obstruction; therefore, an imbalance between oxygen supply and demand results.

Pacing tachycardia has been used as a form of stress test in patients with heart disease for about 30 years, usually in association with simultaneous hemodynamic evaluation (Sowton *et al.* 1967, Rios & Hurwitz 1976, Robson *et al.* 1976, Heller, *et al.* 1984a, Heller, *et al.* 1984a). Sowton *et al.* (1967) noted that artificially increasing the heart rate by pacing the right atrium usually could induce angina in patients with symptomatic coronary artery disease. Since this original paper, other investigators have described characteristic pacing-induced changes in ECG (Parker *et al.* 1969, Helfant *et al.* 1970, Rios & Hurwitz 1976, Robson *et al.* 1976, Heller, *et al.* 1984a), adenosine production (Fox *et al.* 1974, Feldman *et al.* 1992), lactate metabolism (Parker *et al.* 1969, Helfant *et al.* 1970), regional wall motion (Dwyer 1970, Tzivoni *et al.* 1981), and defects in thallium scintigraphy (Weiss *et al.* 1983, Heller *et al.* 1984b, McKay *et al.* 1984). The incorporation of thallium scintigraphy into pacing protocols has improved the overall utility of atrial pacing as a stress test. This approach is successful in detecting both reversible ischaemia and infarcted myocardium (Weiss *et al.* 1983, Heller *et al.* 1984b, McKay *et al.* 1984).

1.1.3. Tachycardia-independent myocardial ischaemia with subtotal coronary flow reduction.

There is evidence that angina may also be caused by transient reductions of oxygen supply as a consequence of coronary vasoconstriction (spasm) (Hills & Braunwald 1978, Epstein & Talbot 1981, Ganz *et al.* 1987). In patients with severe, although subcritical, fixed obstruction to coronary flow, only a minor increase in dynamic obstruction may result in an absolute fall in coronary blood flow and thus myocardial ischaemia (Muller 1980).

Variant angina pectoris (Prinzmetal's angina) was characterized as an unusual syndrome of cardiac pain that occurs almost exclusively at rest, usually is not precipitated by emotional stress, and is often associated with ST-segment elevations on ECG (Prinzmetal *et al.* 1959). This type of ischaemia is due to coronary artery spasm (Oliva *et al.* 1973), which is defined as a transient, abrupt, marked and potentially reversible reduction in the diameter of at least one large coronary artery (normal or diseased) (Conti 1986). Intravenously administered ergonovine or acetylcholine provides a sensitive and specific test for provoking coronary artery spasm in patients so predisposed (Winniford *et al.* 1983, Lagerqvist *et al.* 1991).

In the case of *unstable angina*, myocardial ischaemia can be induced by an increase in oxygen demand (exertion), but more usually by a decrease in oxygen supply (coronary vasoconstriction and/or platelet aggregation). Accompanied with ECG changes, these patients may present with one or more of following features: 1) crescendo angina (more severe, prolonged, or frequent) superimposed on a preexisting pattern of relatively stable, exertion-related angina; 2) angina at rest as well as with minimal exertion; or 3) new-onset angina which is brought on by minimal exertion (Braunwald 1988). Most patients with unstable angina have severe fixed obstructive coronary artery disease. unstable angina involves an associated risk of progression to acute myocardial infarction via occlusion of affected vessels, and in severe ischaemic circumstances is associated with transient disturbance of both systolic and diastolic ventricular function (Vatner *et al.* 1986)

1.1.4. Incipient necrosis with occlusion of one of the epicardial coronary arteries.

This is a condition commonly termed acute myocardial infarction. In most of the cases, it is a result of plaque rupture atheromatous/tissue and the subsequent thrombus formation with resultant occlusion of the coronary artery (Freifeld *et al.* 1983, de Wood *et al.*

1986). In some cases, a prolonged epicardial coronary spasm can also contribute to ischaemia causing irreversible damage (Benacerraf *et al.* 1983, Vincent *et al.* 1983).

A critical issue in this regard is the relationship between extent of tissue hypoperfusion, duration of interruption of flow and extent of resultant necrosis within the ischaemic zone. Information in this area is fragmentary because (1) there is no adequate mechanisms to investigating this fully in human subjects, (2) a lack of any “ideal” animal model, (3) complicating factors, such as variable collateralization, ischaemic “preconditioning” (Murry & Jennings 1986), variable diastolic dysfunction due to left ventricular hypertrophy, and finally the potential for a post-ischaemic necrotic phase (“reperfusion injury”). However, it is clear that the occurrence of some degree of subendocardial necrosis need not reflect complete occlusion of any epicardial artery, provided the imbalance between oxygen supply and demand is sufficiently great.

1.1.5. Conventional therapy in the management of myocardial ischaemia and infarction.

Therapeutic options in patients with acute or chronic myocardial ischaemia are now extensive. These are reviewed only in brief, as therapy of ischaemia/infarction is to some extent incidental to the subject matter of this thesis.

1.1.5.1 Anti-ischaemic and anticoagulant drugs

1.1.5.1.1. Nitrates

Organic nitrates, such as glyceryl trinitrate (GTN), isosorbide dinitrate and isosorbide mononitrate, are the most commonly used anti-anginal drugs. Organic nitrates act as exogenous nitric oxide (NO) donors. Therefore their various actions are related to the

effects of NO on vasculature and platelets, including coronary and peripheral arterial and venous vasodilatation and anti-aggregatory effects (Horowitz & Henry 1987, Chirkov *et al.* 1993). The most significant problem related to the clinical use of nitrate therapy is nitrate tolerance, meaning a diminution of clinical effect of drug, which is engendered at least in part via reduced rate of NO generation, after continuous use (Horowitz & Henry 1987).

1.1.5.1.2. Calcium channel antagonists

This type of vasodilators, such as verapamil, diltiazem and nifedipine, have been extensively utilised for the treatment of myocardial ischaemia. These agents are selective for the L-type slow calcium channels, interfering with the entry of calcium into myocytes (negative inotropy) and vascular smooth muscle cells (vasodilator).

1.1.5.1.3. β -adrenoceptor antagonists

These agents competitively inhibit binding of catecholamines on β -adrenoceptors. They are extremely effective in the management of exertional angina via reducing myocardial oxygen demand by causing relative bradycardia and negative inotropy.

1.1.5.1.4. anti-aggregatory and anti-coagulant agents

Given extensive evidence that platelet aggregation/thrombus formation are centrally involved in the pathogenesis of acute myocardial ischaemia, there have been extensive developments as regards the therapeutic utility of agents interfering with this process. In brief:

(1) Aspirin (cyclooxygenase inhibitor) is used most in cardiology for both prevention and treatment of myocardial ischaemia.

(2) Other anti-aggregation agents (ADP antagonists, Glycoprotein IIb/IIIa inhibitors) are under investigation as incremental/alternative therapy to aspirin.

(3) Anti-thrombin anticoagulants (heparin, hirudin) are in extensive use for management of unstable angina pectoris. Recently, evidence suggests that various low molecular weight heparins may be of advantage in this condition, via a combination of inhibition of factor X and thrombin formation.

(4) Oral anticoagulants (warfarin) currently are not routinely used during acute ischaemia, but rather during follow-up in selected patients.

(5) Thrombolytic agents are utilized only in the presence of threatened acute transmural infarction.

1.1.5.2. Percutaneous transluminal coronary angioplasty (PTCA).

PTCA is a technique to re-open (or dilate) the stenosed lumen of coronary artery by inflation of an intraluminal balloon. It has been used as a treatment for both chronic and acute ischaemia, including evolving infarction, and occupies an intermediate position in the spectrum between medical therapy and coronary artery surgery.

During balloon inflation (usually 30 seconds to 2 minutes), the stenosed coronary artery is by definition completely occluded. The artery is then reperfused immediately after balloon deflation. Thus the transient myocardial ischaemia followed by reperfusion is inevitable during this type of treatment.

1.1.5.3. Coronary artery bypass grafting (CABG)

CABG remains the usual treatment for extensive myocardial ischaemia. The process in this treatment involves a circulatory arrest followed by application of a combination of arterial and venous by-pass grafts. Therefore, this surgery inevitably involves the potential induction of myocardial ischaemia. Thus can be analogous to a model of ischaemia followed by reperfusion (Ferrari *et al.* 1993).

1.1.6. Sequelae of ischaemia/reperfusion: biochemical and haemodynamic.

1.1.6.1. Effects of ischaemia on myocardial metabolism

Myocardium requires adenosine triphosphate (ATP) for its contractile process. Under physiological aerobic conditions, the myocardium derives its energy supply from oxidation of free fatty acids (FFAs) (approximately 65%), glucose (15%), lactate and pyruvate (12%), and amino acid (5%) (Lassers *et al.* 1972). Long-chain and short chain FFAs are broken down to acetyl CoA which is further metabolized via the tricarboxylic acid (TCA) cycle. Glycolysis plays only a minor role. Lactate is not normally accumulated in the heart because in the presence of O₂ it can be converted to pyruvate, and metabolized via the TCA cycle.

During ischaemia, oxidative phosphorylation cannot proceed at the normal rate, thus inducing a decrease in ATP production. Acetyl CoA can not be oxidized at the normal rate and therefore FFAs accumulate (Owen *et al.* 1970). In contrast, glycolytic flux is increased (Brachfeld & Sheuer 1964). AMP is further metabolized to adenosine, hypoxanthine and xanthine (Jennings *et al.* 1981). Hypoxia also decreases pyruvate-acetyl CoA conversion rate, while the pyruvate-lactate equilibrium shifts toward lactate formation which freely diffuses out of the cells (Jackson 1985). Decrease in ATP content further reduces Na⁺/K⁺ gradients resulting in cell swelling and leading to cell death (Reimer *et al.* 1981, Steenberger *et al.* 1985).

With reversible ischaemia, oxidative metabolic rates recover rapidly after the onset of reperfusion (Goodwin & Taegtmeier 1994, Lopaschuk *et al.* 1990). TCA cycle is replenished through carboxylation of lactate-derived pyruvate (Goodwin & Taegtmeier 1994). However, late reperfusion may cause further damage. As a consequence,

reperfusion results in a large increase in intracellular calcium, leading to mitochondrial calcium overload with subsequent damage to the mitochondrial structure and loss of ability to produce ATP (Ferrari *et al.* 1993). It has also been suggested (Arstall *et al.* 1995, Ohoi *et al.* 1993, Zweir *et al.* 1987) that oxygen free radicals contribute to the membrane and cellular damage associated with reperfusion-induced dysfunction.

1.1.6.2. Effects of ischaemia on myocardial function

1.1.6.2.1. Systolic function

Myocardial ischaemia is generally associated with impairment of the systolic performance in a localized area of myocardium (Tennant & Wiggers 1935, Ross *et al.* 1986, Osakada *et al.* 1983). A reduction of blood flow of 80% results in regional akinesis (Vatner 1980), while the residual myocardium hyperfunctions to maintain global left ventricular systolic function.

Transient episodes of myocardial ischaemia-induced left ventricular dysfunction may be quite prolonged, and post-ischaemic depression ("stunning") of the myocardium, with impaired mechanical performance, reduced high-energy phosphate stores, and abnormal ultrastructure may persist for several days following a 15-minute coronary occlusion (Braunwald & Kloner 1982, Vatner *et al.* 1986). Accumulation of oxygen-derived free radicals may play a role in myocardial stunning (see also section 1.1.6.1), since oxygen free radical scavengers improved the recovery of heart function post ischaemia (Myers *et al.* 1985, Przyklenk *et al.* 1986).

Regional loss of myocardial contractile activity, whether sustained or transient, if sufficiently widespread, depresses overall left ventricular function, results in heart failure.

Haemodynamic evidence of left ventricular failure develops when contraction ceases in 20-25% of the left ventricle; with loss of >40% left ventricular myocardium, severe pump failure ensues and, if this loss is acute, fatal or near-fatal, cardiogenic shock usually develops (Braunwald 1988).

1.1.6.2.2. Diastolic function

Myocardial ischaemia and infarction impairs not only the contractile properties of the heart but also the ventricular relaxation (Gewirtz *et al.* 1983, Carroll *et al.* 1983, Visner *et al.* 1985) as evidenced by a decreased rate of left ventricular pressure decline (negative dP/dt) and ventricular wall thinning and a prolonged isovolumetric relaxation period (Visner *et al.* 1985, Grossman & McLaurin 1976, Bourdillon *et al.* 1983, Carroll *et al.* 1983). In turn, the ischaemia-induced changes in diastolic properties increase resistance to ventricular filling together with the reduced systolic function of the ventricle contribute to the elevation of left ventricular diastolic pressure during ischaemia.

1.2. Physiology and pathophysiology of natriuretic peptides

1.2.1. Structure, synthesis, and release of natriuretic peptides

The granules in the atrial myocytes were first described by Kisch (1956) utilizing electron microscopy. These granules were identified in the cytoplasm of atrial cardiocytes with more granules in the right than in the left atrium. Morphologically and histochemically, the granules resemble the secretory granules found in peptide-secreting cells (de Bold & Bencosme 1975). Changes in dietary sodium and water were shown to alter the granularity of atrial myocytes (de Bold 1979).

Intravenous administration of rat atrial extracts into intact rats produces natriuresis and diuresis (de Bold *et al.* 1981). They suggested that the granules contained a natriuretic substance which they later named Atrial Natriuretic Factor (ANF). This hormone initially had many names, including atrial natriuretic hormone, atrial natriuretic substance, atriopeptin, atrin, cardiodilatin, or cardionatrin. Atrial Natriuretic Peptide (ANP) and ANF are two most widely used acronyms for this peptide. The molecular structures of ANP and its precursors are illustrated in Fig 1.1. The discovery of ANP led to a search for other peptides with natriuretic activity. The two additional peptides discovered were Brain Natriuretic Peptide (BNP) and C-type Natriuretic Peptide (CNP). These natriuretic peptides are a family of hormones sharing a common structural motif consisting of a 17-amino acid loop formed by an intramolecular disulphide linkage (Fig 1.2.) (Lang *et al.* 1992). Only 5 of the 17 amino acids in the ring differ among the three peptides isolated from pig, whereas the *N*- and *C*-terminals vary in both amino acid composition and length (Sudoh *et al.* 1988, 1990). The *C*-terminal tail commonly found in ANP and BNP is completely lost in the case of CNP. The kidney, besides being a



Fig 1.1. The biosynthetic pathway of atrial natriuretic factor (ANF)

The structure of human pre-pro-ANF, pro-ANF₁₋₁₂₆ and ANF₉₉₋₁₂₆ are shown. The amino acid substitutions found in the rat peptide are shown in parentheses (Rayner, 1992).

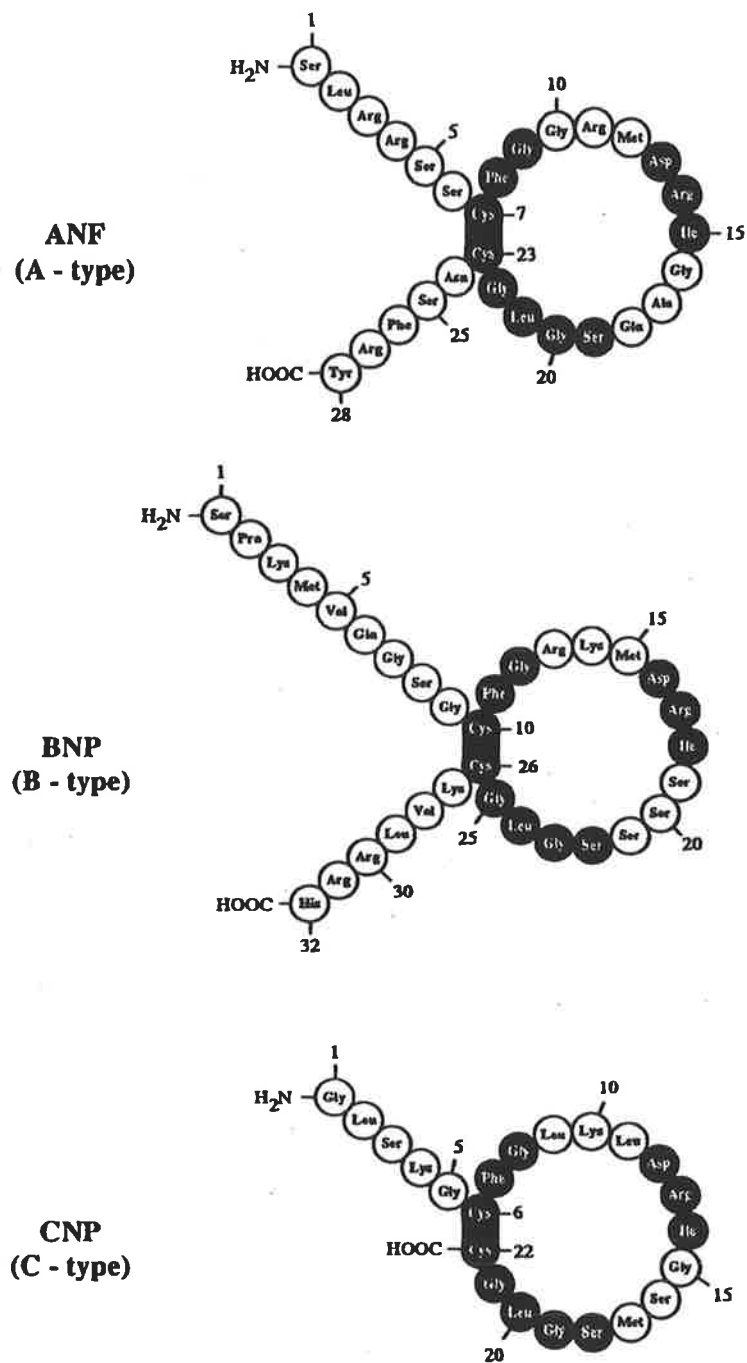


Fig 1.2. Homology of the three major natriuretic peptides (Jamison *et al.* 1992).

major target organ for ANP, may also be the production site of an additional natriuretic peptide: urodilatin, a 32-amino acid hormone corresponding to ANP₉₅₋₁₂₆ was identified in the rat kidney (Schulz-Knappe *et al.* 1988).

1.2.1.1. ANP

ANP₉₅₋₁₂₆ is derived from preproANP, which contains between 149 and 153 amino acids depending upon the species. The human sequence consists of 151 amino acids (Zivin *et al.* 1984) and shares strong homology with peptides from other species including rat (152 amino acids) (Yamanaka *et al.* 1984, Zivin *et al.* 1984). ANP is derived mainly from atrium. The ANP mRNA content in the ventricle is only 7% of that in the atrium (Ruskoaho 1992)

It is generally believed that atrial wall stretch is the predominant stimulus for ANP release (Edwards *et al.* 1988). *In vitro*, mechanical stretch-induced ANP release was observed in isolated myocytes (Gardner *et al.* 1991), isolated rat atria (Bilder *et al.* 1986) and isolated perfused rat hearts. Results were consistent with release of ANP via increases in either right or left heart distension (Lang *et al.* 1985, Mantymaa *et al.* 1991). The concentration of ANP in the medium increased similarly in proportion to the degree of stretch both from left and right atria; this increase in ANP release is temperature dependent (Bilder *et al.* 1986). In the normal Langendorff heart, the atria do not fill, because they are open, and thus the effect of atrial stretch is minimized.

In vivo, high sodium intake for 3 to 5 days also increases ANP concentrations (Hollister *et al.* 1986). In anesthetized rats, infusion of 2 ml of 0.9% saline within 1 min produced a 2 to 3-fold increase in plasma ANP (Lang *et al.* 1985). Human plasma ANP levels

increased 3-fold after 2 L of 0.9% saline infusion or high sodium intake (Sagnella *et al.* 1985).

In healthy humans, basal plasma ANP levels range from approximately 10 to 100 ng/L (3.3 to 33 pmol/L) (Tan *et al.* 1993). Increased plasma ANP levels occur in animals and humans with congestive heart failure (CHF) (Naruse *et al.* 1994; Bilder *et al.* 1989; Shenker *et al.* 1985; Yasue *et al.* 1989 and 1994; Tsutamoto *et al.* 1989; Lewis *et al.* 1992), chronic renal failure (Naruse *et al.* 1994), aortic stenosis (Ikeda *et al.* 1989), myocardial infarction (MI) (Tikkanen *et al.* 1987; Wang *et al.* 1989, Ferrari *et al.* 1990; Tomoda 1988; Gutierrez-Marcos *et al.* 1991; Niitsuma *et al.* 1991; Sigurdsson *et al.* 1993; Ngo *et al.* 1989; Kyriakidis *et al.* 1993; Fontana *et al.* 1990; Arnal *et al.* 1993; Bellone *et al.* 1993; Svanegaard *et al.* 1989; Kettunen *et al.* 1994), after cardiac transplantation (Ganel *et al.* 1997) and atrial or ventricular tachycardia (Twidale *et al.* 1993, Oliver *et al.* 1988, Schiebinger *et al.* 1994). ANP is secreted in increased amounts from the ventricle as well as the atrium in patients with heart failure (Yasue *et al.* 1989).

Other previously described stimuli for ANP synthesis/release include thyroid hormones (Kohno *et al.* 1986), glucocorticoids (Gardner *et al.* 1986), products of arachidonic acid metabolism (PGE₂, PGF₂α) (Gardner & Schulz 1990) and growth factors (Parker *et al.* 1990). Furthermore, the effects of endothelin on ANP syntheses/release are discussed in section 1.4. The influence of various drugs on ANP levels is discussed in section 1.5.

1.2.1.2. BNP

Although BNP was initially identified in the porcine brain, concentrations of BNP are much higher in the heart than in the brain (Struthers 1993). Studies in the rat (Nakao *et al.* 1990) and in man (Kambayashi *et al.* 1990) have shown that BNP is barely detectable

in the brain and is distributed only in the spinal cord, and that the highest tissue concentration of BNP is still present in the heart. In contrast to the conserved amino acid sequence of ANP (Kangawa & Matsuo 1984), the molecular sequence of BNP varies among species (Sudoh *et al.* 1988; Kojima *et al.* 1989; Kambayashi *et al.* 1989, 1990; Sudoh *et al.* 1989; Nguyen *et al.* 1989). Rat BNP, a 45-amino acid residue peptide, has been isolated from the heart (Kojima *et al.* 1989). Human BNP comprise 32 amino acid residues (Kambayashi *et al.* 1990, Sudoh *et al.* 1989) (Fig 1.2.). BNP, derived from the C-terminus of the pro-BNP molecule, shares remarkable similarity to the structure of ANP. However, the amino acid sequence of BNP may vary as much as 50% among different species (Lang *et al.* 1992).

In contrast to ANP, BNP is predominantly synthesized in the ventricle. BNP mRNA may be approximately three-fold greater in the ventricle than in the atrium. Approximately 60% of BNP secreted is derived from ventricle, although the tissue BNP level in the ventricle is only 1% of that in atrium (Ogawa *et al.* 1991, Mukoyama *et al.* 1991b, Hosoda *et al.* 1991), indicating a more rapid release and a small storage of BNP in ventricular myocytes than that in atrial myocytes. The BNP mRNA level is increased in the ventricles of failing hearts (Mukoyama *et al.* 1991a), or MI (Hama *et al.* 1995). Ventricular ANP and BNP gene expression were augmented in hamsters with hypertrophic cardiomyopathy (Tamura *et al.* 1994).

Plasma BNP concentrations in normal subjects range from 0.9 to 1.5 pmol/L (Lang *et al.* 1992b). It is likely that, like ANP, BNP release may also involve a stretch-release coupling mechanism. Plasma BNP levels were increased in normal subjects with chronic dietary sodium loading (Lang *et al.* 1991). Furthermore, haemodialysis decreased plasma BNP level in patients with renal failure (Lang *et al.* 1992a, Buckley *et al.* 1992) and aortic stenosis (Ikeda *et al.* 1989). A higher percentage increase in plasma BNP

concentration was also found in patients with CHF (Yoshimura *et al.* 1993, Mukoyama *et al.* 1991a, Davis *et al.* 1994; Yasue *et al.* 1994, Arad *et al.* 1996), post myocardial ischaemia (Nicholson *et al.* 1993, Mair *et al.* 1997) or MI (Mukoyama *et al.* 1991b, Morita *et al.* 1993, Horio *et al.* 1993, Hama *et al.* 1995, Foy *et al.* 1995, See section 1.2.4.2.), after cardiac transplantation (Ganel *et al.* 1997) and coronary artery bypass grafting (CABG) (Mair *et al.* 1997). The effects of endothelin on BNP synthese/release are discussed in section 1.4.

1.2.1.3. CNP

CNP is the newest member of natriuretic peptide family. It lacks the C-terminal tail which exists in ANP and BNP (Lang *et al.* 1992). CNP is mainly located in the central nervous system (Komatsu *et al.* 1991b, Sudoh *et al.* 1990). One study showed that some cytokines (IL-1a, IL-1b and tumour necrosis factor- α) can stimulate CNP secretion in cultured endothelial cells (Suga *et al.* 1993). CNP has been also isolated from kidney, intestine and lung in both humans and rats (Komatsu *et al.* 1991a); and from European dogfish heart (Schofield *et al.* 1991, Suzuki *et al.* 1991). The sequence of mammalian CNP is highly conserved among species (Sudoh *et al.* 1990, Kojima *et al.* 1990, Tawaragi *et al.* 1991, Suga *et al.* 1992).

1.2.2. Natriuretic peptide receptors

Two types of receptors for natriuretic peptides have been identified (Suga *et al.* 1992, Nakao *et al.* 1992, Anand-Srivastava & Trachte 1993).

1.2.2.1. Natriuretic Peptide Receptor R₁

Natriuretic peptide receptors R₁ (NP R₁) with molecular weight of 120,000-180,000 are coupled to particulate guanylate cyclase (GC) and thus induce cGMP formation (Waldman *et al.* 1984, Kuno *et al.* 1986). These biologically active receptors can be divided into two subtypes based on affinities for different subtypes of natriuretic peptides: the GC-A and the GC-B receptors (or NPR-A and B) (Schulz *et al.* 1989, Chang *et al.* 1989). Suga *et al.* (1992) demonstrated that the rank order of binding affinity for the GC-A was ANP > BNP >> CNP, and CNP > ANP > BNP for GC-B.

1.2.2.2. Natriuretic Peptide Receptor R₂

The other type of natriuretic peptide receptor, NP R₂, with molecular weight of 60,000-70,000 is not coupled to GC (Schenk *et al.* 1985, Shimonaka *et al.* 1987, Fuller *et al.* 1988). The density of these receptors in most tissues is higher than that of NP R₁ receptors. For example, in endothelium, the ratio of NP R₂:R₁ was 94:6 (Leitman *et al.* 1986). This type of receptor is widely distributed but is considered biologically silent (regarding its lack of renal action) and designated as a clearance receptor (C-receptor) (Maack *et al.* 1987). However, using cells lacking R₁ receptor (eg. platelets and thyroid cultured cells), ANP inhibited cAMP production, suggesting that NP R₂ receptors may be coupled to the adenylyl cyclase/cAMP system (Tseng *et al.* 1990, Anand-Srivastava *et al.* 1991).

1.2.3. Biological effects of natriuretic peptides

Proposed biological effects of ANP include natriuresis, diuresis, vasodilation and inhibition of the release and/or actions of several hormones, such as renin, angiotensin II, aldosterone, endothelin and vasopressin (Ruskoaho 1992, Yoshimura *et al.* 1991,

Wilkins *et al.* 1997) (Fig 1.3). The lack of specific natriuretic peptide receptor antagonists has made it difficult to study the physiological actions of natriuretic peptides, but alternative inferential techniques are available (see below).

1.2.3.1. Renal actions of natriuretic peptides

The kidney is a major target organ for ANP (Knepper *et al.* 1991). The original investigation by de Bold *et al.* (1981) showed atrial myocardial extracts induced a rapid, large increase in renal salt and water excretion when administered intravenously to rats. Studies in healthy humans confirmed that low doses (0.15 to 1.0 µg/min) of ANP induced a significant increase in diuresis, natriuresis and extravasation of fluid accompanied by increases in chloride, calcium, magnesium, phosphate excretion and cGMP levels (Weidmann *et al.* 1986, Anderson *et al.* 1990, Bidiville *et al.* 1998, Morice *et al.* 1988, Solomon *et al.* 1988, Cogan 1990, Bunner *et al.* 1990, Nakamura *et al.* 1990). These effects are caused by changes in renal haemodynamics, in particular, a renal vasodilator effect and an increase in glomerular filtration rate, and by effects of ANP on the renal tubules (Oshima *et al.* 1984, Cogan 1990, Knepper *et al.* 1991). Furthermore, studies have also shown ANP increases urinary sodium excretion by inhibiting sodium reabsorption across the inner medullary collecting duct via cGMP-dependent mechanisms (Gunning *et al.* 1988; Stanton 1991). NPR-A gene knockout mice are resistant to the natriuretic effect of exogenous ANP or acute volume expansion, suggesting the natriuretic effects of ANP and BNP are mediated by NPR-A (Wilkins *et al.* 1997, Kishimoto *et al.* 1996). This helps explain the lack of natriuretic effect of CNP, since CNP has much less affinity for NPR-A receptors (refer to section 1.2.2.).

Intravascular administration of porcine BNP to rats also induced diuretic and natriuretic responses which are similar to those for ANP (Sudoh *et al.* 1988). In an kidney epithelial

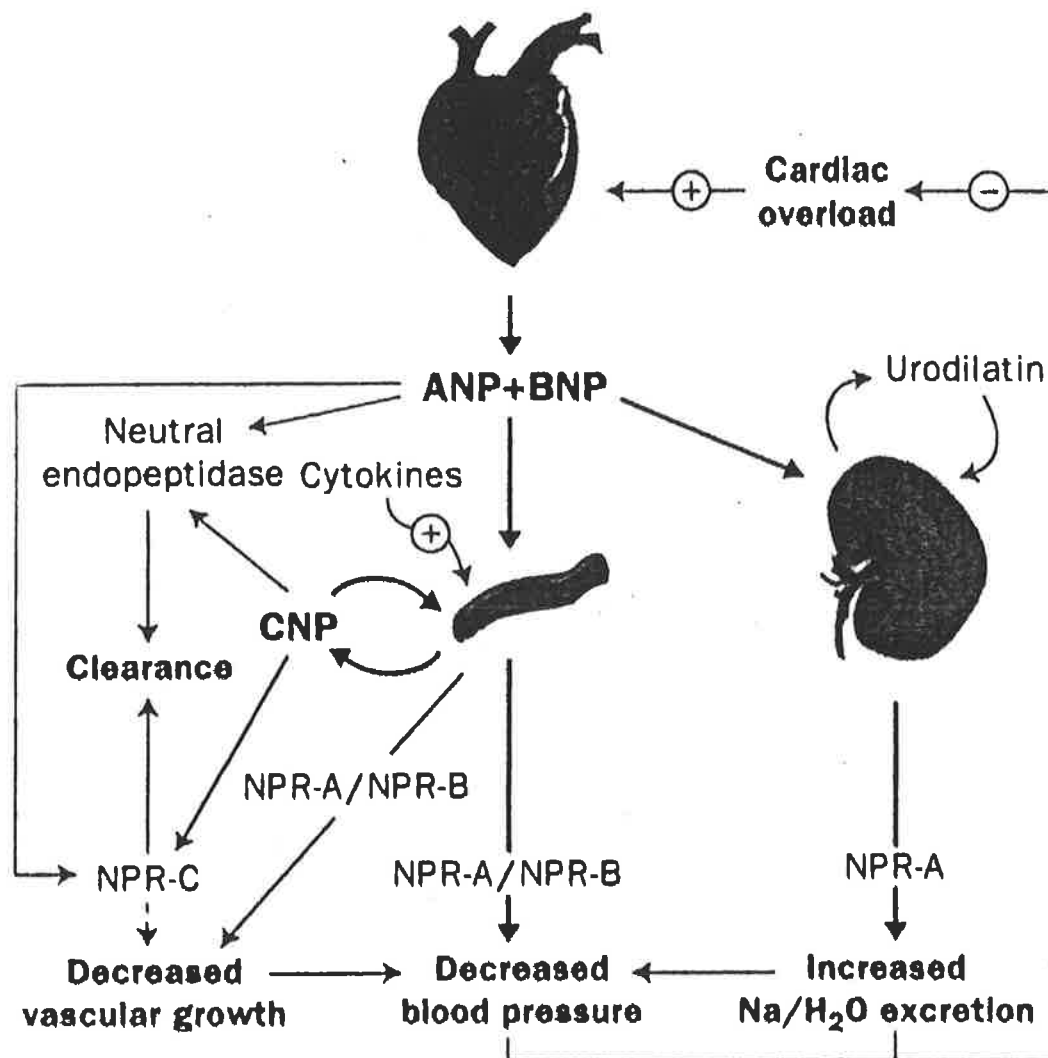


Fig. 1.3. Postulated physiology of the natriuretic peptide family (Wilkins *et al.* 1997)

cell line, BNP has been shown to produce cGMP (Iwata *et al.* 1980). In anaesthetized dogs, an intrarenal infusion of BNP attenuated renin secretion and increased urinary flow rate and sodium excretion without altering blood pressure, glomerular filtration rate or renal blood flow (Akabane *et al.* 1991). In the inner medullary collecting duct system, BNP has been shown to be a potent inhibitor of sodium uptake (Gunning *et al.* 1990).

1.2.3.2. Effect of natriuretic peptides on the cardiovascular system

Mice with ANP gene or NPR-A gene “knockout” have raised blood pressure independent of salt intake (John *et al.* 1995, Lopez *et al.* 1995). In healthy humans, high doses (eg. 1.5 µg/min) of ANP have more pronounced effects on blood pressure reduction, due to the direct arteriole vasodilator effect (Bolli *et al.* 1987, Hughes *et al.* 1987, Jansen *et al.* 1991, Currie *et al.* 1983, Garcia *et al.* 1984) and to a decrease in cardiac output (Oelkers *et al.* 1988, Weidmann *et al.* 1986, Morice *et al.* 1988, Shen *et al.* 1990, Woods & Anderson 1990). The latter effect may be the result of a reduction in both pre-load and after-load (Atlas *et al.* 1992, Allen *et al.* 1989). ANP reduced pre-load by vasodilation (Winqvist *et al.* 1984) and reduction in intravascular volume by inducing diuresis and shifting intravascular fluid to the interstitial space (Huxley *et al.* 1987, Rutlen *et al.* 1990, Trippodo *et al.* 1986); The after-load reduction is achieved by ANP’s direct arteriolar vasodilator effect which is mediated by cGMP (Heim *et al.* 1988, Kawabe *et al.* 1992, Nakagawa *et al.* 1988, Ohlstein & Berkoeitz 1985, Schini *et al.* 1988). HS-142-1, a NP R₁ receptor antagonist, delayed left ventricular relaxation, whilst BNP has the opposite effect (Yamamoto *et al.* 1997).

NP-induced increases in coronary blood flow have been reported in dog (Ertl & Bauer 1990, Chu & Cobb 1987, Chu *et al.* 1990, Nakagawa *et al.* 1988, Yamamoto *et al.* 1997) and human (Serizawa *et al.* 1988, Chu *et al.* 1989a). ANP limited ischaemia-

induced ventricular arrhythmia (Linz *et al.* 1988, Takata *et al.* 1996), and increased the ATP content in the inner layers of the ischaemic myocardium (Takata *et al.* 1996). HS-142-1 decreased coronary flow in anaesthetized dogs (Yamamoto *et al.* 1997). Natriuretic peptides also inhibit ET biosynthesis in rat endothelial cells (Emori *et al.* 1993).

ANP and BNP act as a dual natriuretic peptide system intimately involved in circulatory homoeostasis (Lang *et al.* 1992). BNP has biological effects that are similar, but some elements are different to those of ANP. BNP infusion-induced natriuretic response is greater in patients with CHF than in normal subjects (Yoshimura *et al.* 1991) whereas ANP caused more natriuresis in healthy individuals (Struthers *et al.* 1993). CNP also possesses some vasorelaxant activity (Wei & Aarhus 1993) and has been considered to regulate local vascular tone.

Although the natriuretic peptides' vasodilator effects are well recognized, ANP infusion may paradoxically increase mesenteric vascular resistance (Woods & Anderson 1990), and systemic vascular resistance in intact animals (Shen *et al.* 1990). In isolated guinea-pig heart, low dose (near-physiological) ANP perfusion increased coronary flow rate whereas high dose (nM) decreased it (Di Nardo *et al.* 1993). In conscious dog and isolated perfused rat heart, ANP had biphasic vasoactive effects in a time-dependent manner, ie. initial vasodilation followed by vasoconstriction (Shen *et al.* 1991, Bauer *et al.* 1994). This ANP-induced rise in vascular resistance was attenuated by autonomic blockade except for the mesenteric vascular resistance effects (Woods & Anderson 1990; Shen *et al.* 1990, 1991). Thus, while ANP clearly exerts some vasoconstrictor effects, these are only partially mediated by sympathetic mechanisms; the remainder of these mechanisms remain to be elucidated.

1.2.3.3. Other Physiological actions of natriuretic peptides

ANP inhibits renin and aldosterone secretion (Oppenorth *et al* 1986, Richards *et al.* 1988, Solomon *et al.* 1988, Clinkenbeard *et al.* 1990, Williams *et al.* 1988), suppresses vasopressin and ACTH release (Poole *et al.* 1987, Samson 1985, Allen *et al.* 1988, Williams *et al.* 1988), and inhibits glucose-stimulated insulin secretion in intact mouse and rat (Ahren 1988). A study showed CNP inhibited the ACTH-induced increase in aldosterone, this effect can be reversed by HS-142-1, a R_2 receptor antagonist (Kawai *et al.* 1996). Physiological concentrations of atrial natriuretic factors also inhibit corticotropin releasing factor-stimulated adrenocorticotropin secretion from cultured anterior pituitary cells. (King & Baertschi 1989).

1.2.4. Effect of myocardial ischaemia on release of natriuretic peptides.

1.2.4.1. ANP, BNP and myocardial ischaemia.

Myocardial ischaemia is a potent stimulus for ANP release. However, there is a lack of uniformity regarding the findings of currently published studies in this area. For example Table 1.1. summarizes the results of previously published studies in man concerning this issue. In patients with coronary artery disease undergoing dynamic exercise, plasma ANP levels have generally been reported as being higher than those associated with exercise in the absence of coronary disease (Nicholson *et al.* 1993; Hu *et al.* 1988; de Marzio *et al.* 1992; Nishikimi *et al.* 1988; Marumoto *et al.* 1995a,b, Morimoto *et al.* 1997). However one study failed to show any change in plasma ANP level during ischaemia caused by exercise (Ikaheimo *et al.* 1989). Plasma ANP levels also increased during percutaneous transluminal coronary angioplasty (PTCA) and exercise-induced angina (Ikaheimo *et al.* 1989, Wiedermann *et al.* 1991; Prachar *et al.* 1991; Malatino *et al.* 1992; Gill *et al.* 1995; Mair *et al.* 1997). In patients with unstable angina, plasma BNP levels were higher than those in patients with stable angina or normal subjects (Kikuta *et al.* 1996).

Table 1.1. Effects of ischaemia on plasma ANP and BNP concentrations in humans: summary of previously reported studies.

Authors	Patient group	Stimulus	Result
Nicholson	CAD	exercise	increase [ANP], [BNP]
Hu	CAD	exercise	increase [ANP]
de Marzio	post MI	exercise	increase [ANP]
Nishikimi	post MI	exercise	increase [ANP]
Marumoto	post MI	exercise	increase [ANP] [BNP]
	CAD	exercise	increase [ANP] [BNP]
Morimoto	post MI	exercise	increase [ANP] [BNP]
Ikaheimo	CAD	exercise	[ANP] no change
	CAD	PTCA	increase [ANP]
Wiedermann	CAD	PTCA	increase [ANP]
Prachar	CAD	PTCA	increase [ANP] LAD*
Gill	CAD	PTCA	increase [ANP]
	CAD	CI	[ANP] no change
Malatino	CAD	PTCA	increase [ANP]
	CAD	CI	increase [ANP]
	CAD	angina	increase [ANP]
Kikuta	CAD	angina	increase [BNP]
Mair	CAD	angina	increase [BNP]
	CABG	AO clamp	increase [BNP] not [ANP]
Northridge	CPB	AO clamp	increase [ANP]
Thomassen	CAD	atrial pacing	[ANP] no change

PTCA=percutaneous transluminal coronary angioplasty. CAD=coronary artery disease. MI=myocardial infarction. CI=contrast injection. CPB=cardiacpulmonary bypass. AO clamp=aortic cross-clamping. CABG=coronary artery bypass grafting. * ANP elevation was significant only in patients with left anterior descending artery (LAD) occlusion.

However, one report showed atrial pacing-induced ischaemia did not alter levels of ANP (Thomassen *et al.* 1987). In patients undergoing cardiopulmonary bypass surgery, whose pre-operation plasma ANP levels were in the normal range, there were significant increase in plasma ANP concentration post aortic cross-clamping (Northridge *et al.* 1992). However, a study in patients undergoing CABG showed plasma BNP but not ANP concentration to be increased post aortic cross-clamping (Mair *et al.* 1997). Release of ANP (or N-terminal pro-ANP) and BNP during acute MI has been reported in rat (Tikkanen *et al.* 1987; Wang *et al.* 1989) and human (Arnal *et al.* 1993; Bellone *et al.* 1993; Ferrari *et al.* 1990; Fontana *et al.* 1990; Gutierrez-Marcos *et al.* 1991; Kyriakidis *et al.* 1993; Ngo *et al.* 1989 & 1991; Niitsuma *et al.* 1991; Omland *et al.* 1993a,c; Sigurdsson *et al.* 1993; Svanegaard *et al.* 1989; Tomoda 1988; Arad *et al.* 1996, Huttli *et al.* 1995, Michel *et al.* 1995) with or without left ventricular systolic dysfunction. The increase in ANP secretion is proportional to infarct size and the degree of cardiac overload (Michel *et al.* 1995). In the Survival and Ventricular Enlargement (SAVE) study, plasma ANP concentrations were increased in 522 patients with MI with reduced left ventricular ejection fraction but without overt heart failure, compared with an age-matched control group (Rouleau *et al.* 1991). Plasma ANP (or N-terminal pro-ANP) concentrations have been used as an indicator of mortality, cardiac function and exercise capacity for patients with acute MI (Cohn 1990, Hall *et al.* 1994,1995, Nakamura *et al.* 1993, Omland *et al.* 1993b,c, Svanegaard *et al.* 1992, Livanainen *et al.* 1997, Bonarjee *et al.* 1995).

In patients with acute inferior MI associated with right ventricular infarction (RVMI), plasma ANP levels were twofold higher than those observed in patients without right ventricular involvement. After volume loading, although right atrial pressure increased, only a blunted response was observed in patients with RVMI, while there was a significant increase of ANP levels in patients with acute inferior MI without RVMI (Serra *et al.* 1991). Patients treated with streptokinase or tissue plasminogen activator have lower plasma ANP levels than those not receiving thrombolytic treatment (Ngo *et al.* 1989,

Phillips *et al* 1989), but it has not been determined whether this disparity is due to improved haemodynamic status or to other mechanisms.

1.2.4.2. Involvement of BNP and CNP in ischaemic processes

Unlike ANP, BNP secretion is mainly from the ventricle (Lang *et al.* 1992). This may explain why plasma BNP levels are not increased after exercise in normal subjects (Nicholson *et al.* 1993). Ischaemia-stimulated BNP release has been demonstrated in patients with exercise-induced ischaemia (Nicholson *et al.* 1993, Morimoto *et al.* 1997) and acute MI (Mukoyama *et al.* 1991b; Morita *et al.* 1993; Horio *et al.* 1993, Hama *et al.* 1995, Foy *et al.* 1995). Although plasma BNP concentrations were lower than ANP in normal subjects, BNP levels were higher than ANP in acute uncomplicated MI (Foy *et al.* 1995). In another study of uncomplicated acute MI with early reperfusion, plasma ANP levels returned to the normal range after day 3, while BNP levels remained high for 4 weeks (Morita *et al.* 1993). The increased plasma BNP levels did not correlate with pulmonary capillary wedge pressure but correlated inversely with cardiac index (Mukoyama *et al.* 1991, Morita *et al.* 1993), left ventricular ejection fraction (Foy *et al.* 1995) and infarct size (Horio *et al.* 1993). Plasma BNP concentrations have not yet been investigated in association with left ventricular ischaemia induced by PTCA or atrial pacing. Yoshimura *et al.* reported different secretion patterns of ANP and BNP in patients with CHF (Yoshimura *et al.* 1993). CNP induced relaxation in isolated canine veins and saphenous artery (Wei & Aarhus 1993). Therefore, CNP may contribute to NP-induced decrease in cardiac preload.

1.2.4.3. Mechanisms of NP release in Myocardial ischaemia

Myocardial ischaemia-stimulated ANP release is commonly thought to be mediated by increased atrial distension caused by ventricular failure (Ikaheimo *et al.* 1989; Malatino *et*

al. 1992; Tomoda 1988). A study showed the BNP concentration gradient between anterior interventricular vein and aortic root was greater in patients with anterior MI than in patients with inferior MI, suggesting the regional ventricular wall stretch caused by MI stimulates BNP release (Sumida *et al.* 1995). However, some studies have demonstrated the increase of ANP levels to be correlated with size of infarction but not with left ventricular dysfunction (Ngo *et al* 1989; Kyriakidis *et al* 1993) or atrial stretch (Prachar *et al* 1991). It is therefore possible myocardial ischaemia itself may directly affect ANP release. It has been demonstrated hypoxia or low flow global ischaemia cause release of ANP from the isolated rat heart (Uusimaa *et al* 1989, Chen *et al* 1993, Baertschi *et al* 1986). Correlations between myocardial redox and energy states and ANP release has also been studied (Uusimaa *et al* 1989). The increase in ANP release during the post-ischaemic period showed a positive linear correlation with the atrial and ventricular lactate/pyruvate ratios and negative non-linear correlation with the atrial and ventricular phosphorylation potential. This suggested ANP release during and after ischaemia may be due not only to atrial distension but also to changes in energy metabolism.

The intracellular mechanism of ANP release induced by ischaemia is not yet clear. Since myocardial ischaemia or infarction can cause elevation of plasma norepinephrine, epinephrine, renin, angiotensin II, aldosterone, vasopressin and endothelin (ET) (Wang *et al* 1989, Gutierrez-Marcos *et al* 1991, Sigurdsson *et al* 1993, Yasuda *et al* 1990), increased ANP secretion may therefore be stimulated by vasoconstrictor hormones. ET in particular has been shown to stimulate ANP and BNP secretion in rat myocytes (Thibault *et al* 1994, Horio *et al* 1993) and intact mice (Okumura *et al* 1993). ANP and BNP secretion enhanced by ET is mediated by endothelin A receptors (Thibault *et al* 1994). ET stimulates the hydrolysis of phosphoinositides to IP₃ and DAG, presumably activating PKC in cardiac myocytes to stimulate ANP secretion. Since ET augmented the basal and the stretch-stimulated ANP release in perfused rat heart (Mantymaa *et al.* 1990) and isolated myocytes (Gardner *et al.* 1991), and as BQ-123, a selective ET_A receptor

antagonist, inhibits volume-loading-induced ANP release (Leskinen *et al* 1997), ET may contribute towards ischaemia-induced ANP (and possibly BNP) release.

1.2.5. Available techniques for manipulating natriuretic peptides.

1.2.5.1. Clearance of natriuretic peptides from the circulation.

One of the limitations of using ANP as a therapeutic agent is its short half-life ($t_{1/2}$) which is about 0.5 to 1 min in rats (Luft *et al.* 1986, Barclay *et al* 1991) and 2 to 5 min in humans (Nakao *et al.* 1986, Tan *et al.* 1993). ANP is cleared by almost every tissue (Grebes & Volar 1990). After incubation of ANP with tissue homogenates, Tang *et al.* (1984) found the rank order of degradative potency to be kidney > liver > lung > plasma > heart. Receptor-mediated binding, uptake, and metabolism by target tissues, degradation by enzymes and other processes at plasma membranes, and excretion into non-plasma fluids such as urine may be involved in ANP clearance from the circulation (Roskoaho 1992). The majority of ANP clearance probably involves receptor-mediated endocytosis (Maack *et al.* 1987, Nussenzveig *et al.* 1990b). In isolated cells, ^{125}I -labelled ANP was initially bound to the cell membrane and then rapidly internalized and subsequently degraded (Nussenzveig *et al.* 1990b, Murthy *et al.* 1989).

In addition to the receptor-mediated clearance, ANP is also degraded enzymatically (Fig 1.3.). The most important of the enzymes responsible for the degradation is Neutral endopeptidase EC 3.4.24.11 (NEP, also, called enkephalinase or atriopeptidase) (Olins *et al.* 1987, Berg *et al.* 1988, Koehn *et al.* 1987, Stephenson & Kenny 1987, Sonnenberg *et al.* 1988). NEP, a metallopeptidase with a zinc in its active center, is mainly located in renal brush border and also in brain, lung, thyroid, intestine, seminal vesicles, prostate plasma and vascular tissue (Roskoaho 1992, Erdos & Skidgel 1989, Yandle *et al.* 1989,

Tamburini *et al.* 1989). The endopeptidase also hydrolyzes BNP (Vogt-Schaden *et al.* 1989, Bourne & Kenny 1990, Vanneste *et al.* 1990a), and other vasoactive peptides, including enkephalins, bradykinin, substance P, neurotensin, gastrin and chemotactic peptide (Roskoaho 1992).

Furthermore, other enzymes such as angiotensin-converting enzyme metabolism (Tang *et al.* 1984, Kettunen *et al.* 1994; Omland *et al.* 1993a) and kallikrein (Briggs *et al.* 1984) have also been implicated to a lesser extent in ANP degradation. Aprotinin, a serine protease inhibitor, increased $t_{1/2}$ of ANP (Vanneste *et al.* 1990b) and enhanced natriuretic responses (Briggs *et al.* 1984). In vascular smooth muscle cells, receptor-mediated clearance represented about 55%, whereas the extracellular proteolysis represented approximately 45% of the total clearance of ^{125}I -ANP (Johnson *et al.* 1990).

1.2.5.2. Inhibition of natriuretic peptide elimination

1.2.5.2.1. NP receptor antagonist

Major antagonists for R_1 receptors (see section 1.2.2) synthesized to date include A74186, anatin and HS-142-1 (Anand-Srivastva & Trachte 1993). A74186, A68828 and A71915 are peptide derivatives of ANP (Trachte 1993, von Gelgern *et al.* 1990, Holleman *et al.* 1991). Anantin, which is also a R_2 receptor agonist, is a product of the microorganism *Streptomyces coeruleus* (Weber *et al.* 1991, Trachte 1993). The most widely used inhibitor is HS-142-1, a nonpeptide ANP receptor antagonist (Qiu *et al.* 1996, Sano *et al.* 1992, Imura *et al.* 1992). HS-142-1 reduced ^{125}I ANP binding with a IC_{50} of 10 μM , suppressed cGMP generation in response to ANP, and eliminated renal, anti-steroidogenic, and vasodilator effects of NPs (Ohyama *et al.* 1992, Sano *et al.* 1992, Liu *et al.* 1996, Kawai *et al.* 1996; Yamamoto *et al.* 1997). These receptor antagonists have a number of disadvantages, including low potency, limited availability, and lack of specificity for individual receptors (Anand-Srivastva & Trachte 1993). In

addition, one study identified isatin (idole-2,3-dione), a compound (found in rat brain) with anxiogenic properties, as a endogenous R_1 receptor antagonist (Glover *et al.* 1995).

The major impetus for the development of ANP receptor antagonists is the desire to elevate plasma ANP concentrations by occupying NP R_2 receptors, resulting in suppression of NP clearance. The truncated derivatives of ANP, including C-ANF₄₋₂₃ and SC 46542, were used as selective inhibitors of R_2 receptors (Maack *et al.* 1987, Koepke *et al.* 1989, Souque *et al.* 1992). These molecules were shown to lose their renal activities, but retain high-affinity binding to ANP receptors. Intra venous infusion of C-ANF₄₋₂₃ increased ANP concentrations 2 to 3-fold (Jin *et al.* 1990). However, most of these ligands also possess agonist activities (Anand-Srivastava *et al.* 1990).

1.2.5.2.2. NEP inhibitors

There are currently four different classes of NEP inhibitors: thiols (thiorphan and SQ 29072), carboxyl (SCH 39370, UK 69578), phosphoryl and hydroxamate inhibitors (kelatorphan) (Gerbes & Vollmar 1990, Schwartz, *et al.* 1990, Trapani *et al.* 1989, Seymour *et al.* 1989, Sybertz *et al.* 1989, Danilewicz *et al.* 1989). SQ 29072 potentiated the depressor, diuretic, and natriuretic actions in conscious SHR (Seymour *et al.* 1989). Consistent with inhibition of ANP degradation, NEP inhibitors cause an increase in plasma ANP (Danilewicz *et al.* 1989, Northridge *et al.* 1992, Munzel *et al.* 1992), natriuretic, diuretic (Danilewicz *et al.* 1989, Trapani *et al.* 1989, Sybertz *et al.* 1989, Munzel *et al.* 1992), hypotensive responses (Sybertz, 1991) and increase left ventricular end diastolic pressure (Trippodo *et al.* 1991). Furthermore, UK 69578 (also named candoxatrilat) decreased ¹²⁵I ANP removal from plasma (Barclay *et al.* 1991). Orally effective NEP inhibitors, eg. candoxatril and CGS 25155, have been developed (O'Connell *et al.* 1992, MacPherson *et al.* 1993). The recent development of dual NEP/Angiotensin-converting enzyme (ACE) inhibitors (Seymour *et al.* 1996, Ferro *et*

al. 1998, Cleland *et al.* 1998) also allowed this group of drugs to be utilized clinically in the management of heart failure.

1.2.5.3. ANP antibody

ANP-specific antibody (Ab) has been used as a tool for physiological studies. In intact animals, ANP-Ab suppressed plasma ANP and angiotensin II levels, urine flow and sodium excretion (Rudd *et al.* 1989, Gray 1994, Gray *et al.* 1997). Injection of ANP antiserum into rat third cerebral ventricle inhibited the natriuresis and increase in plasma ANP levels in response to blood volume expansion (Antunes-rodrigues *et al.* 1993). These findings confirmed the natriuretic and diuretic effects of endogenous ANP.

1.2.5.4. Gene manipulation

Mice with genetically altered NP or NPR-A gene have been used recently for investigation of NP's physiological actions. Mice with ANP gene or NPR-A gene deletion (knockout) have raised blood pressure independent of salt intake (John *et al.* 1995, Lopez *et al.* 1995). Conversely, over-expression of the ANP gene lowers systemic blood pressure (Steinhelper *et al.* 1990).

1.3. Physiology and Pathophysiology of Endothelins

1.3.1. Structure, synthesis, and release of endothelins

1.3.1.1. Structure and biosynthesis

Endothelin (ET) is a 21 amino acid polypeptide, first isolated, purified, sequenced and cloned in 1987 (Yanagisawa *et al.* 1988). A little later, the amino acid sequence of a rare snake venom, sarafotoxin, was also reported and shown to be very similar to that of ET (Kloog & Sokolovsky 1989). There are three closely related isoforms of ET: endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3); which differ by only a few amino acids. The fourth member, called vasoactive intestinal constrictor (VIC), is considered to be the murine form of ET-2 (Bloch *et al.* 1991). The ET molecules have several conserved amino acids which form two intrachain disulfide bonds. The main differences in the endothelin isopeptides reside in their N-terminal ends (Fig 1.4.).

ETs are widely distributed in mammalian tissues, and have been demonstrated to be produced from endothelial cells (Yanagisawa *et al.* 1988, Macdonald *et al.* 1988, Boulanger & Luscher 1990, McClellan *et al.* 1994, Haug *et al.* 1998), vascular smooth muscle cells (Sung *et al.* 1994, Haug *et al.* 1998), myocytes (Suzuki *et al.* 1993), kidney mesangial cell (Simonson 1993), neurones and astrocytes in the central nervous system, hepatocytes, Sertoli cells, and breast epithelial cells (Rubanyi & Polokoff 1994, Levin 1995).

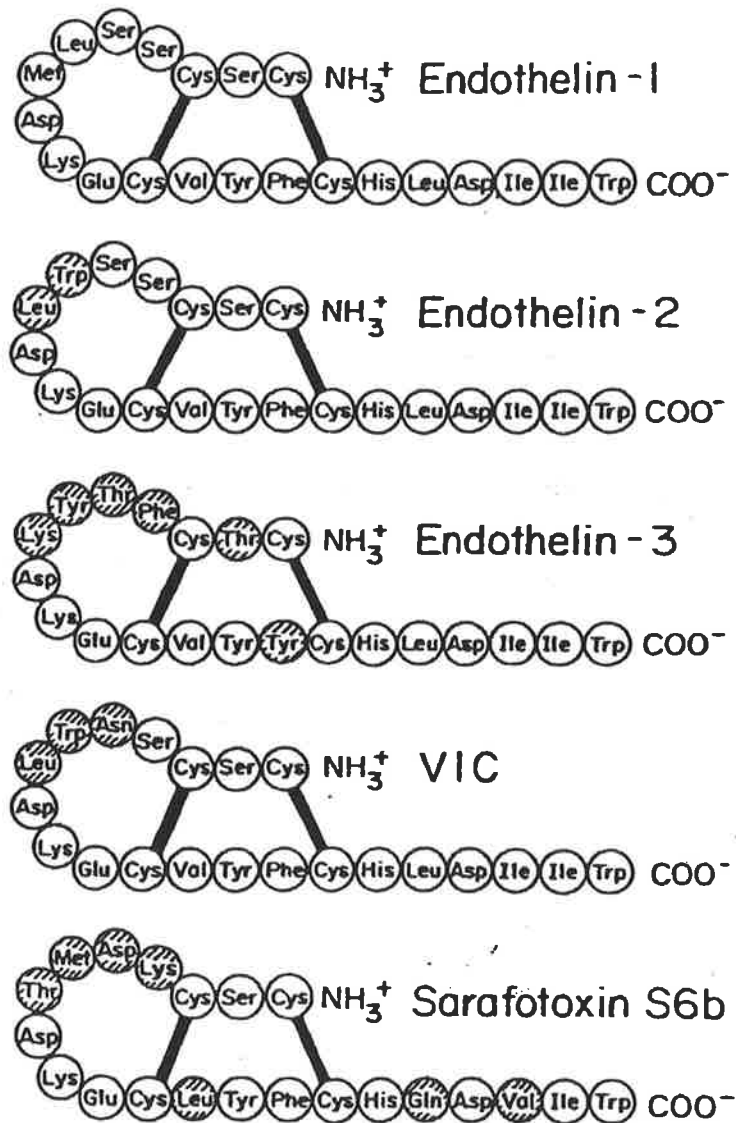


Fig 1.4. Structures of endothelin isoforms and sarafotoxin (Kramer *et al.* 1992).

ET is formed from a 203-amino acid peptide precursor prepro-endothelin by endopeptidase(s), to yield an intermediate 39-residue peptide termed big endothelin (or proendothelin). The latter is cleaved by endothelin-converting enzyme (ECE) to yield endothelin, the active form of the peptide (Yanagisawa *et al.* 1988). The ET is then broken down by proteases (possibly neutral endopeptidase EC 24.11) (Sokolovaky *et al.* 1990, Vijayaraghavan *et al.* 1990) into an inactive form. About 70% of ET-1 secretion from endothelial cells is toward the abluminal side of the cell (Yoshimoto *et al.* 1991).

1.3.1.2. Regulation of ET release

The main stimuli for ET release include thrombin, angiotensin II, arginine vasopressin, noradrenaline, phorbol ester, glucose, tumour necrosis factor- α , interleukin I β , growth factors, insulin, LDL, HDL, and hypoxia (Emori *et al.* 1993; Sung *et al.* 1994, Kohno *et al.* 1992, Yoshimoto *et al.* 1991, Ohta *et al.* 1990, Resink *et al.* 1990, Tamirisa *et al.* 1995, Levin 1995). Changes in shear stress of the vascular wall has been shown to regulate expression of the ET-1 and preproendothelin-1 gene (Yanagisawa *et al.* 1988, Ziegler *et al.* 1998). Mechanical stretch also enhanced ET-1 synthesis/release *in vitro* (Ziegler *et al.* 1998, Yamazaki *et al.* 1996, Hishikawa *et al.* 1995). These stimuli can induce the transcription of ET-1 mRNA and the synthesis and secretion of ET-1 within minutes (Levin 1995).

Nitric oxide (NO) donors: such as organic nitrates, sodium nitroprusside, S-nitroso-N-acetoxy penicillamine (SNAP) and acetoxime; superoxide dismutase (SOD); cGMP; bradykinin; and NPs have been reported to inhibit ET release (Ryan *et al.* 1993, Saijonmaa *et al.* 1990, Monose *et al.* 1993, Boulanger & Luscher 1990). In contrast, NO synthase inhibitors, such as NG-monomethyl-L-arginine (L-NMMA) and methylene blue, potentiated thrombin- and A23187-stimulated ET-1 release (Boulanger & Luscher

1990). Heparin also inhibited both basal and thrombin-stimulated ET-1 release while enhancing NO formation (Ryan *et al.* 1993, Yokokawa *et al.* 1993a,b). This heparin-induced inhibition of ET-1 release was abolished by NG-monomethyl-L-arginine (L-NMMA), a NO synthase inhibitor, indicative of a NO-mediated effect (Yokokawa *et al.* 1993b). Co-culture of endothelial cells with smooth muscle cells and skin fibroblasts significantly inhibited the accumulation of ET-1 release from endothelial cells (Stewart *et al.* 1990, Cade *et al.* 1991).

Although ET secretion is mainly abluminal, plasma ET-1 measurements are nevertheless potentially useful, because plasma ET-1 concentrations have been found to correlate well with the severity of some diseases, such as congestive heart failure (Kiowski *et al.* 1995). Increased plasma ET concentrations are also found in patients with unstable angina, myocardial infarction and in some reports, immediately following percutaneous transluminal coronary angioplasty (PTCA) (Wieczorek *et al.* 1994; Malatino *et al.* 1993, Ray *et al.* 1992), and may have prognostic implications in heart failure.

1.3.2. Endothelin receptors

1.3.2.1. Characterization of ET receptors

Two types of receptors, ET_A and ET_B, have been identified and cloned from mammalian cells (Rubanyi *et al.* 1994). A third receptor subtype, named ET_C receptor (ET-3 selective), has been cloned from the frog *Xenopus laevis*. To date, no evidence of ET_C has been presented for mammalian tissues (Karne *et al.* 1993). The ET_A receptor has greater affinity for ET-1 than ET-2 and ET-3 whereas the ET_B receptor is nonisopeptide-selective. ET_A receptors have been found predominantly in the heart, blood vessels of the brain and vascular smooth muscle, whereas the ET_B receptors are more widely distributed

and predominate in the kidney, uterus, central nervous system and endothelial cells (Tamirisa *et al.* 1995).

ET_A receptor stimulation has generally been found to induce vasoconstriction. The ET_B receptor subtype located on smooth muscle (tentatively termed ET_{B1}) also mediates vasoconstriction. It appears to be functionally distinct from the ET_B subtype located on the vascular endothelium (tentatively termed ET_{B2}) which mediates vasorelaxation via the release of NO (Davenport & Maguire 1994, Pernow & Modin 1993) and prostacyclin (Botting & Vane 1990). Thus ET_B receptor stimulation mediates both vasodilator and vasoconstrictor effects of ET (Clozel *et al.* 1992). ET_B receptors also play a role in clearance of ET from the circulation by endothelial cells (Fukuroda *et al.* 1994). Recently developed gene manipulation methods demonstrated these two pharmacologically predicted ET_B receptor subtypes are derived from the same gene (Mizuguchi *et al.* 1997).

Both ET_A and ET_B receptors have been described to be coupled to phosphatidylinositol (4,5)-bisphosphate hydrolysis via G protein-coupled phospholipase C, and to generation of inositol phosphates and diacylglycerol, resulting in an increased concentration of intracellular Ca²⁺ (Ohnishi *et al.* 1989, Takuwa *et al.* 1989, Chan & Greenberg 1991).

1.3.2.2. Regulation of ET receptors

ET receptors, like many other peptide cell surface receptors, are subject to ligand-induced down-regulation. In vascular smooth muscle cells, ET-1 pre-treatment caused a substantial decrease in ¹²⁵I-ET-1-binding sites as well as ET-1 -induced increase in intracellular calcium (Hirata *et al.* 1988). This homologous down-regulation of ET-1-binding sites in smooth muscle occurred within 30 min of ET-1 exposure and persisted for up to 18 hours (Roubert *et al.* 1990, Miasiro & Paiva 1990). Because the ET receptor

mRNA exhibited a half-life of at least several hours, the ligand-induced downregulation of the message might be due to increased mRNA degradation (Sakurai *et al.* 1992). The sensitivity of cell surface ET receptors to ligand-induced down-regulation may have therapeutic implications. Phorbol ester, a PKC activator, led to a rapid and sustained 50% decrease in ET-1-binding site (Rasink *et al.* 1990). However, phosphoramidon, an ECE inhibitor, decreased ET release but increased receptor binding of exogenous ET in mesangial cells (Clozel *et al.* 1993). Ischaemia also induced externalization of ET-1 binding sites (Liu *et al.* 1989, Nayler *et al.* 1992).

1.3.3. Physiological actions of Endothelins

1.3.3.1. Vasoactive effects

ET-1 is a potent and usually long acting vasoconstrictor (Clark *et al.* 1989, Ezra *et al.* 1989, Dohi & Luscher 1991, Vierhapper *et al.* 1990, Yang *et al.* 1990, Takeshita *et al.* 1991, McGowan *et al.* 1995). The ET-induced vasoconstrictor effects are mediated by both ET_A and ET_B receptors (Fig 1.5.), since ET-3 and sarafotoxin S6c also cause vasoconstriction (Haynes *et al.* 1995, Thompson *et al.* 1995). Selective ET_A or nonselective ET receptor antagonists and ECE inhibitors have been shown to induce vasodilation (Haynes & Webb 1994, Kiowski *et al.* 1995) (See section 1.3.5). In isolated perfused rat heart and anaesthetized rats, bolus injection of endothelin-1 results in biphasic effects on coronary flow: the initial vasodilation followed by a prolonged vasoconstriction (Neubauer *et al.* 1990, Wright & Fozard 1988). As ET is mainly released abluminally, it may act as a paracrine regulator for basal vascular tone (Haynes & Webb 1994, Kiowski *et al.* 1995).

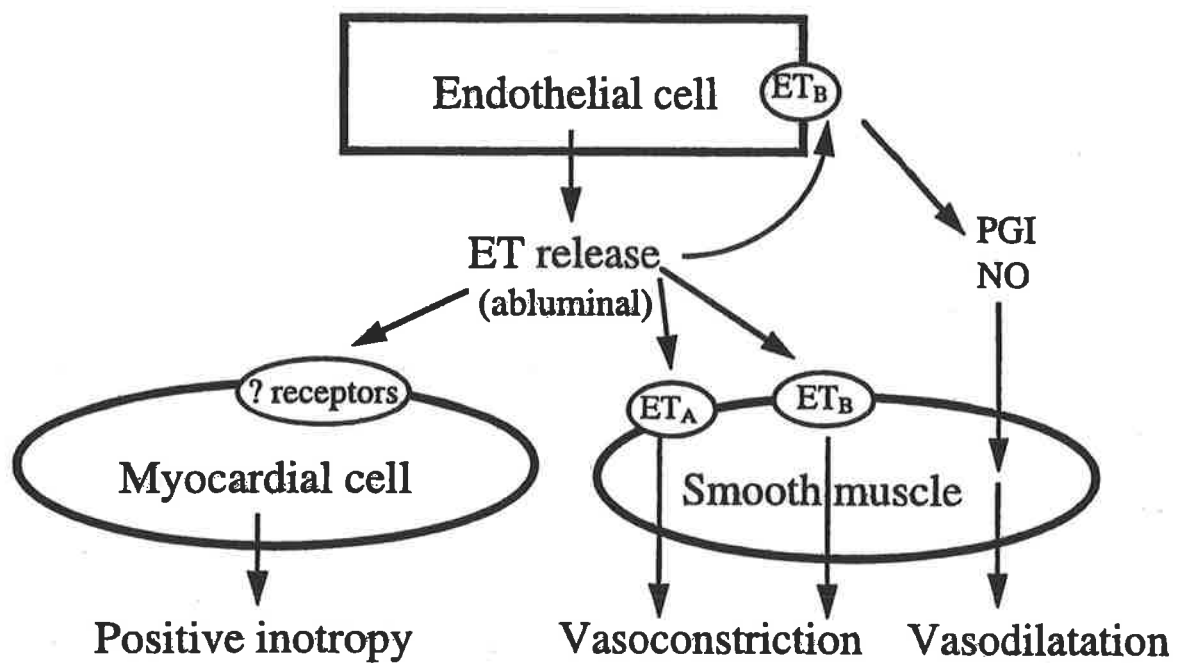


Fig 1.5. Schematic diagram of physiologic actions of endothelin, predicated upon results of experimental work described in this chapter.

1.3.3.2. Inotropic effects

Exogenous endothelin-1 (ET-1) has also been shown to exert a positive inotropic effect on isolated cardiac myocytes and papillary muscle (Kelly *et al* 1990, McClellan *et al* 1996, Evans *et al* 1994, Kasai *et al* 1994, Endoh *et al* 1996). However, *in vivo* studies can not demonstrate the positive inotropic effect of exogenous ET-1, almost certainly because of concomitant induction of myocardial ischaemia via coronary vasoconstriction (Beyer *et al* 1995, Domenech *et al* 1991, Gulati *et al* 1996, Cirino *et al* 1994 & 1997, Donckier *et al* 1994). The development of ET receptor antagonists also offers a means of obtaining inferential information concerning the inotropic effects of ET *in vivo*. Studies with intravenous administration of bosentan, an ET_{A/B} receptor antagonist, in patients with severe chronic heart failure have demonstrated both an increase in cardiac output and a decrease in peripheral vascular resistance (Kiowski *et al* 1995, Sutsch *et al* 1996, Spinal *et al* 1997, Shimoyama *et al* 1996).

1.3.3.3. Other cardiac effects of ET

Studies *in vitro* have shown ET to have a positive chronotropic effect (Ishikawa *et al.* 1988) as well as prolongation of the action potential duration *in vitro* (Watanabe *et al.* 1989a,b). However, studies *in vivo* and in isolated rabbit atria showed ET-1 causes bradycardia that may be secondary to the ET-1 induced ischaemia (Karwatowsk Prokopczuk & Wennmalm 1990, Zhu *et al.* 1997). Studies with cultured myocytes suggest ETs may be involved in cardiac hypertrophy (Suzuki *et al.* 1990, Bogoyevitch *et al.* 1994, Yamazaki *et al.* 1996, Harada *et al* 1998), indicating ET may act as a growth factor.

1.3.4. Effect of myocardial ischaemia on release of ET

Increased plasma ET concentrations may occur in patients with unstable angina, myocardial infarction and immediately following PTCA (Malatino *et al.* 1993, Wieczorek *et al.* 1994, Kyriakides *et al.* 1995). Endogenous ET release into the coronary perfusate of isolated perfused rat hearts was been shown to increase during reperfusion but decrease during severe coronary flow reduction (Brunner *et al.* 1992, Brunner 1995). Ischaemia-induced externalization of ET-1 binding sites (Liu *et al.* 1989, Nayler *et al.* 1992) may result in incremental ET-1 binding to the receptors rather than release into the perfusate. Furthermore, hypoxia increased the maximal responses of ET-1 and ET-3 in the rat isolated perfused mesenteric arterial bed (Douglas *et al.* 1991). However, the relationship between net ET-1 efflux rate and extent of ischaemia has not yet been investigated.

1.3.5. Available techniques for manipulating endothelins

Current methodologies investigating the putative pathophysiological role of endogenous ET includes the use of endothelin converting enzyme (ECE) inhibitor, anti-endothelin antibodies, endothelin receptor antagonists and ET or ET receptor gene deletion. For regulation of ET synthesis/release and ET receptors, see section 1.3.1.2. and 1.3.2.2.

1.3.5.1. Endothelin converting enzyme inhibitor

ECE inhibitors, such as phosphoramidon, thiophan or CGS 26303, abolished big ET-induced vasoconstriction *in vivo* and *in vitro* (Hoffman, *et al.* 1994, McMahon *et al.* 1991, Warner *et al.* 1996), and cause a direct vasodilation (Haynes & Webb 1994). However, these inhibitors seem to lack specificity due to ECE's structural homology to NEP; most of the so-called selective inhibitors of ECE also inhibit other metalloproteases

such as NEP (Wallace *et al.* 1998, Pelletier *et al.* 1998, Maguire *et al.* 1997). Furthermore, endopeptidase 24.11 (also called NEP, or EC 3.4.24.11) can both form and break down ETs; and also break down NPs (Murphy *et al.* 1994, Sokolovsky *et al.* 1990, Vijayaraghavan *et al.* 1990, Gerbes & Vollmar 1990). Recently, an inhibitor, PD 069185 which appears selective for ECE-1 and has also been shown to have little effect on other metalloproteases, and therefore may have therapeutic potential (Ahn *et al.* 1988).

1.3.5.2. Antibody against ET

Antibody against ET antagonized the ET-1-induced vasoconstriction and reduces infarct size (Klemm, *et al.* 1995, Kusumoto *et al.* 1993).

1.3.5.3. Endothelin receptor antagonists

To date, there are many specific ET receptor antagonists available for investigating the physiology of ETs as well as a potential treatment for disease (Fig 1.6.). In cultured human umbilical vein smooth-muscle cells, FR139317, an ET_A receptor antagonist, caused an immediate, transient but marked increase in ET levels, with a decrease in levels of big ET-1 in the supernatant (Yu & Davenport 1995). BQ-123, another specific ET_A receptor antagonist, has been shown to inhibit ET-induced vasoconstriction in porcine coronary artery (Pernow & Modin 1993), and to attenuate the hypertensive response to ET-1 and big ET in rats (McMurdo *et al.* 1993). Despite findings that BQ-123 reduces infarct size and inhibits anoxia-induced coronary vasoconstriction in canine models (Grover *et al.* 1993, Vedernikov *et al.* 1993) specific investigations of the interaction between endogenous ET and ET_A receptor antagonists on vasomotor tone are relatively few. One investigation in isolated isovolumetric rat hearts by Han *et al.* (1995) failed to demonstrate any effects of BQ-123 (estimated at 1 μ M) on coronary vasomotor tone. Furthermore, BQ-123 failed to inhibit ET-induced coronary vasoconstriction in a

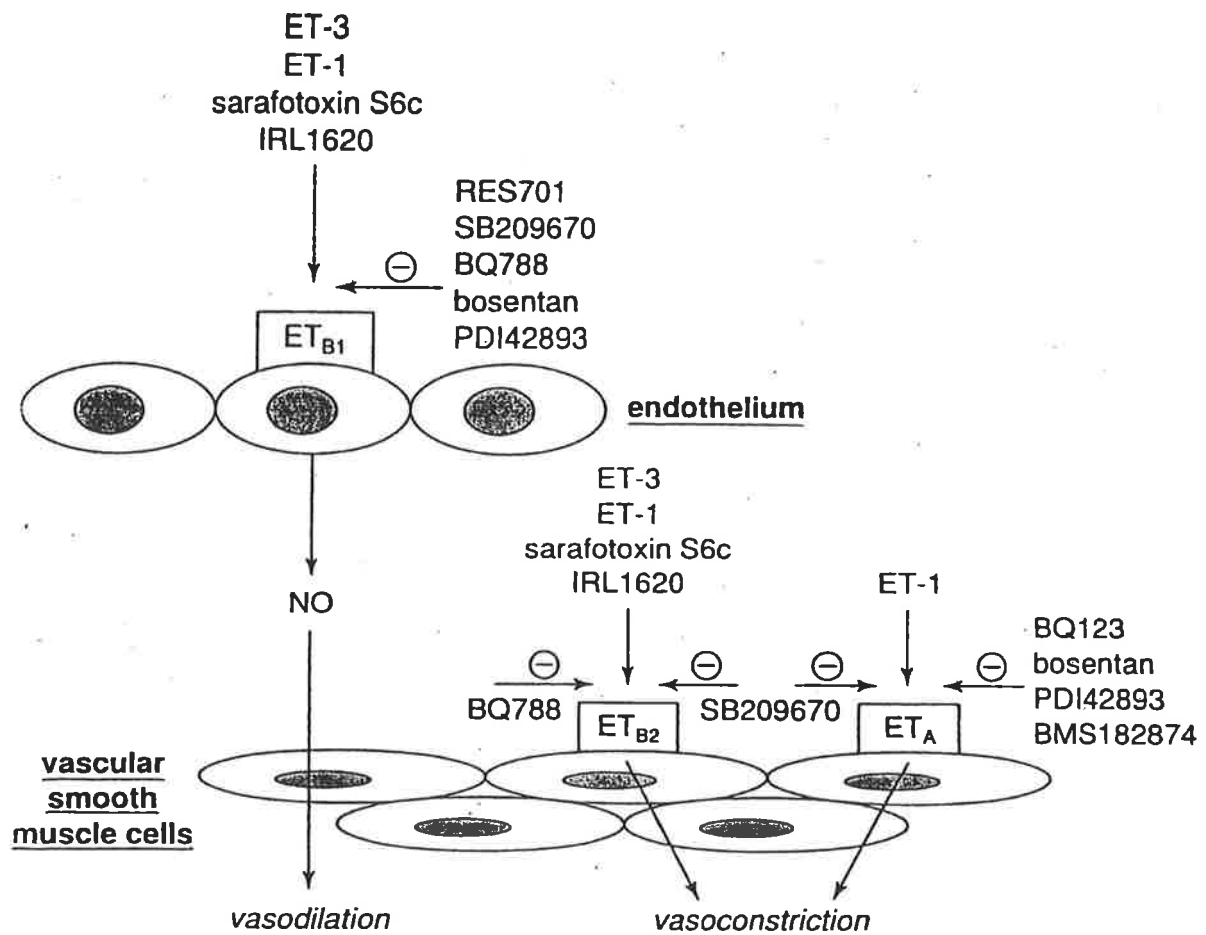


Fig 1.6. The anatomical location of putative subclasses of endothelin receptor subtypes (Douglas *et al.* 1994), including available antagonists.

Langendorff perfused rat heart model (Balwierczak 1993). Neither the recovery of coronary flow after reperfusion, nor the effects of BQ-123 on ET-1 efflux from the heart was previously investigated.

Seo *et al.*, using bosentan (Ro 47-0203), an ET_A/ET_B receptor antagonist, demonstrated that both ET_A and ET_B receptors mediate ET-1 contraction of isolated human blood vessels (Seo *et al* 1994). Dagassan *et al.* (1994) demonstrated a coronary vasodilator effect (coronary flow increased by 14%) of bosentan (10⁻⁵ M) in non-ischaemic rat heart, which did not persist during an immediate post-ischaemic phase. However, to date there are only limited data on the effect of ET_A and ET_{A/B} antagonists on the relationship between endothelin production and coronary vasomotor tone during ischaemia and reperfusion.

There is considerable information showing that ET_B receptors mediate ET clearance from the circulation, especially into pulmonary endothelium (Fukuroda *et al* 1994, Kiowski *et al* 1996, Ozaki *et al* 1995, Dupuis & Gorasky 1996, Sato *et al* 1995, Weber *et al* 1996, Brunner & Doherty 1996). Studies of clearance of ET-1 from plasma into pulmonary tissue during acute ET-1 administration in intact dogs (Dupuis & Gorasky 1996), rats (Fukuroda *et al* 1994) and also isolated perfused rat lungs (Sato *et al* 1995) demonstrate specific inhibition of the clearance process by the selective ET_B receptor antagonist, BQ-788. These findings are also consistent with elevation of plasma ET-1 concentrations induced by the ET_{A/B} antagonist, bosentan (Kiowski *et al* 1996, Weber *et al* 1996). As regards possible specific effects of ET_B receptor stimulation on ET-1 kinetics within the heart, Brunner and Doherty have demonstrated that BQ-788, but not the ET_A antagonist PD 155080, increased ET-1 release for the perfused rat heart (Brunner & Doherty 1996).

1.3.5.4. Gene manipulation

Application of gene “knockout” techniques to endothelin research has led to the definitive of roles for the ET isopeptides and their receptors. Homozygous mice with null ET-1 and ET_A receptors present have craniofacial deformities and defects in the cardiovascular outflow tract, indicating ET_A receptors are essential for the development of the first brachial arch derived from neural crest (Clouthier *et al.* 1998, Hirata 1996). Homozygous mice with null ET-3 and ET_B receptors presented with megacolon (Hirschsprung’s disease) associated with spotted colour coats, indicating that ET_B receptors are essential for the development of neural crest-derived cell lineage, enteric neuron and epidermal melanocytes (Hirata 1996). Furthermore, in normal mice, ET_B agonists, sarafotoxin S6c and IRL1620 caused vasodilation in aorta precontracted with phenylephrine and vasoconstriction in gastric fundus; these effects were abolished in tissues from ET_B receptor gene “knockout” mice (Mizuguchi *et al.* 1998). However, genetic manipulation techniques have not been utilized to date to examine interactions of ET with ischaemic processes.

1.4. Interactions between Natriuretic Peptides and Endothelins

The effects of ANP and ET are mutually antagonistic in many circumstances (Ota *et al.* 1992; Bolger *et al.* 1990). This physiological antagonism extends beyond direct interaction between vasoconstrictor (particularly ET-1) and vasodilator (ANP).

1.4.1. Receptor based interactions

Under normoxic condition, ANP secretion enhanced by exogenous ET was inhibited by BQ-123 (10^{-6} to 10^{-5} M) (Thibault *et al.* 1994, Skvorak *et al.* 1995, Irons *et al.* 1993, Leite *et al.* 1994), but not affected by ET_B agonists (Thibault *et al.* 1994) suggesting that ET_A receptors may mediate the ET-induced ANP release. Furthermore, BQ-123 and bosentan inhibited volume-loading-induced ANP release indicating that endogenous ET plays a role in stretch-induced ANP and BNP synthesis/release (Leskinen *et al.* 1997, Magga, *et al.* 1997). ET-1 has also been shown to inhibit ANP-induced formation of cGMP (Marala *et al.* 1993). However, the role of endogenous ET on ischaemia-induced ANP release has not been determined.

Conversely, NPs and nitro-vasodilators inhibited both basal and thrombin- or angiotensin II stimulated ET-1 release from endothelial cells (Saijonmaa *et al.* 1990, Emori *et al.* 1993; Kohno *et al.* 1992). This effect can be mimicked by 8-bromo-3',5'-cyclic guanosine monophosphate, indicating cGMP mediates the ANP- and nitro-vasodilator-induced suppression of ET-1 release.

1.4.2. Physiological antagonism

ANP-increased coronary blood flow has been reported (Ertl & Bauer 1990, Chu & Cobb 1987, Chu *et al.* 1990, Nakagawa *et al.* 1988, Serizawa *et al.* 1988, Chu *et al.* 1989a). The time course of proximal coronary dilation was related more closely to increased plasma cGMP than ANP concentrations (Chu *et al.* 1989a). Natriuretic peptides also modulate its constrictor effect on aortic ring preparations (Bolger *et al.* 1991). It would therefore be interesting to know whether natriuretic peptides affect ischaemia via limiting ET synthesis and effect.

Exogenous ET-1 (10^{-9} to 10^{-6} M) augmented basal and stretch-induced ANP (and BNP) secretion in rat myocytes (Thibault *et al.* 1994, Horio *et al.* 1993, Leite *et al.* 1994, Irons *et al.* 1993, McDonough *et al.* 1994, Bruneau *et al.* 1997) and rat atria (Skvorak *et al.* 1995, Hu *et al.* 1988). In intact animals, ET-1 infusion increased ANP release as well as arterial pressure (Cheung 1994). In a constant-flow perfusion rat heart model (with intact atrium), where ET-1-induced vasoconstriction did not change the coronary flow rate, ET-1 (2.3×10^{-9} M) perfusion increased ANP release (Mantymaa *et al.* 1990). In the studies presented in this thesis, ET-1 (5×10^{-10} M) perfusion did not induce a significant increase in ANP release rate, although it did cause an immediate decrease in perfusion flow rate. However, there was a significant increase of ANP release post ET-1 perfusion. A higher concentration of ET-1 (10^{-9} M) was tested in a preliminary study but found to cause profound arrhythmia and cessation of coronary flow.

1.5. Effects of anti-ischaemic therapy on release of natriuretic peptides and endothelins

Since natriuretic peptides have potential effects on myocardial ischaemia, it is very important to know the interactions between natriuretic peptides and the drugs which may also affect ischaemia. Nifedipine and felodipine increased plasma ANP levels in rat and humans with hypertension (Knorr *et al.* 1988, Rappelli *et al.* 1987, Iwasaki *et al.* 1989, Cerasola *et al.* 1988). However, in patients with CAD, verapamil abolished the effect of atrial pacing on ANP release (Haufe *et al.* 1988). Other studies confirmed that nifedipine and verapamil inhibited ANP secretion *in vitro* and *in vivo* (Iida & page 1989, Weinmann 1991). β -blockade increased plasma ANP concentrations, secondary to an increase of cardiac chamber volumes (Nakaoka *et al.* 1987, Thamsborg *et al.* 1987, Bouissou *et al.* 1989). Streptokinase therapy was associated with diminished MI-induced ANP release in humans (Phillips *et al.* 1989). Acute nitrate administration suppressed plasma ANP levels in patients with CAD and CHF (Kinoshita *et al.* 1989, Shotan *et al.* 1993). Chronic nitrate administration may induce a variety of neurohumoral changes leading to fluid retention, increased secretion of ANP and increased vasomotor tone (Parker *et al.* 1991).

The influence of other drugs on ANP levels can be attributed to their haemodynamic effects. Noradrenaline and angiotensin induce a pressor effect and hence increase ANP levels (Uehlinger *et al.* 1987). Noradrenaline may also directly stimulate ANP secretion (Wong *et al.* 1988). Angiotensin converting enzyme (ACE) inhibitors decrease plasma ANP concentrations, consistent with decreased cardiac filling pressures (Kettunen *et al.* 1994; Omland *et al.* 1993; Tanabe *et al.* 1996; Huttl *et al.* 1995).

The interaction of diltiazem, verapamil, nitro-vasodilators and heparin with ET-1 release has also been discussed (see section 1.3.1.2). Diltiazem and verapamil inhibit endothelin release from cultured human coronary smooth muscle cells and endothelial cells (Haug *et al.* 1998). Organic nitrates reduce basal and stimulated ET-1 gene expression in cultured endothelial cells (Ryan *et al.* 1993, Saijonmaa *et al.* 1990). Heparin also inhibited both basal and thrombin-stimulated ET-1 release while enhancing NO formation (Ryan *et al.* 1993, Yokokawa *et al.* 1993a,b). This heparin-induced inhibition of ET-1 release was abolished by NG-monomethyl-L-arginine (L-NMMA), a NO synthase inhibitor, indicating NO-mediated effect (Yokokawa *et al.* 1993b). The effects of these drugs on ET may contribute to their anti-ischaemic effect.

1.6. Plan of Research

1.6.1. Hypotheses to be tested

- 1). Ischaemia-induced ANP release may be attenuated by ischaemia-induced bradycardia.
- 2). ET-1 may modulate ischaemia/hypoxia-induced ANP release.
- 3). ET-1 regulates local vasomotion and myocardial contraction during ischaemia or hypoxia.
- 4). PTCA induced ischaemia stimulates ANP and ET-1 release in man.
- 5). Acute nitrate therapy may suppress ANP and ET-1 release in patients with coronary artery disease not associated with CHF.

1.6.2. Experimental design:

1.6.2.1. In vitro studies

Langendorff perfused isolated rat hearts: Male Porton rats will be anaesthetised with an intraperitoneal injection of sodium pentobarbitone and hearts removed via thoracotomy. Isolated rat hearts will be perfused by a modified non-recirculating Langendorff procedure at a constant pressure (70 cm H₂O) with modified Eagle's medium (Chen et al. 1993). The medium will be continually gassed with carbogen (95% O₂, 5% CO₂). Global ischaemia will be induced by decreasing perfusion flow rate from 13±2 ml/min to 4±1 ml/min for 30 minutes followed by 30 minutes reperfusion. Hypoxia will be induced by perfusing hearts with perfusate gassed with a special mixture of gas (5% O₂, 90% N₂ and 5% CO₂) for 30 minutes followed by 30 minutes re-oxygenation.

1.6.2.1.1. Experiments to test hypothesis 1

In order to examine the role of ischaemia-induced bradycardia on ANP release during global ischaemia in this preparation, the following groups of perfused hearts will be compared (n=6-8 for each group):

- (a) Control (normal perfusion without ischaemia) non-paced hearts
- (b) Control hearts with right atrial pacing to keep the heart rate constant.
- (c) Globally ischaemic (low perfusion flow rate) hearts or hypoxic (low O₂ with normal perfusion flow rate) hearts.
- (d) Globally ischaemic or hypoxic hearts with right atrial pacing.

1.6.2.1.2. Experiments to test hypothesis 2 and 3

In order to examine the putative role of ET-1 in modulating ANP release in this preparation, the following groups of Langendorff perfused hearts will be compared (n=6-8 for each group):

- (a) Control hearts (refer to 1.6.2.1.1.b)
- (b) Globally ischaemic or hypoxic hearts (refer to 1.6.2.1.1.d).
- (c) Control with the ET_A-receptor antagonist, BQ-123.
- (d) Globally ischaemic or hypoxic hearts with BQ-123 in perfusate.
- (e) Control with the ET_A/ET_B-receptor antagonist, bosentan.
- (f) Globally ischaemic or hypoxic hearts with bosentan in perfusate.

The effluent of perfusate will be collected for the assay of ANP, BNP and ET-1 during baseline, ischaemia and reperfusion; or hypoxia and re-oxygenation. The force of contraction, negative dF/dt , coronary flow rate and heart rate will be monitored. ANP, BNP and ET-1 will be quantified using radioimmunoassay methods.

1.6.2.2. In vivo studies

1.6.2.2.1. PTCA-induced ischaemia in human heart (to test hypothesis 4)

Patients (n=30) undergoing PTCA and right heart catheterization will be enrolled. Blood samples will be taken from femoral artery during baseline at the end of the first balloon inflation (occlusion of the coronary artery) and 1 minute after balloon deflation (reperfusion) to measure ANP, BNP and ET-1 concentrations, results will be correlated with the extent of ischaemic change on continuous ECG and patients' symptom (angina).

1.6.2.2.2. Stable angina patients: (to test hypothesis 5)

Patients (n=30 for each group) undergoing diagnostic coronary angiograms will be enrolled in this study. Blood samples will be taken from aorta, pulmonary artery, femoral artery and vein for measurement of ANP, BNP, ET-1. Cardiac output, left ventricular ejection fraction (LVEF), pulmonary capillary wedge pressure (PCWP), arterial pressure and heart rate will be monitored.

Group-1:

Patients who are not on nitrate therapy before angiogram will receive acute intravenous (iv) infusion of glyceryl trinitrate (GTN) 10 μ g/min for 10 min. Blood sampling and haemodynamic measurement will be performed during baseline and at the end of the 10 minutes of GTN administration.

Group-2:

Blood sampling and haemodynamic measurement will be performed prior and at the end of the acute GTN administration in patients receiving chronic administration of nitrates.

CHAPTER 2

MATERIALS AND METHODS

2.1. ANIMAL STUDIES.

2.1.1. MATERIALS AND ANIMALS

The male Porton rats used in all animal studies detailed in this thesis were obtained from the Institute of Medical and Veterinary Science (Adelaide, Australia). Animals were housed in an air-conditioned room maintained at 22°C, on a 12 hour light-12 hour dark cycle with food and tap water available ad libitum. The details of weight ranges of rats and administered substances are described in the relevant methods section of each chapter corresponding to their utilisation. All experimental procedures performed on animals were approved by the Institutional Animal Ethics Committee in accordance with the guidelines set by the National Health and Medical Research Council (NH&MRC) of Australia. Experiments were performed at laboratory temperature (22-25°C). Materials and chemical substances used were of the highest possible purity and quality.

2.1.2. THE ISOLATED PERFUSED RAT HEART PREPARATION

The isolated coronary-perfused whole rat heart preparation used in the following studies is an adaptation of the original Langendorff retrograde perfusion heart model (Langendorff, 1895). The heart is isolated without neural, hormonal or any other functional inputs; and studies with this preparation rely primarily on the unique myotonic nature of individual cardiocytes.

2.1.2.1. “Langendorff” Retrograde Perfusion

The modified Langendorff perfusion set-up was configured as previously described (Rayner 1992, Chen 1994). The ascending aorta was cannulated, and the hearts perfused with an oxygenated solution (perfusate), without cannulation of the atrium and in the absence of pulmonary or systemic circulation. The coronary circulation was maintained under constant pressure via retrograde flow through the aortic cannula (Fig 2.1). Coronary effluent from coronary sinus and other coronary veins passed through the right atrium and left the heart via superior and inferior venae cavae or through the right ventricle and pulmonary artery. Since the venae cavae, pulmonary arteries and veins were open in this model, the intra-atrial pressure was virtually zero.

In the original Langendorff model, the aortic valves remain intact. Thus the left atrial and ventricular chambers were not perfused. Although they still contract rhythmically, there is no left ventricular output. However, the adaptation utilised for the studies involved rupturing the aortic valve with the cannula, and maintaining a constant perfusion pressure. Thus, in our paradigm, the left ventricle, as well as coronary arteries, was perfused under a diastolic pressure of 70 cm H₂O which subsequently became the left ventricular after-load during systole. The left ventricular perfusate was then ejected back into the aorta and through the coronary arteries. The aim of this modification is to approximate to the “working heart” preparation, without compromising the closed nature of the Langendorff coronary perfusion system.

2.1.2.2. Perfusion Procedure

2.1.2.2.1. Perfusion Apparatus

A diagram of the apparatus used for these studies is shown in Fig. 2.2. The stock of

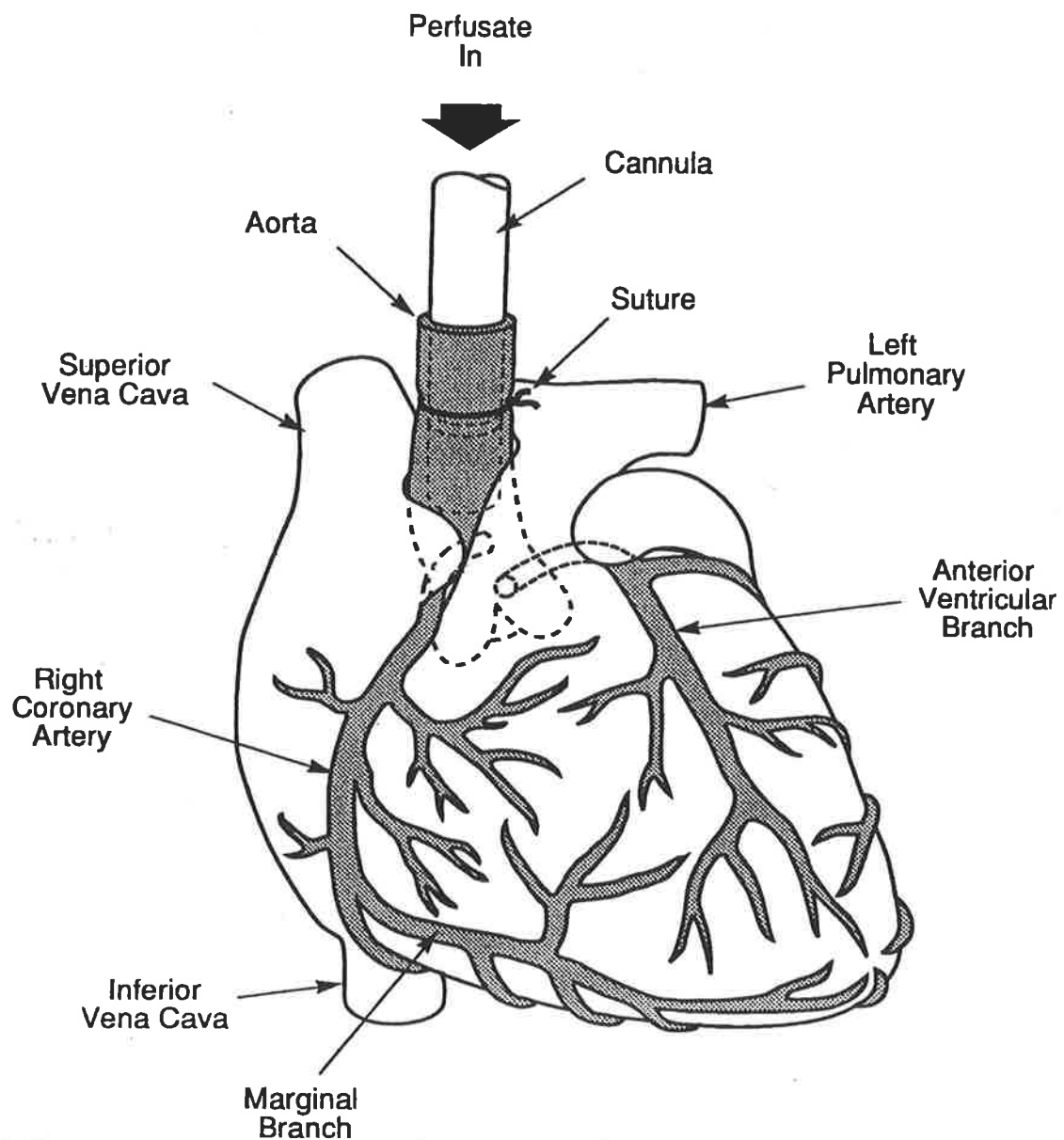


Fig 2.1. Diagram of the modified Langendorff coronary-perfused heart model.

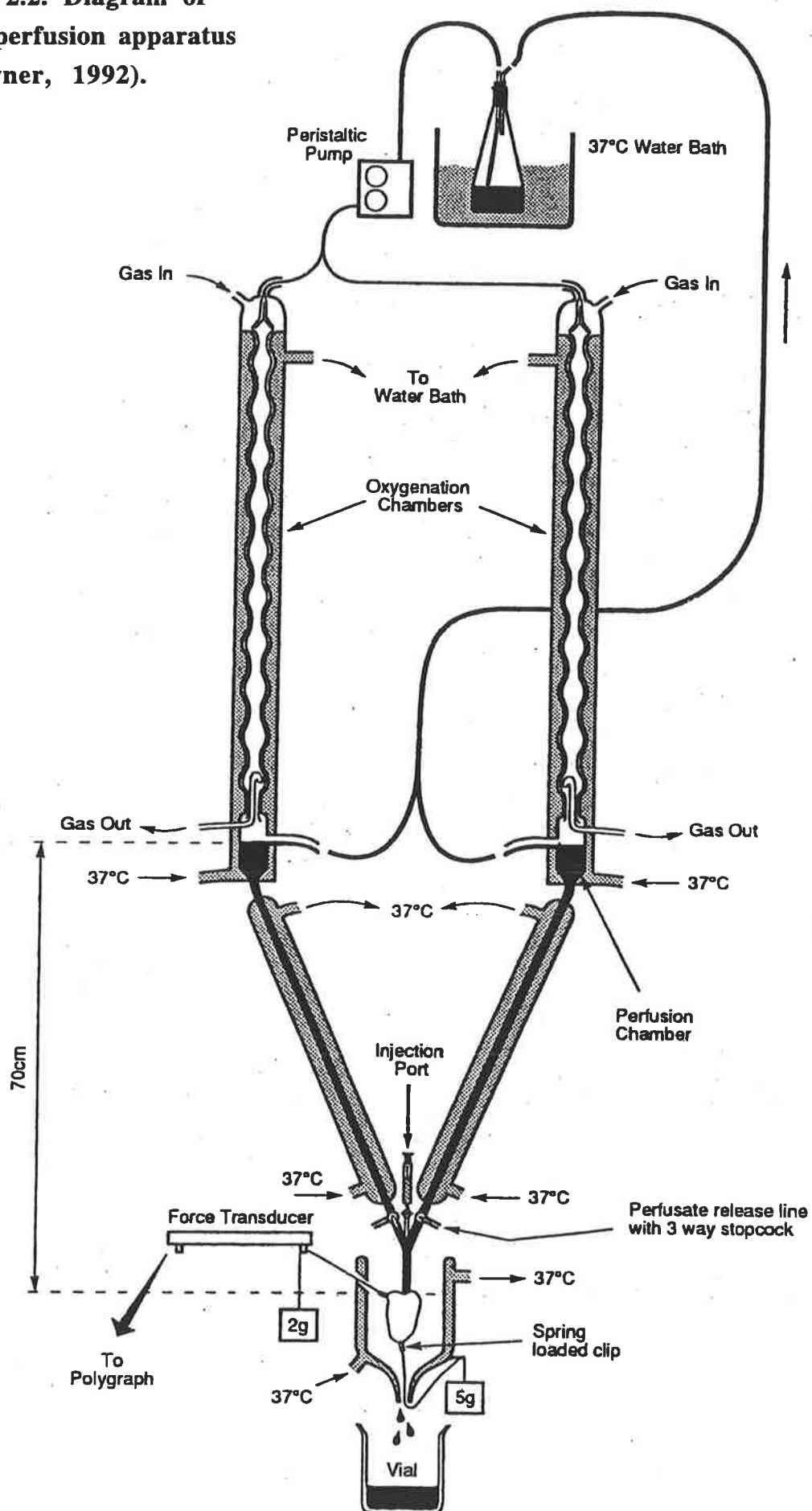
perfusate was maintained at 37°C by a water bath and gassed with carbogen (95% O₂; 5% CO₂) or a special gas mixture for hypoxia (see below). The perfusate was pumped to the top of the oxygenation chamber(s) by a rotary pump at a constant flow rate of approximately 0.5 L/min (Rayner, 1992). A flange then directed the buffer over the inner surface of the oxygenation chamber to increase the surface area available for absorption of O₂ and CO₂ from carbogen to maintain oxygen saturation and pH. A special gas mixture comprising 5% O₂; 5% CO₂; 90% N₂ was utilised for hypoxic experiments.

At the base of the oxygenation chamber was a reservoir, positioned 70 cm above the heart, connected to a delivery tube. The reservoir had a total capacity of 20 ml and was connected to the heart via a delivery tube, any excess perfusate escaped via an overflow outlet and was returned to the stock perfusate. This ensured the level within the reservoir remained constant and allowed the perfusate to be delivered to the heart at a constant pressure of 70 cm H₂O. Perfusate temperature was maintained at 37°C by circulating water from the water bath through the jacketed oxygenation chambers, buffer reservoirs and delivery tubes. The isolated heart was also encased in a jacketed incubation chamber designed to allow perfusate collection by way of an open drainage port at its base and experimental manipulations through two 5 mm wide vertical slits extending half way down the chamber. The aortic cannula was located 70 cm below the overflow outlet of the reservoir and joined at a four-way junction. Perfusate movement was controlled by two three-way stopcocks.

2.1.2.2.2. Perfusate Composition

A modified buffer based on Eagles culture media (Minimum Essential Medium; MEM) utilised in these studies was obtained from Cyto-Systems (Sydney, Australia). The medium was composed of modified Eagles MEM with Earles balance salts, L-glutamine

Fig. 2.2. Diagram of the perfusion apparatus (Rayner, 1992).



and non-essential amino acids (Eagles). The detailed composition of this modified MEM is shown in the Appendix.

2.1.2.2.3. Heart Isolation

Male Porton rats weighing 406 ± 9 g ($n=144$) were anaesthetised with an intraperitoneal injection of sodium pentobarbitone 50 mg/kg (Boehringer Ingelheim Pty. Ltd, Australia). The abdominal cavity was opened through a transverse incision with scissors. The diaphragm was transected and a lateral incision made along the right side of the rib cage in the anterior chest wall. The heart was rapidly excised through an incision made by a single cut with scissors through the arch of the aorta approximately 5 mm from the beating heart. The heart was then arrested by placing it into a ice chilled beaker containing 0.9% NaCl. After trimming connective tissue, lung and excess fat, clips attached with lengths of silk ligature were placed on the apex of the heart and the top of the right ventricle. These attachments allowed for the measurement of cardiac force of contraction.

2.1.2.2.4. Initiation of perfusion

Retrograde perfusion through the aorta was begun from the reservoir as soon as the heart was positioned. The aorta was held by forceps and slipped approximately 3 mm onto a grooved stainless steel perfusion cannula (external diameter 3 mm), within 1-2 minutes of excision from the rat. The aortic valve was disrupted by drawing the heart up the cannula until it had penetrated into the left ventricle. The aorta was secured with a ligature tight around the aorta 2 mm above the left ventricle in order not to occlude the origin of coronary arteries. As soon as the aorta was positioned on the cannula, perfusate was introduced to the heart by turning a three-way stopcock. All air bubbles were carefully removed from the buffer delivery system before cannulation in order to prevent myocardial infarction from air embolism.

In order to allow the heart to recover totally from the period of anoxia associated with excision, perfused hearts were allowed to equilibrate for 40 minutes before sampling began. Coronary effluent from the heart was collected, the volume of coronary effluent was measured by calibrated pipette, and 1 ml aliquots of effluent were collected into Eppendorff tubes and snap frozen on dry ice. Samples were stored at -80° C for ANP, BNP and ET-1 measurement.

2.1.2.2.5. Right Atrial Pacing

Hearts were paced by placing electrodes (silver or platinum) against the right atrium in order to minimise rate-related effects on myocardial oxygen demand and coronary vasomotor tone. Pacing was performed at a rate $8.7 \pm 7.6\%$ above the spontaneous heart rate (264 ± 42 beat per min, $n=70$) with square wave pulses of 1 mS at a voltage 100% above threshold. The major purpose of achievement of constant heart rate was to avoid the emergence of contractile changes during ischaemia partially mediated via the Treppe effect (Borzak *et al.* 1991).

2.1.3. EXPERIMENTAL PROTOCOL

All experiments were performed within 2-2.5 hours of set up, a period known to have no significant changes in basal coronary perfusion flow or cardiac activity (Aronson & Serlick, 1976). The experimental protocols were as follows: (1) initiation of perfusion; (2) a 40 min equilibration period; (3) a 20 min sample collection period with basal perfusion; (4) a 30 minute experimental intervention period followed by (5) a 30 minute return to basal perfusion. All samples were collected over a 1 min period

2.1.3.1. Endothelin (ET), ET Receptor Antagonists and Nitric Oxide (NO) Inhibitor

Whenever a fixed concentration of substance (e.g. ET-1, ET receptor antagonists and NO inhibitor) was perfused through the isolated heart, the dual perfusion apparatus described in figure 2.2. was used. One chamber was used for delivering normal medium while the other was reserved for perfusate containing either test substances equilibrated with carbogen or the special gas mixture (for hypoxia groups), or normal medium (for control group). The dual chambers were used to overcome any possible intrinsic differences associated with their assembly, although none were detected during characterization trials (Rayner 1992). When inhibitors or antagonists were added, a 10 min pre-perfusion of these substances was performed before the initiation of ischaemia.

2.1.3.2. Global Ischaemia

Following collection of baseline samples, the test condition of 30 min ischaemia and 30 minutes reperfusion was performed. Hearts were subjected to different levels of global ischaemia by decreasing perfusion flow rate from that of basal flow rate established for each individual heart via a three-way stopcock. The final phase of the experimental protocol involved reperfusion, by returning to normal perfusion pressure and flow rate for 30 min.

2.1.3.3. Hypoxia

At the end of baseline sample collection, 30 min of hypoxia followed by 30 min of re-oxygenation was performed. Hypoxia was induced in hearts by rapid switching to the second chamber with perfusate buffer gassed with 5% O₂, 5% CO₂ and 90% N₂ for 30 min. The re-oxygenation was introduced by perfusing normal medium gassed with carbogen.

2.1.4. CARDIAC PARAMETERS

The volume of coronary perfusion flow was measured by a calibrated pipette and perfusion flow rate was calculated as mls/min/g wet weight heart. In some of isolated rat heart experiments, a flow probe was used above the cannula to measure the coronary flow. However, this flow rate measurement by the flow probe interfered with atrial pacing. Thus all the perfusion flow measurements were therefore done by volumetric measurement.

The heart rate and developed tension were monitored by a force displacement transducer via the clip(s) attached to the heart and recorded by a polygraph chart recorder (Model 7D, Grass Instrument Co. Quincy, Mass, USA) or MacLab (model 4e, ADInstruments, Australia). Calibration of recording apparatus was performed prior to commencing each experiment.

2.1.5. ATRIAL NATRIURETIC PEPTIDE (ANP) RADIOIMMUNOASSAY

ANP from coronary effluent was quantified by radioimmunoassay (RIA).

2.1.5.1. Reagents

2.1.5.1.1. Assay buffer

NaFam buffer (sodium phosphate 0.04 M; BSA, 0.25 %; EDTA 10 mM and trasylol, 500 IU/ml) adjusted to pH 7.4. The final salt concentrations were (mM): Na_2HPO_4 , 28; NaH_2PO_4 , 7.6; NaCl, 102.5 and thimerosal, 0.006 (as a preservative).

2.1.5.1.2. ANP Standard

Rat α ANP₉₉₋₁₂₆ from Auspep, Australia.

2.1.5.1.3. Antibody

ANP antibody was raised in our laboratory by immunizing rabbits with rat α ANP₉₉₋₁₂₆ conjugated to bovine thyroglobulin (Sigma Chemical, USA). The antibody is directed to the mid portion of α ANP. The rabbit antiserum was diluted in assay buffer to give a final dilution in the incubation mixture of 1:30,000 in the assay tube.

2.1.5.1.4. Tracer

¹²⁵I-ANP₉₉₋₁₂₆ was prepared in our own laboratory using the iodogen method modified by Oliver *et al.* (1988). Rat ANP₉₉₋₁₂₆ 10 μ g was pipetted into a 1.5 ml Eppendorf tube coated with 40 μ g of Iodogen (Pierce, Chemical Co. USA), and mixed with 20 μ l sodium phosphate buffer (0.5 M, pH 7.4) and 1 mCi NaI¹²⁵ (Radiochemical Centre, NSW, Australia). After 20 minutes incubation at room temperature, the reaction was stopped by addition of 200 μ l sodium phosphate buffer. Radiolabelled peptide was separated from radioactive iodide using a Sephadex G-25 column. The column was eluted with NaFam/BSA buffer and each 1 ml fraction was collected and counted by a gamma (γ) counter (Canberra Packard, Australia). A representative radioactivity profile of fractions is shown in Fig 2.3.

2.1.5.1.5. Human γ Globulin

The human γ globulin (CSL Ltd, Vic. Australia) was diluted 1:20 in assay buffer and stored at 4° C until use.

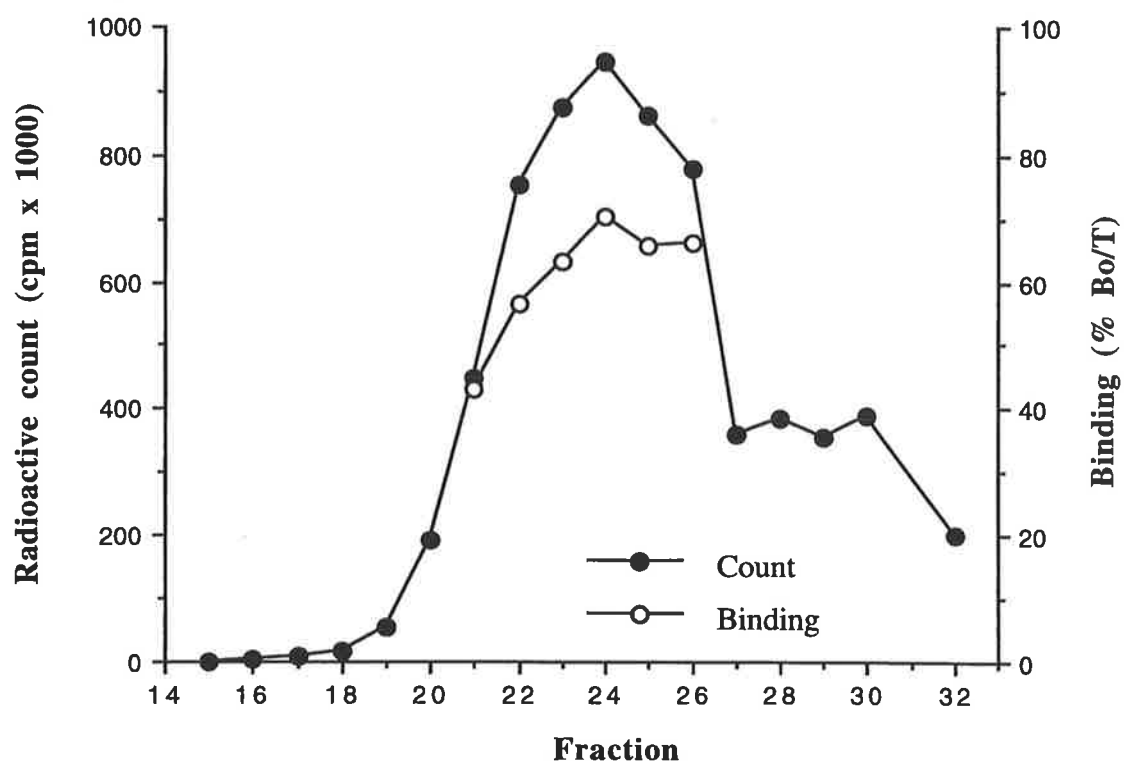


Fig 2.3. A representative profile of radioactivity and binding capacity of ^{125}I labelled ANP.

2.1.5.1.6. Polyethylene Glycol (PEG)

PEG 6000 was from BDH Laboratory supplies, Poole, England, and a 17.6% solution was used.

2.1.5.2. Selection of ANF tracer

Each fraction of tracer diluted to approximately 20,000 count per minute (cpm) was tested for its ability to bind to rabbit anti-rat ANP antiserum by incubation with the antibody for 48 hours in a volume of 400 μ l at 4°C. Bound ^{125} I-ANP was precipitated by incubation with 100 μ l diluted human γ globulin and 1.25 ml PEG (17.6%) at room temperature for 30 minutes. The samples were then centrifuged for 30 minutes at 2800 g at 4°C. Supernatant was discarded and the pellet counted in a γ counter. The bound ^{125} I-ANP was expressed as a percentage of total amount of tracer, shown in Fig 2.3. The fraction with the highest binding capacity and radioactivity was then diluted in assay buffer to achieve 10,000 cpm/tube and used for assay.

2.1.5.3. Assay Procedure

ANP was measured directly from coronary effluent without extraction. The assay was performed at room temperature using glass tubes (Oliver *et al.* 1988). The standard curve was a serial dilution of rat α ANP₉₉₋₁₂₆ in the range of 0-500 pg/tube (Fig 2.4.). 50 μ l of perfusate medium was added to each tube for non-specific binding (NSB) and standard curve. 50 μ l samples followed by 100 μ l antiserum were aliquoted to test tubes except for total count (TC) and NSB and all tubes were incubated for 48 hours at 4°C. 100 μ l of tracer was then added into all tubes, mixed and incubated for a further 16-24 hours at 4°C. The samples were then precipitated as for the binding test (see section 2.1.5).

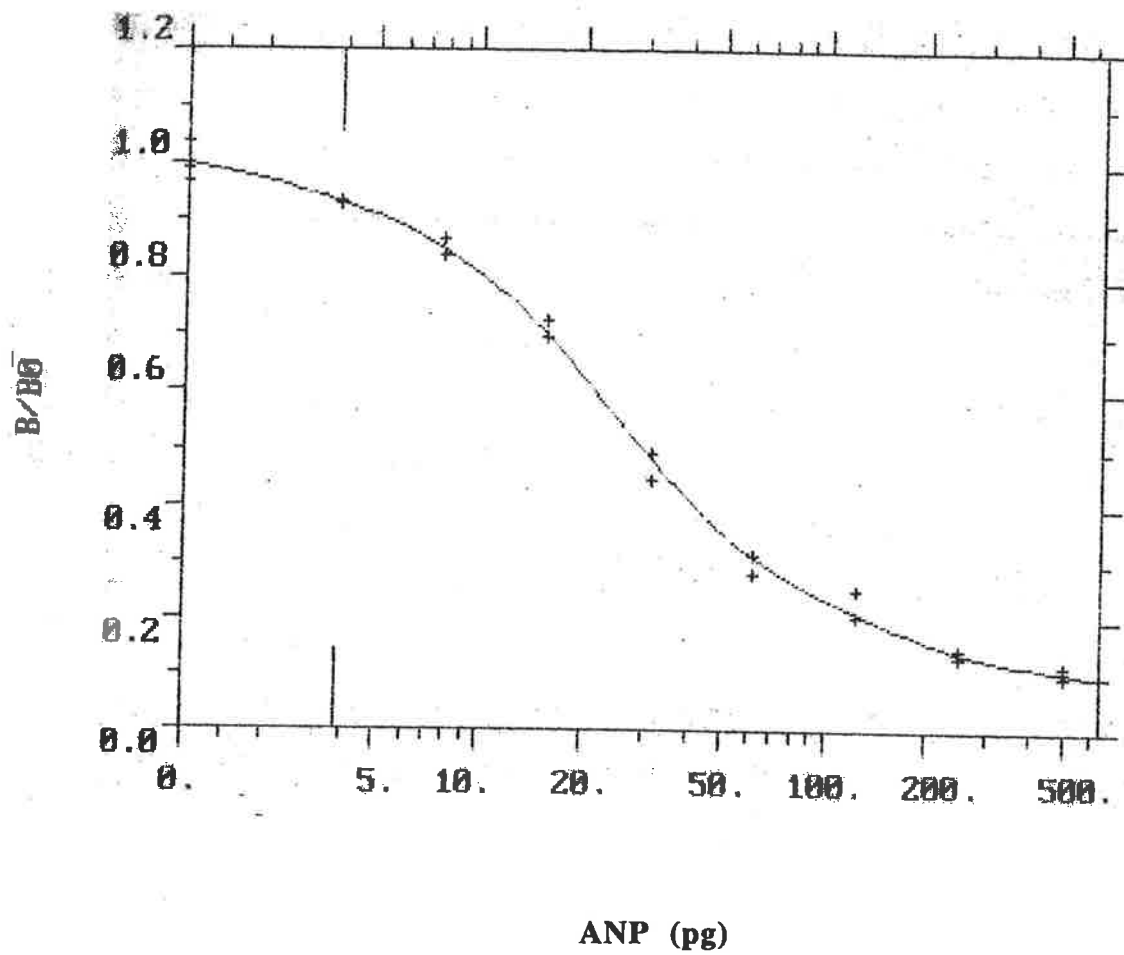


Fig. 2.4. A representative standard curve for ANP RIA.

2.1.5.4. Assay Characteristics

The antibody used for this study had a cross-reactivity less than 0.002% with a range of unrelated peptides including neuropeptide Y, substance P, vasopressin and angiotensin II (Rayner *et al.* 1993). The antibody had cross-reactivity with β ANP and BNP (45% and 12% respectively) (Rayner *et al.* 1993). The cross-reactivity with ET-1 was 6%. The ET-1 concentration used was 50,000 times higher than the ET-1 concentration in the effluent; Hence, it was unlikely that the endogenous ET-1 in rat heart effluent would interfere the ANP assay.

The intra assay coefficient of variation (CV) at 658 pg/ml was 3.9% (n=6) and the inter assay CV at 282 pg/ml was 15.3% (n=52). The mean $\pm$ SEM ED₅₀ of the standard curve was 32.8 $\pm$ 1.4 pg/tube (n=59).

2.1.6. Brain Natriuretic Peptide (BNP) RIA

2.1.6.1. Reagents

2.1.6.1.1. Assay buffer

Assay buffer contained NaH₂PO₄2H₂O, 2.96; Na₂HPO₄, 11.50; NaCl, 2.92; and NaN₃ (azide), 0.1 g/L; 0.1% Triton and 0.25% BSA. The buffer was adjusted to pH 7.4.

2.1.6.1.2. BNP Standard, antibody and tracer

Rat BNP-45, rabbit anti-BNP-45 (Rat) serum and ¹²⁵I-BNP-45 (5 μ Ci) were obtained from Peninsula, USA. The tracer was diluted in assay buffer to obtain 10,000 cpm. The

antiserum was also diluted in 0.1% Triton to obtain a binding of 30-40% of the total count.

2.1.6.1.3. Human γ Globulin and PEG (precipitating agents)

As for ANP assay.

2.1.6.2. Assay Procedure

2.1.6.2.1. Sample extraction

2 mls perfusate was transferred into ice cold 5 ml polypropylene tubes containing 2 ml 0.1% Trifluoro acetic acid (TFA). Sep-Pak Vac C18 cartridges columns (Waters, USA) were activated with 1 ml acetonitrile (60%) and TFA (0.1%) before loading acidified sample on to columns. Columns were washed with TFA (0.1%) then, eluted with 3 mls of acetonitrile (60%) in TFA (0.1%) into polypropylene tubes. The eluates were dried under N₂ at room temperature and reconstituted in 250 μ l assay buffer.

2.1.6.2.2. RIA procedure

The assay was performed at room temperature in polypropylene tubes. The standard curve was prepared by serial dilution of rat BNP-45 in the range of 0-500 pg/tube (Fig. 2.5.). 100 μ l of standard or reconstituted sample followed by 100 μ l antiserum were aliquoted into test tubes. The incubation and precipitation procedure was the same as for the ANP RIA procedure previously described.

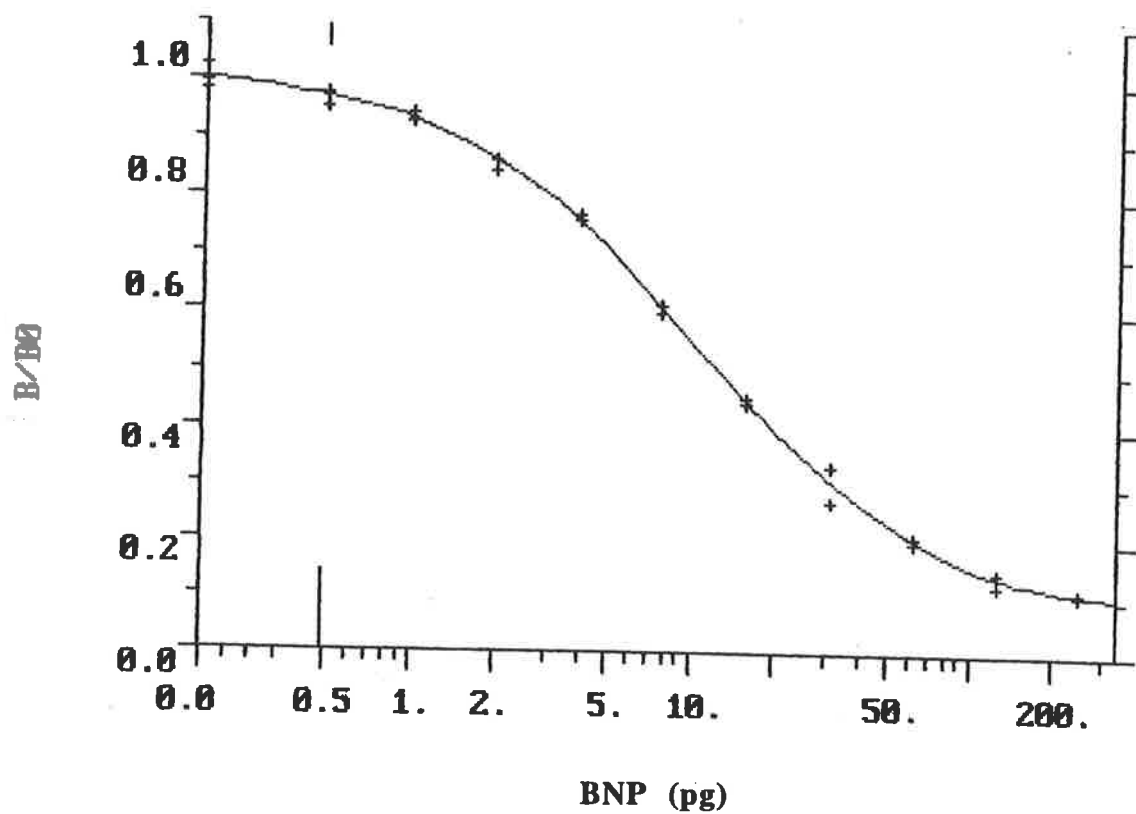


Fig. 2.5. A representative standard curve for BNP RIA.

2.1.6.2.3. Assay Characteristics

The antibody used for this study had a cross-reactivity of 100% with BNP-32 (rat) and BNP-10-32 (rat). There was no cross-reactivity with rat pre-pro-BNP, human BNP-32, human α ANP, endothelin-1 (human, porcine), angiotensin II and porcine BNP (Peninsula, USA).

The mean $\pm$ s.e.m. ED₅₀ of standard curve was 24.3 $\pm$ 19.6 pg/tube (n=6). Recovery was 78.6 $\pm$ 24.6 %. The intra assay CV at 24.7 pg/ml was 10.7% (n=3) and the inter assay CV at 64.1 pg/ml was 44.5% (n=6). All the BNP results included in this thesis were expressed as percentage of baseline due to this poor inter-assay CV. Samples from each experiment (each rat) were assayed within one assay.

2.1.7. Endothelin-1 (ET-1) RIA

2.1.7.1. Reagents

2.1.7.1.1. Assay buffer

Refer to BNP assay buffer.

2.1.7.1.2. ET-1 Standard

ET-1 (human, porcine) was obtained from Auspep, Australia.

2.1.7.1.3. Antibody

Rabbit anti-ET-1 (Porcine, Human) serum was obtained from Peninsula Laboratories Inc, USA. Antiserum was diluted in 0.1% Triton to obtain a binding of 30-40% of total count. The ET-1 antibody exhibited cross reactivities of ET-2, ET-3 and big ET-1 (human) are 7%, 7% and 17% respectively.

2.1.7.1.4. Tracer

¹²⁵I Endothelin-1 was obtained from Amersham, UK. The tracer dilution was calculated to give approximately 10,000 cpm/100 µl/assay tube.

2.1.7.1.5. Human γ Globulin and PEG (precipitation agent)

Refer to ANP assay.

2.1.7.2. Assay Procedure

2.1.7.2.1. Sample collection

Approximal 45 mls of coronary effluent was collected in 50 ml polypropylene centrifuge tubes containing 4.5 ml of 1 % TFA. This volume represented a 5 minutes sample collection.

2.1.7.2.2. Sample extraction

Refer to BNP assay. Each sample (50 ml) was acidified and loaded onto a C₁₈ column.

2.1.7.2.3. RIA procedure

Refer to BNP assay. A representative standard curve is shown in Fig. 2.6.

2.1.7.2.4. Assay Characteristics

The antibody used for this study had a cross-reactivity of 7% with ET-2 (human) and ET-3 (rat, human); 35% with big ET (porcine), 17% with Big ET (human); 3% with sarafotoxin S6b. There is no cross-reactivity with big ET 22-38 (human), BNP 26 (porcine), human α ANP, angiotensin I, II and III or vasopressin. The intra assay CV at 2 pg/tube was 12 % (n=5) and the inter assay CV at 2.9 pg/tube was 13.3% (n=14). The mean $\pm$ SEM ED₅₀ of the standard curve was 3.1 $\pm$ 0.1 pg/tube (n=33). The sensitivity of the assay was 0.4 pg ET-1. Recovery was 93.0 $\pm$ 17.7 %.

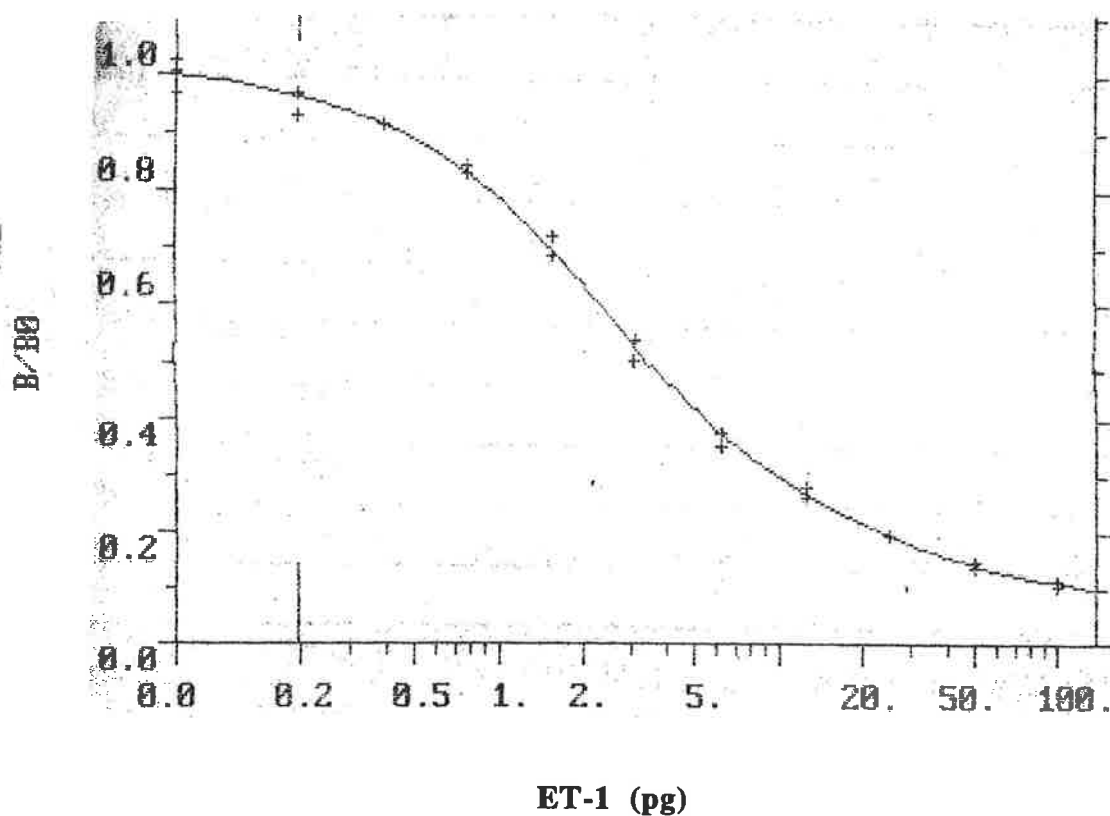


Fig. 2.6. A representative standard curve for ET-1 RIA.

2.2. HUMAN STUDIES.

All studies were approved by the Queen Elizabeth Hospital Human Ethics Committee and informed consent was obtained prior to study in all cases.

2.2.1. PATIENTS UNDERGOING PERCUTANEOUS TRANSLUMINAL CORONARY ANGIOPLASTY (PTCA)

2.2.1.1. Patient Selection

Patients undergoing elective PTCA were included for the study.

- Inclusion criteria:
- 1). Male or female of age 18 years and < 80 years.
 - 2). Stable or unstable angina.
 - 3). Haemodynamically significant stenosis in a major coronary artery demonstrated at coronary angiography.
 - 4). Intention to undergo PTCA with signed consent.

- Exclusion criteria:
- 1). Significant left main coronary artery disease.
 - 2). Haemodynamically significant valvular heart disease.
 - 3). Acute myocardial infarction within one month prior to procedure.

2.2.1.2. Protocol

Right heart catheterization was performed with Swan-Ganz catheter via a femoral vein sheath. Left ventricular catheterization and coronary angiography were performed via the femoral artery sheath. Arterial pressure was monitored both through the femoral artery sheath and the coronary catheter. A three lead electrocardiogram (ECG) was recorded

during the procedure including all periods of balloon inflation. Blood samples were collected simultaneously from femoral artery and pulmonary artery for ANP and ET-1 RIA prior to initial balloon inflation, at the end of this balloon inflation and one minute thereafter. Only samples from this first balloon inflation were used for the study in order to minimise effects of ischaemic preconditioning (Murry & Jennings 1986). Data were collected as regards presence/absence of ischaemic pain and/or ECG changes during this initial period of coronary occlusion.

2.2.2. PATIENTS INVOLVED IN INVESTIGATION OF GLYCERYL TRINITRATE-ANP-ET INTERACTION.

2.2.2.1. Patient Selection

Twenty-seven patients undergoing elective coronary angiography were selected for this study.

Inclusion criteria:

- 1). Male or female of age > 18 years and < 80 years.
- 2). Stable angina pectoris.
- 3). Intention to undergo coronary angiography.

Exclusion criteria:

- 1). Significant left main coronary artery disease.
- 2). Haemodynamically significant valvular heart disease.
- 3). Acute myocardial infarction within one month prior to procedure.
- 4). Unstable angina.
- 5). Severe systolic heart failure (New York Heart Association class III, IV).
- 6). Systemic hypotension.
- 7). Atrial fibrillation or flutter.

2.2.2.2. Protocol

As for the PTCA group, a femoral approach was used. A 15 min interval was allowed to elapse from the termination of angiography to that of haemodynamic/biochemical assessment, in order to exclude potential haemodynamic effects of contrast media. Cardiac output was measured by thermodilution method (Ganz *et al* 1971).

Intravenous glyceryl trinitrate (GTN; 10 µg/min) was infused through an antecubital vein for 10 min via a non-adsorbent delivery system. The arterial pressure, pulmonary capillary wedge pressure, heart rate and cardiac output were measured at baseline, and 5 min and 10 min after GTN infusion. Blood samples from pulmonary artery, femoral artery and vein were collected for ANP and ET-1 RIA assay before and after GTN infusion.

2.2.3. BLOOD COLLECTION

Blood was drawn into chilled syringes and transferred to the chilled tubes containing both EDTA (1 mg/ml) & Aprotinin (500-1000 KIU/ml). Samples were then centrifuged at 2800 g and 4° C for 15 min. Plasma was snap frozen and stored at -80° C until assay.

2.2.4. PLASMA ANP RIA.

Extraction of plasma ANP was performed according to the Amersham sample treatment protocol using C₈ columns (AmprepTM, Amersham, UK). Plasma (1 ml) was acidified with 0.25 ml of 2 M HCl and centrifuged at 2800 g and 4° C for 15 min prior to loading onto the columns. Interfering compounds were removed by wash with 15 ml of 0.1% trifluoroacetic acid (TFA) and ANP was eluted with 4 ml of 60 % acetonitrile acidified with 0.1 % TFA. The eluate was then dried under nitrogen overnight at room temperature, reconstituted in 0.25 ml assay buffer (pH 7.4) and stored at 4° C for at least

one hour to dissolve the dried pellet. The reconstituted samples were centrifuged at 2800 g and 4° C for 15 minutes. The supernatant was used for assay. The RIA assay was performed using ANP RIA kits obtained from Amersham, UK. International standard of human α ANP was obtained from the National Institute for Biological Standards and Control (NIBSC, UK).

ANP concentrations were expressed in fmol/ml and corrected for an extraction recovery (68.5 ± 1.0 %, $n=10$) estimated at the time of each assay by adding known amounts of standard to plasma. The intra-assay CV at 15.1 fmol/ml was 6.7% ($n=5$) and the inter-assay CV at 19.1 fmol/ml was 12.1% ($n=10$).

2.2.5. PLASMA ET-1 RIA.

The assay reagents and sample extraction procedures refer to perfusate ET-1 assay (see 2.1.7.1). 1 ml plasma was used for each sample extraction. The intra assay CV at 4.1 pg/ml was 7.8% ($n=6$) and the inter assay CV at 11.0 pg/ml was 10.9% ($n=7$). The mean $\pm$ SEM ED₅₀ of the standard curve was 3.3 ± 0.15 pg/tube ($n=8$). The sensitivity of the assay was 0.4 pg ET-1. Recovery was 56.7 ± 3.4 %.

2.2.6. PLASMA CGMP RIA.

Plasma cGMP was extracted according to Amersham's sample treatment protocol using trimethylaminopropyl SAX minicolumns (Amprep TM, Amersham Int plc, UK). The eluted analyte was dried under nitrogen at 37°C, reconstituted in one ml assay buffer and either stored at 4° C for same day assay or stored at -20°C for up to one week. The reconstituted samples were spun at 12,000g for 2 minutes prior to RIA using the Amersham cGMP [¹²⁵I] assay system's non-acetylation procedure (Amersham, UK). cGMP concentrations were expressed in pmol/ml. Extraction recovery was greater than

90%, intra-assay reproducibility at 7.2 pmol/ml was 5.6% (n=5) and the inter-assay coefficient of variation at 10.8 pmol/ml was 6.6% (n=7).

2.3. DATA ANALYSIS

Data were expressed throughout as mean $\pm$ SEM. ANP and ET-1 release rates were calculated as pg (or fg) of peptide per minute per gram wet heart weight. Coronary perfusion flow rate was expressed as ml/min/g wet heart weight. The heart rate and developed tension (cardiac force of contraction) were expressed as beats per minute (bpm) and grams respectively. In the human studies, plasma ANP and ET-1 concentrations were expressed as fmol/ml and pg/ml respectively.

Data were processed using Microsoft Excel 2.2 and MacLab v 3.5.2. The variation between individual hearts was normalized as percentage change from the baseline mean. To compare the changes of peptide release rate, or the impairment of cardiac force of contraction, the area under the curve (AUC) (arbitrary integrated units) was calculated by MultiFit 2.02 using the trapezoidal method.

Comparison of patient characteristics between acute and chronic nitrate therapy groups utilized unpaired *t* test (quantitative parameters) and chi-squared analysis (categorical parameters). Correlations between haemodynamic data and biochemical changes, or biochemical parameters were made utilizing linear regression analysis. Comparison of area under curves and human haemodynamic data were performed using unpaired or paired Student's *t* test for normally distributed data. Data involving multiple comparisons were evaluated utilizing two-factor analysis of variance (ANOVA), with repeated measure where appropriate for multiple comparisons. Tests for deviations from Gaussian distribution were performed using the Kolmogorov-Smirnov test. The nonparametric test used in this study was Mann-Whitney U.

A *P* value of <0.05 was considered statistically significant.

CHAPTER 3 - RESULTS

DETERMINANTS OF ANP/BNP/ET-1 RELEASE DURING ISCHAEMIA.

3.1 Introduction

Myocardial ischaemia *in vivo* appears to provide a strong stimulus for ANP release, although the precise mechanism remains to be delineated. Patients with coronary artery disease, undergoing dynamic exercise, plasma ANP levels have generally been reported as being higher than those associated with exercise in the absence of coronary disease (Nicholson *et al.* 1993; Hu *et al.* 1988; De Marzio *et al.* 1992; Nishikimi *et al.* 1988). Plasma ANP levels also increased at the end of (or 2 min post) percutaneous transluminal coronary angioplasty (PTCA), and following angina (Ikaheimo *et al.* 1989, Chenu *et al.* 1991; Prachar *et al.* 1991; Wiedermann *et al.* 1991; Malatino *et al.* 1992; Gasser *et al.* 1993). ANP release has also been reported to increase during acute myocardial infarction (MI) in rat (Tikkanen *et al.* 1987; Wang *et al.* 1989) and human (Ferrari *et al.* 1990; Tomoda 1988; Gutierrez-Marcos *et al.* 1991; Niitsuma *et al.* 1991; Sigurdsson *et al.* 1993; Ngo *et al.* 1989; Kyriakidis *et al.* 1993; Fontana *et al.* 1990; Arnal *et al.* 1993; Bellone *et al.* 1993; Svanegaard *et al.* 1989) with or without left ventricular systolic dysfunction. Furthermore, patients treated with streptokinase or tissue plasminogen activator (tPA) had lower plasma ANP levels than those not receiving thrombolytic treatment (Ngo *et al.* 1989, Phillips *et al.* 1989) indicating a possible correlation between extent of infarction and that of ANP release.

Myocardial ischaemia-stimulated ANP release is commonly thought to be mediated by increased atrial distension caused by ventricular diastolic failure (Ikaheimo *et al.* 1989; Malatino *et al.* 1992; Tomoda 1988). Moreover, plasma ANP levels were greater in patients who had inferior myocardial infarction with right ventricular infarction than without (Serra *et al.* 1991). However, some studies have demonstrated the increase of ANP levels to be correlated with size of infarction but not with resultant left ventricular dysfunction (Ngo *et al.* 1989; Kyriakidis *et al.* 1993) or atrial stretch (Prachar *et al.*

1991). This raises the possibility that myocardial ischaemia *per se* may directly affect ANP release.

The physiology and pathophysiology of brain natriuretic peptide (BNP) have been discussed in Chapter 1. ANP and BNP probably act as a dual natriuretic peptide system intimately involved in circulatory homoeostasis (Lang *et al.* 1992). Unlike ANP, BNP secretion is mainly from the ventricle (Lang *et al.* 1992, Roskoaho, 1992). Ischaemia-associated rises in plasma BNP concentrations have been demonstrated in patients with ischaemia (Nicholson *et al.* 1993, Mair *et al.* 1997) and acute MI (Mukoyama *et al.* 1991; Morita *et al.* 1993; Horio *et al.* 1993, Hama *et al.* 1995, Foy *et al.* 1995,). In another study of uncomplicated acute MI with early reperfusion, plasma ANP levels returned to the normal range after day 3, while BNP levels remained high for 4 weeks (Morita *et al.* 1993). The increased plasma BNP levels did not correlate with pulmonary capillary wedge pressure but positively correlated with infarct size (Horio *et al.* 1993). A recent study in patients undergoing CABG showed plasma BNP but not ANP concentration increased post aortic cross-clamping (Mair *et al.* 1997). The pathophysiological mechanisms which lead to BNP release during ischaemia remain poorly investigated to date. In particular, it remains uncertain whether abnormal ventricular function and /or the biochemistry of ischaemic *per se* are primary mediator(s) of the release process.

Endothelin (ET) is another cardioactive peptide that is affected by myocardial ischaemia (see chapter 1). Increased plasma ET concentrations are found in patients with unstable angina, myocardial infarction and immediately following percutaneous transluminal coronary angioplasty (Wieczorek *et al.* 1994; Malatino *et al.* 1993, Kyriakides *et al.* 1995). Endogenous ET release into the coronary perfusate of isolated perfused rat hearts was been shown to increase during reperfusion but decrease during severe coronary flow reduction (Brunner *et al.* 1992). The relationship between net ET-1 efflux rate and extent

of ischaemia has not yet been investigated. Ischaemia causes an increase in externalization of ET-1 binding sites (Liu *et al.* 1989; Nayler *et al.* 1992).

In previous studies, it has been possible to develop a model of myocardial ischaemia associated with the isolated rat heart (Chen *et al.* 1993). This model is noteworthy for the absence of atrial distension. However, it was found that a coronary perfusion reduction of 55-60% was associated with an increase of $125.6 \pm 23.2\%$ (peak) in ANP efflux from the perfused heart. It was found that high concentrations (10^{-5} M) of meclofenamate or nordihydroguaiaretic acid inhibited ANP release in this model, raising the possibility that cyclo-oxygenase and lipoxygenase may contribute to ischaemia-induced ANP release.

In the currently described experiments, the relationship between extent of ischaemia, associated changes in heart rate and contractile function, and cardiac release of ANP, BNP and ET-1 was examined further.

3.2. Method

3.2.1. Isolated heart perfusion:

Refer to Chapter 2.1.2. Briefly, isolated rat hearts (n=82) were perfused by a modified Langendorff procedure with modified Eagles medium and at constant pressure. The medium was continually gassed with carbogen (95% O₂, 5% CO₂). Global ischaemia was induced by decreasing perfusion flow rate for 30 min. This ischaemia phase was then followed by 30 minutes' reperfusion.

3.2.2. Protocol

After 40 minutes equilibration, baseline samples were collected every 2 minutes for 10 minutes. In some hearts (n=59), global ischaemia (perfusion flow reduced by 27-87% from basal perfusion flow rate) was induced. A 30 minute global ischaemia period was followed by 30 minutes reperfusion (ie. returning the perfusion pressure to the same as basal perfusion). It was intended prospectively to compare three degrees of coronary flow reduction of approximately 35,55 and 75% respectively, with control data and with each other. Force of contraction and spontaneous heart rate (for non-paced hearts) were monitored. Right atrial pacing was used in some experiments to keep the heart rate constant during the experimental period: pacing was achieved by placing electrodes against the right atrium, and was performed at a rate $9 \pm 1\%$ above the spontaneous heart rate with square wave pulses of 1 mS at a voltage 100% above non-ischaemic threshold. Volume of perfusate was measured by calibrated pipette as an index of coronary flow rate. The perfusate effluent was collected for the measurement of ANP, BNP and ET-1 during baseline, ischaemia and reperfusion. Peptides were measured using radioimmunoassay (refer to chapter 2.1.5 to 2.1.7.). BNP results were expressed as percentage of baseline due to the high inter-assay variability.

3.3. Results:

3.3.1. Extent of Coronary Flow Reduction

Basal coronary perfusion rate was 7.46 ± 0.15 ml/min/g wet heart weight. In control hearts ($n=6$), there was no significant fluctuation in perfusion rate over the duration of the experiment. As intended (section 3.2.2), three different degrees of coronary flow reduction were induced for 30 minutes (Fig 3.1.). Specifically, these three groups of flow reduction were of mean extent $34.6 \pm 3.1\%$ (range 27-48%), $55.9 \pm 1.1\%$ (range 51-61%) and $76.8 \pm 1.9\%$ (range 70-87%). This induced temporary decrease in flow rate was followed by a phase of hyper-perfusion regardless of the severity of ischaemia. During reperfusion, the hearts were perfused under the same pressure as basal perfusion. Thus, changes in coronary perfusion flow rate reflected changes in coronary vessel resistance throughout.

3.3.2. Determinants of ANP release

3.3.2.1. Perfusion flow reduction

The mean basal ANP release rate was 6.50 ± 0.30 pg/min/g wet heart weight. Fig 3.2. illustrates the ANP release in response to different rates of reduced flow. Mild ischaemia (coronary flow reduction of $34.6 \pm 3.1\%$, $n=6$) did not affect ANP release rate compared with the control group, although there was a mild transient increase of ANP wash-out during early reperfusion (Fig 3.2.a.). Moderate ischaemia (coronary flow reduced by $55.9 \pm 1.1\%$) induced a significant increase of ANP efflux rate ($P < 0.005$, unpaired t test of AUC, $n=10$) with a peak of $78.1 \pm 13.0\%$ over baseline at 20 min. During reperfusion, there was a rapid wash-out phase of ANP into effluent ($155.8 \pm 25.8\%$ over basal ANP release rate) within 1 min post-ischaemia, and ANP release rate returned to baseline after

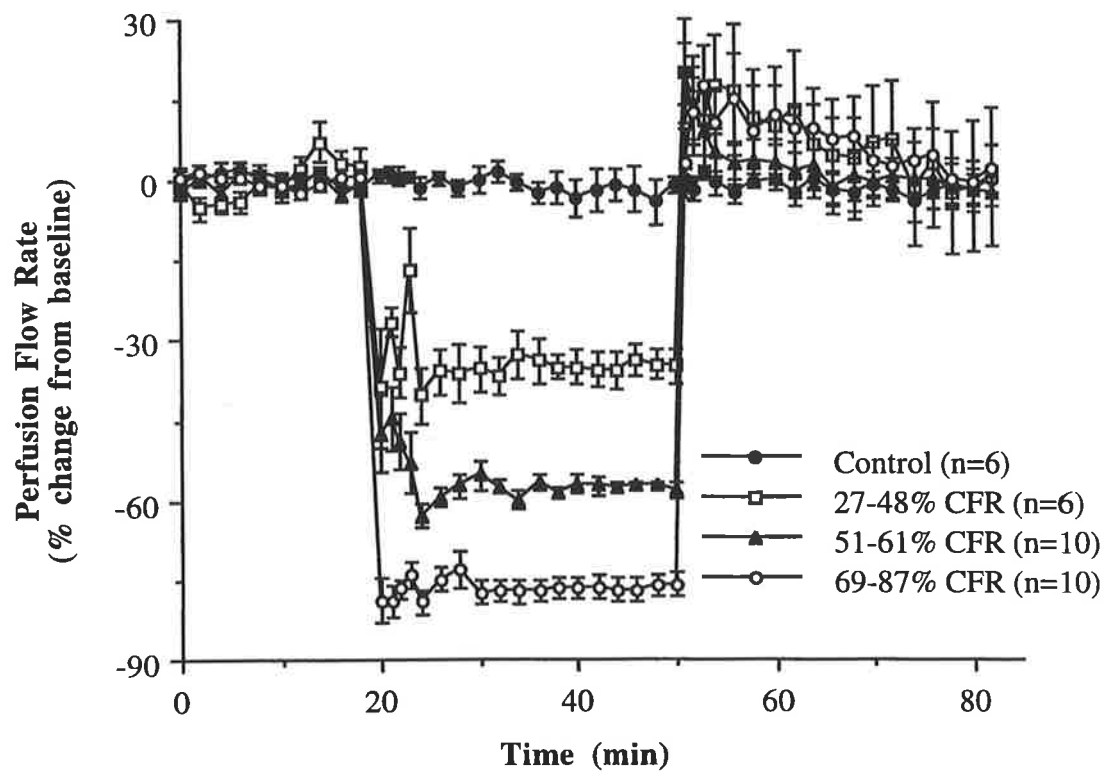


Fig. 3.1. Coronary flow reduction (CFR) compared with control.

10 min of reperfusion (Fig 3.2.b). Severe ischaemia (coronary flow reduced by $76.8 \pm 1.9\%$) resulted in a sustained increase ($P < 0.001$, unpaired t test of AUC, $n=10$) of ANP release rate which reached a plateau after 6 minutes of ischaemia with a peak of $52.7 \pm 18.9\%$ over baseline. The ANP wash-out during reperfusion reached a peak of $286.7 \pm 61.8\%$ over baseline and returned to baseline following 14 minutes of reperfusion.

Fig 3.3. summarises the effect of these different degrees of coronary flow reduction on ANP release during 30 minutes ischaemia, expressed as the integrated areas under the ANP release curves with time. Mild ischaemia did not significantly affect ANP release. Moderate and severe ischaemia (coronary flow reduction $>50\%$ of baseline) caused a significant increase ($P < 0.005$, one-factor ANOVA) of ANP release from isolated rat heart compared with control group. There was no significant difference in ANP release between the moderate and severe ischaemia groups.

3.3.2.2. Role of Bradycardia

The spontaneous heart rate was decreased during moderate and particularly during severe ischaemia (Fig 3.4.). The heart rate appears inversely correlated with severity of ischaemia. Since ANP release has been reported to be affected by heart rate (Twidale *et al.* 1993, Oliver *et al.* 1988, Schiebinger *et al.* 1994), this ischaemia-induced bradycardia may therefore affect the net ANP release during experimentally induced ischaemia. Thus, additional experiments were performed with right atrial pacing at the rate of $9 \pm 1\%$ above the spontaneous heart rate so as to maintain the heart rate constant during ischaemia, in order to investigate the effect of ischaemia *per se* on ANP release.

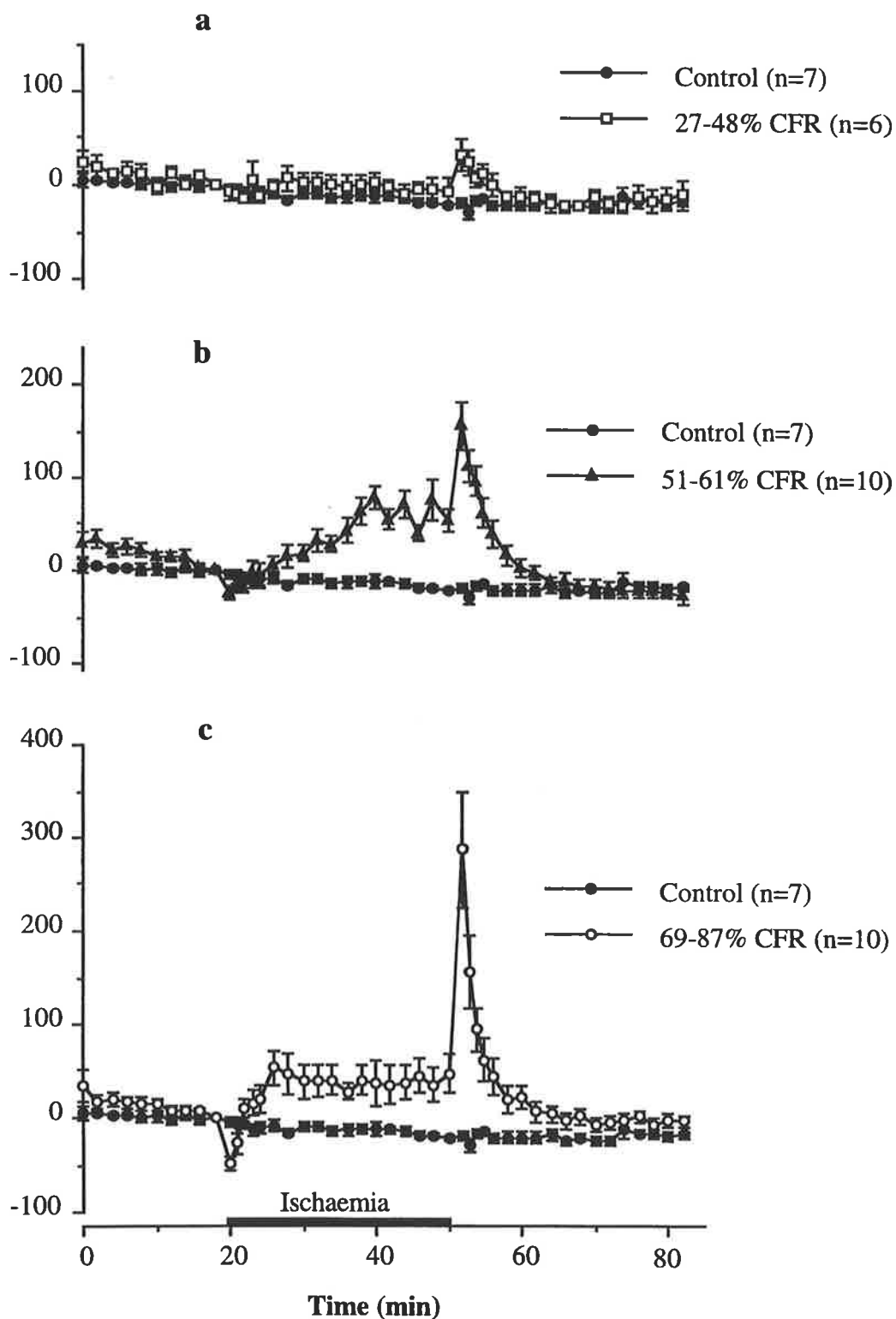
**ANP release rate
(% change)**

Fig 3.2. The effects of coronary flow reduction on ANP release from isolated rat hearts.

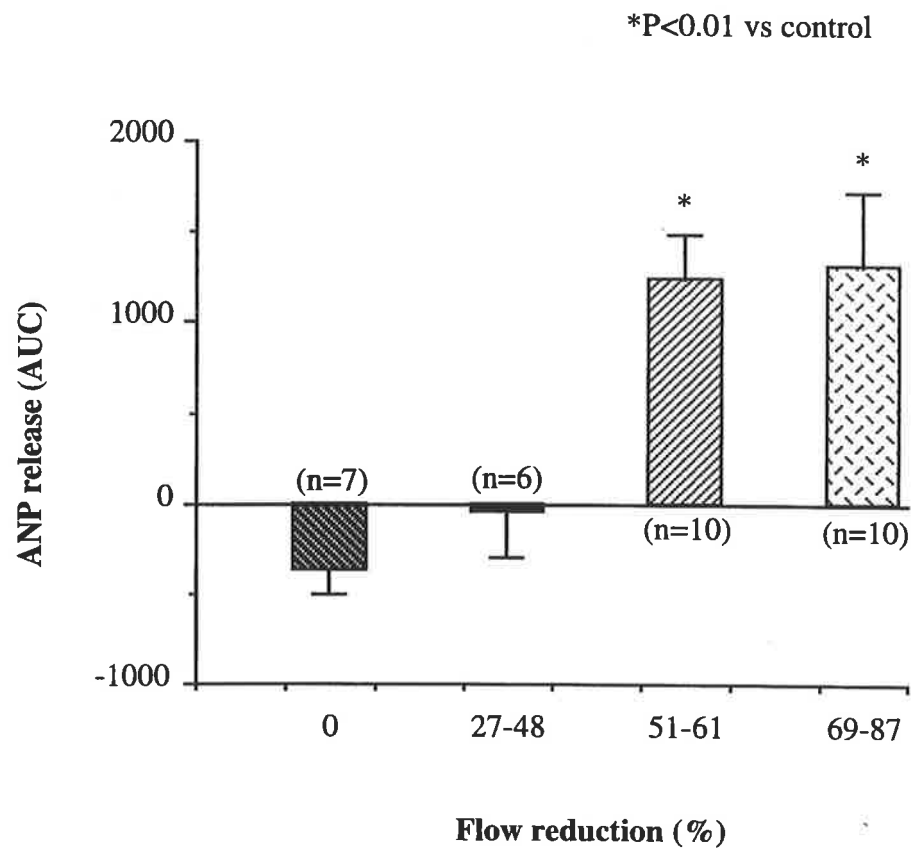


Fig 3.3. ANP release expressed as area under curve (AUC, arbitrary units) for different degrees of coronary flow reduction.

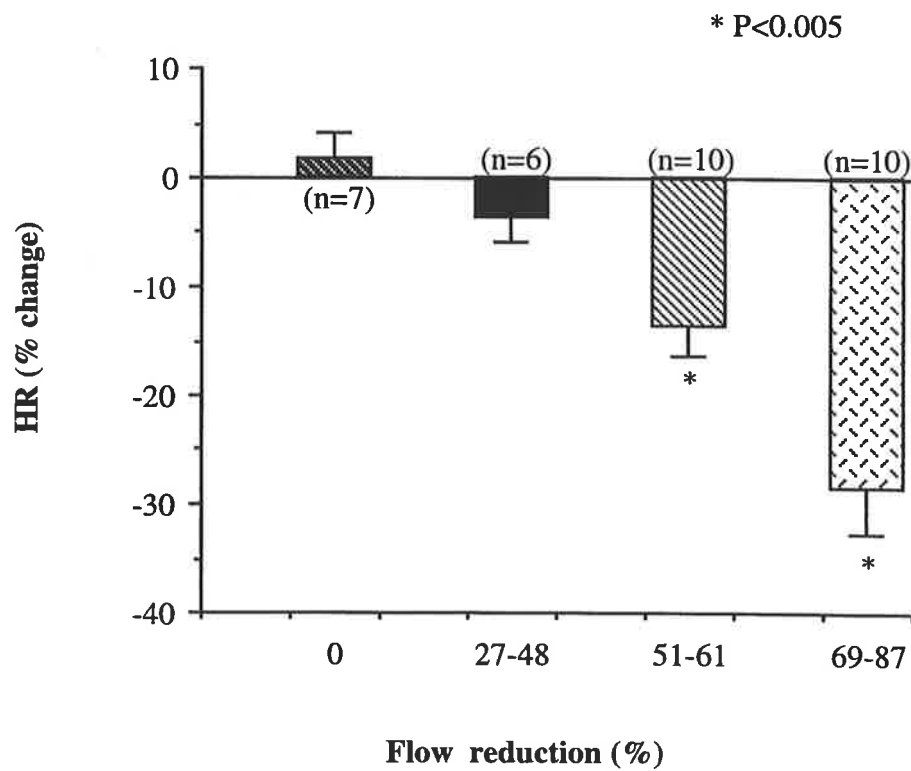


Fig 3.4. Effect of coronary flow reduction on spontaneous heart rate (mean $\pm$ SEM of % change from baseline).

Right atrial pacing did not alter the basal ANP secretion rate (Fig 3.5.). There were no significant differences in ANP release rate between paced (n=6) and non-paced (n=7) hearts in the absence of ischaemia (control). Furthermore, ANP release rate during ischaemia (expressed as AUC) from paced hearts was not significantly different from the non-paced hearts with same degree of ischaemia. Thus, ischaemia-induced bradycardia had no effect on ANP release. Thus, in paced hearts, both moderate (n=17) and severe (n=16) ischaemia induced significant increases ($P<0.01$, one-factor ANOVA) in ANP release compared with control.

3.3.3. BNP release

In non-ischaemic hearts (n=6), BNP release rate gradually increased during the experimental period. At the end of the experiment, BNP release rate had increased by $56.2\pm14.3\%$. In ischaemic hearts (n=5), severe coronary flow reduction (mean $75.3\pm0.9\%$) did not affect BNP release during the ischaemic period. Although there was a transient increase of BNP release at the beginning of reperfusion, the BNP release rate during the 30 min reperfusion period was lower ($P<0.05$, two-way ANOVA with repeated measure) in ischaemic hearts compared with control group (Fig 3.6.).

3.3.4. ET-1 release

Basal ET-1 release rate was 204 ± 30 fg/min/g wet heart weight. In the absence of ischaemia (n=6), this basal rate progressively increased during the experimental period: at the end of 60 minutes, ET-1 release rate had increased by $145\pm40\%$. In ischaemic hearts, moderate ischaemia ($58.2\pm0.9\%$ flow reduction, n=6) did not significantly affect ET-1 efflux. The peak ET-1 efflux of $44.3\pm35.4\%$ above baseline occurred at 22 minute of reperfusion, In severe ischaemia ($75.3\pm0.9\%$ perfusion flow reduction, n=10) there was a trend towards a decrease ($P=0.055$, unpaired *t* test of AUC) in ET-1 efflux during the

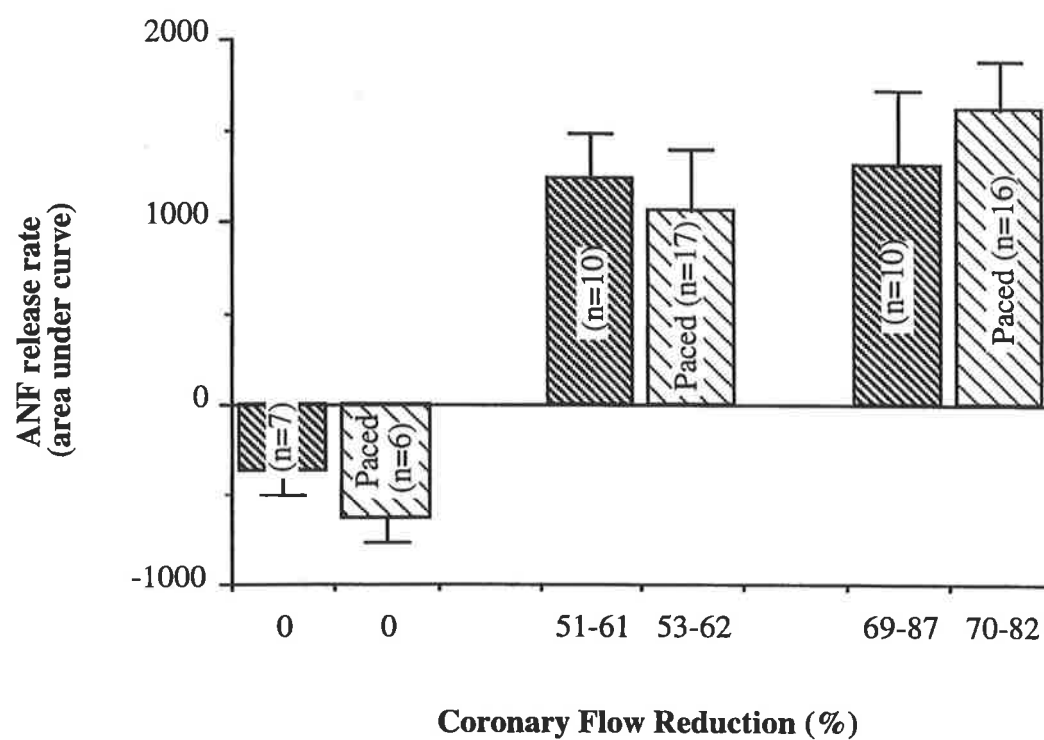


Fig 3.5. Effect of right atrial pacing on coronary flow reduction-induced ANP release (mean $\pm$ SEM of AUC) from isolated rat hearts.

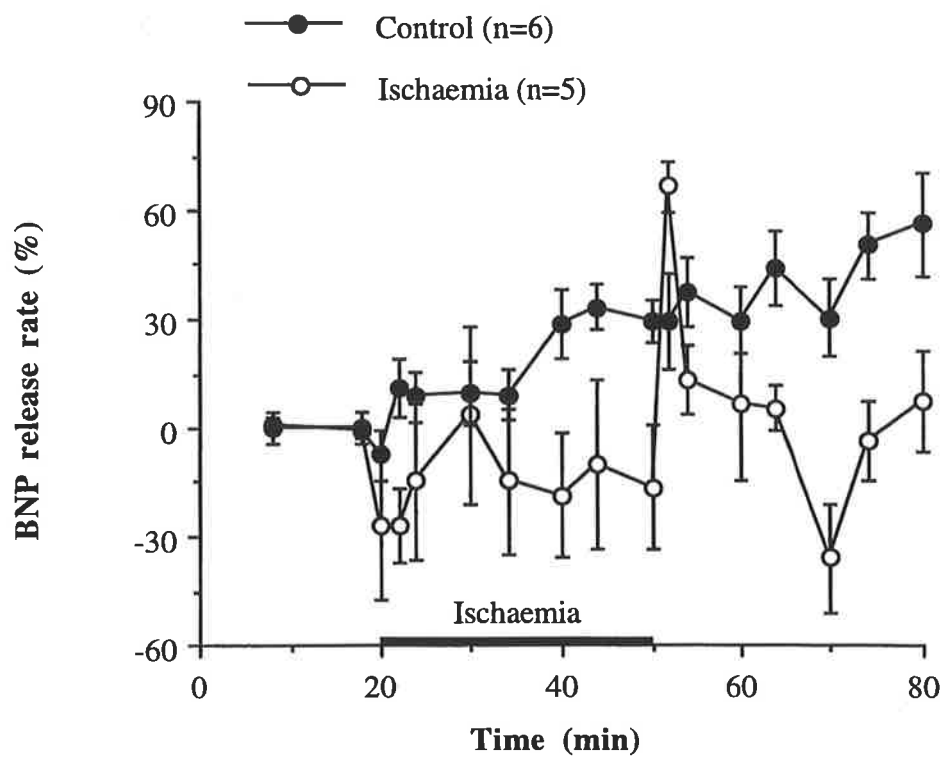


Fig 3.6. Effect of right atrial pacing on coronary flow reduction induced BNP release (% change from baseline).

ischaemic period compared with control hearts. However, there was a rapid transient increase ($71.9 \pm 26.3\%$ above baseline) in ET-1 efflux during reperfusion (Fig 3.7.). In both moderate and severely ischaemia hearts, ET-1 efflux rate during the 30 min reperfusion period was lower than that in control hearts ($P < 0.005$, two-way ANOVA with repeated measure). In contrast, ET-1 concentration in coronary effluent was increased ($P < 0.05$, two-way ANOVA with repeated measure) during moderate (49.8 ± 7.8 fg/ml) and severe (82.8 ± 16.4 fg/ml) ischaemia, compared with control (22.4 ± 4.6 fg/ml). Therefore, the reduction in net efflux reflected the reduced perfusion rate.

3.3.5. Correlation with Cardiac Function

3.3.5.1. Effect of ischaemia on cardiac contractile function

In non-ischaemic hearts, there was no significant change in force of contraction during the experimental period. Right atrial pacing did not affect the cardiac systolic function in control hearts. In non-paced hearts, the force of contraction was not significantly different between ischaemic and control hearts. In paced hearts, both moderate and severe ischaemia induced decreases ($P < 0.005$ vs control, one-factor ANOVA of AUC) in the force of contraction. Atrial pacing therefore unmasked the ischaemia-induced impairment in cardiac force of contraction compared with the same degree of global ischaemia ($P < 0.005$, unpaired t test of AUC) in non-paced hearts.

3.3.5.2. Effect of ischaemia on cardiac diastolic function

Fig 3.9. illustrates the effects of global ischaemia and reperfusion on myocardial relaxation (shown as dF/dt minimum). We examined 17 atrial paced rat hearts where 6 of these were analysed for effect of moderate coronary flow reduction on cardiac relaxation.

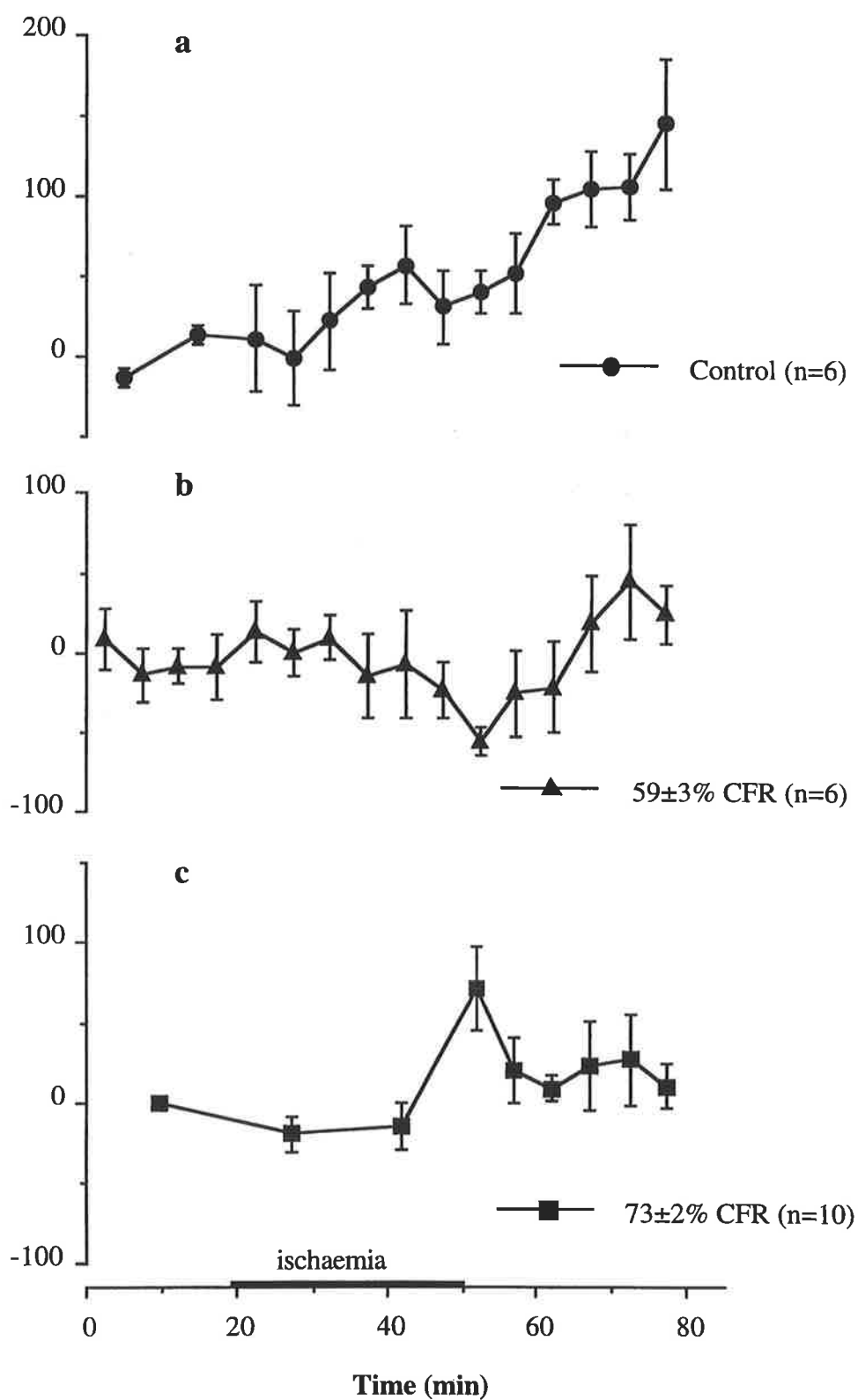
ET-1 efflux (% change)

Fig 3.7. Time course of ET-1 efflux (mean±SEM) in control hearts (a), compared with moderate (b) and severe (c) coronary flow reduction (CFR).

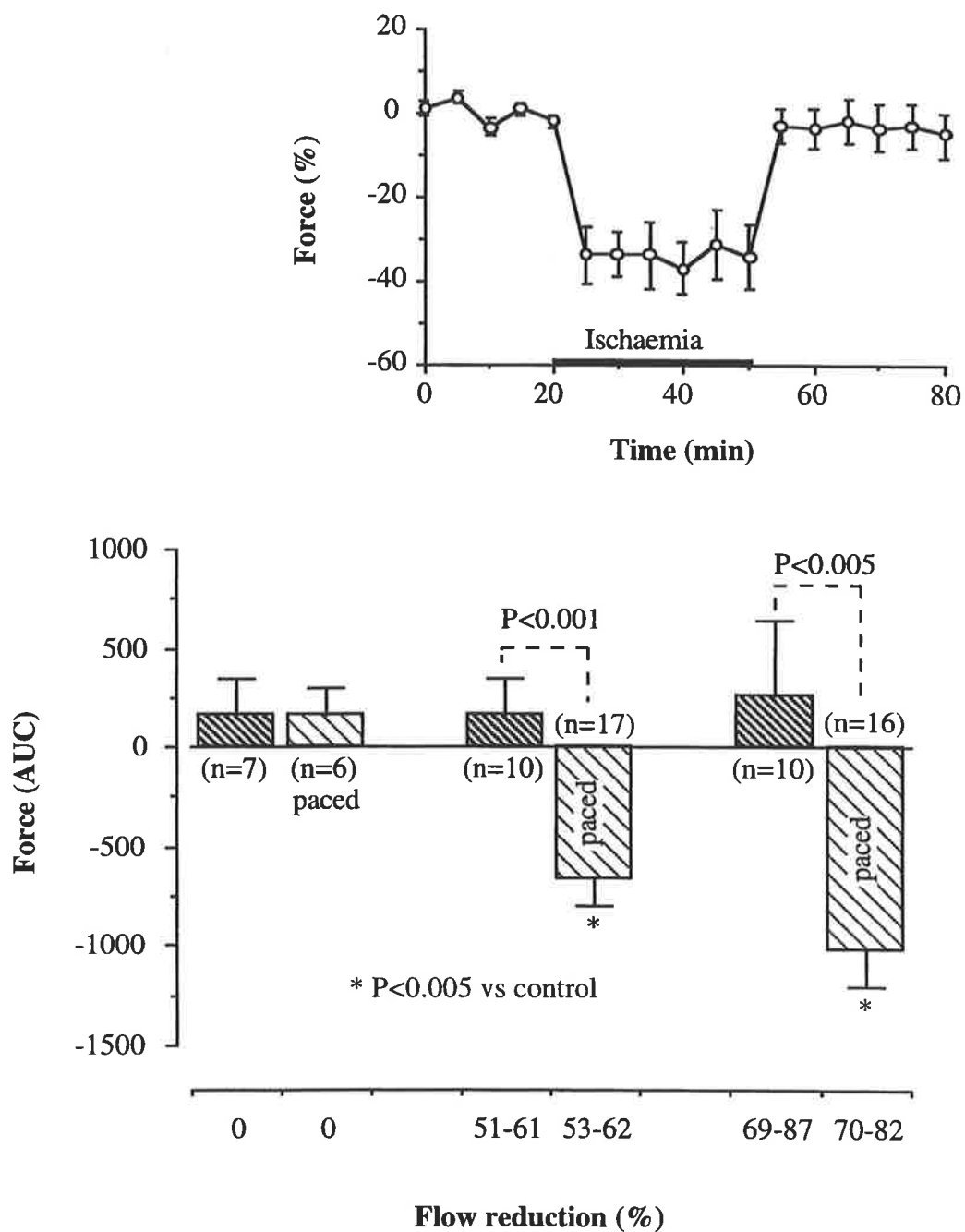


Fig 3.8. Effect of coronary flow reduction on cardiac force of contraction (mean $\pm$ SEM, of AUC) - comparison of pace and non-paced hearts with similar degree of coronary flow reduction.

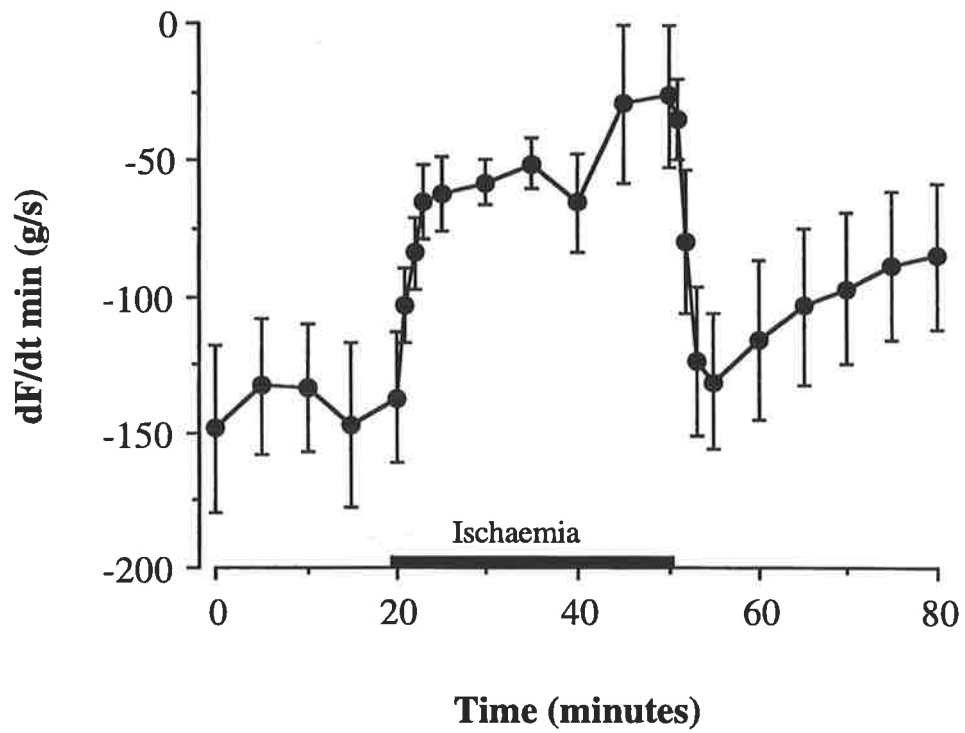


Fig 3.9. Effect of coronary flow reduction on dF/dt minimum (mean $\pm$ SEM).



Moderate ischaemia induced a rapid increase in absolute value of dF/dt minimum, an inverse correlate of the speed of myocardium relaxation, compared with basal values. This impairment of cardiac relaxation thereafter was sustained until reperfusion. However, recovery of relaxation was incomplete.

3.4. Discussion

The current study showed that ANP but not ET-1 release was increased during acute low flow ischaemia in the isolated perfused rat hearts. This increase in ANP release was independent of atrial distension and ischaemia-induced bradycardia. Prevention of ischaemia-induced bradycardia did not affect this incremental ANP release. However, the maintenance of constant heart rate via right atrial pacing facilitated examination of changes in contractile function during ischaemia; hence all subsequent investigations were performed utilizing this methodology whenever practicable.

Although ANP release associated with myocardial ischaemia is commonly thought to be mediated by increased atrial distension caused by ventricular diastolic failure (Ikaheimo *et al.* 1989; Malatino *et al.* 1992; Tomoda 1988), the results of the present study, where atrial stretch does not occur, confirmed that myocardial ischaemia/reperfusion (no-flow) or low coronary flow rate “directly” stimulates ANP release (Ussimaa *et al.* 1992a & b, Chen *et al.* 1993, Arad *et al.* 1994). Furthermore, these studies demonstrate ANP release increased only when coronary flow reduction was more than 50%. This is consistent with the previous findings of Chen *et al.* (1993). However, Arad *et al.* demonstrated that ligation of the left coronary artery in Langendorff-perfused rat hearts, resulting in an average reduction of $44 \pm 3\%$ in the coronary flow, decreased the ANP release rate ($18 \pm 5\%$) (Arad *et al.* 1994). The difference between the results presented by Arad *et al.* from those presented here may reflect the different effects of global ischaemia and regional ischaemia on ANP release (e.g. the ligation of the left coronary artery may not cause right atrial ischaemia). However, more importantly, Arad’s model would eliminate perfusion of the ischaemic zone; coronary effluent would be from essentially non-ischaemic myocardium.

Uusimaa *et al.* demonstrated that ANP release rate was decreased during 90% coronary flow reduction (Uusimaa *et al.* 1992a). The difference between their experiments and these presented here are (i) they used colloid-free Krebs solution (versus albumin-containing medium), (ii) they used constant flow of 5 ml/min (versus constant pressure and a basal coronary perfusion flow rate of approximately 13.5 ml/min). Furthermore, a 90% reduction in flow rate (Uusimaa *et al.* 1992a) was not attempted in the current experiment due to the difficulty of keeping the heart rate constant during this degree of ischaemia.

In this isolated perfused rat heart model, there was no significant difference in ANP release between moderate (51-61%) and severe (69-87%) coronary flow reduction, with severe ischaemia causing a more severe bradycardia in the absence of pacing (Fig 3.4.). An increased frequency of atrial contraction has been shown to be an effective stimulus for the release of ANP both *in vivo* (Oliver *et al.* 1988) and *in vitro* (Schiebinger *et al.* 1994). In this study, right atrial pacing using a rate of 10% above the spontaneous heart rate did not affect the basal ANP release rate. This result confirmed a previous study showing an atrial pacing frequency up to 67% above the spontaneous heart rate had no effect on ANP release from the isolated perfused rat heart (Kato *et al.* 1990). While ischaemia-induced bradycardia may act as a homeostatic myocardial protection, the effect of bradycardia on ANP release is unknown. In the studies presented here for the isolated perfused rat heart, the increment in ANP release rate for paced and non-paced groups were not significantly different indicating that low frequency atrial pacing and ischaemia-induced bradycardia did not affect ANP release.

In contrast to ANP, BNP secretion rate was not affected by 30 minutes of 75% coronary flow reduction in this system. In patients with exercise-induced ischaemia (Nicholson *et*

al. 1993) and acute MI (Mukoyama *et al.* 1991; Morita *et al.* 1993; Horio *et al.* 1993) plasma BNP concentrations were increased. In patients with uncomplicated acute MI and early reperfusion, plasma ANP levels were increased and reached a peak value within 4 hours after onset of symptoms, returning to the normal range after day 3. In contrast, plasma BNP concentrations increased slowly and reached peak approximately 20 hours after onset of symptoms, remaining elevated for 4 weeks. These data therefore provide additional evidence for different secretion patterns of ANP and BNP in response to ischaemia (Morita *et al.* 1993). Similarly, a study in rats demonstrated that mRNA for BNP increased 4 hours post-infarction whereas tissue BNP concentrations increased only 12 hours post infarction (Hama *et al.* 1995).

The studies included in this chapter have demonstrated that a reduction in coronary flow of $58 \pm 1\%$ does not affect ET-1 release rate; and a flow reduction of $75 \pm 1\%$ caused a small decrease in ET-1 release rate during 30 minutes ischaemia followed by a rapid transient increase in ET-1 release rate occurring within 5 minutes of reperfusion. Brunner *et al.* has observed a similar pattern in ET-1 and total ET release rate from isolated rat heart during a more prolonged (90-180 min) and severe (90%) flow reduction followed by reperfusion (Brunner 1995, Brunner *et al.* 1992). Studies *in vivo* also show increased plasma ET concentrations during unstable angina, myocardial infarction and immediately following percutaneous transluminal coronary angioplasty (Wieczorek *et al.* 1994; Malatino *et al.* 1993). In this paradigm, ET-1 concentration was increased during ischaemia. However, the release rate did not increase during the ischaemic period. Ischaemia-induced externalization of ET-1 binding sites (Liu *et al.* 1989) may result in incremental ET-1 binding to the receptors rather than release to the perfusate. Therefore, the net ET-1 efflux into coronary perfusate may be unchanged or even decreased during global ischaemia. In the control group (non-ischaemic hearts), ET-1 release rate progressively increased by about 150% during the experimental period. The mechanism for this slow rise in basal ET-1 release rate is unknown; however, it is possible that it

could result from progressive perturbation of endothelial function during *in vitro* perfusion.

Myocardial ischaemia is commonly associated with impairment of cardiac diastolic and systolic function (Vatner 1980, Bourdillon *et al.* 1983). Impairment of cardiac relaxation during ischaemia was also noted in the studies presented here. However, in non-paced hearts, there was no significant decrease in cardiac force of contraction during ischaemia. A previous study has shown that the contractile tension increases with simultaneous amplitude decrease during global ischaemia in the isolated perfused rat heart (Uusimaa *et al.* 1992b). This "increase" of force of contraction during ischaemia may be due to ischaemia-induced bradycardia by virtue of a negative staircase phenomenon (Borzak *et al.* 1991). Atrial pacing unmasked ischaemia-induced impairment in cardiac systolic function.

In conclusion, studies included in this chapter have shown 1) acute coronary flow reduction up to 50% increased ANP but not BNP or ET-1 release; 2) atrial pacing at a rate 10% over spontaneous heart rate did not affect this ischaemia-induced ANP release, but unmasked ischaemia-induced impairment in cardiac systolic function. These results clearly indicate ischaemia is a stimulus for ANP release independent of ischaemia-induced atrial stretch and bradycardia. Since this atrial pacing technique did not affect ANP release, but unmasked ischaemia-induced cardiac dysfunction, this procedure of moderate or severe coronary flow reduction with atrial pacing was used for further studies directed at the mechanism of ischaemia-induced ANP release.

3.5. Summary

The current study evaluated the relationship between degrees of coronary flow reduction (CFR), impairment of spontaneous contractile and relaxation function, heart rate (HR), and ANP/BNP/ET-1 release in the Langendorff-perfused rat heart. Thirty minutes global ischaemia was followed by 30 minutes reperfusion, with HR either spontaneous, or constant via right atrial pacing.

Results: Basal perfusion flow rate was 7.46 ± 0.15 ml/min/g wet weight (ww) (n=82), ANP release rate was 6.5 ± 0.3 ng/min/g ww and ET-1 release rate was 204 ± 30 fg/min/g ww. CFR less than 50% did not affect ANP release. Moderate (50-60% CFR) and severe (70-87% CFR) ischaemia for 30 minutes increased ANP, but not BNP or ET-1 release. There was no significant difference in the incremental ANP release between these two groups. Right atrial pacing at a rate 10% over spontaneous HR did not affect basal or ischaemia-induced ANP release. However, it unmasked ischaemia-induced impairment of cardiac systolic function where moderate ischaemia induced an impairment in dF/dt minimum as well as dF/dt maximum.

Conclusions: In this system, where atrial distension does not occur, ANP but not ET-1 release is stimulated by moderate (or severe) ischaemia. The relationship between the degree of ischaemia and extent of ANP release is not significantly modulated by presence/absence of ischaemia-induced bradycardia. Cardiac systolic and diastolic function was impaired by <50% CFR.

CHAPTER 4 - RESULTS

MECHANISMS OF ISCHAEMIA ASSOCIATED ANP RELEASE.

4.1. Introduction

Both atrial natriuretic peptide (ANP) and endothelins (ET) are potent vasoactive hormones which may have pathogenic relevance during myocardial ischaemia. Increased plasma ET concentrations are found in patients with unstable angina, myocardial infarction and immediately following percutaneous transluminal coronary angioplasty (PTCA) (Wieczorek *et al.* 1994; Malatino *et al.* 1993). ANP is also released under similar condition, i.e. during myocardial hypoxia (Toth *et al.* 1994), ischaemia (Malatino *et al.* 1992) and/or infarction (Tikkanen *et al.* 1987; Gutierrez-Marcos *et al.* 1991) with or without left ventricular systolic dysfunction. ANP release rate is also increased during ischaemia (as discussed in chapter 3). A potential mechanism for ANP release *in vivo* during ischaemia is atrial distension causing stretch of atrial myocytes. However, in the isolated perfused rat heart, Chen *et al.* (1993) have demonstrated ischaemia-induced ANP release may also occur independent of atrial distension; this has been confirmed by some of the studies included in chapter 3 of this thesis. The mechanism of this ischaemia-induced ANP release is not yet clear. However, one possibility is that ET-1 may stimulate ANP release, as previously demonstrated in non-ischaemic rat myocytes (Thibault *et al.* 1994), and that this might be accentuated during ischaemia.

ET's vasoactive effects are mediated by two types of receptors, ET_A and ET_B (Rubanyi & Polokoff 1994) (refer to chapter 1.3.). Endothelin receptor antagonists may therefore provide an opportunity to investigate the putative pathophysiological roles of endogenous ET. The selective ET_A receptor antagonist, BQ-123, inhibited ET-induced vasoconstriction in porcine coronary artery (Pernow *et al.* 1993), attenuated ET-1 and big ET-induced blood pressure rise and reduced infarct size in a canine model (Grover *et al.* 1993). Exogenous ET also stimulated secretion of natriuretic peptides by rat myocytes,

and this was blocked by BQ-123 (Thibault *et al.* 1994) indicating that ET_A receptor stimulation may mediate release of ANP.

Endothelial cells, in addition to ET, also release a vasodilator factor named endothelium-derived relaxing factor (EDRF), which possesses chemical and pharmacological activities that are identical with or closely similar to those of nitric oxide (NO). One important physiological stimulant of EDRF release is increased flow rate (Furchgott & Vanhoutte 1989). Co-culture of bovine aortic endothelial cells with neonatal rat atrial myocytes increased ANP release. This increased ANP release was inhibited partially (47%) by acetylcholine, which is known to evoke the release of EDRF (Lew & Baertschi 1989). However, methylene blue, oxyhaemoglobin, or hydroquinone, agents that are known to inhibit the release or effects of EDRF, caused an increase in ANP release (Sanchez-Ferrer *et al.* 1990). These findings therefore raise the possibility that endogenous EDRF or NO may also affect ANP release, but are inconsistent as regards the nett effect of NO on ANP release.

The aim of the present study was to investigate the putative role of NO and ET in ischaemia-induced ANP release, utilizing a selective NO synthase inhibitor, NG-nitro-L-arginine methyl ester (L-NAME), exogenous ET-1 and ET receptor antagonists (BQ-123 and bosentan) in the isolated perfused rat heart.

4.2. Methods

4.2.1. Isolated heart preparation

This preparation was identical to that described in chapter 2.1.2. Briefly, male Porton rats were anaesthetised and the heart rapidly excised. The aorta was cannulated, and the heart transferred to the Langendorff apparatus perfused with modified Eagles minimum essential medium. Medium was equilibrated with O₂-CO₂ (95:5 vol/vol) at 37°C for 40 min.

4.2.2. Protocol

Refer to chapter 2.1.3. Right atrium pacing was performed. After baseline sample collecting, hearts were perfused with L-NAME, (10⁻⁴ M), BQ-123 (10⁻⁶ M), or bosentan (10⁻⁵ M) for 40 min followed by 30 minutes perfusion with normal medium. BQ-123 (Auspep, Australia) and L-NAME (Sigma, MO, U.S.A.) were dissolved directly in Eagles minimum essential medium. Bosentan (Roche, Switzerland) was dissolved in 1 ml of distilled water at 37° C before addition to Eagles minimum essential medium. Global ischaemia (range 50-85% flow reduction) was induced 10 min after perfusion with ET or NO inhibitors had commenced. Exogenous ET-1 (5x10⁻¹⁰ M) was perfused for 30 min. Force of contraction and heart rate were monitored; volume of perfusate was measured; and coronary effluent collected for the measurement of ANP and ET-1 by RIA (refer to section 2.1.5 and 2.1.7.).

4.3. Results

4.3.1. Interaction between nitric oxide and ischaemia-induced ANP release

4.3.1.1. L-NAME effects on ANP release

Non-ischaemic hearts (Fig 4.1.a): In the absence of ischaemia and/or L-NAME (n=6), the basal ANP release rate did not fluctuate significantly (refer to chapter 3.3.). Forty minutes of L-NAME (10^{-4} M) perfusion (n=6) did not significantly affect ANP release rate as assessed utilizing the two-way ANOVA with repeated measure. However, it was noted that there was a transient increase in ANP efflux 20-30 minutes after initiation of L-NAME perfusion.

Ischaemic hearts: In the absence of L-NAME (n=22), ischaemia (56.9-76.6% coronary flow reduction) *per se* induced an increase in ANP release. The ANP release rate progressively increased after an initial decrease during the first 6 min of ischaemia. At the end of the 30 min ischaemic period, ANP release rate was $81.6 \pm 22.9\%$ over basal level. The peak ANP efflux rate was 1.94 ± 0.43 fold over basal efflux rate and occurred within the first minute of reperfusion. This ischaemia-stimulated ANP release was not significantly affected by the L-NAME (10^{-4} M) (unpaired *t* test, AUC) in the hearts with matched degree of ischaemia ($67.5 \pm 3.4\%$ coronary flow reduction, n=5) (Fig 4.1.b).

4.3.1.2. L-NAME effects on ET-1 release

Non-ischaemic hearts (Fig 4.2.a): In the absence of L-NAME (n=6), the basal ET-1 release rate did not fluctuate significantly. L-NAME (n=5) did not significantly affect ET-

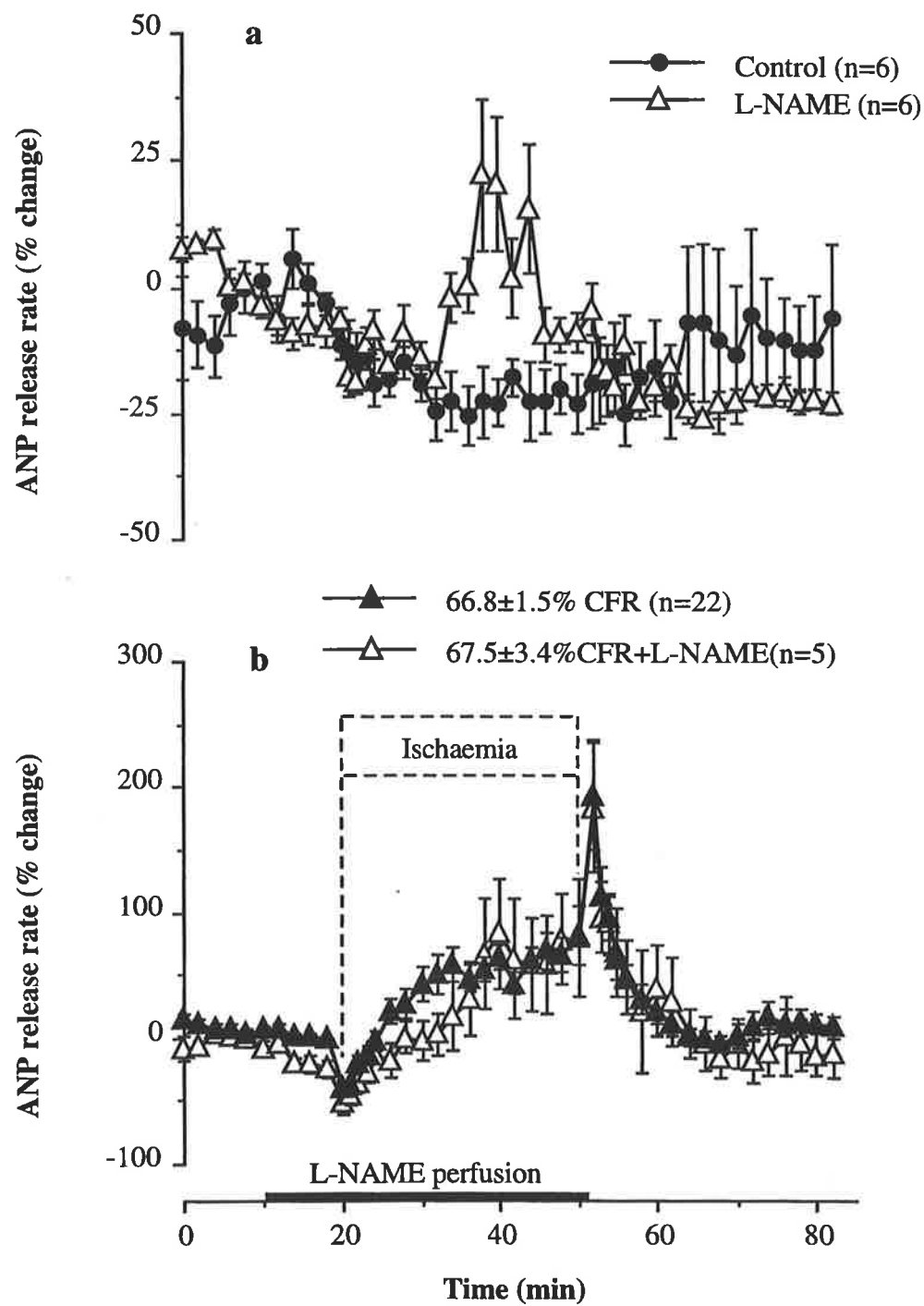


Fig 4.1. Effect of L-NAME on ANP release rate during normal perfusion (a) and ischaemia (b).

1 release rate.

Ischaemic hearts: In the absence of L-NAME (n=10), ischaemia did not significantly alter ET-1 efflux. L-NAME (n=5) did not affect ET-1 efflux rate (Fig 4.2.b).

4.3.1.3. Effect of L-NAME on coronary flow rate

Non-ischaemic hearts: In control hearts (n=6), there was no significant fluctuation in perfusion rate over the duration of the experiment (refer to 3.3.). During perfusion with L-NAME, there was a decrease of approximately 25% ($P<0.001$, two-way ANOVA with repeated measure, n=5) in perfusion rate. This difference between perfusion rates for L-NAME and control hearts persisted ($P<0.001$, two-way ANOVA with repeated measure) after wash-out of L-NAME (Fig 4.3.a).

Ischaemic hearts: In the absence of L-NAME (n=22), the induced decrease in flow rate was followed by a phase of post-reperfusion increase in perfusion flow rate. After perfusion with L-NAME, there was a pre-ischaemic flow reduction ($P<0.001$, two-way ANOVA with repeated measure). There was no significant difference in perfusion flow rate during the ischaemic period between the two groups. The post-ischaemic flow rate was significantly lower ($P<0.01$, two-way ANOVA with repeated measure) than for control hearts (Fig 4.3.b).

4.3.1.4. Effect of L-NAME on cardiac force of contraction

Non-ischaemic hearts: In the absence of L-NAME, there was no significant change in

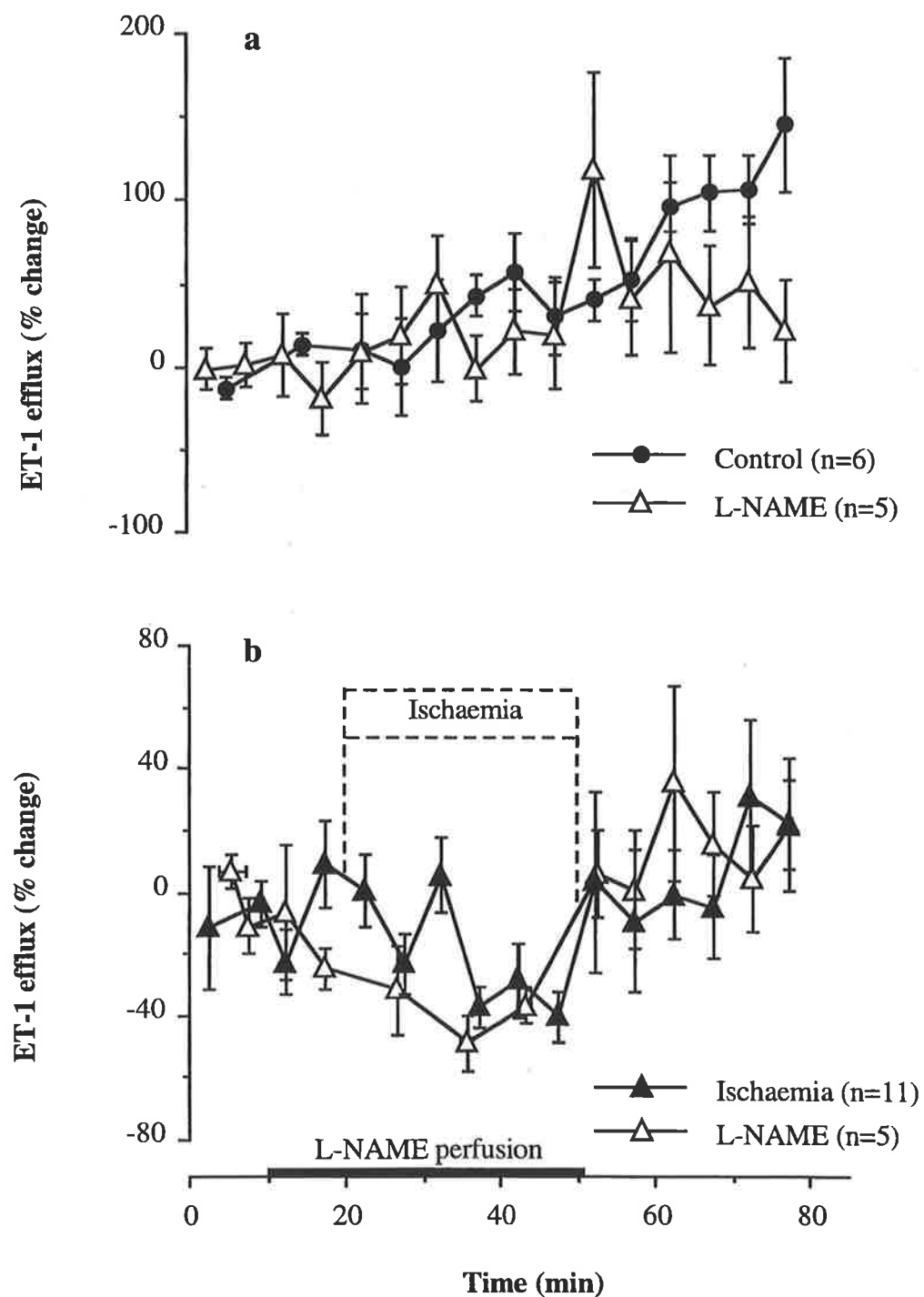


Fig 4.2. Effect of L-NAME on ET-1 release rate during normal perfusion (a) and ischaemia (b).

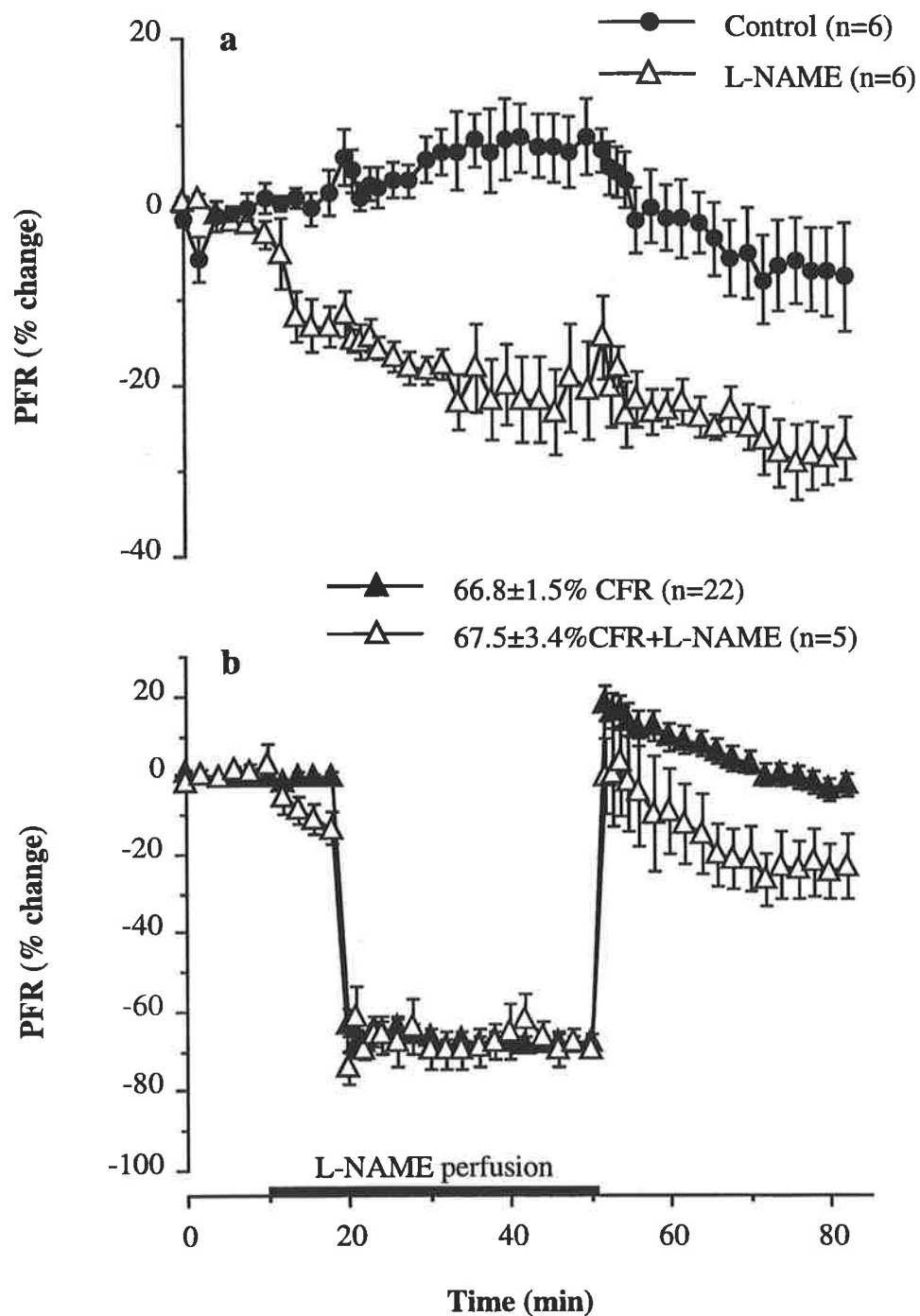


Fig 4.3. Effects of L-NAME (10⁻⁴ M) on coronary perfusion flow rate in normal perfusion hearts (a) and hearts with fixed coronary flow reduction (PFR, b).

force of contraction during the experimental period. L-NAME perfusion, started 10 min after the first baseline measurement, had no detectable effect on cardiac force of contraction (Fig 4.4.).

Ischaemic hearts: Ischaemia (n=22) *per se* induced a transient decrease in force of contraction ($P<0.05$, unpaired *t* test of AUC). L-NAME perfusion for 10 min prior to ischaemia had no significant effect on cardiac force of contraction. During global ischaemia, L-NAME tended to accentuate this ischaemia-induced decrease in cardiac force of contraction (Fig 4.4.), but the difference was not statistically significant ($P=0.096$, unpaired *t* test of AUC).

4.3.2. Interaction of endothelins with ischaemia-induced ANP release

4.3.2.1. Effect of exogenous ET-1 on ANP release

4.3.2.1.1. Exogenous ET-1 on perfusion flow rate

In normal perfused hearts (n=6), there was no significant change in perfusion flow rate over the duration of the experiment (refer to 3.3.). The perfusion flow rate decreased ($P<0.001$, two-way ANOVA with repeated measures) beginning within 2 min of ET-1 (5×10^{-10} M) perfusion (Fig 4.5.). This ET-induced coronary flow reduction ($61.1 \pm 6.6\%$ at 30 min of ET-1 perfusion) persisted during the 30 min post ET-1 perfusion phase.

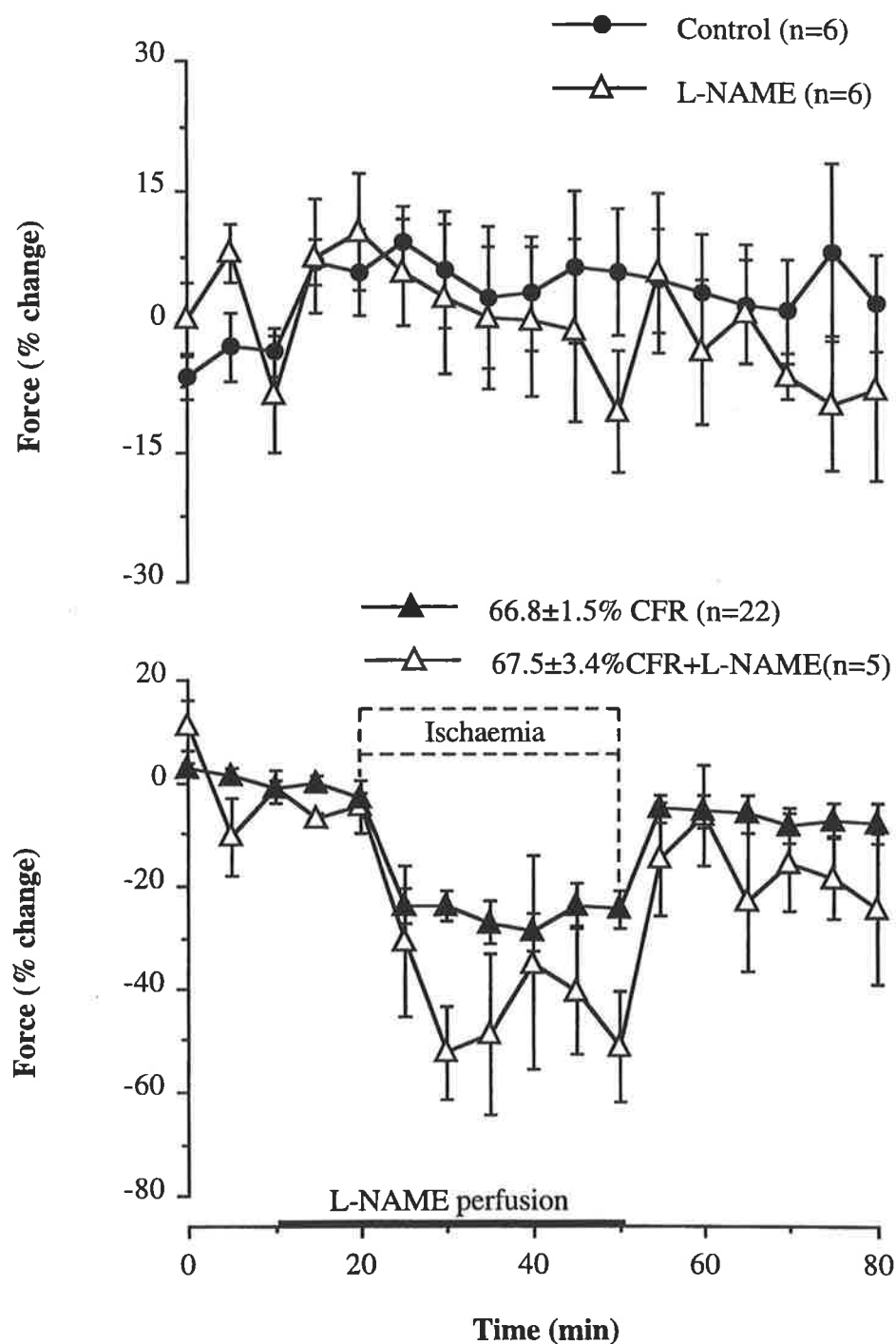


Fig 4.4. Effect of L-NAME on cardiac force of contraction in normal perfusion hearts and ischaemic hearts.

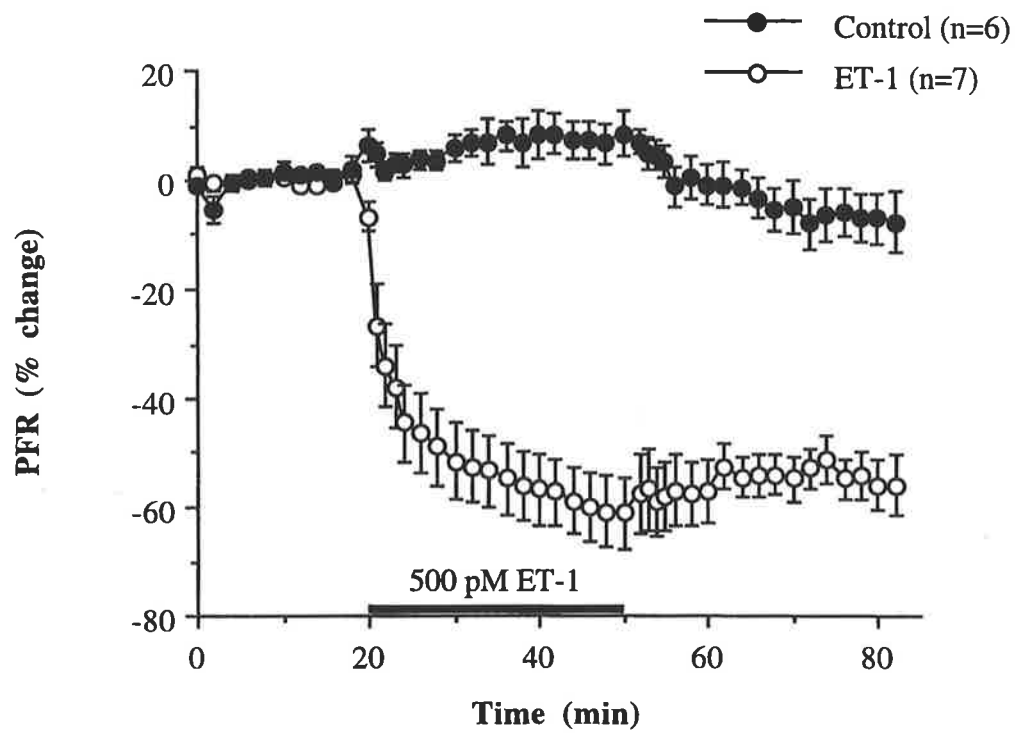


Fig 4.5. The vasoconstrictive effect of 5×10^{-10} M exogenous ET-1 perfusion in isolated perfused rat heart.

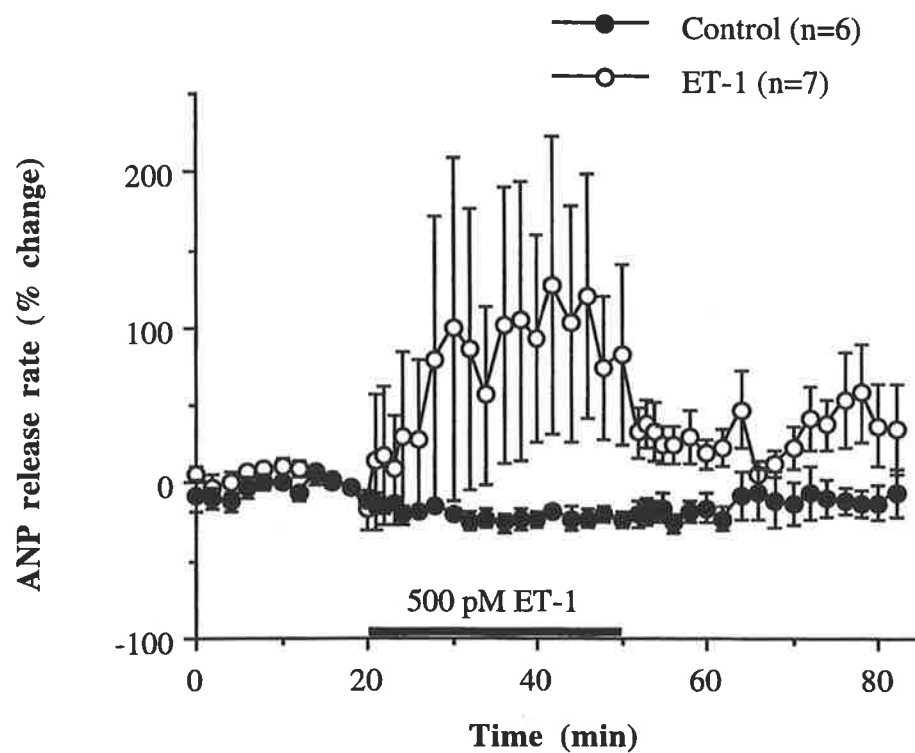


Fig 4.6. Effect of 30 minute exogenous ET-1 (5×10^{-10} M) perfusion on ANP release rate from isolated rat hearts.

4.3.2.1.2. Exogenous ET-1 on ANP release

In normal perfused hearts in the absence of ET-1 (Control group, n=6), the basal ANP release rate did not fluctuate significantly (refer to chapter 3.3.). A 30 min perfusion of ET-1 (5×10^{-10} M, n=7) did not significantly increase ANP release rate ($P=0.23$, unpaired t test of AUC) (Fig 4.6). During the post ET-1 perfusion (normal perfusion) phase, there was a significant increase in ANP release rate compared with that in control hearts ($P<0.01$, two-way ANOVA with repeated measures).

4.3.2.1.3. Exogenous ET-1 on cardiac force of contraction

In the absence of exogenous ET-1, there was no significant change in force of contraction during the experimental period. ET-1 (5×10^{-10} M) had no detectable effect on cardiac force of contraction during and after its perfusion (Fig 4.7.).

4.3.2.2. Effect of ET receptor antagonists on ANP release

In non-ischaemic hearts (Fig 4.8.): Initial basal ANP release rate was 6.5 ± 0.3 ng/min/g ww. This ANP release rate declined marginally throughout the experimental period of 80 min. Neither BQ-123 (10^{-6} M, n=7) nor bosentan (10^{-5} M, n=7) affected ANP release rate.

In ischaemic hearts (Fig 4.9.): During severe ischaemia ($75.3 \pm 0.9\%$ coronary flow reduction), there was increased ANP release ($P<0.0001$, unpaired t test of AUC n=16) with transient post-ischaemic ANP wash-out (refer to chapter 3.3). There was no significant variation in ANP release in the presence of BQ-123 (n=5). Moderate ischaemia ($58.2 \pm 0.9\%$ coronary flow reduction) *per se* also induced a significant increase in ANP

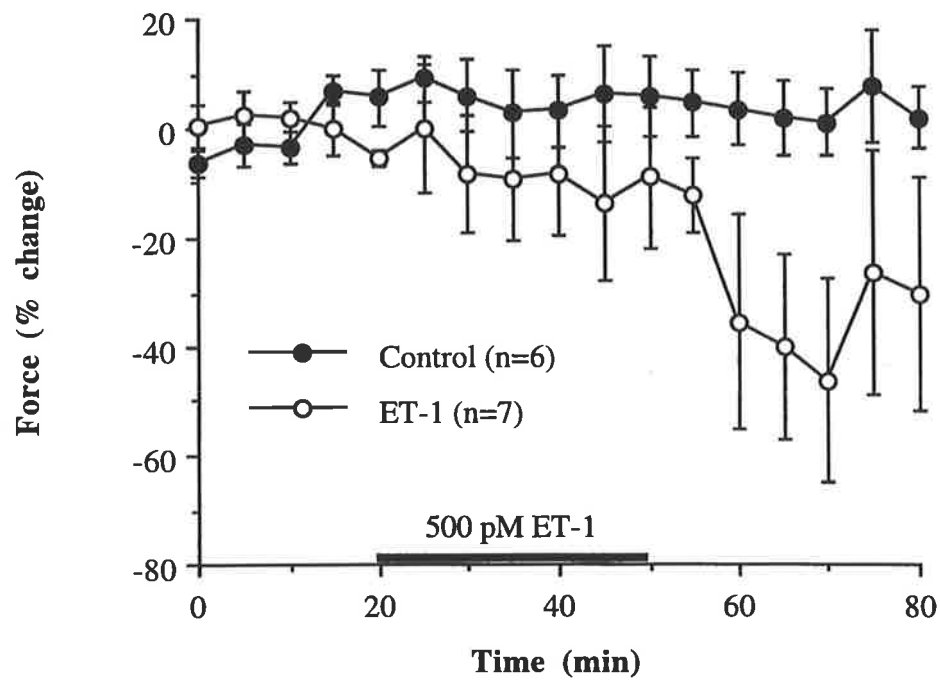


Fig 4.7. Effect of 5×10^{-10} M ET-1 perfusion on contractile force of isolated rat hearts

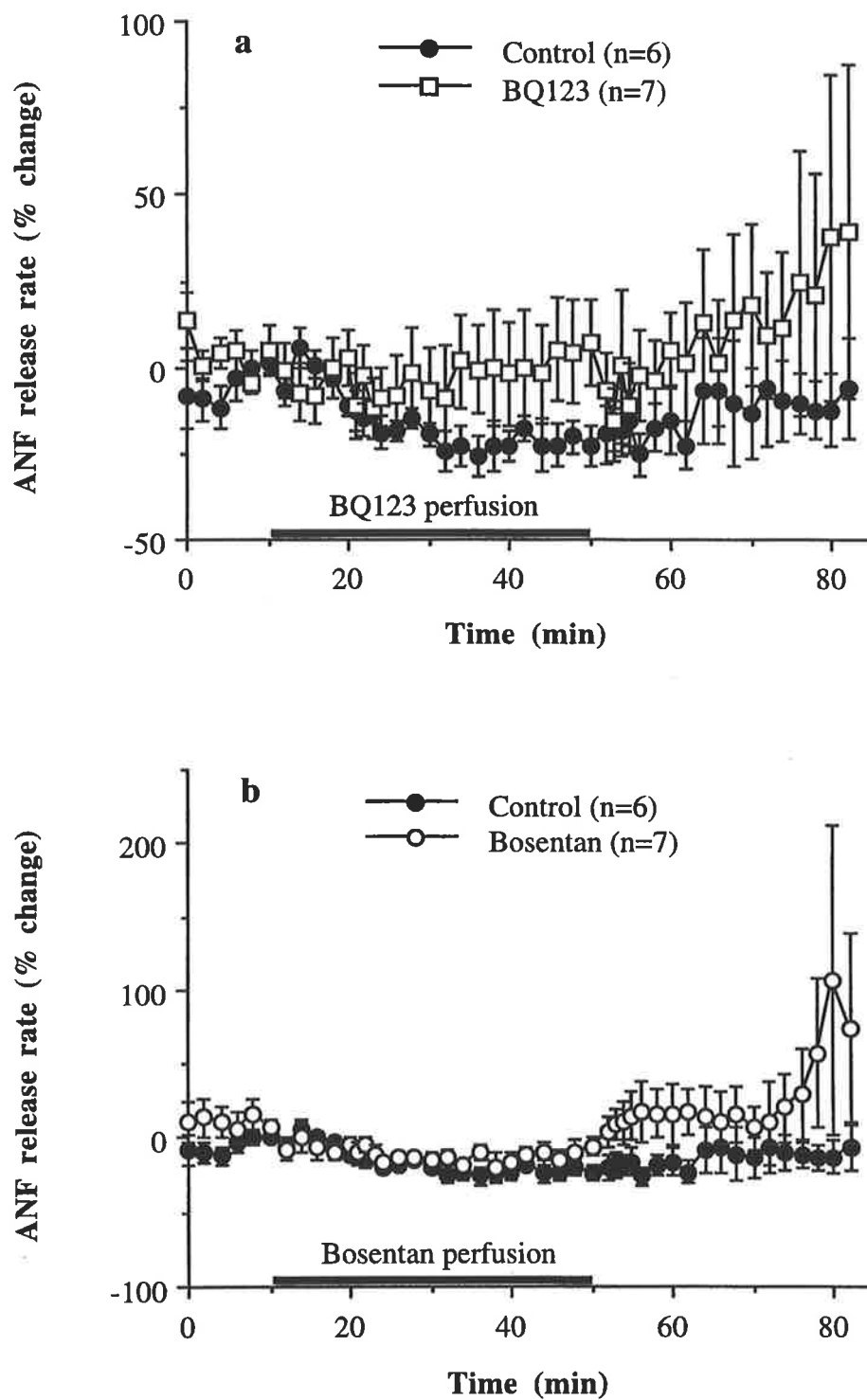


Fig 4.8. The effects of BQ-123 (10^{-6} M) or bosentan (10^{-5} M) perfusion on ANP release rate compared with control hearts.

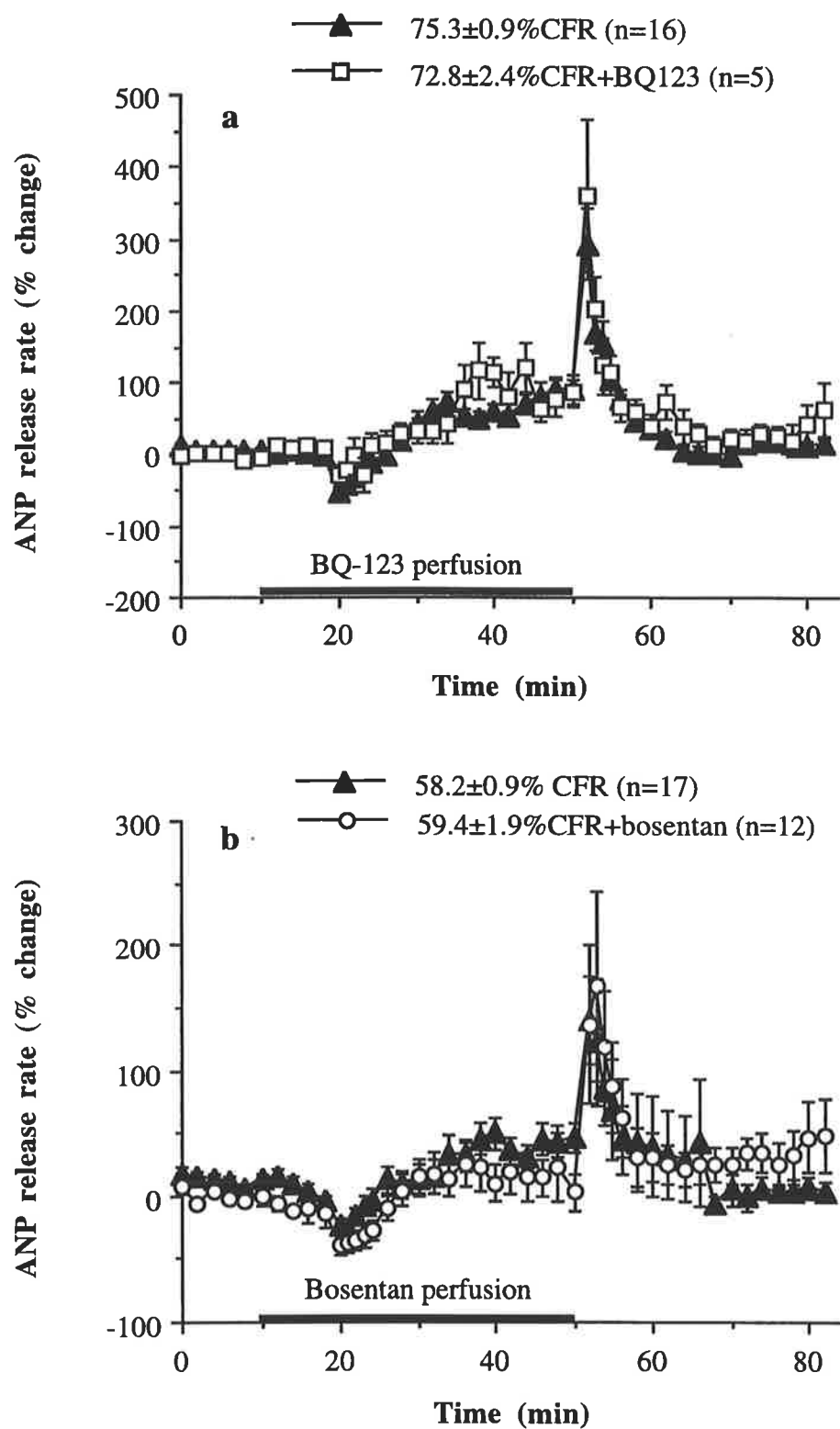


Fig 4.9. The effects of BQ-123 and bosentan on ischaemia-induced ANP release compared to hearts with the same degree of ischaemia.

release rate ($P < 0.01$, $n = 17$). The mean areas under ANP release/time curves for the same degree of ischaemia in the presence or absence of bosentan were 315 ± 1426 and 1074 ± 1363 units respectively ($P = 0.16$, unpaired t test).

4.4. Discussion

The overall major conclusion to be drawn from the experiments described in this chapter is that, despite the *a priori* arguments in favour of putative interactions, neither endothelin nor NO are major modulators of ischaemia-induced ANP release in this paradigm. The concentration of L-NAME used in the current study is high enough to have induced a 20-30% coronary flow reduction, which of itself does not affect ANP efflux (see chapter 3). A previous study showed L-arginine, a NO precursor did not alter plasma ANP level in human subjects (Smulder *et al.* 1994). In conscious rats, L-NAME infusion increased plasma concentrations of vasopressin but not atrial natriuretic peptide (Yamaoto *et al.* 1994). However, in a similar paradigm, bolus intravenous injection of L-NAME increased plasma levels of ANP and mean arterial pressure (Leskinen *et al.* 1995). A further study showed that acetylcholine partially inhibited the increase in ANP release induced by co-culture of endothelial cells with atrial myocytes (Lew & Baertschi 1989). The finding of coronary vasoconstriction during and after L-NAME perfusion in non-ischaemic and ischaemic hearts was consistent with previous studies (Ahlborg & Lundberg 1997, Edwards *et al.* 1996). Thus, as far as ANP : NO interaction is concerned, it must be presumed that any NO-dependent ANP release does not share a common mechanism with that seen in this ischaemic/non-atrial-distended heart.

Exogenous ET-1 (10^{-9} to 10^{-6} M) augmented basal and stretch-induced ANP (and BNP) secretion in rat myocytes (Thibault *et al.* 1994, Horio *et al.* 1993, Leite *et al.* 1994, Irons *et al.* 1993, McDonough *et al.* 1994) and rat atria (Skvorak *et al.* 1995, Hu *et al.* 1988). In intact animals, ET-1 infusion increased ANP release as well as arterial pressure (Cheung 1994). In a constant-flow perfusion rat heart paradigm (with intact atrium), where ET-1-induced vasoconstriction did not change the coronary flow rate, ET-1 (2.3×10^{-9} M) perfusion increased ANP release (Mantymaa *et al.* 1990). In the studies

presented here, ET-1 (5×10^{-10} M) perfusion did not induce a significant increase in ANP release rate, although it did cause an immediate decrease in perfusion flow rate. However, there was a significant increase of ANP release post ET-1 perfusion. A higher concentration of ET-1 (10^{-9} M) was tested in a preliminary study but found to cause profound arrhythmia and cessation of coronary flow.

Under normoxic condition, ANP secretion enhanced by exogenous ET was inhibited by BQ-123 (10^{-6} to 10^{-5} M) (Thibault *et al* 1994, Skvorak *et al* 1995, Irons *et al.* 1993, Leite *et al.* 1994), but not affected by ET_B agonists (Thibault *et al* 1994) suggesting that ET_A receptors may mediate the ET-induced ANP release. Furthermore, BQ-123 and bosentan inhibit volume-loading-induced ANP release indicating that endogenous ET plays a role in stretch-induced ANP release (Leskinen *et al* 1997). However, the role of endogenous ET on ischaemia-induced ANP release has not been determined. In this study, neither bosentan (10^{-5} M) nor BQ-123 (10^{-6} M) affected ischaemia-stimulated ANP release indicating endogenous ET did not mediate ischaemia-induced ANP release. The intracellular mechanism for ischaemia-induced ANP release therefore remains unclear.

The role of NO on ET-1 release is controversial. L-NAME increased plasma ET-1 level *in vivo* (Edwards *et al* 1996, Cao *et al* 1995). In perfused rat adrenal gland, L-NAME stimulated, but L-arginine inhibited ET-1 release (Hinson *et al* 1996). L-NAME also stimulates ET-1 release from cultured human umbilical vein endothelial cells (Cao *et al* 1995). Another NO inhibitor, NG-monomethyl-L-arginine (L-NMMA), has also been reported to stimulate ET-1 release in the human pulmonary artery (Ahlbory & Lundberg 1997). Inhaled NO induced an initial increase and a subsequent decline of plasma ET-1 concentrations in human (Christou *et al* 1997). However, L-NAME inhibited ischaemia-induced ET-1 release from pancreas in humans (Peralta *et al* 1997). In bovine pulmonary

artery endothelial cells, NO donors downregulated ET-1 production (Ryan *et al* 1993). In the studies presented here, neither basal nor ischaemia-induced ET-1 release rate were affected by L-NAME suggesting NO did not modify the ET-1 release from coronary artery endothelium and endocardium. The exogenous ET-1 perfusion increased ET-1 concentration to about 100-fold in the coronary effluent (data not shown). The effects of BQ-123 and bosentan on ET-1 efflux are presented in chapter 5.

The phenomenon of increased ANP release associated with infusion of ET-1 in concentrations sufficient to decrease coronary flow by approximately 60% is likely to reflect the reduction in coronary perfusion rather than any specific ET-1 effect. Similarly, it is possible that accentuation of flow reduction (say from 35% in controls to 55% in L-NAME-treated hearts) might have (in therapy) led to a similar effect.

Although the accentuation of negative inotropic effects of ischaemia with L-NAME did not reach statistical significance, these may be of considerable interest as regards understanding of the physiological effects of endogenous NO, and are worthy of detailed examination as a future project.

The studies performed in this thesis therefore eliminated some potential mechanisms of ANP release induced by ischaemia. It can now be stated with some certainty, that neither ET-1 nor NO modulate this phenomenon. Previous investigations (Chen *et al.* 1993) showed that ischaemia-induced ANP release was completely abolished by either meclofenamate (10^{-5} M), a cyclo-oxygenase inhibitor, or nordihydroguaiartaric acid (10^{-5} M), a lipoxygenase inhibitor, superficially suggesting the possible involvement of either cyclo-oxygenase or lipoxygenase in the modulation of ischaemia-induced ANP release, if one of the inhibitors was specific. These postulated mechanisms of effect are mutually

exclusive; hence there is considerable uncertainty as to the specificity of these interventions.

Hypoxia has been shown to induce ANP release in the isolated perfused rat and rabbit hearts (Lew & Baertschi 1989, Baertschi *et al.* 1986). Myocyte stretch increases myocardial oxygen consumption (Ruskoaho 1992). Although atrial pressure did not change in the experiments included in this chapter, tissue hypoxia may represent an underlying mechanism for ANP release. For further discussion of hypoxia-induced ANP release refer to chapter 6.

Calcium (Ca^{2+}) is an important second messenger in regulating secretion of ANP (Schiebinger *et al.* 1994). Plasma ANP concentrations were significantly higher in rats after CaCl_2 infusion and in the absence of significant changes in mean arterial blood pressure (Fujimura *et al.* 1989). In isolated rat hearts, the Ca^{2+} ionophore A23187, which introduces free Ca^{2+} into the cell, increased ANP release (Ruskoaho *et al.* 1985). Calcium antagonists, such as nifedipine and verapamil, inhibited ANP secretion (Iida & page 1989, Weinmann 1991). During ischaemia, intracellular Ca^{2+} was increased (Elz & Nayler 1987). Therefore, there is a possibility that increases in intracellular Ca^{2+} during ischaemia may contribute to the ischaemia-induced ANP release. However, the current experiments in beating hearts were not designed to test this hypothesis.

4.5. Summary

The studies presented here used a modified Langendorff perfused rat heart with experimentally induced low flow ischaemia in the presence or absence of the L-NAME, exogenous ET-1 and ET receptor antagonists, BQ-123 or bosentan, in order to define the putative roles for NO and ET in mediating ANP release.

Isolated rat hearts were perfused using a Langendorff apparatus with tissue culture medium under a constant pressure of 70 cm H₂O and heart rate maintained constant via atrial pacing. Exogenous ET-1 (5×10^{-10} M) was perfused for 30 minutes. L-NAME (10^{-4} M), BQ-123 (10^{-6} M) and bosentan (10^{-5} M) were perfused for 40 minutes. Global ischaemia was induced 10 minute after L-NAME, BQ-123 or bosentan perfusion. ET-1 and ANP were measured by RIA.

Results are expressed as mean $\pm$ SEM. L-NAME, BQ-123 and bosentan did not affect basal or ischaemia-induced ANP release. Exogenous ET-1 perfusion induced a late (i.e. post ET-1 perfusion) increase in ANP release ($P < 0.01$). L-NAME perfusion did not alter ET-1 release rate from rat heart. Both L-NAME and exogenous ET-1 induced significant perfusion flow reduction, but none of them significantly affected cardiac force of contraction.

These results suggested endogenous ET and NO do not mediate basal and low flow ischaemia-induced ANP release.

CHAPTER 5 - RESULTS

EFFECTS OF ENDOTHELIN ANTAGONISTS ON CORONARY FLOW, ENDOTHELIN-1 RELEASE AND SYSTOLIC FUNCTION.

5.1. Introduction

Endothelins (ET) are a family of vasoactive hormones secreted mainly from endothelium. Their vasoconstrictor and vasodilator effects are mediated by two types of receptors, ET_A and ET_B (Rubanyi & Polokoff 1994). The ET_A receptor is classified by having greater affinity for ET-1 than ET-3 whereas the ET_B receptor is nonisopeptide-selective. ET_A receptor stimulation has generally been found to mediate vasoconstriction. The ET_B receptor subtype located on smooth muscle also mediates vasoconstriction. It appears to be functionally distinct from the ET_B subtype located on the vascular endothelium which mediates vasorelaxation via the release of NO (Davenport & Maguire 1994, Pernow & Modin 1993). ET_B receptors play a role in clearance of ET from the circulation by endothelial cells (Fukuroda *et al* 1994).

ET's potent vasoactive effects may have pathogenic relevance during myocardial ischaemia. Increased plasma ET concentrations are found in patients with unstable angina, myocardial infarction and immediately following percutaneous transluminal coronary angioplasty (Wieczorek *et al* 1994, Malatino *et al* 1993). Endogenous ET is also released into the coronary perfusate of isolated perfused rat hearts during ischaemia/reperfusion (Brunner *et al* 1992). In cultured human endothelium, hypoxia induces ET gene expression and secretion (Kourembanas *et al* 1991). Whilst ischaemia caused an increase in ET-1 binding site density (Liu *et al* 1989, Nayler *et al* 1992). Furthermore, hypoxia increased the maximal responses of ET-1 and ET-3 in the rat isolated perfused mesenteric arterial bed (Douglas *et al* 1991).

Currently available methodologies for investigating the putative pathophysiological role of endogenous ET include the use of endothelin converting enzyme inhibitors, anti-

endothelin antibodies and endothelin receptor antagonists. A specific ET_A receptor antagonist, BQ-123, has been shown to inhibit ET-induced vasoconstriction in porcine coronary artery (Pernow & Modin 1993), to attenuate the hypertensive response to ET-1 and big ET in rats (McMurdo *et al* 1993), and to reduce infarct size in a canine model (Grover *et al* 1993). However, BQ-123 failed to inhibit ET-induced coronary vasoconstriction in the Langendorff perfused rat heart (Balwierczak 1993). Seo *et al.* using bosentan (Ro 47-0203), an ET_A/ET_B receptor antagonist, demonstrated that both ET_A and ET_B receptors mediate contraction to ET-1 in isolated human blood vessels (Seo *et al* 1994). In isolated rat hearts, bosentan (10⁻⁵ M) increased coronary flow but did not influence contractility (Dagassan *et al* 1994). However, to date there are only limited data on the effect of ET_A and ET_{A/B} antagonists on the relationship between endothelin production and coronary vasomotor tone during ischaemia and reperfusion.

Exogenous endothelin-1 (ET-1) has also been shown to exert a positive inotropic effect on isolated cardiac myocytes and papillary muscles (Kelly *et al* 1990, McClellan *et al* 1996, Evans *et al* 1994, Kasai *et al* 1994, Endoh *et al* 1996). However, *in vivo* studies could not demonstrate a positive inotropic effect of exogenous ET-1, almost certainly because of concomitant induction of myocardial ischaemia via coronary vasoconstriction. (Beyer *et al* 1995, Domenech *et al* 1991, Gulati *et al* 1996, Cirino *et al* 1994 & 1997, Donckier *et al* 1994). The development of ET receptor antagonists also offers a means of obtaining inferential information concerning the inotropic effects of ET *in vivo*. To date; studies with intravenous administration of bosentan in patients with severe chronic heart failure have demonstrated both an increase in cardiac output and a decrease in peripheral vascular resistance (Kiowski *et al* 1995, Sutsch *et al* 1996, Spinal *et al* 1997, Shimoyama *et al* 1996); these findings suggest little fluctuation in underlying inotropic state. However, it is known that ET-1 is released during acute myocardial ischaemia (Ray *et al* 1992). Hence the inotropic effects of ET may fluctuate during

ischaemia, with consequent variability in effects of ET antagonists in modifying contractile state during severe myocardial ischaemia.

The aim of the present study was to examine, in the perfused rat heart, the effects of BQ-123 and bosentan on coronary flow, release of ET-1 into coronary effluent and the contractile state during, and following global myocardial ischaemia.

5.2. Methods

Refer to chapter 2.1.

Protocol

Isolated hearts were perfused and paced following the procedure outlined in chapter 2.1. After 40 min equilibration, baseline samples of coronary effluent were collected for 10 minutes. All agents were soluble in perfusate. Hearts were perfused with BQ-123 (10^{-6} M) or bosentan (10^{-5} M) for 40 min followed by 30 min with normal medium, minus ET receptor antagonist. In some hearts ($n=5$ for BQ-123; $n=12$ for bosentan), global ischaemia (perfusion flow reduced by $72.8 \pm 2.4\%$ for BQ-123; $59.4 \pm 1.9\%$ for bosentan) was induced 10 min after ET receptor antagonist perfusion had commenced. The basis for the disparate reductions in coronary perfusion rate with the two ET receptor antagonists was the development of bradyarrhythmias when bosentan-pretreated hearts were subjected to $\geq 67\%$ flow reduction. While the basis for this phenomenon was not elucidated, the reductions in perfusion rate were maximal for maintenance of constant heart rate for both agents tested. Coronary flow rate was measured. Force of contraction and heart rate were monitored. Coronary effluent was collected for ET-1 RIA.

5.3. Results

5.3.1. Effect of BQ-123 and bosentan on coronary perfusion flow rate

Non-ischaemic hearts: Basal coronary perfusion rate was 7.5 ± 0.2 ml/min/g wet heart weight. In control hearts ($n=6$), there was no significant fluctuation in perfusion rate over the duration of the experiment. During perfusion with BQ-123 (10^{-6} M) for 40 min, there was a progressive ($P < 0.0001$, two-way ANOVA with repeated measures) decrease in perfusion rate. The resultant difference between perfusion rates for BQ-123 and control hearts persisted ($P = 0.01$, two-way ANOVA with repeated measures) after wash-out of BQ-123. In contrast, neither bosentan (10^{-5} M) perfusion, nor subsequent wash-out induced any significant changes in perfusion rate (Fig 5.1.).

Ischaemic hearts: In the absence of BQ-123 or bosentan, the induced decrease in flow rate was followed by a phase of post-reperfusion increase in perfusion flow rate ($P < 0.0001$, two-way ANOVA with repeated measures) (Fig 5.2.a and 5.2.b). After perfusion with BQ-123 (Fig 5.2.a), post-ischaemic flow rate was significantly ($P < 0.05$, two-way ANOVA with repeated measures) lower than for control hearts. Although bosentan tended to cause a mild increase in perfusion flow rate during the pre-ischaemic phase, the effect was not significant. Bosentan did not significantly affect the post-ischaemic flow rates (Fig 5.2.b).

5.3.2. Effect of BQ-123 and bosentan on ET-1 release

Non-ischaemic hearts (Fig 5.3.): Basal ET-1 release rate was 211 ± 14 pg/min/g wet

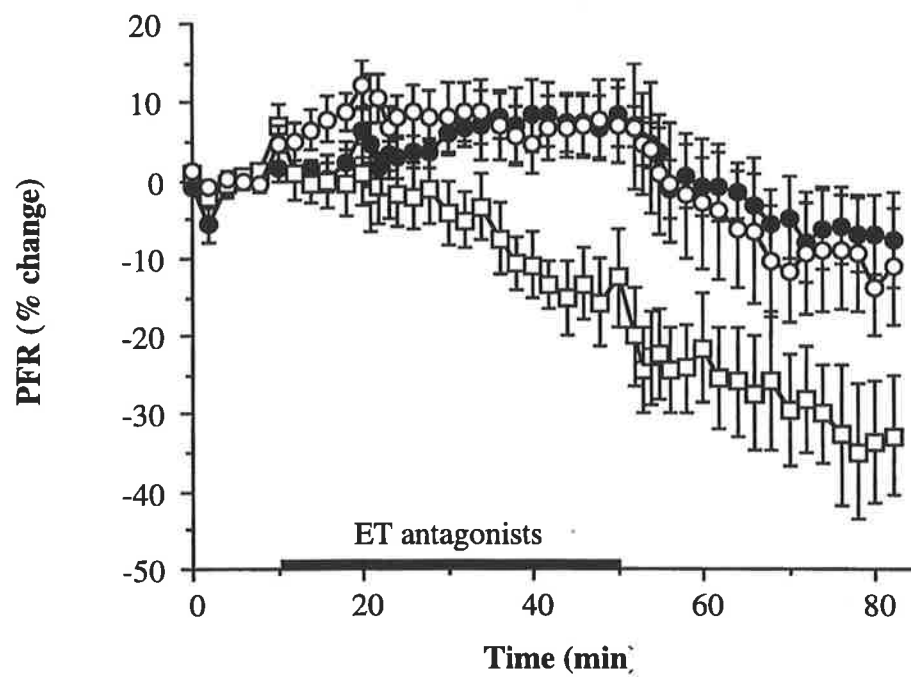


Fig 5.1. Effects in non-ischaemic hearts of BQ-123 (10^{-6} M) (□, n=7) and bosentan (10^{-5} M) (○, n=7) on perfusion flow rate (PFR) compared with control (●, n=6).

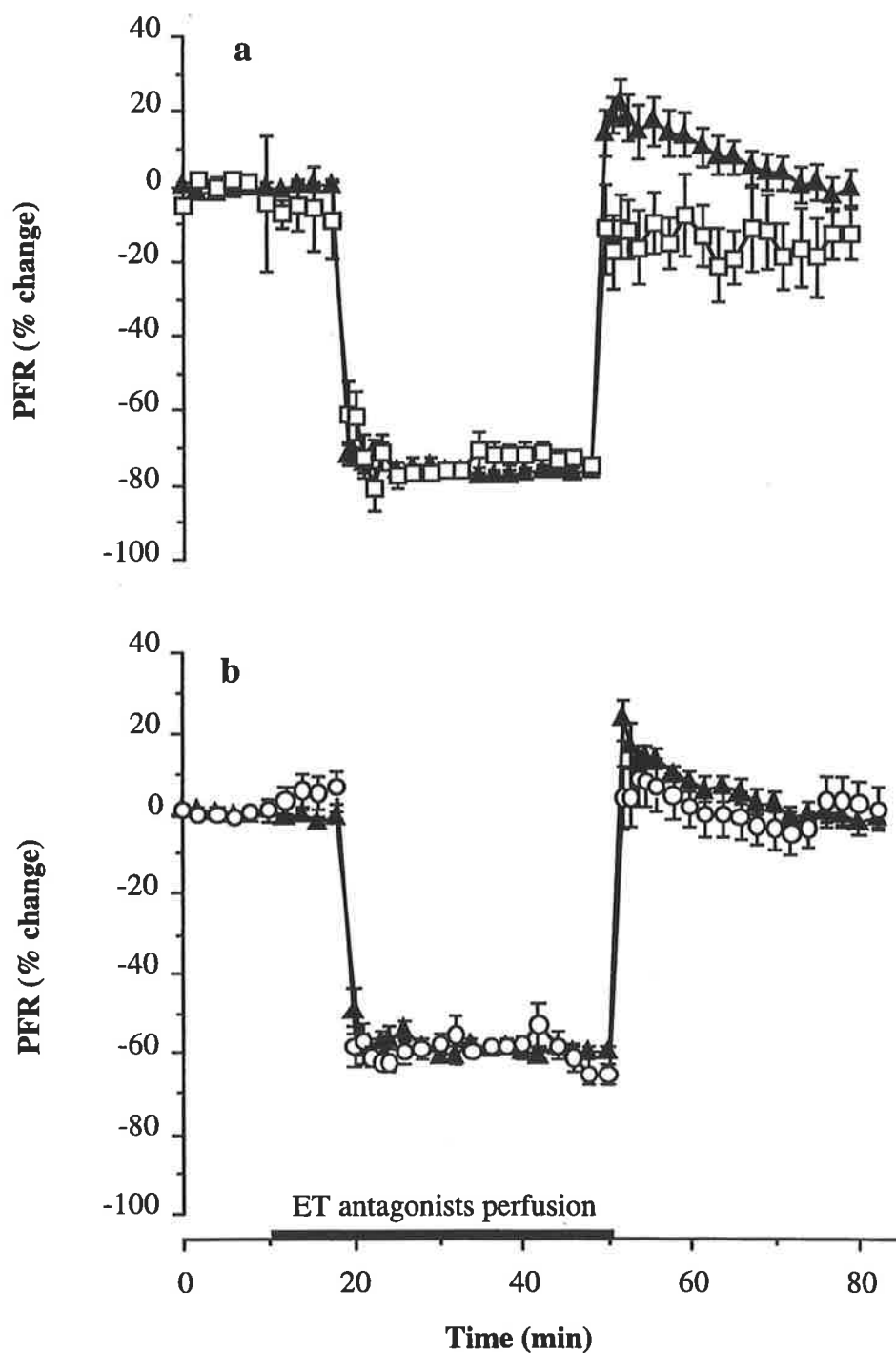


Fig 5.2. Effect of BQ-123 (10^{-6} M) (□, n=5) and bosentan (10^{-5} M) (○, n=12) on perfusion flow rate (PFR) in ischaemic hearts compared with the same degree of ischaemia in the absence of ET receptor antagonists (▲, n=16-17).

heart weight. In the absence of ischaemia and/or ET receptor antagonists, this basal rate did not fluctuate significantly. Almost immediately after BQ-123 perfusion had commenced, ET-1 efflux rate increased ($P<0.05$, unpaired t test of AUC), the increase within 5 min being $730\pm188\%$. This increase in ET-1 efflux was sustained throughout the BQ-123 perfusion period. Ten min post BQ-123 wash-out, the ET-1 efflux rate had returned to baseline. Bosentan caused a gradual but sustained increase ($P<0.0001$, unpaired t test of AUC) in ET-1 efflux rate with a peak increase of $315\pm81\%$ after 30 minutes. The increase was significantly less than the increase in ET-1 efflux induced by BQ-123 ($P<0.05$, two-way ANOVA with repeated measures). Following wash-out of either BQ-123 or bosentan, the ET-1 efflux rate returned towards baseline levels.

Ischaemic hearts: In the absence of BQ-123, ischaemia ($75.3\pm0.9\%$ perfusion flow reduction) did not alter ET-1 efflux, but there was a transient increase in ET-1 efflux during reperfusion. The increase in efflux induced by BQ-123 was less marked during ischaemia than in its absence ($P<0.05$, unpaired t test of AUC) (Fig 5.4.a). In the absence of bosentan, moderate ischaemia ($58.2\pm0.9\%$ flow reduction) did not affect ET-1 efflux. However, with the same level of ischaemia, ET-1 efflux in the bosentan-treated group was significantly increased ($P<0.001$, unpaired t test of AUC) (Fig 5.4.b), with a small secondary increase ($P<0.0001$, two-way ANOVA with repeated measures) in ET-1 efflux following reversal of ischaemia.

5.3.3. Effect of BQ-123 and bosentan on force of contraction

Non-ischaemic hearts: In the absence of ET-1 receptor antagonists, there was no significant change in force of contraction during the experimental period. BQ-123 or bosentan perfusion, started ten minutes after the first baseline measurement, had no detectable effect on cardiac force of contraction. However, bosentan induced a significant

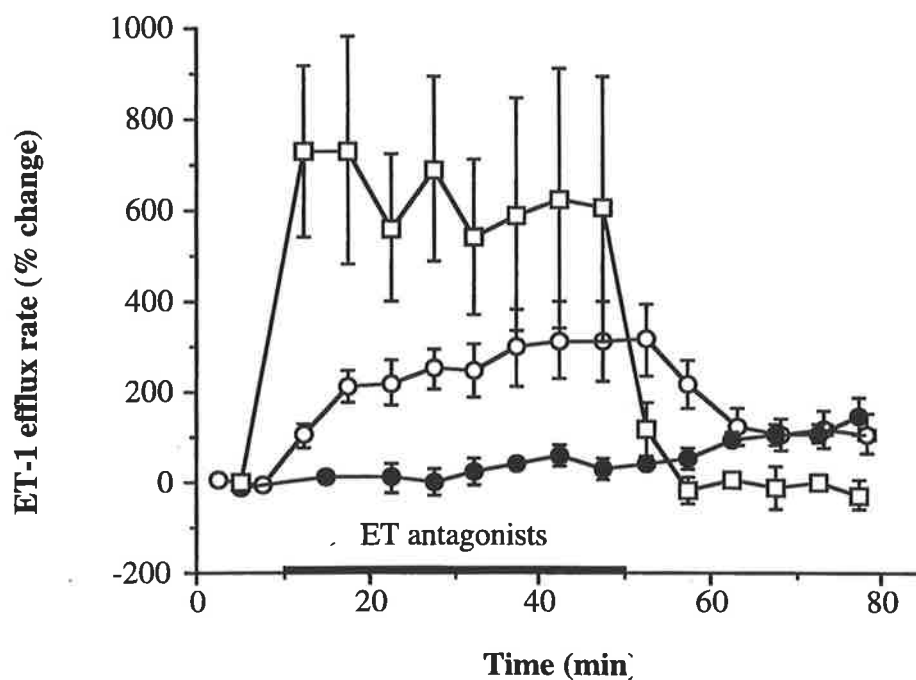


Fig 5.3. Effect of BQ-123 (10^{-6} M) ($\square$, $n=7$) and bosentan (10^{-5} M) ($\circ$, $n=7$) compared with control ($\bullet$, $n=6$) on endothelin-1 (ET-1) efflux rate in non-ischaemic hearts.

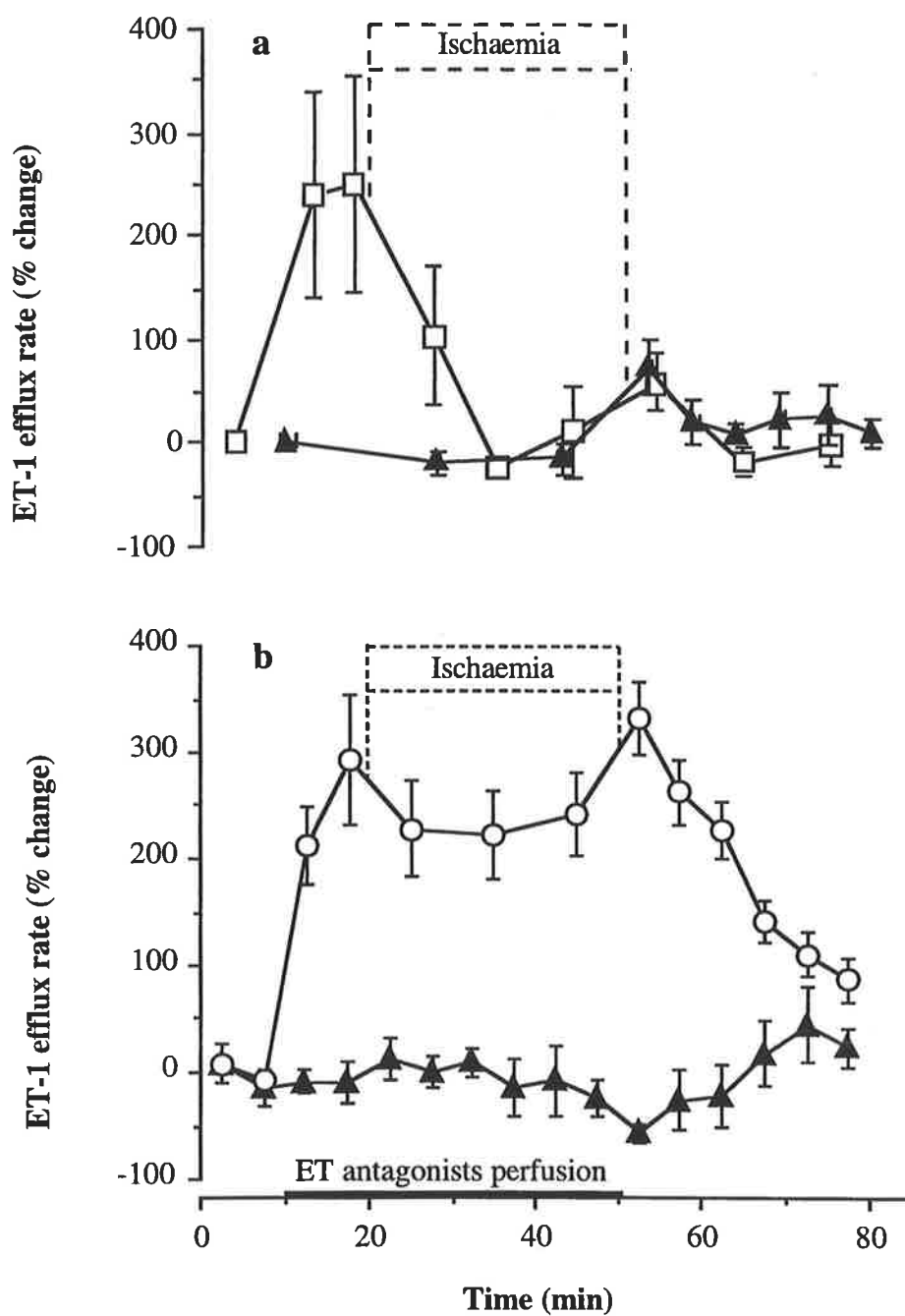


Fig 5.4. Effects in ischaemic-reperfused hearts of BQ-123 (10^{-6} M) (□, n=5) and bosentan (10^{-5} M) (○, n=12) on endothelin-1 (ET-1) efflux rate compared with control ischaemia/reperfusion(▲, n=16-17).

change in the force-time interaction relative to that seen in control hearts (Two-way ANOVA with repeated measures; $F=2.977$, $P=0.008$). This implies a trend towards progressive reduction in force of contraction during bosentan infusion (Fig 5.5.).

Ischaemic hearts: Severe ischaemia ($n=16$) *per se* induced a transient decrease in force of contraction ($P<0.005$, unpaired t test of AUC). BQ-123 perfusion for 10 minutes prior to ischaemia had no effect on cardiac force of contraction. However, during global ischaemia, BQ-123 showed a tendency to accentuate ($P=0.06$, unpaired t test of AUC, $n=5$) the ischaemia-induced decrease in cardiac force of contraction (Fig 5.6.a). Fig 5.6.b illustrates the effect of bosentan on cardiac force of contraction in ischaemic hearts. In the absence of bosentan, coronary flow reduction induced a transient decrease in force of contraction ($P<0.05$ vs control, unpaired t test of AUC, $n=17$). Ten min pre-ischaemic bosentan perfusion did not alter the cardiac force of contraction. However, bosentan induced a further decrease in force of contraction ($P<0.05$, unpaired t test of AUC, $n=12$) during the ischaemic phase. During reperfusion, the recovery of cardiac systolic function was impaired in bosentan-treated groups ($P<0.05$, two-way ANOVA with repeated measures).

5.3.4. Effect of BQ-123 and bosentan on cardiac relaxation in ischaemic hearts

Fig 5.7. illustrates the effects of global ischaemia and reperfusion on myocardial relaxation (shown as dF/dt min) in the presence or absence of bosentan (refer to chapter 3.3.3.2). The impairment of relaxation documented during ischaemia utilizing this measure was not significantly affected by perfusion with bosentan. Furthermore, recovery of relaxation during reperfusion was similarly incomplete for bosentan and control hearts.

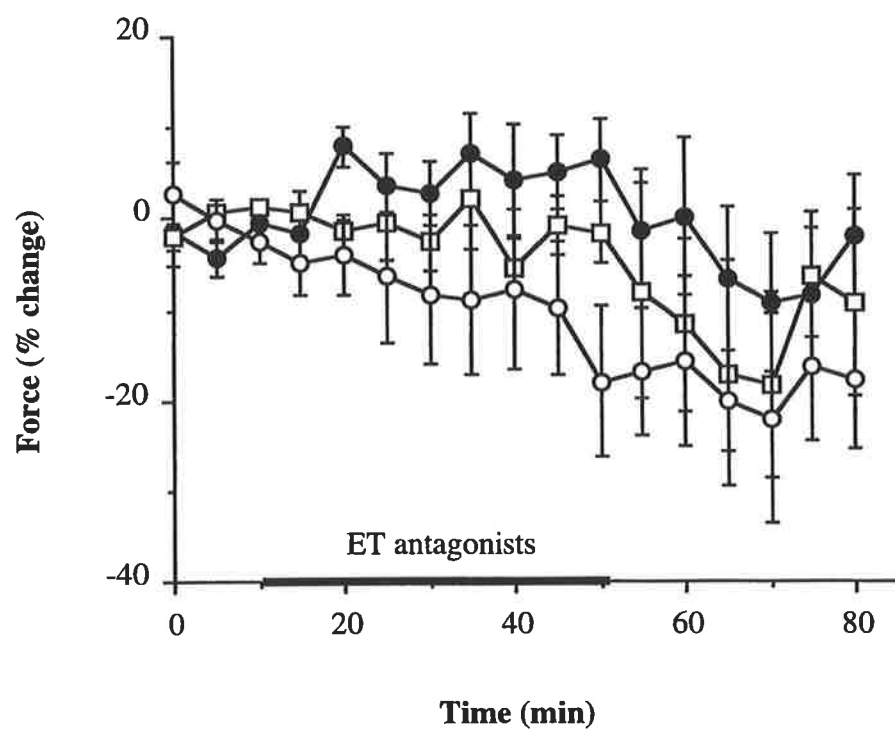


Fig 5.5. Effects in non-ischaemic hearts of bosentan (10^{-5} M) (○, n=7) on force of contraction (Force) compared with control (●, n=6).

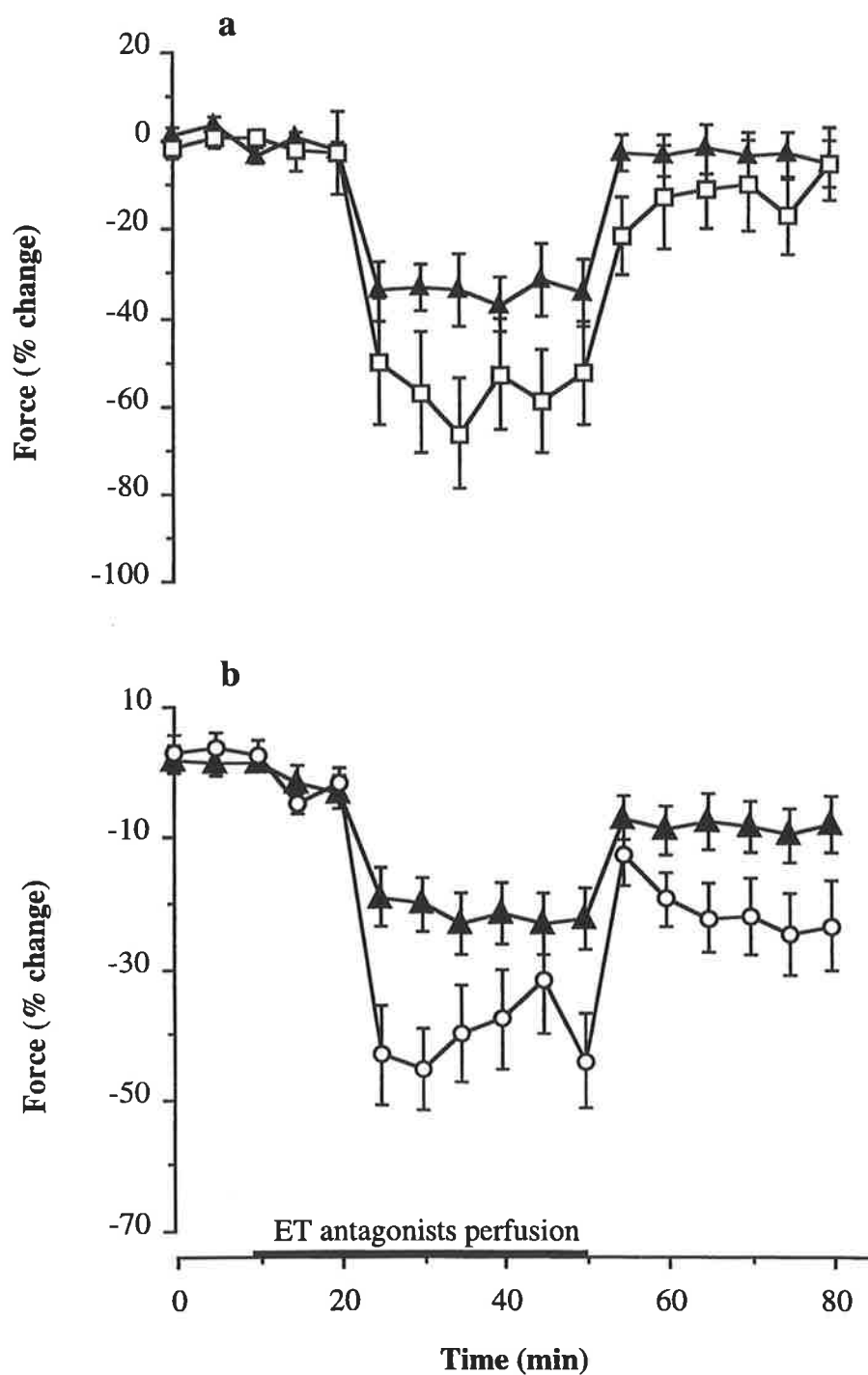


Fig 5.6. Effects in ischaemic-reperfused hearts of BQ-123 (10^{-6} M) ($\square$, $n=5$) and bosentan (10^{-5} M) ($\circ$, $n=12$) on cardiac force of contraction compared with control ischaemia/reperfusion($\blacktriangle$, $n=16-17$).

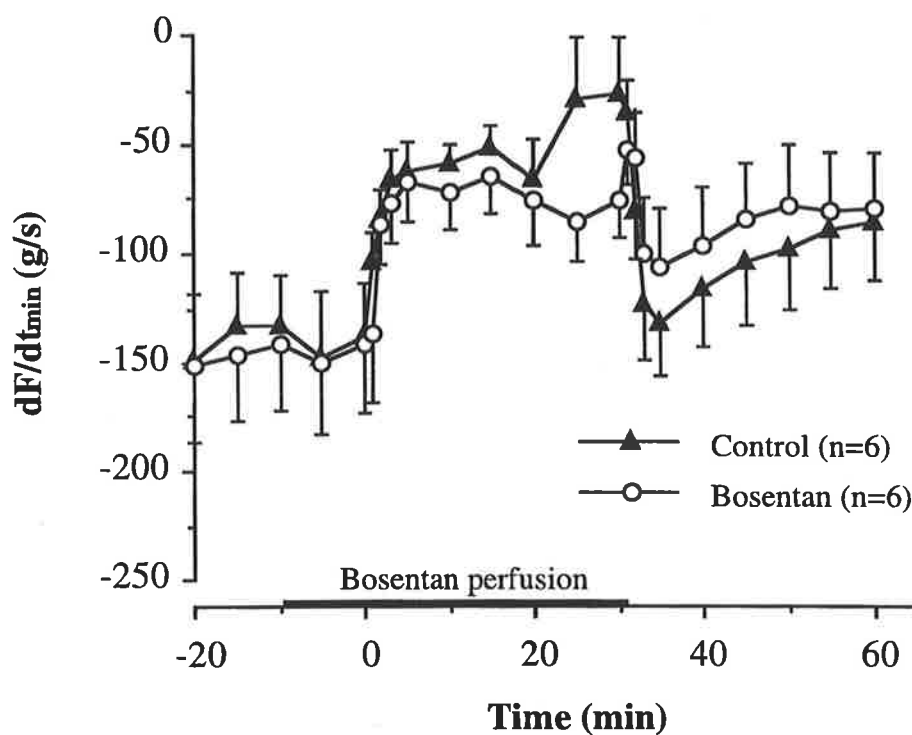


Fig 5.7. Effects in ischaemic-reperfused hearts of BQ-123 (10^{-6} M) ($\square$, $n=5$) and bosentan (10^{-5} M) ($\circ$, $n=12$) on cardiac relaxation (expressed as dF/dt min) compared with control ischaemia/reperfusion($\blacktriangle$, $n=12$).

Discussion

The results of the current study indicate that the effects of the selective ET_A receptor antagonist BQ-123 on coronary vasomotion and ET-1 efflux differ markedly from those of the ET_A/ET_B antagonist bosentan in the Langendorff-perfused rat heart. These differences are evident both in the presence and absence of transient myocardial ischaemia. Major differences were (i) coronary vasoconstriction occurred during and after perfusion with BQ-123 but not bosentan; (ii) post-ischaemic perfusion flow rate was significantly lower with BQ-123 than during control perfusion, while there were no significant differences between bosentan and control during this phase; and (iii) bosentan but not BQ-123 increased ET-1 efflux during ischaemia. Furthermore, while neither agent markedly affected contractile performance behaviour in the absence of ischaemia, bosentan significantly reduced force of cardiac contraction during myocardial ischaemia; BQ-123 tended to exert a similar effect ($P=0.06$). These findings have both physiological and pharmacological implications.

This is the first study demonstrating coronary vasoconstrictor effects of an ET_A antagonist. Previous studies with BQ-123 have primarily addressed its inhibition of exogenous ET-induced vasoconstriction which is usually evident in the 0.1-10 μM range (Pernow *et al* 1993) The concentration used in the present study (1 μM) is assumed to be an effective concentration for the blockade of ET_A receptors. Despite findings that BQ-123 reduces infarct size and inhibits anoxia-induced coronary vasoconstriction in canine models (Grover *et al* 1993, Vedernikov *et al* 1993) specific investigations of the interaction between endogenous ET and ET_A receptor antagonists on vasomotor tone are relatively few. One investigation in isolated isovolumetric rat hearts by Han *et al* (1995) failed to demonstrate any effects of BQ-123 (estimated at 1 μM) on coronary vasomotor tone. However, in their study, effects of BQ-123 infusion on coronary flow were

examined only after five minutes of infusion: in the current experiment no vasoconstriction was detectable at that stage. Furthermore, these investigations did not examine either recovery of coronary flow after reperfusion, or effects of BQ-123 on ET-1 efflux from the heart. Information related to coronary vasomotor effects of bosentan is also somewhat limited. However, Dagassan *et al.* (1994) demonstrated a coronary vasodilator effect (coronary flow increased by 14%) of bosentan in non-ischaemic rat heart, which did not persist during an immediate post-ischaemic phase; these results are similar to those of the current study.

In this study, both BQ-123 and bosentan induced an increase in ET-1 efflux. The effect of BQ-123 accords with a previous finding in cultured human umbilical vein smooth-muscle cells where FR139317, an ET_A receptor antagonist, caused an immediate, transient but marked increase in ET levels, with a decrease in levels of big ET-1 in the supernatant (Yu & Davenport 1995). With the recent identification of ET_A receptors on endothelial cells (Nishimura *et al* 1995, Ozaki *et al* 1995), it is possible that ET antagonists might inhibit a negative feedback mechanism involving ET-1 regulating its own release. There are no previous studies which have specifically investigated this possibility. Nevertheless, the implication that endogenously released ET might in some way exert homeostatic autocrine control on tissue ET concentrations, presumably via interaction with cardiac ET_A receptors, is of considerable physiological interest.

In the specific case of bosentan, an additional mechanism might theoretically contribute to increased ET-1 efflux. There is considerable information showing that ET_B receptors mediate ET clearance from the circulation, especially in pulmonary endothelium (Fukuroda *et al* 1994, Kiowski *et al* 1996, Ozaki *et al* 1995, Dupuis & Gorasky 1996, Sato *et al* 1995, Weber *et al* 1996, Brunner & Doherty 1996). Studies on clearance of ET-1 from plasma by pulmonary tissue during acute ET-1 administration in intact dogs

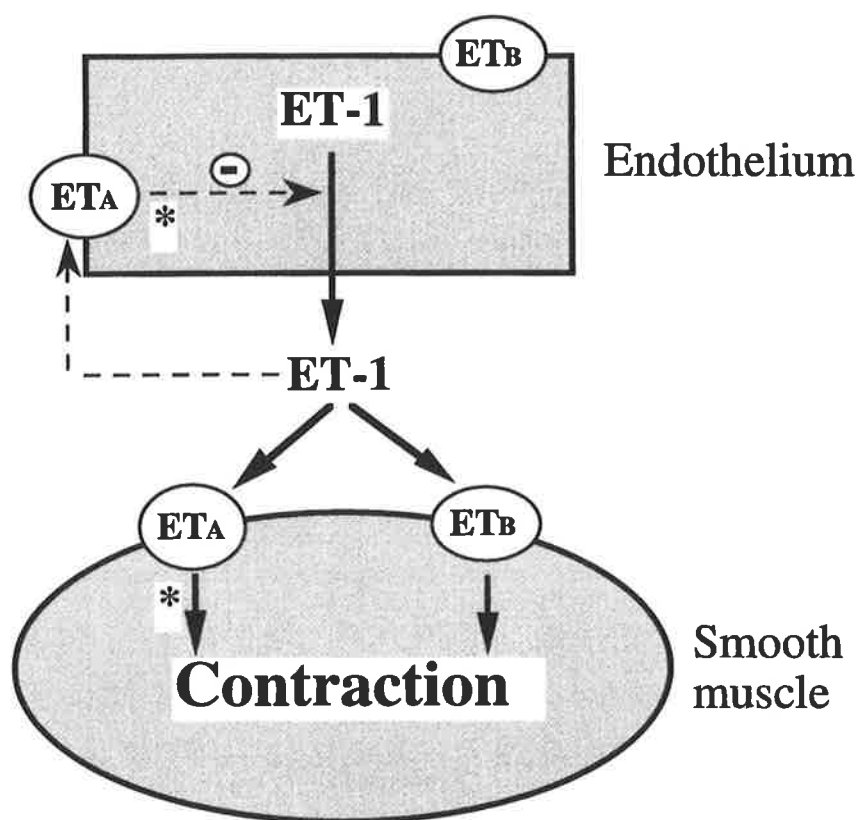
(Dupuis & Gorasky 1996), rats (Fukuroda *et al* 1994) and also in isolated perfused rat lungs (Sato *et al* 1995) demonstrate specific inhibition of the clearance process by the selective ET_B receptor antagonist. BQ-788. These findings are also consistent with elevation of plasma ET-1 concentrations induced by bosentan (Kiowski *et al* 1996, Weber *et al* 1996). As regards possible specific effects of ET_B receptor stimulation on ET-1 kinetics within the heart, Brunner and Doherty have demonstrated that BQ-788, but not the ET_A antagonist PD 155080, increased ET-1 release using the perfused rat heart (Brunner & Doherty 1996): these results are at variance with the findings in our current experiments, which suggest primary or sole modulation of ET-1 efflux via ET_A-receptor-dependent mechanism. While the basis for this discrepancy is not clear, there are a number of significant differences in experimental conditions which may account for the difference of the results. The utilization in Brunner and Doherty's experiments of (i) colloid-free Krebs solution (vs albumin-containing medium), (ii) exclusion of potential effects of ventricular endocardial endothelium on ET-1 kinetics (Brunner & Doherty 1996, Brunner 1995) (vs ventricular perfusion utilizing current experimental design) and (iii) constant flow (vs constant pressure) perfusion may all contribute to this discrepancy. However, in their experiments, significant increases in ET-1 efflux with BQ-788 appeared only after 75 minutes of perfusion, while the current experiments involved shorter duration of ET antagonist exposure. Irrespective of the reason, it must be emphasized that we have found no evidence of an ET_B-selective mechanism for increased ET-1 efflux in the current experiments: the observation that stimulated ET-1 efflux is more pronounced with BQ-123 than with bosentan implies that, at least over the first 40 minutes of ET receptor antagonist perfusion, this phenomenon is ET_A-receptor mediated.

The finding of coronary vasoconstriction during and after ET_A receptor antagonist perfusion appears paradoxical since it implies activation of vasoconstrictor mechanisms both in the absence and presence of transient coronary flow reduction. It is conceivable the increase in ET-1 efflux induced by BQ-123 mediated the observed vasoconstriction

and that the total absence of vasoconstriction despite ET-1 release with bosentan is an indication that the increased ET-1 exerted its effects via the ET_B receptors alone. Although the selectivity can be attributed to blockade of the ET_A receptors mediating vasoconstriction, an additional factor may be the selectivity of the ET_B receptor for ET-1 in low concentrations (Balwierczak 1993, McClullich *et al* 1996); which would render ET_A antagonists to be relatively ineffective.

After BQ-123 wash-out, in the non-ischaemic heart, despite return of the ET-1 efflux to baseline, the vasoconstrictor effect of BQ-123 persisted. This effect is likely to reflect persistent stimulation of ET_B receptors by ET-1, given the extremely slow dissociation rate of ET-1 from ET_B receptor (Waggoner *et al* 1992). This hypothesis is also consistent with the lack of “rebound” vasoconstriction with the ET_A/ET_B antagonist bosentan, since this agent, although accelerating ET release, would have prevented binding of ET with ET_B receptors. We are not aware of previous reports of “rebound” vasoconstriction after wash-out of an ET receptor antagonist. A model which takes into account the current observations is shown in Figure 5.8. It is emphasised that this model is speculative and more detailed experiments exploring the source of the ET-1 will be required to assess its validity. Similarly, during post-ischaemic reperfusion, there was a significantly lower coronary flow for BQ-123 than control, while there was no such effect for bosentan (Fig 5.2.). The experimental design did not permit us to determine whether this difference reflected progressive differential effects of ET receptor antagonists on coronary vasomotor tone (i.e. continuation of the slight pre-ischaemic vasoconstriction with BQ-123 and vasodilation with bosentan), or whether it might represent a specific interaction with post-ischaemic vasodilator physiology.

The results of contractile performance of left ventricle also have implications regarding both the receptor mechanisms underlying positive inotropic effects of ET, and also the



*** Inhibited in presence of BQ-123**

Fig 5.8. Schema of proposed mechanisms underlying incremental ET-1 efflux and resultant ET_B receptor-mediated coronary smooth muscle contraction in the presence of selective ET_A antagonist (BQ-123).

potential homeostatic effects of these mechanisms during severe myocardial ischaemia. Furthermore, the effects observed with BQ-123 and bosentan are relevant to the use of ET receptor antagonists in patients prone to episodic myocardial ischaemia. In the current experiments, neither BQ-123 nor bosentan affected force of contraction during normal perfusion. However, during a moderate reduction in coronary flow, bosentan (10^{-5} M) tended to accentuate the ischaemia-induced reduction in force of contraction. These changes were not mediated via ET-induced ischaemia, as coronary flow was externally adjusted in order to eliminate variability between groups. Furthermore, heart rate was kept constant. The results therefore strongly imply a differential effect of endogenous ET on cardiac systolic function, consistent with incremental ET release (Endoh *et al* 1996) and/or effect during the ischaemic period.

The inconclusive results as regards BQ-123 may imply type 2 error; supplies of this agent were limited. No experiments with selective ET_B antagonists were performed in this series. Nevertheless, the magnitude of the observed changes with bosentan (10^{-5} M) was no greater than that seen with BQ-123. Therefore, the results remain consistent with previous observations in isolated ferret papillary muscle (Kourembanas *et al* 1991) that the positive inotropic effects of ET are entirely mediated by ET_A receptor stimulation. Surprisingly, in rabbit papillary (or ventricular) muscle, myocardial ET_A receptors appear to mediate inotropic responses to ET-3 (Liu *et al* 1989, Nayler *et al* 1992), but not ET-1 (Liu *et al* 1989). A previous study in rat isolated right ventricular strips revealed that the ET_A receptor antagonist BQ-123 exerted a moderate negative inotropic effect in the absence of ischaemia (Domenech *et al* 1991). This indicates that ET_A receptors mediate at least a component of the inotropic effect of endogenous ET in the rat. However, in the current study, no significant negative inotropic effects of BQ-123 or bosentan were detected during non-ischaemic perfusion.

The implied existence of an accentuated positive inotropic effect of endogenous ET during acute global ischaemia and/or early reperfusion is a novel finding of this study. Such an effect could be considered homeostatic, and its disruption therefore is an area of potential concern regarding the future clinical use of ET receptor antagonists. However, currently available studies with whole animal models of myocardial ischaemia and reperfusion do not reveal any deleterious effect of ET receptor antagonists (Gulati *et al* 1996, Cirino *et al* 1994); indeed BQ-123 reduced infarct size in a canine model (Cirino *et al* 1997). These results are not necessarily paradoxical. First, ET receptor antagonists may exert beneficial effects on coronary perfusion (direct or via collaterals) and on cardiac work (via afterload reduction); neither of these circumstances could be examined with the current experimental design. Furthermore, no studies to date have included detailed examination of ET receptor antagonist effects on haemodynamic status during myocardial ischaemia.

One of the unexpected results of the current study was the abolition of ET-1 efflux during ischaemia by BQ-123, with minimal change during the ischaemic period in the presence of bosentan (Fig 4). The mechanism of this effect was not elucidated in the current study. As the experimental design resulted in somewhat greater flow reductions during BQ-123 than during bosentan perfusion experiments, as discussed previously, it is possible that this small differential degree of ischaemia may have contributed to the observed effects. However, an alternative explanation would be an acute reduction in extent of ET_A receptor occupancy during ischaemia in the case of 10⁻⁶ M BQ-123 in particular. Previous investigations have demonstrated externalization of ET receptors in rat heart during ischaemia, with a consequent increase in B_{max}. (Liu *et al* 1989) Hence, if ET-1 release in the presence of ET_A antagonists were modulated by an ET_A-receptor-mediated negative “feedback” mechanism, an increase in ET_A receptor density would provide a basis for decreased extent of ET by low concentration of ET_A antagonists. The observed effects of BQ-123 in particular on contractile performance during ischaemia may reflect these mechanisms.

In conclusion, in isolated perfused rat hearts, both BQ-123 and bosentan increased ET-1 efflux, but only BQ-123 exerted vasoconstrictor effects. Furthermore, during ischaemia the effects of BQ-123 on ET-1 efflux were abolished, and bosentan induced a significant negative inotropic effect. These results offer new insights into possible local cardiac mechanisms of ET-1 release, and also indicate that transient exposure to the ET_A antagonist BQ-123 may induce coronary vasoconstriction which persists unchanged during a wash-out phase. The results also strongly suggest that the importance of ET-induced positive inotropic effects is markedly accentuated during ischaemia. Clinical implications are that selective ET_A antagonists may be disadvantageous relative to ET_A/ET_B receptor antagonists in the presence of myocardial ischaemia, and that if such agents were withdrawn, a gradual rather than an abrupt cessation would be preferable. Furthermore, the negative inotropic effects of ET receptor antagonists (especially selective ET_A receptor antagonists) during ischaemia may limit their safety in patients with combined ischaemia and impaired left ventricular function.

5.5. SUMMARY

The aim of the present study was to evaluate and compare the effects of BQ-123, an ET_A receptor antagonist, and bosentan, an ET_A and ET_B receptor antagonist, on coronary vasomotor tone, left ventricular systolic and diastolic function and ET-1 efflux in the presence or absence of myocardial ischaemia/reperfusion.

Isolated rat hearts were perfused using a Langendorff preparation. Global ischaemia was induced on average by $68 \pm 2\%$ (SEM) reduction of a baseline perfusion flow rate 10 minutes after introduction of ET antagonists. Thirty minutes of ischaemia was followed by 30 minutes reperfusion. ET-1 efflux in coronary perfusate was measured by radioimmunoassay.

In non-ischaemic hearts (n=7), BQ-123 (10^{-6} M) perfusion induced a progressive decrease in coronary flow rate compared with a control group. This flow reduction persisted after wash-out of BQ-123. In contrast, bosentan (10^{-5} M, n=7) induced no change in perfusion rate. In the absence of ET antagonists (n=16), there was a $22 \pm 6\%$ post-ischaemic increase in perfusion flow rate. BQ-123 (n=5) but not bosentan (n=12) abolished this post-ischaemic increase in flow rate. Neither BQ-123 nor bosentan induced significant variation in force of contraction. In ischaemic hearts, ischaemia *per se* induced a transient decrease in force of contraction. Bosentan significantly ($P < 0.05$) accentuated and BQ-123 tended to accentuate ($P = 0.06$) this decrease in force of contraction during ischaemia. Bosentan but not BQ-123 significantly impaired the recovery of systolic function during reperfusion ($P < 0.05$). However, bosentan had no effect on cardiac relaxation. Both BQ-123 and bosentan perfusion increased ET-1 efflux rate to $730 \pm 188\%$

and $315 \pm 81\%$ respectively. This effect was abolished during ischaemia for BQ-123, but not for bosentan.

Conclusions: In isolated perfused rat hearts, both BQ-123 and bosentan increased ET-1 efflux, but only BQ-123 exerted vasoconstrictor effects. These results thus generated the hypothesis that (1) ET-1 release within the coronary vascular bed may be subject to negative feedback regulation mediated via ET_A receptors. (2) ET_A receptor antagonists increase ET-1 efflux, which may lead to net vasoconstriction via unopposed ET_B stimulation. Furthermore, the negative inotropic effects observed during ischaemia suggest that ET is critical to the maintenance of systolic function during ischaemia.

CHAPTER 6 - RESULTS

EFFECT OF HYPOXIA ON ANP/BNP/ET-1 RELEASE FROM ISOLATED PERFUSED RAT HEART.

6.1. Introduction

There is also strong evidence that hypoxia stimulates ANP release *in vitro* and *in vivo*. Studies *in vivo* have shown that acute and chronic alveolar hypoxia increase plasma ANP concentration in lambs (Baertschi & Teague 1989), rats (Albert *et al.* 1997) and humans (Cargill *et al.* 1996, De Angelis *et al.* 1996, Lordick *et al.* 1995). Another study showed that acute hypoxia (10% O₂) induced an increase in plasma ANP level without any increase in atrial pressure (Kawashima *et al.* 1992). Thus, hypoxia-induced ANP release seems independent of atrial stretch. This was confirmed by studies *in vitro* where anoxia increased ANP and BNP release from isolated perfused rat ventricles (Toth *et al.* 1994) and isolated perfused rat and rabbit heart (Lew & Baertschi 1989, Baertschi *et al.* 1986, Focaccio *et al.* 1995). However, the mechanism of hypoxia-induced ANP release is unclear. Exposure to hypoxia (1% O₂) for 36 hours resulted in an increase in ANP mRNA levels in cultured atrial cardiocytes (Chen *et al.* 1997); thus this hypoxia-induced eventual increase in ANP gene expression can not contribute to the rapid release of ANP during acute hypoxia. Since this hypoxia-induced ANP release was reproducible and reversible (Baertschi *et al.* 1990, Chen 1994), it is also unlikely the released ANP was caused by cell damage during hypoxia. It has been suggested that both α - and β -adrenergic mechanisms may account for the ANP response to hypoxia in isolated rat hearts (Lew & Baertschi 1989). Nordihydroguaiaretic acid, a lipoxygenase inhibitor, also partially attenuates hypoxia-induced ANP release (Chen 1994), subject to the specificity caveats discussed in Chapter 4.4.

Elevated plasma endothelin-1 levels were also found in conscious rats following 60 minutes of pulmonary alveolar hypoxia (5-10% O₂) (Shirakami *et al.* 1991). In cultured human umbilical vein endothelium, hypoxia induces ET gene expression and ET secretion

within 1 hour of exposure to hypoxia (Kourembanas *et al.* 1991). The relationship (if any) between hypoxia-induced ET-1 and ANP release has not yet been investigated.

This present study investigated, 1) the effects of hypoxia (5% O₂) on ANP, BNP and ET-1 release, heart rate; and cardiac contractile function; and

2) the role of endogenous endothelin in hypoxia-induced ANP release (using BQ-123, a selective ET_A receptor antagonist, and bosentan, an ET_A/ET_B receptor antagonist).

6.2. Method

6.2.1. Isolated heart perfusion:

Details refer to Chapter 2.1.2. In particular, twenty rat hearts were perfused with modified Eagles medium. The medium for basal perfusion and re-oxygenation was continually gassed with carbogen (95% O₂, 5% CO₂). Hypoxia was induced by perfusing medium gassed with a mixture of gases (5% O₂, 90% N₂, 5% CO₂).

6.2.2. Protocol

Refer to chapter 2.1.

A 30 min hypoxia was followed by 30 min of re-oxygenation. In ET receptor antagonists treated groups (n=6 for BQ-123, and n=4 for bosentan respectively), hypoxia was induced at the same time as the ET receptor antagonist perfusion had commenced. ET receptor antagonists were not perfused during re-oxygenation phase. Right atrial pacing could not be utilized during hypoxia due to elevation of pacing threshold during this phase. The spontaneous heart rate and force of contraction were monitored, and coronary flow rate was measured. The effluent was collected for the RIA assay of ANP, BNP and ET-1 (refer to chapter 2.1.5 to 2.1.7.). BNP results were expressed as percentage of baseline due to high inter-assay variability.

6.3. Results:

6.3.1. Effects of hypoxia on ANP, BNP and ET-1 release

6.3.1.1. Effect of hypoxia on ANP release

The mean basal ANP release rate was 6.50 ± 0.30 pg/min/g wet weight. ANP efflux rate increased ($P < 0.005$, unpaired t test of AUC, $n=10$) rapidly after perfusate gassed with 5% O₂ was introduced (Fig 6.1.). The increase in peak ANP release rate was $794 \pm 163\%$ beyond basal ANP release rate following 18 min of hypoxia. This increase of ANP release persisted in the first 6 min of re-oxygenation, and then returned towards basal level, but remained mildly elevated until the end of 30 min re-oxygenation period.

6.3.1.2. Effect of hypoxia on BNP release

Hypoxia had a tendency to increase BNP release rate compared with the control group, but this increase was not statistically significant ($P=0.071$, two way ANOVA with repeated measures, $n=6$). The peak BNP release rate during hypoxia was $145 \pm 56\%$ over baseline, and occurred at 10 min of hypoxia. There was no significant change in BNP release rate during re-oxygenation (Fig 6.2.).

6.3.1.3. Effect of hypoxia on ET-1 release

Perfusate from 5 rat hearts were collected for ET-1 measurement. The mean basal ET-1 release rate was 204 ± 30 fg/min/g ww. Hypoxia tended to induce an initial increase ($36 \pm 11\%$ in first 5 min hypoxia) followed by a decrease ($P=0.056$, two way ANOVA with repeated measures) in ET-1 release. During re-oxygenation, the ET-1 release did not

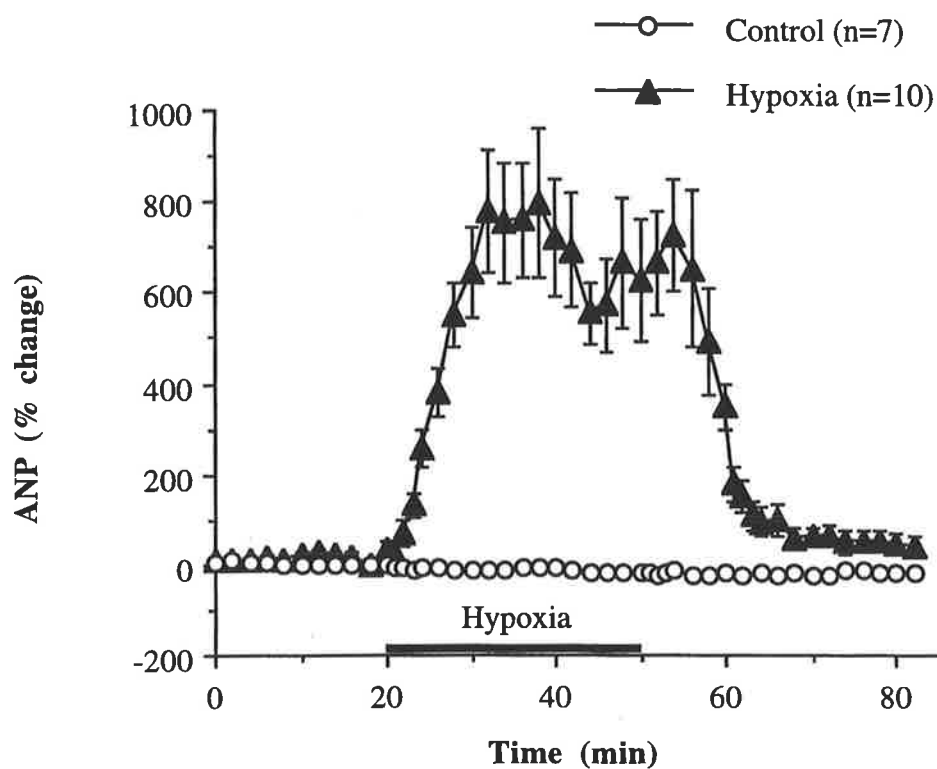


Fig 6.1. Effect of hypoxia (5% O₂, n=10) on ANP release from isolated perfused rat hearts, compared with normoxic hearts (n=7).

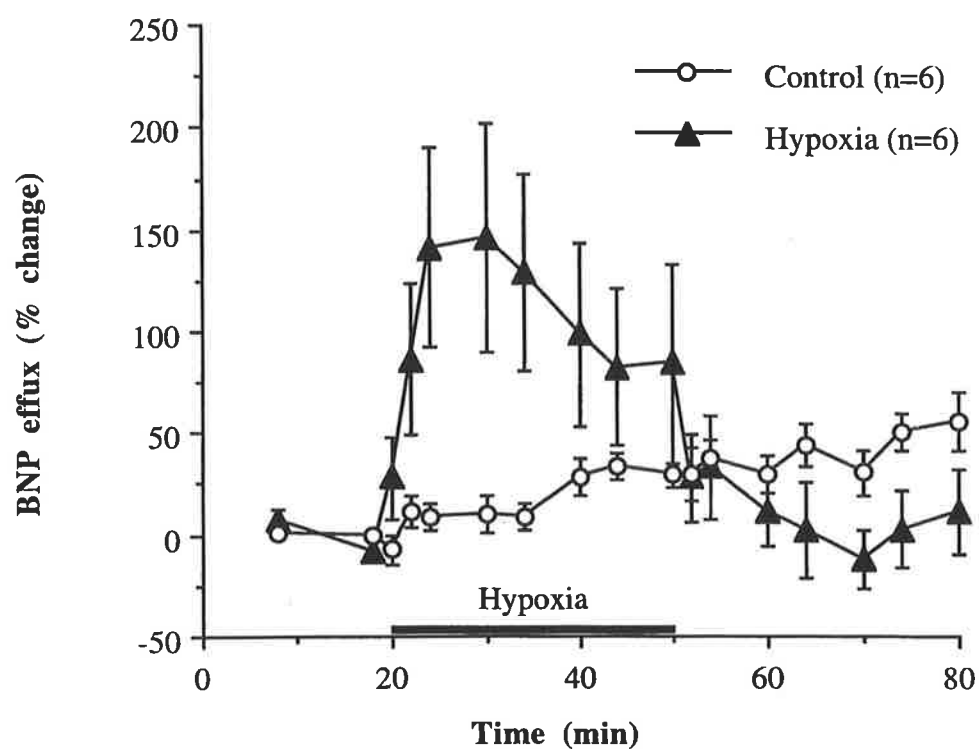


Fig 6.2. Effect of hypoxia (5% O₂, n=6) on BNP release from isolated perfused rat hearts, compared with normoxic hearts (n=6).

completely recover ($P<0.01$, two way ANOVA with repeated measures) compared with control (paced, $n=6$) hearts (Fig 6.3.).

6.3.2. Changes in cardiac performance and coronary flow

Hypoxia induced a significant bradycardia. The mean heart rate during the hypoxic period was decreased by $71.4\pm4.0\%$ ($P<0.0001$ vs control, unpaired t test on mean decreases).

Despite the reduction in heart rate, perfusion flow rate increased (mean $28.2\pm9.2\%$, $P<0.05$ unpaired t test, two-way ANOVA with repeated measures) immediately after the hearts were exposed to perfusate gassed with 5% O_2 . Maximal increase in coronary perfusion flow rate ($43.9\pm17.1\%$) occurred within the first minute of hypoxia. This increase of perfusion flow rate was partially sustained until re-oxygenation (Fig 6.4.).

Hypoxia also induced a significant decrease ($59.4\pm5.1\%$, $P<0.0001$ vs control, unpaired t test on AUC) in the cardiac force of contraction. This impairment in cardiac force of contraction induced by hypoxia occurred within the first 5 min of the hypoxic period and was sustained until re-oxygenation. Cardiac systolic function did not fully recover during the 30 min normoxic period after re-oxygenation (Fig 6.5.)

6.3.3. Effect of BQ-123 and bosentan on ANP, ET-1 release during hypoxia/ re-oxygenation.

6.3.3.1. Effect of BQ-123 and bosentan on ANP release.

In normally perfused hearts, neither BQ-123 ($n=7$) nor bosentan ($n=7$) significantly

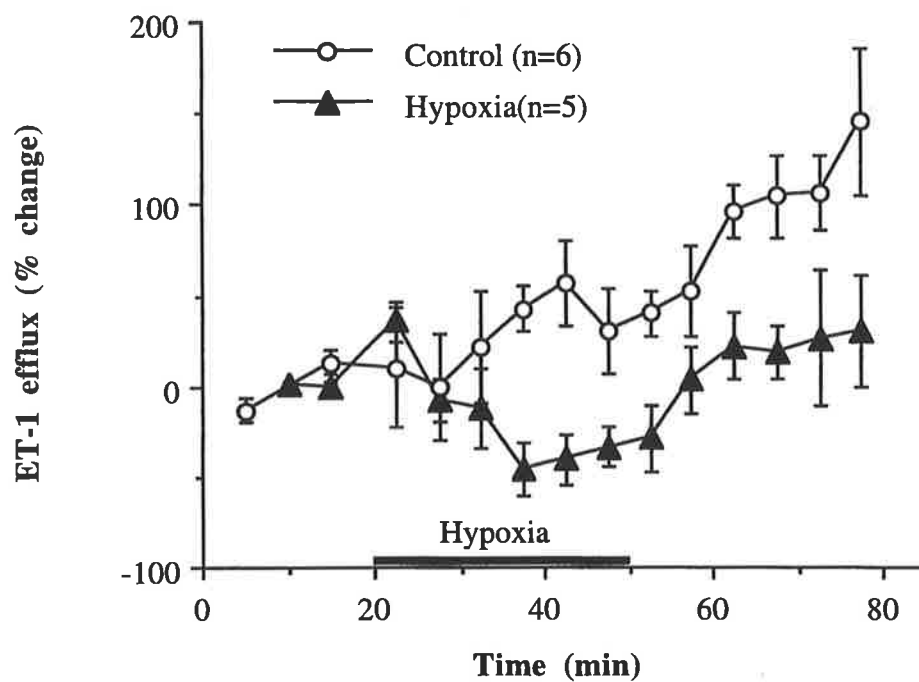


Fig 6.3. Effect of hypoxia (5% O₂, n=5) on endothelin-1 (ET-1) efflux rate from isolated perfused rat hearts, compared with normoxic, control hearts (n=6).

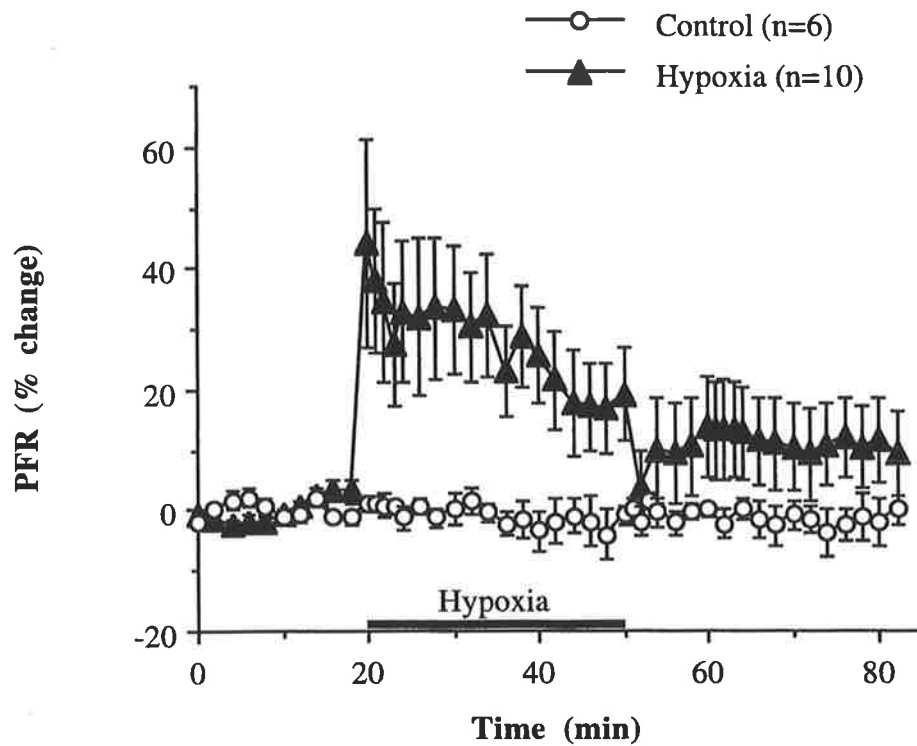


Fig 6.4. Effect of hypoxia (5% O₂, n=10) on perfusion flow rate (PFR) compared with normoxic, control hearts (n=7).

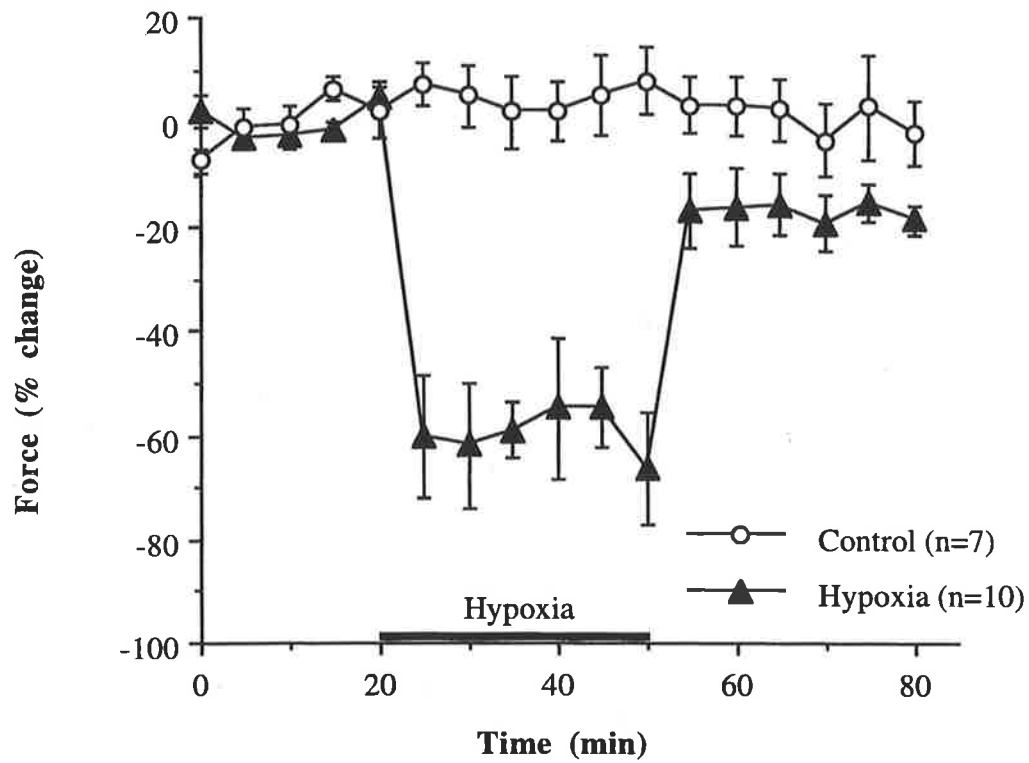


Fig 6.5. Effect of hypoxia (5% O₂, n=10) on cardiac force of contraction (Force) compared with normoxic, control hearts (n=7).

affected ANP release compared with control hearts (n=6) (refer to chapter 4.3.2.2.). In hypoxic hearts, BQ-123 (10^{-6} M) did not affect hypoxia-induced ANP release. ANP release during hypoxia, expressed as mean $\pm$ SEM for area under the ANP/time curve, for hypoxia and hypoxia+BQ-123 groups was 18200 $\pm$ 2840 (n=10) and 13900 $\pm$ 2670 (n=6) units respectively. However, bosentan (10^{-5} M) significantly suppressed hypoxia-induced ANP release ($P<0.05$, unpaired t test of AUC, n=4); ANP release during 30 min of hypoxia in the bosentan group was 7670 $\pm$ 2510 units (AUC). There was no difference between bosentan-treated and untreated hypoxic hearts in ANP release during re-oxygenation (Fig 6.6.b). Fig 6.7. summarises the effect of hypoxia $\pm$ ET receptor antagonists on ANP release.

6.3.3.2. Effect of BQ-123 and bosentan on ET-1 release

In normoxic hearts, both BQ-123 and bosentan perfusion increased ET-1 efflux rate to 730 $\pm$ 188% and 315 $\pm$ 81% respectively (refer to chapter 5.3.2.). During hypoxia and while perfusing with ET receptor antagonists, BQ-123 significantly increased ($P<0.05$, Mann-Whitney U on AUC) bosentan tends to increase ET-1 efflux rate ($P=0.069$, unpaired t test on AUC). However, the increase did not attain a statistically significant level compared with ET-1 efflux from hypoxic hearts in the absence of ET receptor antagonists. The area under the ET-1/time curves for hypoxia, hypoxia+BQ-123 and hypoxia+bosentan were -562 $\pm$ 328, 21700 $\pm$ 12400 and 2280 $\pm$ 1450 units respectively. The ET-1 efflux rates returned to basal levels after 12.5 min of re-oxygenation (Fig 6.8.).

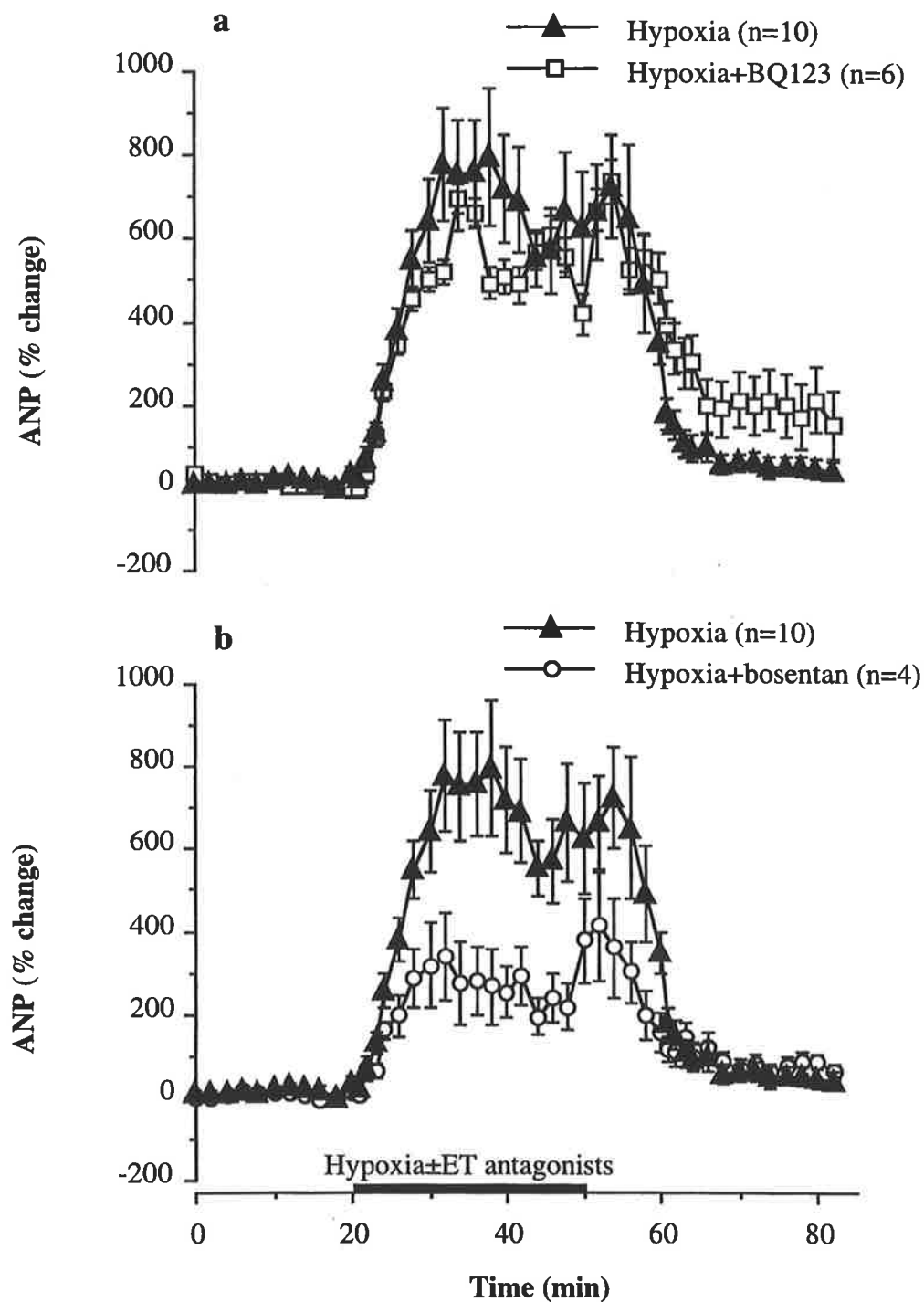


Fig 6.6. Effect of BQ-123 (n=6) and Bosentan (n=4) on hypoxia-induced ANP release from isolated perfused rat hearts, compared with hypoxic hearts in absence of ET receptor antagonists (n=10).

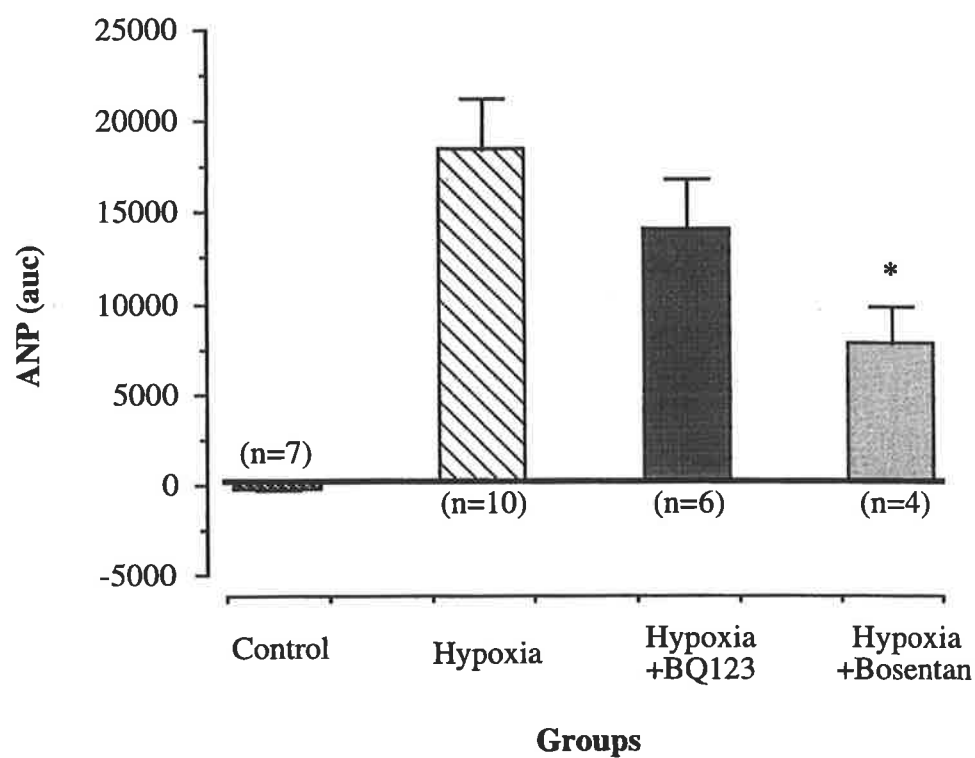


Fig 6.7. ANP release expressed as area under curve (AUC, arbitrary units) from hypoxia (n=10), hypoxia+BQ-123 (n=6) and hypoxia+bosentan (n=4) hearts compared with normoxic hearts (control, n=7). * $P<0.05$ vs hypoxia.

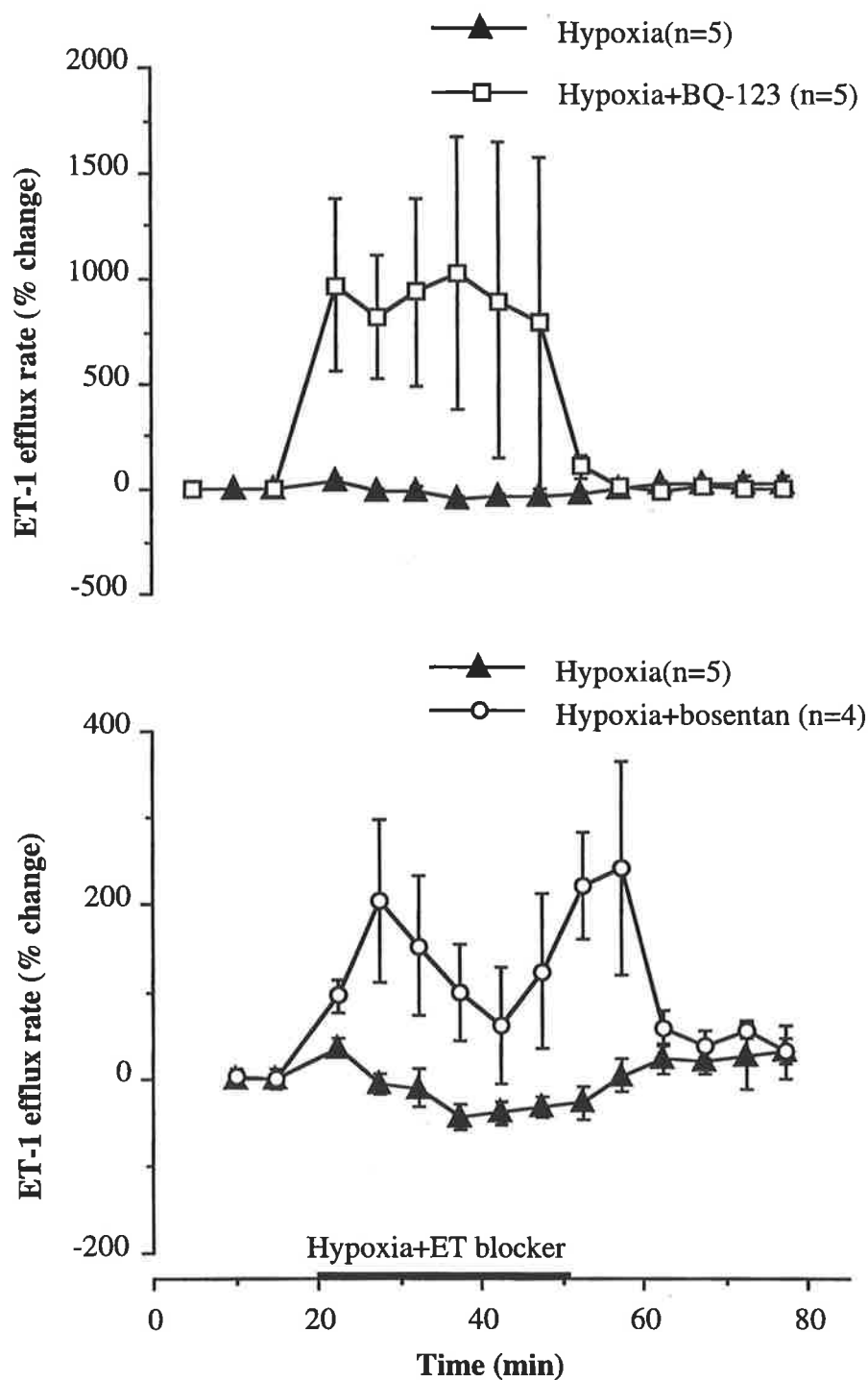


Fig 6.8. Effect of BQ-123 (n=5) and Bosentan (n=4) on ET-1 release, compared with hypoxic hearts in absence of ET receptor antagonists (n=5).

6.3.4. Effect of BQ-123 and bosentan on hypoxia-induced changes in flow rate, heart rate and force of contraction

6.3.4.1. Coronary flow rate

In non-hypoxic hearts, BQ-123 perfusion induced a progressive decrease in coronary flow rate compared with the control group. This reduction in flow persisted after wash-out of BQ-123. In contrast, bosentan induced no change in the perfusion rate (refer to chapter 5.3.1.). BQ-123 attenuated ($P < 0.05$, unpaired t test on AUC) hypoxia-induced increase in perfusion flow rate, whereas bosentan had no effect on coronary flow rate during hypoxia (Fig 6.9.). During re-oxygenation period, neither bosentan nor BQ-123 significantly affect flow rate.

6.3.4.2. Heart rate and cardiac force of contraction

In non-hypoxic hearts, the heart rate was maintained constant via right atrial pacing in the presence of BQ-123 or bosentan. In hypoxic hearts, hypoxia induced a significant bradycardia (mean heart rate decreased by $71.4 \pm 4.0\%$). Both BQ-123 and bosentan did not affect this hypoxia-induced bradycardia.

Neither BQ-123 nor bosentan induced any significant variation in the force of contraction in normal perfused paced hearts (refer to chapter 5.3.3.). In non-paced hearts, however, hypoxia induced a significant decrease in cardiac force of contraction. BQ-123 (10^{-6} M) and bosentan (10^{-5} M) perfusion for 10 min prior to ischaemia had no effect on cardiac force of contraction. With both BQ-123 and bosentan ($P = 0.270$ and $P = 0.059$ respectively, unpaired t test on AUC), the hypoxia-induced decrease in force of contraction tended to be accentuated, but this did not reach statistical significance (Fig 6.10.).

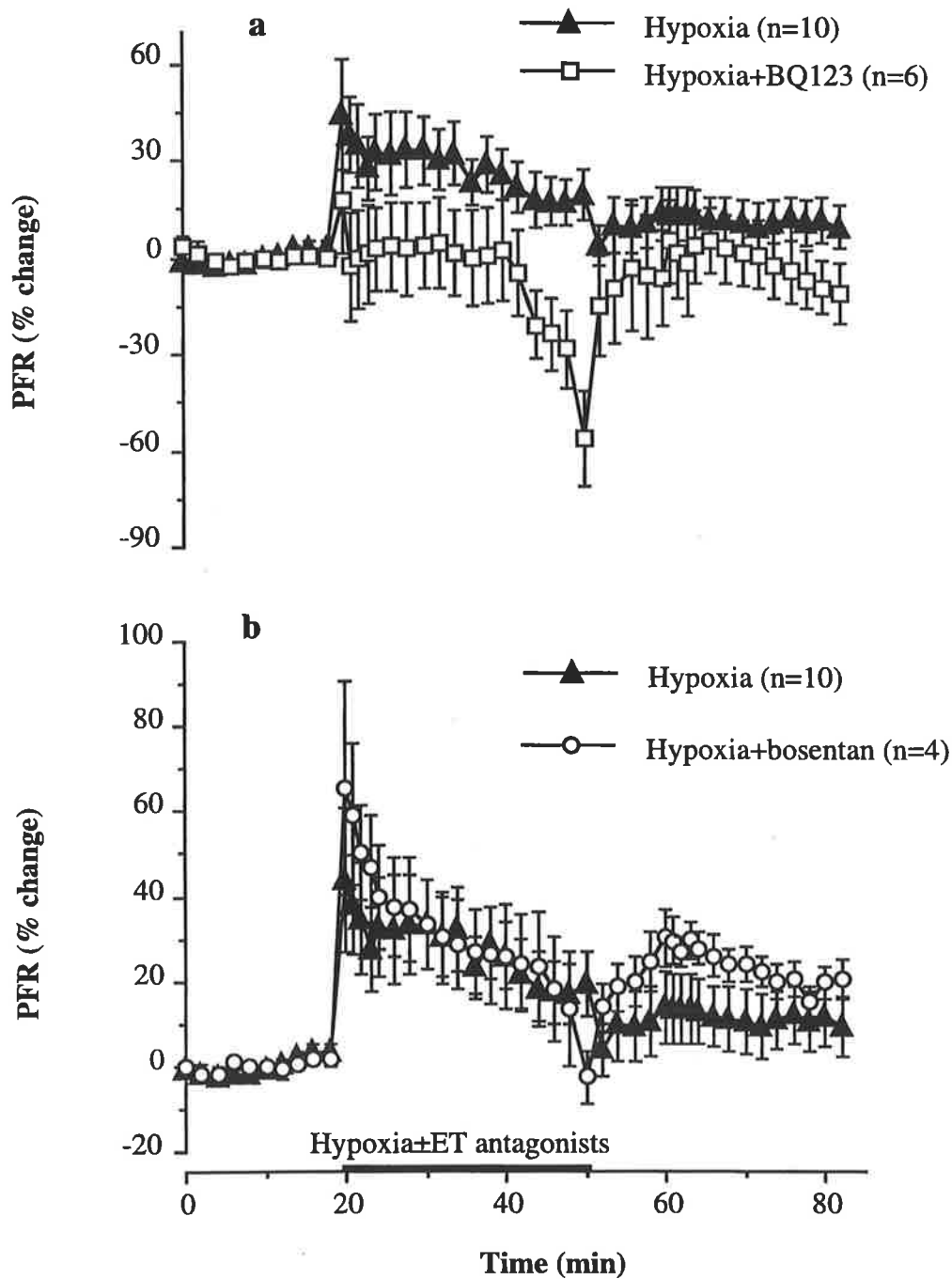


Fig 6.9. Effect of BQ-123 (10^{-6} M, n=5) and Bosentan (10^{-5} M, n=4) on perfusion flow rate (PFR) during hypoxia and re-oxygenation, compared with hypoxic hearts in absence of ET antagonists.

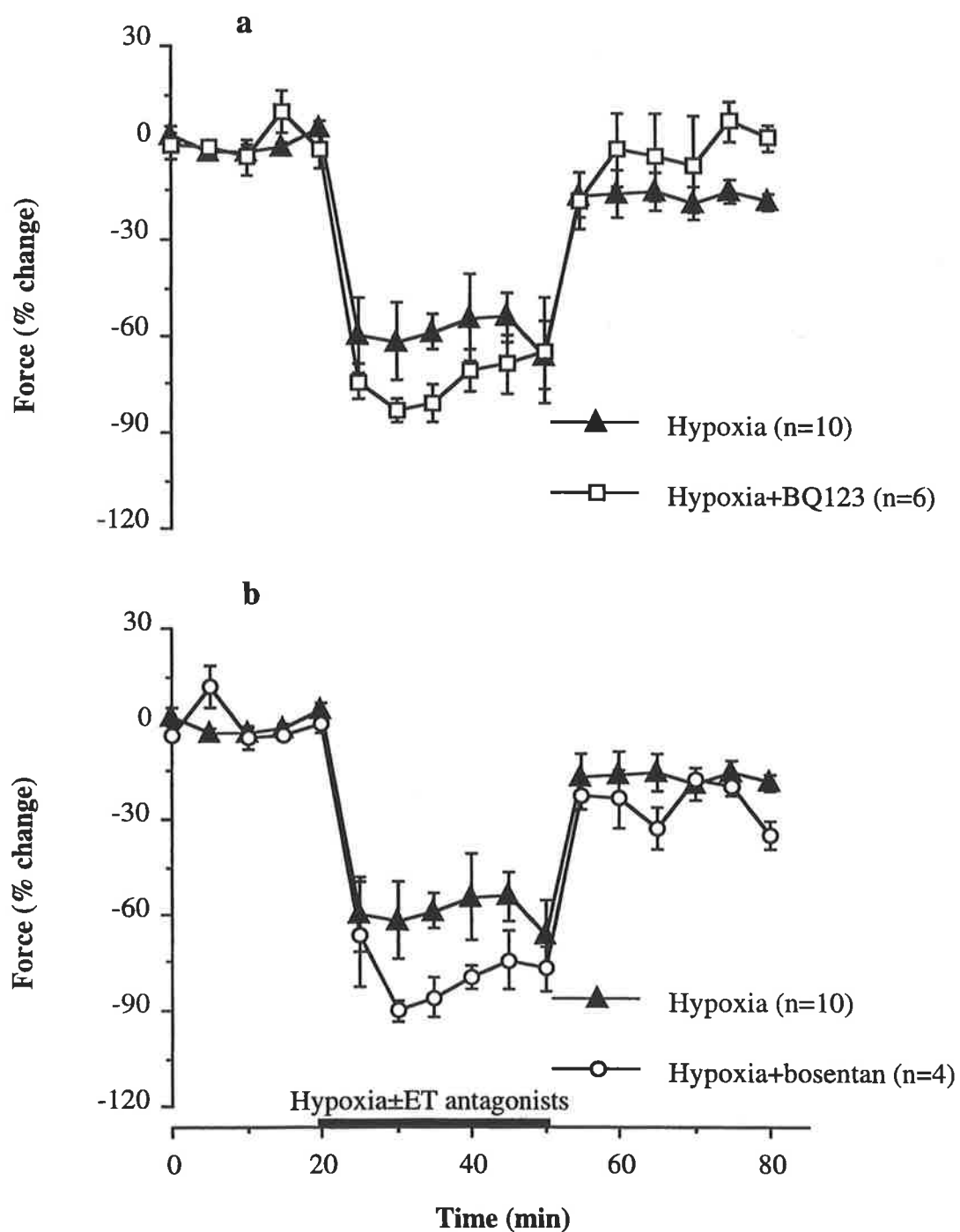


Fig 6.10. Effect of BQ-123 (n=5) and Bosentan (n=4) on hypoxia-induced impairment in cardiac force of contraction (Force), compared with hypoxic hearts in absence of ET receptor antagonists (n=10).

6.4. Discussion

The studies included in this chapter indicate that acute hypoxia (5% O₂) is a powerful stimulus for ANP release from the isolated rat heart. This increased ANP release, like that occurring with ischaemia, is independent of atrial stretch; and bosentan, a non-selective ET receptor antagonist, attenuated this hypoxia-stimulated ANP release, indicating that the hypoxia-induced ANP release is at least partially mediated by endothelin, despite the absence of any “net” increase in ET-1 efflux during the period of hypoxia.

Acute and chronic alveolar hypoxia increases plasma ANP concentration in animals (Baertschi & Teague 1989, Albert *et al.* 1997) and humans (Cargill *et al.* 1996, De Angelis *et al.* 1996, Lordick *et al.* 1995) *in vivo*. The results from the current study demonstrated that hypoxia-induced ANP release was independent of atrial stretch. These findings were consistent with previous studies that have shown acute hypoxia induced an increase in plasma ANP level without any increase in atrial pressure (Kawashima *et al.* 1992); studies *in vitro* have also shown anoxia increased ANP in isolated perfused rat and rabbit heart (Toth *et al.* 1994, Lew & Baertschi 1989, Baertschi *et al.* 1986). Since it takes 36 hours to show any increase in ANP mRNA levels after exposure to hypoxia (Chen *et al.* 1997), the increase in ANP gene expression is unlikely to contribute to the rapid release of ANP during acute hypoxia. Lew & Baertschi (1989) demonstrated that both α - and β -adrenergic mechanisms may mediate at least part of the ANP response to hypoxia.

In addition, the studies presented here have demonstrated that endogenous endothelin may also play a role mediating part of the acute hypoxia-stimulated ANP release from isolated perfused rat hearts. Exogenous ET augmented basal and stretch-induced ANP (and BNP)

secretion from rat myocytes (Thibault *et al* 1994, Horio *et al* 1993, Leite *et al* 1994, McDonough *et al* 1994) rat atria (Skvorak *et al* 1995, Hu *et al* 1988), perfused rat heart (Mantymaa *et al.* 1990), and intact animals (Okumura *et al* 1993, Cheung 1994). ANP (and BNP) secretion enhanced by exogenous ET was inhibited by BQ-123 (Thibault *et al* 1994, Skvorak *et al* 1995, Irons *et al.* 1993, Leite *et al.* 1994), but not affected by ET_B antagonists (Thibault *et al* 1994) suggesting that ET_A receptors mediate the ET-activated ANP (or BNP) release. Furthermore, observations that BQ-123 and bosentan inhibit volume-loading-induced ANP release, indicate that endogenous ET may play a role in ANP release (Leskinen *et al* 1997). Myocyte stretch increases myocardial oxygen consumption, and tissue hypoxia may represent the underlying mechanisms for ANP release (Ruskoaho 1992). However, there has been no previous study to show directly that endogenous ET mediates a component of hypoxia-activated ANP release. In the study presented here, bosentan (10^{-5} M) but not BQ-123 (10^{-6} M) attenuated hypoxia-stimulated ANP release.

Although elevated plasma endothelin-1 levels were found in an *in vivo* study after 60 min alveolar hypoxia (Shirakami *et al.* 1991), the “net” ET-1 release rate was not affected by 30 min anoxia (no O₂) (Toth *et al.* 1994). The current study also suggests that 30 minute low O₂ hypoxia did not affect ET-1 efflux rate.

In these studies, the constant pressure perfusion system used enabled us to investigate hypoxia-induced changes in coronary perfusion flow rate and the effect of ET receptor antagonists on this change in flow rate during hypoxia. The results indicate that hypoxia induced an increase in coronary perfusion flow rate. This hypoxia-induced vasodilation was abolished by BQ-123 but not bosentan, suggesting primarily ET_B-receptor mediated vasoconstriction. Furthermore, BQ-123 increased and bosentan tended to increase the ET-1 efflux rate during hypoxic period (possible via blockade of a negative feedback

pathway). This increased ET-1 efflux may also contribute to BQ-123-induced vasoconstriction effect (See chapter 5 for further discussion).

Although hypoxia significantly decreased heart rate, a significant decrease in cardiac force of contraction still occurred during hypoxia. In this case, the negative staircase effect may be counterbalanced by severe hypoxia-induced negative inotropy. Bosentan tended to accentuate this hypoxia-induced negative inotropy, suggesting a role for endogenous ET in maintaining cardiac contractile function during hypoxia (See Chapter 5 for analogous data during ischaemia).

In conclusion, these studies have shown, 1) hypoxia is a powerful stimulus for ANP release. This increased ANP release is independent of any change in atrial distension; 2) Bosentan attenuated the hypoxia-induced ANP release indicating that this increment of ANP release during hypoxia was mediated at least partially by ET; 3) BQ-123, but not bosentan, abolished hypoxia-induced vasodilation. BQ-123 increased and bosentan tended to increase the ET-1 efflux rate. 4) Bosentan had a tendency to accentuate the hypoxia-induced impairment in cardiac systolic function, suggesting that endogenous ET may play a role in maintaining cardiac systolic function during hypoxia.

6.4. Summary

It was previously documented that coronary flow reduction stimulated ANP release in the isolated Langendorff-perfused rat heart. Using the same model, 1) the effects of hypoxia on ANP, BNP and ET-1 release, heart rate; and cardiac contractile function; and 2) the role of endogenous endothelin in hypoxia-induced ANP release (using BQ-123 and bosentan) were further investigated.

Hypoxia induced an 8-fold increase in ANP release rate, but did not affect BNP and ET-1 efflux rate. The ANP release rate returned towards the basal level after re-oxygenation. Bosentan (10^{-5} M) but not BQ-123 (10^{-6} M) significantly attenuated ($P<0.05$) hypoxia-induced ANP release. During hypoxia, heart rate was reduced by $71.4\pm4.0\%$ ($P<0.0001$), force of contraction was reduced by $59.4\pm5.1\%$ ($P<0.0001$), however, coronary flow rate increased ($P<0.05$). BQ-123 increased ($P<0.05$) and bosentan tended to increase ET-1 release and to decrease cardiac force of contraction during the hypoxic period. BQ-123 but not bosentan abolished the hypoxia-induced increase in perfusion flow rate.

These results suggest that 1) hypoxia stimulates ANP release independent of change in atrial distension and the increment of ANP release during hypoxia was partially mediated by endogenous ET; 2) ET_A receptor antagonism may lead to a “net” vasoconstriction via unopposed ET_B stimulation; and 3) that endogenous ET may play a role in maintaining cardiac systolic function during hypoxia.

CHAPTER 7 - RESULTS

TRANSIENT MYOCARDIAL ISCHAEMIA IN MAN: EFFECTS ON ET-1 AND ANP RELEASE

7.1. Introduction

Percutaneous transluminal coronary angioplasty (PTCA) has been used as a treatment for both chronic and acute myocardial ischaemia, including evolving infarction. Inherent in this procedure, the stenosed coronary artery is completely occluded during balloon inflation and then reperfused following balloon deflation. Thus, in the absence of extensive collateralization, transient myocardial ischaemia/reperfusion is inevitable during this type of treatment (refer to chapter 1.1.5.2.). As previously discussed, myocardial ischaemia is a potent stimulus for ANP (BNP) release both directly (see chapter 3) and indirectly via diastolic left ventricular dysfunction and consequent atrial distension. Plasma ANP levels were elevated in patients with unstable angina (Kikuta *et al.* 1996), ischaemia caused by exercise (Nishikimi *et al.* 1988; Marumoto *et al.* 1995a,b, Morimoto *et al.* 1997), and MI (Fontana *et al.* 1990; Gutierrez-Marcos *et al.* 1991; Kyriakidis *et al.* 1993; Arad *et al.* 1996) (refer to 1.2.4.). Previous investigations also showed increased plasma ANP levels (in coronary sinus, femoral artery and vein, and pulmonary artery blood) post PTCA (Ikaheimo *et al.* 1989, Wiedermann *et al.* 1991; Prachar *et al.* 1991; Malatino *et al.* 1992; Gill *et al.* 1995, Kyriakides *et al.* 1995).

Increased plasma ET concentrations are found in patients with unstable angina, and MI (Malatino *et al.* 1993, Wieczorek *et al.* 1994). Although some studies demonstrated plasma ET-1 to be increased at the end of PTCA (Ameli *et al.* 1993, Kruger *et al.* 1998), other studies failed to detect any significant change in plasma ET-1 levels immediately following PTCA (Malatino *et al.* 1993, Montalescot *et al.* 1994, Hasdai *et al.* 1997), or at the end of the first balloon inflation (Kyriakides *et al.* 1995, 1996).

Despite these reported data, there is little information available concerning either the determinants or mechanisms of release of ANP and ET-1 following PTCA. Specifically, Ikaheimo *et al.* (1989) have demonstrated that increased ANP concentrations tended to be associated with elevated pulmonary capillary wedge pressures (PCWP) during PTCA. However, whether this represents specifically incremental release from distended left atrium, or the end-product of a large ischaemic zone, was uncertain from this study. Nevertheless, one implication of these findings is that the creation of a large ischaemic zone (for example with occlusion of the proximal left anterior descending coronary artery) is likely to increase both ANP and ET release through the above mentioned mechanisms.

However, patients undergoing PTCA in coronary vessels of moderate size do not necessarily develop global impairment of left ventricular diastolic function, and hence may not develop stimuli for atrial distension. In the current study, the primary objective was to exam the possible impact of PTCA of such vessels (right coronary and circumflex arteries) on ANP and ET-1 release into peripheral blood; it would have been anticipated that such release might have reflected to a greater extent the induction of ischaemia rather than atrial distension.

7.2. Methods

7.2.1. Patient selection

Seven patients (five male and two female, mean age 57) with haemodynamically significant stenosis in one major epicardial coronary artery and undergoing non-emergency PTCA were selected for study. Patients with significant left main coronary artery stenosis were excluded. All patients received verapamil prior to PTCA and intravenous GTN prior to and during PTCA. Informed consent was obtained prior to study (refer to chapter 2.2.2.1.).

7.2.2. Protocol

A femoral approach was used in all cases. The arterial pressure, pulmonary capillary wedge pressure (PCWP), mean pulmonary pressure (PAP) and mean right atrial pressure (RAP), were measured prior to balloon inflation (refer to chapter 2.2.1.2.). A three lead ECG was recorded throughout the procedure. Blood samples were collected simultaneously from femoral artery and pulmonary artery for ANP and ET-1 RIA prior to initial balloon inflation, at the end of this balloon inflation (about 2 min) and 1 min thereafter. Sample preparation was as described in chapter 2.2.2.3. For plasma ANP and ET-1 RIA, see chapter 2.2.3 and 2.2.4. The ET-1 antibody exhibited cross reactivities of human ET-2, ET-3 and big ET-1 of 7, 7 and 17% respectively.

7.2.3. Analysis of data

Data were expressed throughout as mean $\pm$ SEM. Plasma ANP and ET-1 concentrations were expressed as fmol/ml and pg/ml respectively. Comparison of data was performed using one-way ANOVA repeated measure. A *P* value of <0.05 was considered statistically significant (refer to chapter 2.3.).

7.3. Results

7.3.1. Patient characteristics and haemodynamic parameters.

Seven patients (five male and two female, mean age 57), who presented with stable (n=4) or unstable (n=3) angina prior to PTCA, were studied. Five of these patients had coronary disease limited to the single vessel chosen for PTCA; 2 patients had a further 60-70% stenosis in a non-dilated vessel. Five patients received angioplasty to their right coronary artery (RCA), and 2 patients had circumflex artery (Cx) angioplasty. Pulmonary capillary wedge pressure (PCWP) was elevated prior to PTCA in one patient (Table 7.1.). Balloon inflation resulted in ST-segment elevation and chest pain in only one patient (patient No 2). There were no clinical complications during the procedure.

7.3.2. Effects of PTCA on plasma ANP concentrations

Basal plasma ANP concentrations in femoral arteries (FA) and pulmonary arteries (PA) were 16.2 ± 3.2 and 17.8 ± 3.6 fmol/ml respectively. At the end of the 2 min initial balloon inflation, plasma ANP concentrations in FA and PA were 15.6 ± 3.1 and 19.5 ± 3.3 fmol/ml respectively. Post PTCA (1 min of balloon deflation) ANP levels were 16.4 ± 3.5 and 20.3 ± 2.9 fmol/ml in FA and PA respectively (Fig 7.1.).

7.3.3. Effects of PTCA on plasma ET-1 concentrations

Basal plasma ET-1 concentration in FA and PA were 3.59 ± 0.31 and 3.3 ± 0.23 pg/ml respectively. ET-1 levels did not change following a 2 min initial balloon inflation, nor after 1 min of balloon deflation (Fig 7.2.). In patient 2, who developed ST elevation and pain during balloon inflation, there were also no changes in either ANP or ET-1 concentrations.

Table 7.1. Patient characteristic and haemodynamic parameters.

Patient No	Age	Sex	PTCA vessel	Angina type	MAP	PCWP
1	64	M	Cx	unstable	115	7
2	69	M	RCA	stable	81	3
3	46	F	RCA	unstable	91	18
4	62	M	RCA	stable	100	NR
5	51	M	Cx	unstable	118	14
6	44	M	RCA	stable	93	5
7	66	F	RCA	stable	116	9
Mean	57				102.0	9.33
SEM	4				5.49	2.32

Cx, circumflex artery; RCA, Right coronary artery; MAP, mean arterial pressure; PAP, mean pulmonary arterial pressure; RAP, mean right atrial pressure; PCWP, mean pulmonary capillary wedge pressure. NR, not recorded.

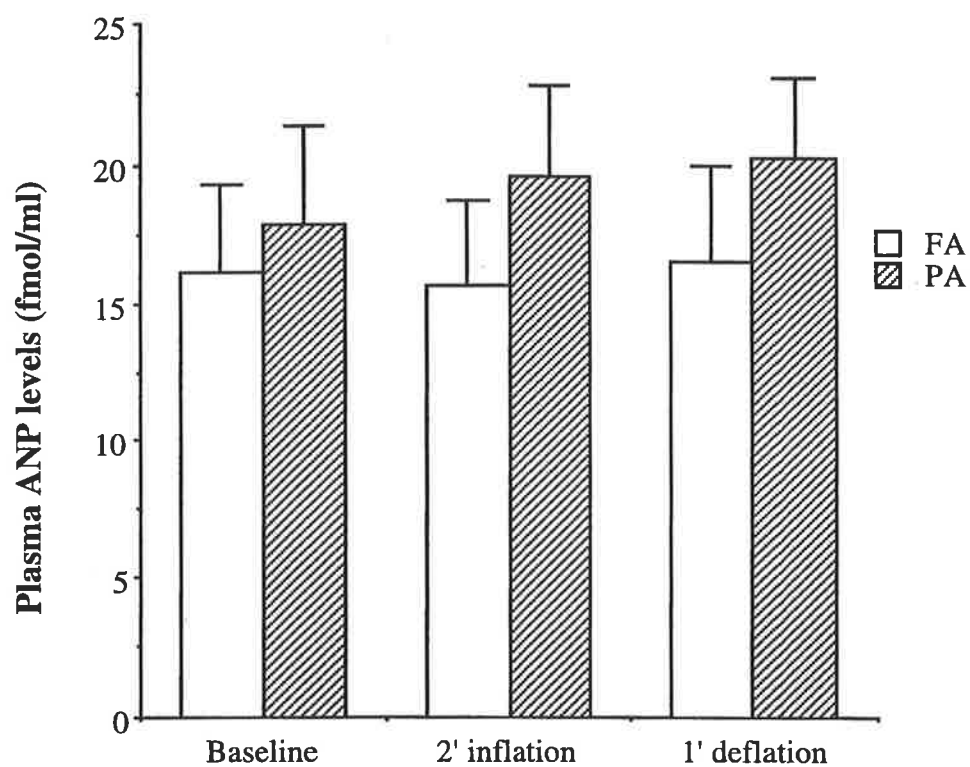


Fig 7.1. Plasma ANP concentrations (mean $\pm$ SEM) before PTCA, at the end of (2 min) initial balloon inflation, and 1 min post the first balloon deflation. FA=femoral artery, PA=pulmonary artery.

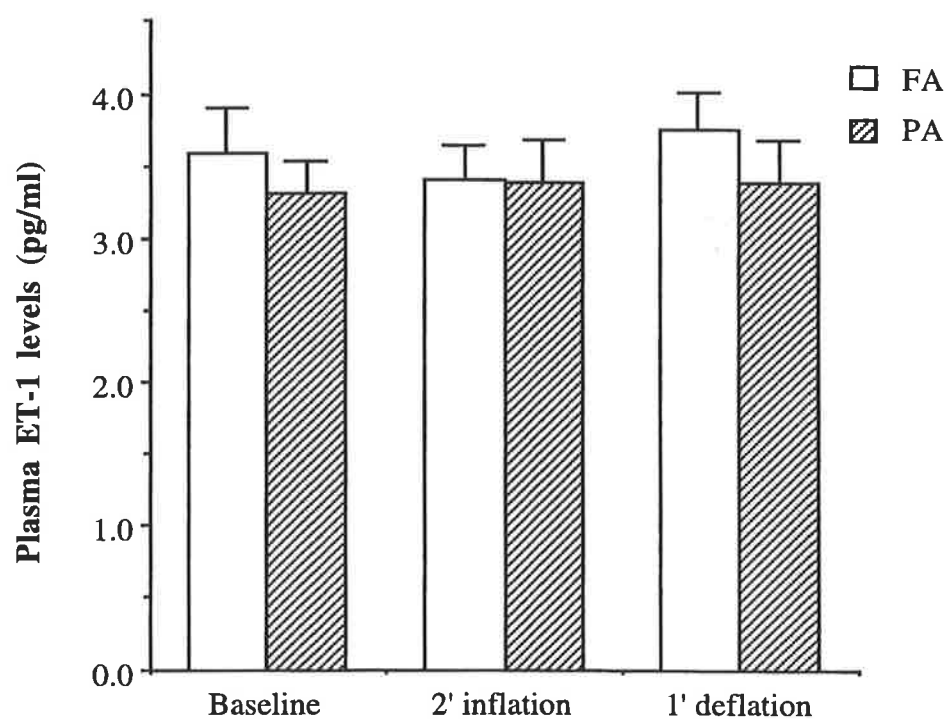


Fig 7.2. Plasma ET-1 concentrations (mean $\pm$ SEM) before PTCA, at the end of (2 min) initial balloon inflation, and 1 min post the first balloon deflation. FA=femoral artery, PA=pulmonary artery.

7.4. Discussion

In this study, plasma ANP and ET-1 concentrations did not alter significantly immediately subsequent to the initial intra-coronary balloon inflation during PTCA. These results therefore differ from the majority of previously published data; the basis for this difference merits discussion, especially as previously all reported studies have demonstrated increased ANP concentrations (Table 7.2.) and the majority increased ET-1 concentrations (Table 7.3.).

Changes in shear stress on the vascular wall has been shown to regulate expression of the ET-1 and pre-pro-endothelin-1 gene (Yanagisawa *et al.* 1988, Ziegler *et al.* 1998). Mechanical stretch also enhanced ET-1 synthesis/release *in vitro* (Ziegler *et al.* 1998, Yamazaki *et al.* 1996, Hishikawa *et al.* 1995). Therefore, in theory, the stress on vascular wall induced by intravascular balloon inflation during PTCA should increase ET-1 release.

As regards previous ANP-related investigations it is clear from Table 7.2 that increases in peripheral and coronary sinus ANP concentrations are generally observed. It is not clear from these data to what extent the increase in ANP concentrations was related to the size of the induced ischaemic zone, nor to the consequent extent of left ($\pm$ right) atrial distension. By comparison, the results of the current study suggest, at most, a minimal increase in ANP concentrations in PA (mixed venous) blood.

The data regarding previous ET-related experiments are more heterogeneous (Table 7.3): 3 investigations failed to demonstrate an increase in peripheral ET-1 concentrations. One

Table 7.2. Summary of previous studies of effect of PTCA on plasma ANP concentrations.

Studies	PTCA vessel	Sample site	ANP (% increase)	Comments
Ikaheimo 1989	LAD, RCA	FV PA	600 800	Increased ANP only found in patient with increased PCWP during PTCA
Malatino 1992	LAD, Cx, RCA	PA	60	ANP elevation at the end of balloon inflation. Both basal and post PTCA ANP levels correlated with PCWP.
Prachar 1991	LAD, Cx, RCA	RA FA	30 NS	ANP elevation only in patients with LAD occlusion in RA blood, after 1 min balloon inflation.
Gassser 1990	LAD	FA CS	83 112	ANP increase within minutes after PTCA
Weidermann 1991	LAD, RCA	PA	188	ANP elevation 5 min after PTCA
Chenu 1991	LAD, Cx, RCA	CA	50	ANP elevation only after the last balloon inflation. ANP correlates with PCWP. No Ca ⁺⁺ or β -blocker.
Uusimaa 1993	LAD, Cx, RCA	CS FA	60 NS	ANP elevation only in patients with increased lactate in CS blood.
Ameli 1993	LAD, Cx, RCA	FA	65	ANP increase at the end of PTCA. High basal level. Most of patients had unstable angina or AMI.
Nigri 1996	LAD, CX	CS FV	80 NS	ANP elevation only in CS blood during balloon inflation.
Gill 1995	LAD	CS	30	Lactate increased.
Kyriakides 1995	LAD	FV	8	ANP increase at the end of PTCA. No change after initial inflation.
Gasser 1993	LAD	FV CS	83 11	The increase in ANP independent of changes in PCWP.

PCWP=pulmonary capillary wedge pressure, LAD=left anterior descending coronary artery, Cx=circumflex coronary artery, RCA=right coronary artery, FA=femoral artery, FV=femoral vein, PA=pulmonary artery, CA=coronary artery, CS=coronary sinus, RA=right atrium, AMI=acute myocardial infarction, NS=not significant.

Table 7.3. Summary of previous studies of effect of PTCA on plasma ET-1 concentrations.

Studies	PTCA vessel	Sample site	ET-1 (% increase)	Comments
Kyriakides 1995*	LAD	FV	30	ET-1 increase at the end of PTCA. No change after <u>initial</u> inflation.
Kyriakides 1996	LAD	PV	10-40	ET-1 increase only at the end of PTCA. No change after the initial balloon inflation.
Ameli 1993*	LAD, Cx, RCA	FA	10-20	ET-1 increase at the end of PTCA. High basal level. Most patients had unstable angina or AMI.
Malatino 1993	LAD, RCA	CS AO	90 NS	CS ET-1 increase at the end of balloon inflation only for LAD lesion. ET-1 (V-A) also increased.
Hasdai 1997		CA FA	90 NS	ET-1 increase only in distal CA blood.
Kruger 1998	LAD, Cx, RCA	PV CS	90 230	Total ET instead of ET-1.
Montalescot 1994	LAD	CS, FA	no change	Plasma ET-1 no change during the first 24 hours post PTCA.

LAD=left anterior descending coronary artery, Cx=circumflex coronary artery, RCA=right coronary artery, FA=femoral artery, CA=coronary artery, CS=coronary sinus, AO=aorta, PV=peripheral vein, AMI=acute myocardial infarction. * ANP was also measured, NS=not significant.

investigation revealed that peak ET-1 concentrations in peripheral blood occurred more than 5 min post initial balloon inflation (specific times were not stated; hence it is possible that sampling in the current study was suboptimal). However, in the current study, no suggestion of ET-1 elevation was observed.

The current study was ceased after the first 7 patients when it was clear there was no significant change in either ANP or ET-1 levels. However, if such a change had been observed, it might have been possible to delineate (1) a dissociation between atrial distension and PTCA-induced ANP release (via serial RAP and PCWP monitoring): this would have been an interesting correlate with ischaemia-induced ANP release (chapter 3); (2) the impact of predominant right atrial distension on ANP release (eg with proximal right coronary artery occlusion); (3) the relationship between ET and ANP release in the presence and absence of atrial distension. The design of the study therefore offered the potential opportunity to advance current understanding of the pathophysiology of ANP and ET-1 in human subjects.

The negative findings in this work mabe related to Type II error (ie, false negative results due to the small sample size). Thus possible interpretations are (1) insufficient ischaemia: only one patient had ST elevation or chest pain, and it is possible that collateralization or consequences (2) specific effects of verapamil and/or glyceryl trinitrate (GTN): there is some evidence to suggest that both calcium antagonists (Iida & page 1989, Weinmann 1991) and GTN (see chapter 8) may limit ANP release. All of the patients in the current study had been pre-treated with both verapamil and GTN, as is usual practise prior to PTCA in many units (3) insufficient atrial distension: this was not measured routinely during PTCA; however, in 2 patients, PCWP did not rise following balloon inflation (4) sub-optimal timing of sampling; this may be applicable for ET-1 (Malatino *et al.* 1993, Kyriakides *et al.* 1995, 1996, as mentioned above).

Thus, in order to dissociate between ischaemia and atrial stretch as stimuli for ANP and ET-1, the current study proved inadequate. The issue might be better addressed by the utilization of coronary venous sampling (hence excluding right coronary artery PTCA) with carefully paired haemodynamic determinations. Such a study creates distinct ethical issues.

7.5. Summary

Concentrations of ANP and ET-1, measured prior and subsequent to intra-coronary balloon inflation in patients undergoing elective PTCA, did not change in plasma samples from femoral and pulmonary arteries. Thus, transient single coronary occlusion during PTCA did not alter systemic plasma ANP and ET-1 concentrations immediately.

CHAPTER 8 - RESULTS

**INTERACTIONS BETWEEN ANTI-ISCHAEMIC
THERAPY WITH GLYCERYL TRINITRATE,
HAEMODYNAMIC EFFECTS AND RELEASE OF
ENDOTHELIN-1/ATRIAL NATRIURETIC PEPTIDE**

8.1. Introduction

Organic nitrates, such as glyceryl trinitrate (GTN), isosorbide dinitrate and isosorbide mononitrate, are among the most commonly used anti-anginal drugs. Organic nitrates undergo sulfhydryl-mediated bioconversion to nitric oxide (NO) and/or S-nitrosothiols, which induce activation of soluble guanylate cyclase (GC) leading to formation of cGMP.

Animal studies *in vitro* and *in vivo* have suggested that endothelium derived relaxing factors (EDRF) (and thus presumably NO) inhibit ANP release (Lew & Baertschi 1989, Leskinen *et al.* 1995, Sanchez-Ferrer *et al.* 1990). In patients with CAD and chronic CHF, acute nitrate administration suppressed plasma ANP levels, consistent with the effects of a marked reduction in filling pressures in such patients (Tsutamoto *et al.* 1994a,b, Dakak *et al.* 1993). The effects of nitrates in patients with well-preserved left ventricular function are also relevant. However, there are no data concerning the possible acute interactions with ANP. Furthermore, in healthy volunteers, chronic nitrate administration may induce a variety of neurohumoral changes leading to fluid retention, and increased secretion of ANP (Parker *et al.* 1991).

Nitric oxide (NO) donors: (nitrovasodilators, sodium nitroprusside, S-nitroso-N-acetoxypenicillamine and cGMP) have also been reported to inhibit ET release (Ryan *et al.* 1993, Saijonmaa *et al.* 1990, Monose *et al.* 1993, Boulanger & Luscher 1990). Conversely, NO synthase inhibitors, such as NG-monomethyl-L-arginine (L-NMMA) and methylene blue potentiated thrombin- and A23187-stimulated ET-1 release (Boulanger & Luscher 1990). However, there is little available information on the determinants of this interaction in human subjects.

The aims of the current study were 1) to investigate the effect of acute nitrate therapy on plasma ANP and ET-1 concentrations and gradients across the femoral vascular bed, 2) to compare the effects of acute/chronic nitrate therapy on plasma ANP and ET-1 concentrations in patients with coronary artery disease but without overt heart failure. Since both ANP and nitrates induce cGMP production, plasma cGMP levels were also measured.

8.2. Methods

8.2.1. Patient selection

Patients were selected from those undergoing cardiac catheterization for the investigation of chest pain. We excluded patients with severe systolic hypotension, haemodynamically significant left main coronary artery stenosis, or individuals known to be intolerant of nitrates. Informed consent was obtained prior to study (refer to chapter 2.2.2.1.).

8.2.2. Protocol

8.2.2.1. Acute GTN infusion

The arterial pressure, right atrial pressure (RAP), pulmonary capillary wedge pressure (PCWP), heart rate (HR) and cardiac output (CO) were measured at baseline, and 10 min after GTN infusion (refer to chapter 2.2.2.2.). Blood samples from pulmonary artery (PA), femoral artery (FA) and vein (FV) were collected for ANP and ET-1 RIA. For details of sample preparation refer to chapter 2.2.2.3. For plasma ANP, ET-1 and cGMP RIA, see chapter 2.2.4, 2.2.5., 2.2.6.

After baseline sampling and haemodynamic measurement, GTN (10 µg/min) was infused for 10 min utilizing a non-adsorbent delivery system. Haemodynamic measurements and blood sampling were repeated at 10 min after commencement of GTN infusion. If a patient developed hypotension (ie, systolic blood pressure <90 mm Hg), GTN infusion was ceased immediately.

8.2.2.2. Chronic nitrate therapy

Patients who had been on chronic long-acting nitrate therapy for more than 24 hours were studied during such therapy. Haemodynamic and biochemical measurements were obtained during cardiac catheterization. Blood samples were taken as for 8.2.2.1.

8.2.3. Analysis of data

Data were expressed throughout as mean $\pm$ SEM. Plasma ANP and ET-1 concentrations were expressed as fmol/ml and pg/ml respectively. Comparison of patient characteristics between acute and chronic nitrate therapy groups utilized unpaired *t* test (quantitative parameters) and chi-squared analysis (categorical parameters). Correlations between haemodynamic and biochemical parameters were made utilizing linear regression analysis. Comparisons of haemodynamic data or biochemical changes were performed using paired or unpaired Student's *t* test for normally distributed data. Non-parametric data were compared using Wilcoxon signed-rank, Mann-Whitney U and Spearman rank test for correlations. A *P* value of <0.05 was considered statistically significant (refer to chapter 2.3.).

8.3. Results

8.3.1. Effect of acute GTN infusion on plasma ANP and ET-1 concentrations.

8.3.1.1. Patient characteristics

Twenty-seven patients (17 male and 10 female) were studied. Among them, 13 patients were receiving calcium antagonists and 5 β -adrenoceptor antagonists. Seventeen had haemodynamically significant stenosis (ie, >50% stenosis) in at least one of the major epicardial coronary arteries. Overall, although there were mild LVEF reduction, associated with PCWP elevation in two cases, RAP and cardiac systolic function were normal (Table 8.1.). None of the study subjects experienced symptomatic adverse effects from GTN infusion. In 2 patients, GTN infusion was terminated after 5.5 and 7 min respectively due to emerging hypotension. In one patient, systolic blood pressure increased from 112 to 153 mm Hg after GTN infusion; blood samples were drawn at this time. As this response is inconsistent with haemodynamic effects of GTN alone, and suggests variable autonomic tone, data from this patient were excluded from haemodynamic analyses and correlations

8.3.1.2. Haemodynamic responses to 10 min iv GTN

GTN infusion (10 μ g/min for 10 min) induced significant decreases in SBP ($8.1 \pm 2.3\%$, $P < 0.0001$, paired t test), mean arterial pressure (MAP) ($4.6 \pm 1.9\%$, $P < 0.01$, paired t test) and mean PCWP. The change in mean PCWP was most marked ($-38.3 \pm 4.6\%$, $P < 0.0001$, paired t test). The SBP reduction was more pronounced in subjects without significant coronary artery disease (CAD) than in patients with CAD (14.3 ± 9.7 vs

Table 8.1. Patient characteristics

Pt	Sex	Age (yr)	Wt (Kg)	EF (%)	PCWP (mm Hg)	RAP (mm Hg)	CAD (>50% stenosis)
1	M	60	70	76	13	8	none
2	F	64	68	83	5.0	3	LAD, Cx, RCA
3	M	58	98	57	7.0	1	LAD, Cx
4	M	60	58	79	9.0	2	Cx
5	F	44	55	68	12	5	none
6	M	51	95	70	11	3	Cx
7	F	46	74	77	8.0	8	none
8	M	55	92	59	15	6	Cx
9	F	43	81	68	9.5	NR	none
10	F	54	87	67	6.0	2	none
11	M	63	67	70	12	7	none
12	M	41	76	58	9.5	5	none
13	M	64	76	70	1.5	3	none
14	M	44	70	59	11	4	Cx, RCA
*(15	F	70	67	40	15	11	LAD, RCA)
16	F	49	92	40	20	10	LAD, Cx, RCA
17	F	54	66	50	15	6	LAD, Cx
18	M	60	93	60	3	1	RCA
19	M	58	95	49	4	2	Cx, RCA
20	M	69	71	60	4	4	LAD, Cx, RCA
21	M	40	95	56	11	6	LAD, RCA
22	F	70	69	NR	1	1	none
23	M	63	70	63	13	6	LAD, Cx, RCA
24	M	68	78	62	8	6	LAD
25	M	60	89	56	7	3	LAD
26	M	35	110	64	8	4	Cx
27	F	65	80	75	4	2	none
Mean		56	79	63	9.1	4.4	
SEM		2	3	2.1	0.9	0.5	

EF = Left ventricular ejection fraction, PCWP = Pulmonary capillary wedge pressure, RAP = Mean right atrial pressure, CAD = Coronary artery disease, LAD = Left anterior descending coronary artery, Cx = Circumflex coronary artery, RCA = Right coronary artery. NR = Not recorded. * excluded from haemodynamic analysis, see text.

7.1±5.9%, $P<0.05$, unpaired t test). There was a statistically significant increase in HR ($P<0.01$, paired t test) but not in cardiac index (Table 8.2.).

8.3.1.3. Effects of GTN on plasma ANP and cGMP levels

8.3.1.3.1. ANP

Basal plasma ANP concentrations in FA, FV and PA were 35.3±6.7, 20.3±3.6 and 32.9±6.6 fmol/ml respectively. ANP concentrations in FV were significantly less than those in FA and PA ($P<0.0005$). There was no significant difference in basal plasma ANP concentrations in patients with or without CAD. Baseline ANP concentrations in FA and PA correlated significantly with PCWP ($r=0.44$, $P=0.03$ and $r=0.51$, $P=0.01$ respectively; linear regression). In response to GTN infusion, plasma ANP concentration significantly decreased in FA ($P<0.0005$, Fig 8.1.), FV ($P<0.05$) and PA ($P<0.005$, paired t test; Table 8.3.). The reduction in ANP concentrations was disproportionately great in the case of FA, so that the gradients of ANP concentration across the femoral vascular bed (FA-FV) were also reduced ($P<0.01$, Wilcoxon signed-rank) post GTN, suggesting that ANP extraction across the femoral bed was decreased during GTN infusion. There was no correlation between ANP concentrations/gradients and the changes in SBP or PCWP post GTN infusion.

8.3.1.3.2. cGMP

Basal plasma cGMP concentrations (Table 8.4) were significantly greater in FV than FA ($P<0.01$), while cGMP concentrations in FA were significantly greater than that in PA ($P<0.01$). There was no significant difference in either resting plasma cGMP concentrations, or in cGMP concentration gradient across the femoral vascular bed in patients with and without significant coronary artery stenosis. After infusion of GTN,

Table 8.2. Haemodynamic changes after iv GTN infusion[Ⓜ]

Pt	SBP (mm Hg)		MAP (mm Hg)		PCWP (mm Hg)		HR (beats/min)		CI (L/min/m ²)	
	B	N	B	N	B	N	B	N	B	N
1	174	156	115	111	13	6.0	63	65	2.3	2.1
2	156	136	96	89	5.0	3.0	62	64	2.5	2.4
3	122	124	83	87	7.0	4.0	82	83	2.6	2.2
4	135	131	87	88	9.0	5.0	64	63	2.6	3.1
5	125	115	88	85	12	6.0	60	75	2.9	2.9
6	160	150	104	102	11	5.5	64	70	5.6	NR
7	164	135	119	105	8.0	7.0	84	90	2.8	2.9
8	156	135	110	102	15	5.0	61	64	2.5	2.4
9	114	102	80	75	9.5	4.0	62	63	3.0	4.5
10	130	124	90	88	6.0	7.0	59	61	2.7	2.0
11	142	122	88	85	12	8.0	61	70	3.4	2.6
12	154	131	120	103	9.5	4.0	66	74	3.0	2.1
13	120	95	90	75	1.5	1.5	96	108	1.9	1.9
14	140	137	102	98	11	7	63	65	3.1	2.9
16	148	138	110	100	20	11	84	84	2.8	2.7
17	162	158	110	111	15	5	70	70	2.7	2.2
18	142	132	100	88	3	1	75	77	2.9	2.3
19	155	132	110	94	4	2	78	87	2.8	2.4
20	140	115	101	90	4	3.5	98	101	2.5	2.3
21	139	135	94	97	11	7	67	67	2.9	2.9
22	160	100	102	67	NR	NR	83	78	2.3	1.8
23	180	158	108	100	13	13	54	55	3.2	2.9
24	168	150	103	98	8	3	55	58	2.9	3.0
25	136	138	95	94	7	6	62	55	2.7	2.9
26	130	125	80	95	8	5	74	77	3.6	3.5
27	170	162	117	120	4	2.5	82	80	3.4	3.6
Mean	147	132*	100	94*	9.1	5.3*	70	73*	2.9	2.7
SEM	4	4	2	2	0.9	0.6	3	3	0.1	0.1

Pt=Patient, SBP=Systolic blood pressure, MAP=Mean arterial pressure, PCWP=Pulmonary capillary wedge pressure, HR=Heart rate, CI=Cardiac index, B=Baseline, N=GTN. [Ⓜ] These data represent 10 min of GTN infusion except for pts 12 and 13. * $P < 0.01$ vs baseline. NR=Not recorded.

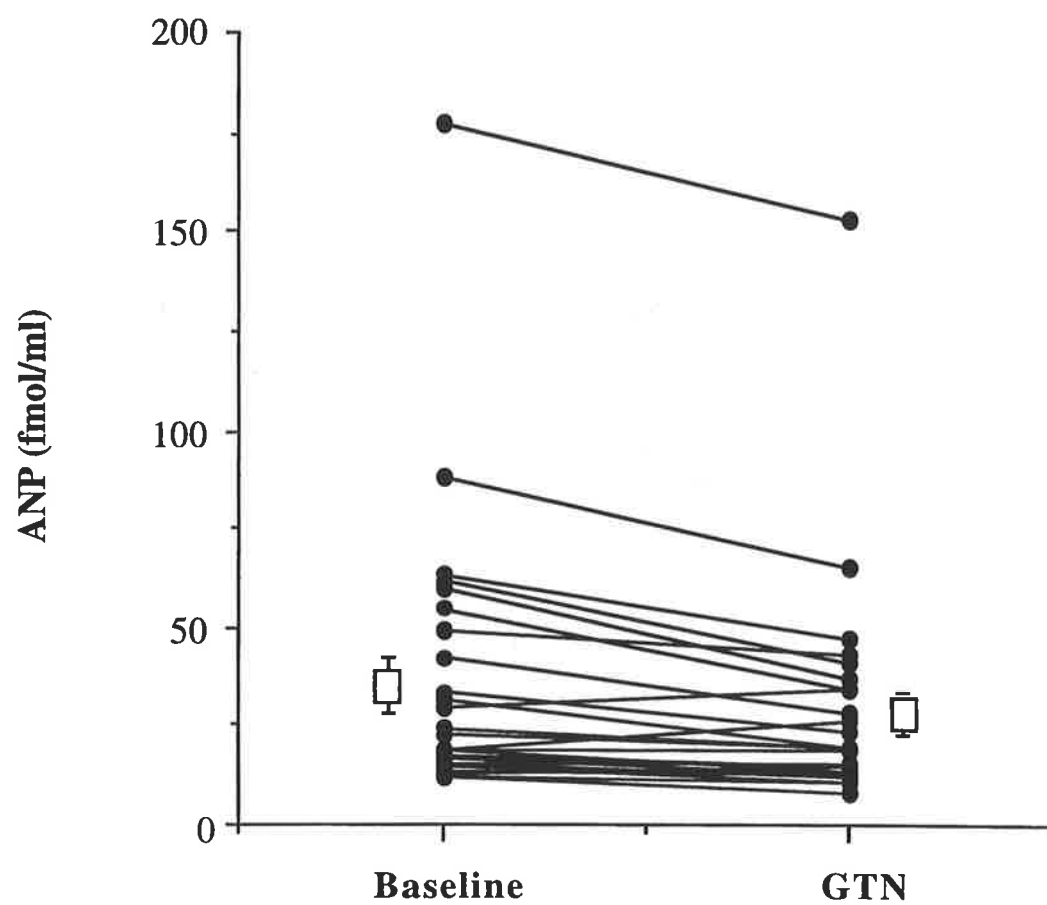


Fig 8.1. Effect of acute iv GTN on femoral artery ANP concentrations (n=27).

Table 8.3. Plasma ANP concentrations before and after GTN infusion[Ⓜ]

Pt	ANP (fmol/ml)							
	FA		FV		PA		(FA-FV)	
	B	N	B	N	B	N	B	N
1	33.5	23.7	17.8	13.8	32.0	24.2	15.6	9.8
2	17.5	14.4	15.4	9.9	15.9	11.8	2.2	4.4
3	17.0	14.9	8.3	7.8	13.6	10.3	8.7	7.1
4	29.0	34.6	15.8	21.6	35.0	38.4	13.2	13.0
5	24.3	19.3	14.7	10.9	30.3	23.4	9.6	8.4
6	42.1	28.4	22.7	21.4	42.6	29.9	19.4	7.0
7	12.9	14.6	9.0	8.8	12.0	16.4	3.9	5.7
8	22.9	19.3	16.3	13.9	21.4	16.4	6.5	5.4
9	18.2	13.3	10.7	9.4	22.8	18.3	7.6	3.9
10	15.1	12.2	9.5	8.3	16.9	16.0	5.6	3.9
11	14.6	11.1	9.6	8.6	15.4	15.4	5.0	2.5
12	16.7	12.9	9.9	8.5	18.3	14.5	6.9	4.4
13	18.8	26.0	8.7	9.4	22.2	25.7	10.1	16.5
14	87.5	65.3	39.8	47.2	@	50.2	47.7	18.1
15	177.9	153.0	96.2	101.1	183.5	165.1	81.7	51.9
16	54.9	34.2	38.6	28.7	55.4	30.4	16.3	5.4
17	61.1	40.8	36.8	28.3	58.4	43.4	24.4	12.5
18	15.6	15.9	12.3	8.7	18.6	13.9	3.3	7.1
19	11.5	10.5	8.7	6.8	11.8	15.0	2.8	3.7
20	18.4	18.9	13.7	11.5	21.5	18.9	4.7	7.4
21	11.9	7.4	6.7	5.2	17.6	11.3	5.3	2.3
22	31.2	19.3	17.9	11.9	32.0	23.0	13.3	7.4
23	59.1	37.4	39.1	33.5	52.1	50.2	20.0	3.9
24	63.3	46.5	27.9	17.0	42.5	40.3	35.4	29.6
25	13.4	12.3	8.8	8.1	18.1	18.4	4.7	4.2
26	15.3	11.4	4.9	6.8	15.0	10.6	10.9	4.6
27	48.7	42.5	27.8	21.5	50.1	45.2	20.9	20.9
Mean	35.3	28.2*	20.3*‡	18.1*‡	33.7	29.5*	15.0	10.0*
SEM	6.7	5.5	3.7	3.7	6.5	5.7	3.3	2.0

Pt=Patient, FA=Femoral artery, FV=Femoral vein, PA=Pulmonary artery, B=Baseline, N=GTN. ‡ $P<0.01$ vs FA, * $P<0.05$ vs baseline, @Sample lost. [Ⓜ]These data represent 10 min of GTN infusion except for pts 12 and 13.

Table 8.4. Plasma cGMP concentrations before and after GTN infusion[□].

Pt	cGMP (pmol/ml)							
	FA		FV		PA		FV-FA	
	B	N	B	N	B	N	B	N
1	7.9	7.1	11.9	12.4	6.6	10.6	3.9	5.3
2	4.6	4.3	5.5	6.1	4.3	5.6	0.9	1.8
3	11.3	7.9	13.3	11.5	8.8	8.3	2.0	3.6
4	10.2	8.8	10.3	11.2	7.7	7.8	0.1	2.4
5	8.7	14.3	9.4	14.7	9.1	12.2	0.7	0.4
6	14.6	16.9	16.1	19.9	12.5	13.3	1.5	3.0
7	12.0	8.1	9.5	8.9	7.0	6.2	-2.5	0.8
8	8.8	7.6	11.6	13.8	8.2	10.3	2.8	6.2
9	10.0	7.9	10.6	12.8	11.1	6.9	0.6	4.9
10	2.6	2.7	2.6	3.1	2.5	2.8	0.0	0.4
11	4.9	3.6	5.9	4.9	4.4	3.7	1.0	1.3
12	5.6	5.3	6.5	6.7	5.5	6.3	0.9	1.4
13	5.5	5.9	6.8	6.9	4.9	5.3	1.3	1.0
14	18.7	16.3	19.6	18.9	15.1	13.9	0.9	2.8
15	12.6	15.6	16.1	20.8	12.8	13.5	3.5	5.2
16	10.3	10.7	12.2	13.7	9.9	10.9	1.9	3.0
17	6.9	6.4	7.8	7.2	5.7	5.9	0.8	0.8
18	3.7	4.1	5.0	5.5	4.1	3.7	1.3	1.3
19	5.3	3.8	5.0	5.2	3.9	3.9	-0.3	1.4
20	3.9	4.2	5.7	6.1	4.4	4.4	1.7	1.8
21	7.7	6.6	8.7	9.0	6.2	4.7	1.0	2.4
22	7.2	6.3	9.3	9.2	6.9	5.7	2.0	2.9
23	13.4	11.8	13.5	14.0	13.0	12.9	0.1	2.2
24	10.8	9.1	11.4	10.6	9.9	8.6	0.6	1.5
25	4.1	3.7	4.5	3.9	3.6	3.6	0.3	0.3
26	5.2	4.8	6.8	6.8	4.9	3.8	1.6	2.0
27	14.6	13.6	14.4	15.1	11.4	10.2	-0.1	1.3
Mean	8.6	8.1	9.6*	10.3 [#]	7.4*	7.6	1.2	2.2**
SEM	0.78	0.81	0.80	0.94	0.66	0.68	0.20	0.31

Pt=Patient, FA=Femoral artery, FV=Femoral vein, PA=Pulmonary artery, B=Baseline,

N=GTN. [□]These data represent 10 min of GTN infusion except for pts 12 and 13.

* $P < 0.01$ vs FA; [#] $P < 0.05$ baseline; ** $P < 0.005$ vs baseline.

there was a significant increase in plasma cGMP concentration gradient between FV and FA (1.2 ± 0.31 to 2.4 ± 0.39 pmol/ml, $P < 0.005$). There was also a small increase in FV plasma cGMP ($P < 0.05$, paired t test). There was no significant correlation between the increased ANP extraction ($[\text{ANP}]_{\text{FA-FV}}$) and decreased cGMP “net” production across femoral vascular bed ($[\text{cGMP}]_{\text{FV-FA}}$) post GTN infusion.

8.3.1.4. Effects of GTN on plasma ET-1 levels

Plasma ET-1 concentrations were measured in 16 patients. Basal plasma ET-1 concentrations in FA, FV and PA were 3.49 ± 0.12 , 3.32 ± 0.30 and 3.37 ± 0.18 pg/ml respectively. There was no significant difference in either resting plasma ET-1 concentrations or in ET-1 concentration gradient across the femoral vascular bed between patients with and without significant coronary artery stenosis. After GTN infusion, ET-1 concentrations in FA and PA did not change significantly, whereas FV ET-1 concentrations tend to increase ($P = 0.052$, paired t test). The ET-1 concentration gradient across the femoral vascular bed ($[\text{ET-1}]_{\text{FV-FA}}$) was increased ($P = 0.017$, Wilcoxon signed-rank) post GTN (Table 8.5). There was no correlation between the gradients of ET-1 level across the femoral vascular bed and the magnitude of changes in SBP or cGMP post GTN.

8.3.2. Effects of chronic nitrate therapy on plasma ANP and ET-1 concentrations.

8.3.2.1. Patient characteristics

Table 8.6. summarizes patient characteristics and haemodynamic parameters of the 23 patients (15 male, 8 female) studied. Among them, 3 patients were receiving β -adrenoceptor antagonists, 18 calcium antagonists and 3 ACE-inhibitors at the time of

Table 8.5. Plasma ET-1 concentrations (pg/ml) before and after GTN infusion[⌘]

Pt No	FA B	FA N	FV B	FV N	PA B	PA N	FV-FA B	FV-FA N
1	3.67	4.26	3.84	4.29	3.84	3.42	0.182	0.033
7	4.01	3.95	3.75	5.16	4.29	3.39	-0.255	1.213
8	3.30	4.47	3.29	3.06	4.25	3.36	-0.014	-1.413
9	4.19	3.23	4.21	3.59	3.58	2.70	0.019	0.362
12	3.57	3.50	3.77	3.95	2.52	2.41	0.222	0.447
16	3.75	2.52	1.79	17.5	2.17	8.13	-1.950	14.97
18	3.12	3.05	4.02	17.5	3.45	3.23	0.900	14.49
19	2.59	2.49	2.80	13.7	2.42	2.12	0.217	11.173
20	3.47	2.54	2.25	2.76	2.89	2.97	-1.222	0.219
21	3.61	2.46	4.54	4.47	3.71	3.86	0.931	2.013
22	2.71	2.85	3.28	2.25	2.33	3.44	0.568	-0.601
23	3.17	3.41	0.00	2.68	4.19	3.40	-3.170	-0.731
24	4.27	4.20	4.93	6.42	4.15	3.58	0.661	2.225
25	3.75	3.42	3.07	3.97	3.39	3.00	-0.673	0.542
26	3.68	3.49	3.99	3.96	3.33	3.12	0.308	0.467
27	2.97	3.55	3.61	3.23	3.46	8.74	0.639	-0.32
Mean	3.49	3.34	3.32	6.16	3.37	3.80	-0.165	2.818*
SEM	0.12	0.16	0.30	1.29	0.18	0.47	0.278	1.364

Pt=Patient, FA=Femoral artery, FV=Femoral vein, PA=Pulmonary artery, B=Baseline, N=GTN. * $P < 0.05$ vs baseline. [⌘]These data represent 10 min of GTN infusion except for pt 12.

Table 8.6. Patient characteristics: chronic nitrate therapy group.

Pt	Sex	Age (year)	HR (beat/min)	LVEF (%)	RAP (mm Hg)	PCWP (mm Hg)	CAD (>50% stenosis)
1	F	74	104	92	0	3	none
2	F	79	100	30	9	28	LAD, Cx, RCA
3	M	68	70	58	4	11	LAD, Cx, RCA
4	M	62	81	47	5	12	LAD, Cx, RCA
5	F	76	78	91	7	19	LAD, RCA
6	F	68	111	43	5	9	LAD, Cx, RCA
7	M	42	77	ND	4	10	LMCA,LAD,Cx,RCA
8	M	67	78	62	5	11	LAD, Cx, RCA
9	F	70	80	57	2	7	LAD
10	M	46	70	36	ND	ND	LAD
11	M	44	60	67	8	6	RCA
12	M	77	78	48	8	12	LAD, Cx, RCA
13	F	70	84	50	2	7	LAD
14	M	63	111	38	9	26	Cx, RCA
15	M	63	90	37	3	14	LAD, Cx, RCA
16	M	74	78	ND	5	7	LMCA,LAD,Cx,RCA
17	M	79	65	45	2	2	Cx, RCA
18	M	72	53	56	9	13	LAD, Cx
19	F	60	NR	ND	ND	24	LMCA, LAD, RCA
20	M	66	51	42	3	4	LAD, Cx, RCA
21	m	52	73	69	5	8	LAD
22	F	58	NR	58	4	16	LAD
23	M	48	78	54	ND	ND	none
Mean		64.3	79.5	53.9	4.95	11.9	
SEM		2.3	3.3	3.3	0.53	1.5	

LVEF=left ventricular ejection fraction, HR=heart rate, PCWP=pulmonary capillary wedge pressure, RAP=mean right atrial pressure, CAD=coronary artery disease, LAD=left anterior descending coronary artery, Cx=circumflex coronary artery, RCA=right coronary artery, LMCA=left main coronary artery, ND=Not determined.

study. Chronic nitrate therapy including isosorbide dinitrate (n=11), isosorbide mononitrate (n=3), transdermal GTN (n=7), or iv GTN (infusion for >24 hours) (n=2). Despite this therapy, five patients had elevated pulmonary capillary wedge pressure (ranging from 16 to 28 mm Hg). Systolic function was impaired in 9 cases (LVEF range 30-48). Twenty-one patients receiving chronic nitrate therapy had haemodynamically significant stenosis in at least one major epicardial coronary artery.

8.3.2.2. Comparisons of haemodynamic parameters between acute and chronic nitrate administration.

Table 8.7 compares the patient characteristics of acute vs chronic nitrate therapy groups. The mean age of patients with chronic nitrate therapy was greater than the acute therapy group ($P<0.01$, unpaired t test). More patients had significant coronary artery disease in chronic (91%) than in acute (63%) therapy group ($P<0.05$, *Chi-square* test). The PCWP was significantly higher ($P<0.0005$), whereas left ventricular ejection fraction was lower ($P<0.05$, unpaired t test) in patients receiving chronic nitrate therapy compared with patients with acute nitrate therapy.

8.3.2.3. Comparisons of plasma ANP concentrations between acute and chronic nitrate administration.

Plasma ANP concentrations in FA and the gradient of ANP levels across the femoral vascular bed were higher during chronic nitrate therapy than those with acute GTN infusion (57.9 ± 11.3 vs 28.8 ± 5.6 , $P<0.05$; and 25.1 ± 5.2 vs 10.0 ± 2.0 , $P<0.01$ respectively; unpaired t test; Fig 8.2.). The ANP concentrations in FA were correlated with PCWP ($r=0.805$, $P<0.0001$, linear regression), RAP ($r=0.489$, $P<0.05$, linear regression), and heart rate ($r=0.472$, $P<0.05$, linear regression); but not with LVEF.

Table 8.7. Patient characteristics - acute vs chronic study. Haemodynamic data reflect post-GTN administration for acute study.

	Acute (n=27)	Chronic (n=23)
Age (years)	56±2	64±2*
Sex (M/F)	17/10	14/8
IHD (%)	63	91*
HR (beat/min)	73±3	80±3
PCWP (mm Hg)	5.5±0.6	11.9±1.5*
LVEF (%)	63±2	54±3*
Nitrate therapy	none	ISDN (n=11) ISMN (n=3) GTN (n=9)

HR=Heart rate, PCWP=Pulmonary capillary wedge pressure, LVEF=Left ventricular ejection fraction, IHD=Ischaemic heart disease, ISDN=Isosorbide dinitrate, ISMN=Isosorbide mononitrate, GTN=Glyceryl trinitrate. * $P<0.05$ vs acute.

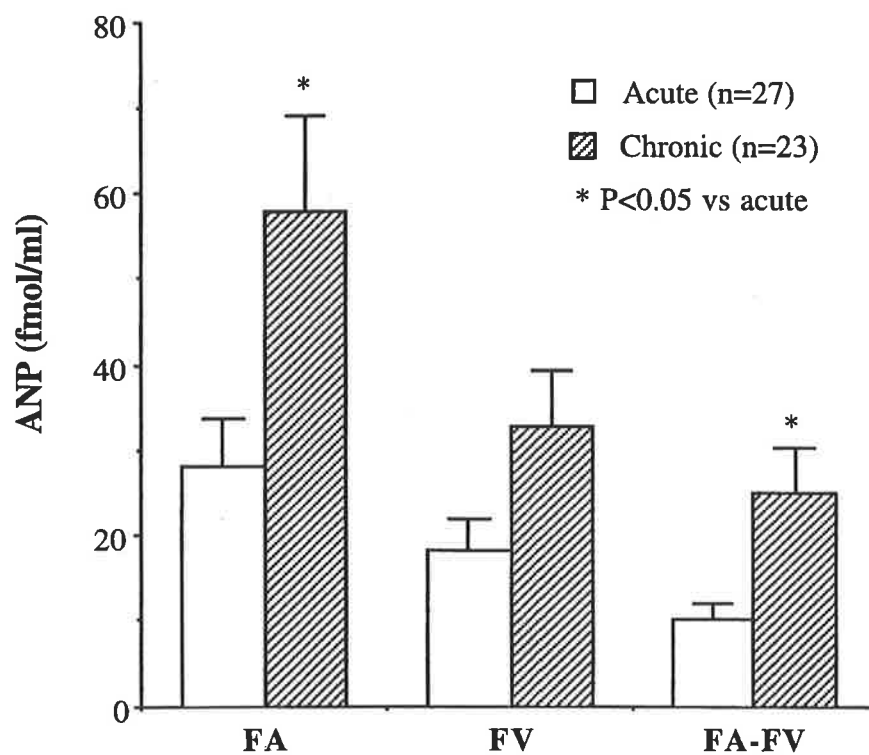


Fig 8.2. Comparison of the plasma ANP concentrations in patients receiving acute or chronic nitrate therapy.

8.3.2.4. Comparisons of plasma ET-1 concentrations between acute and chronic nitrate administration.

In the chronic nitrate therapy group, FA plasma ET-1 levels were significantly higher than those in acute GTN treated patients (4.05 ± 0.32 vs 3.36 ± 0.16 pg/ml, $P < 0.05$, unpaired t test). There was no significant difference of ET-1 concentrations in FV and gradient across the femoral vascular bed (FV-FA) between chronic and acute nitrate therapy groups (Fig 8.3.).

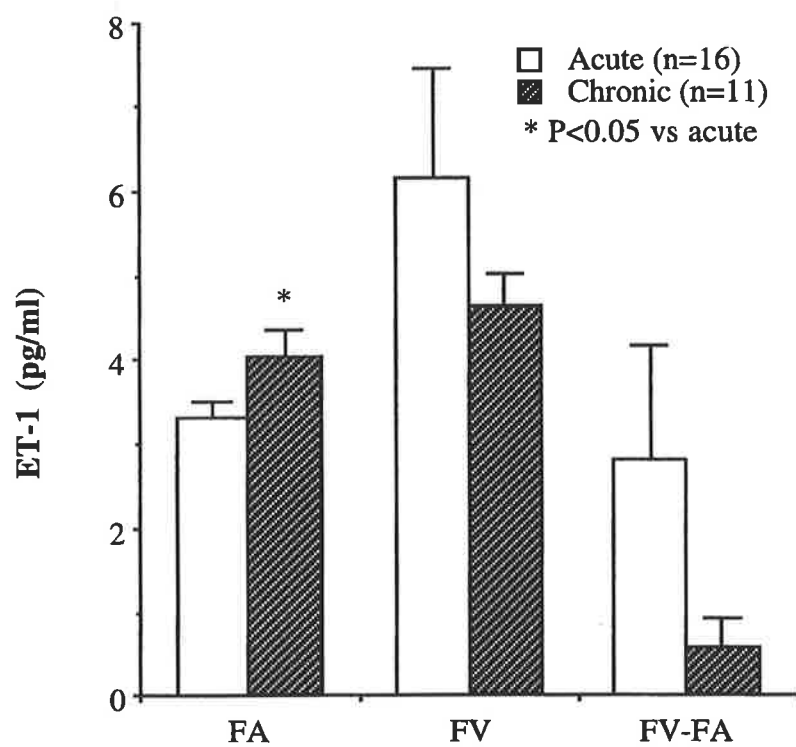


Fig 8.3. Comparison of plasma ET-1 concentration (mean±SEM) in patients with acute or chronic nitrate therapy.

8.4. Discussion

Acute GTN Therapy

In the current study, patients with ischaemic heart disease without overt CHF, acute GTN infusion caused a rise in cGMP generation as expected. This finding is consistent with that of a previous study (Tsutamoto *et al.* 1994b). There are three potential sources of cGMP in this circumstance: exogenous NO from GTN; endogenous NO (EDRF) which is difficult to measure; and ANP which was suppressed by acute GTN administration. Thus, in the current study, the increase in cGMP production represents the effect of GTN attenuated by reduced ANP effect. During acute GTN infusion, ANP release was reduced despite a decrease in heart rate and decreased clearance (extraction across femoral vascular bed). Possible mechanisms to explain this are that GTN decreases ANP production by decreasing atrial stretch (reflected by low PCWP), or by reducing myocardial ischaemia.

After 10 min GTN infusion, plasma ET-1 gradient across the femoral vascular bed was increased. This result contradicts those previous studies *in vitro* utilizing NO donors: (such as the nitro-vasodilators, sodium nitroprusside, S-nitroso-N-acetyl penicillamine and cGMP) which all show inhibition of ET release (Ryan *et al.* 1993, Saijonmaa *et al.* 1990, Boulanger & Luscher 1990). Thus the distinction between *in vivo* (current study) and *in vitro* (previous studies) investigation is particularly important. Since ANP also inhibits ET-1 release (Emori *et al.* 1993; Kohno *et al.* 1992, 1993a,b; Fujisaki *et al.* 1995; Neuser *et al.* 1990; Hu *et al.* 1992), the decrease in plasma ANP concentrations may contribute to this increase in ET-1 efflux. Another possible mechanism for the increase in ET-1 gradient across the femoral vascular bed may be due to the decrease in ET-1 binding (clearance) during GTN infusion. There is evidence which shows ischaemia causes externalization of endothelin-1 binding sites in rat cardiac membranes (Liu *et al.*

1989). The clinical implication of this increased ET-1 release is that it may attenuate the vasodilator effect of GTN and contribute to early onset of the phenomenon of pseudo-tolerance. The increase in ET-1 with GTN is an exception to the usual "biological rule" of increased NO effect being associated with reduced ET effect (Anonymous, Lancet, 1988); the implications of this exception merit wider examination.

Overall, in patients without CHF, biochemically/haemodynamically therapeutic concentrations of GTN induced ANP "withdrawal" and ET-1 "formation". These changes may account for the early pseudo-tolerance to organic nitrates: any increase in ET-1 may limit the anti-ischaemic effects of GTN.

Chronic Nitrate Therapy

The major limitation of this study is differences in patient characteristics between acute and chronic nitrate therapy groups. Many patients in the chronic nitrate therapy group had evidence of asymptomatic left ventricular dysfunction: - PCWP was significantly higher in patients undergoing chronic nitrate therapy. From the correlation between ANP_{FA} concentrations and PCWP post acute/chronic nitrate therapy shown in Fig 8.4, it is apparent that the significant relationship between PCWP and ANP concentration is largely due to elevated ANP concentrations in patients with high PCWP; there may be no significant relationship within the normal PCWP range in either group (ie the PCWP : ANP relationship may actually be curvi-linear). The data for acute and chronic patients can be superimposed at comparable PCWP values.

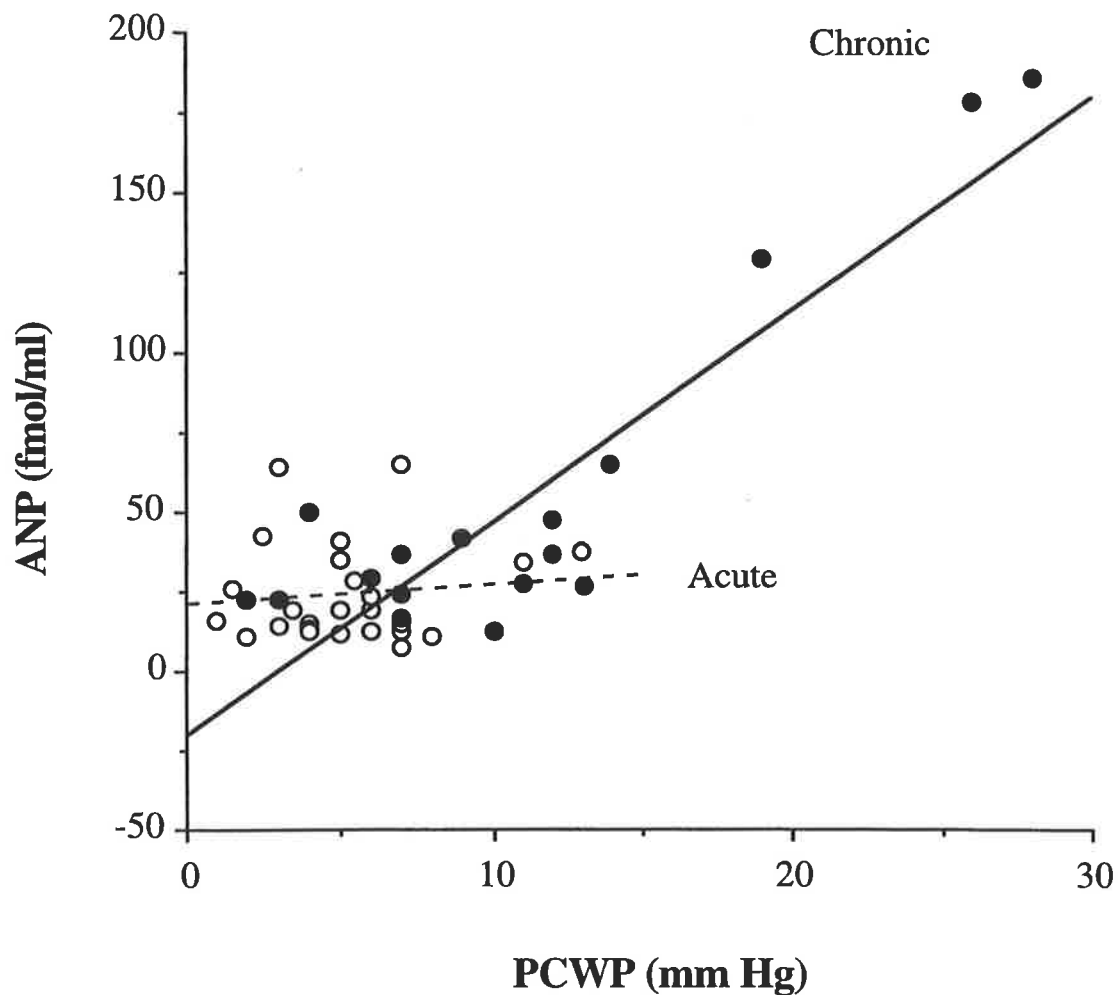


Fig 8.4. Correlations between PCWP and ANP concentration in femoral arterial blood: comparison of acute (open circles) vs chronic nitrate therapy (closed circles). Linear regression, best fit data are shown for both acute (dashed line) and chronic (solid line).

8.5. SUMMARY

In 27 patients undergoing cardiac catheterization for investigation of chest pain, brief intravenous GTN infusion (10 $\mu\text{g}/\text{min}$) significantly decreased pulmonary capillary wedge and systolic arterial pressures, and increased cGMP production across the femoral vascular bed. Plasma ANP concentrations in FA, FV and PA and the ANP gradient across femoral vascular bed (FA-FV) decreased following GTN infusion. ET-1 concentrations in FA and PA plasma did not change significantly in the presence of GTN. However, plasma ET-1 concentration gradient across the femoral vascular bed (FV-FA) increased. Thus acute GTN infusion decreased ANP release and femoral vascular extraction of ANP, but increased plasma ET-1 efflux across the femoral vascular bed.

During chronic nitrate therapy ($n=23$), plasma ANP and ET-1 levels in FA were higher than that in acute nitrate therapy group. However, this group of patients had higher PCWP and lower LVEF than those undergoing acute nitrate therapy. The $[\text{ANP}]_{\text{FA}}$ is correlated with PCWP in chronic nitrate therapy patients, while there was no correlation in acute nitrate therapy group. Thus chronic nitrate therapy had no direct effect on ANP release.

CHAPTER 9

CONCLUSIONS AND FUTURE DIRECTIONS

9.1. Conclusions

In this study, the following major findings have been demonstrated:

Isolated Langendorff-perfused rat hearts,

1) Right atrial pacing at a rate 10% above spontaneous heart rate did not affect basal or ischaemia-induced ANP release.

2) Neither NO synthase inhibitor, L-NAME, nor ET receptor antagonists, BQ-123 and bosentan, affect basal or ischaemia-induced ANP release. Thus endogenous NO and ET did not contribute to the ischaemia-induced ANP release. Beyond these data, the mechanisms of ischaemia-induced ANP release remain unknown.

3) Hypoxia-induced increase in ANP release was partially attenuated by bosentan, indicating a possible role for endogenous ET mediated ANP release during hypoxia.

4) Both BQ-123 and bosentan perfusion increased ET-1 efflux rate. BQ-123 decreased coronary flow rate. Bosentan accentuated the ischaemia-induced impairment in force of contraction. These results provide evidence that ET-1 release within the coronary vascular bed is subject to autocrine negative feedback regulation mediated via ET_A receptors; and that the increased ET-1 efflux may lead to net vasoconstriction via unopposed ET_B stimulation. Furthermore, the negative inotropic effects observed during ischaemia suggest that ET is critical in the maintenance of systolic function during ischaemia

In patients undergoing coronary catheterization,

1) Transient single coronary artery occlusion during PTCA did not immediately alter systemic plasma ANP and ET-1 concentrations. These findings vary from those of the majority of previous studies; the possible basis of these “negative” results has been discussed.

2) Acute GTN infusion decreased ANP release and femoral vascular extraction of ANP, but increased plasma ET-1 efflux across the femoral vascular bed. Thus withdrawal of ANP effect and augmentation of ET effect may be rapidly emerging components of the phenomenon of “pseudo-tolerance” to organic nitrates in patients with angina pectoris.

9.2. Future Directions

9.2.1. Mechanisms of ischaemia-induced ANP release

The current study showed that neither endogenous NO nor endogenous ET mediate ischaemia-induced ANP release. This study therefore eliminated some potential mechanisms of ANP release induced by ischaemia. Previous investigations (Chen *et al.* 1993) showed that ischaemia-induced ANP release was completely abolished by either meclofenamate (10^{-5} M), a cyclo-oxygenase inhibitor, or nordihydroguaiartaric acid (10^{-5} M), a lipoxygenase inhibitor, superficially suggesting the possible involvement of either cyclo-oxygenase or lipoxygenase in the modulation of ischaemia-induced ANP release. Further investigations using specific enzyme blockade are required to dissect the specific pathways involved in ischaemia-induced ANP release.

9.2.2. Implication of ischaemia/hypoxia-induced ANP release.

The current study demonstrated ischaemia/hypoxia significantly increased ANP release. Further studies should focus on the impact of incremental ANP release on coronary flow and myocardial performance. Previous studies have shown that ANP increases coronary blood flow in dog (Ertl & Bauer 1990, Chu & Cobb 1987, Chu *et al.* 1990, Nakagawa *et al.* 1988) and humans (Serizawa *et al.* 1988, Chu *et al.* 1989a). The time course of proximal coronary dilation was related more closely to increased plasma cGMP concentration than that of ANP (Chu *et al.* 1989a). ANP also dilates intramural vessels, causing favourable redistribution of flow to the subendocardium during ischaemia, similar to the effects of GTN (Chu & Cobb 1987, Chu *et al.* 1989a,b). ANP limited the ischaemia-induced ventricular arrhythmia (Linz *et al.* 1988, Takata *et al.* 1996), and the ANP treated group had higher ATP content in the inner layers of the ischaemic

myocardium (Takata *et al.* 1996). Natriuretic peptides (NPs) also inhibit ET biosynthesis in rat endothelial cells (Emori *et al.* 1993). It would therefore be interesting to know whether natriuretic peptides affect ischaemia via limiting ET synthesis and effect.

Using an established Langendorff-perfused rat heart paradigm, the effect of endogenous NPs on coronary flow and cardiac force of contraction can be studied utilizing ANP antibody or NP receptor antagonists during ischaemic/hypoxic perfusion period. In addition, exogenous ANP, BNP perfusion can be used to investigate the direct cardiac effect of NPs. Endopeptidase inhibitor can also be used to increase endogenous NPs.

9.2.3. Effect of endogenous ET on coronary flow and cardiac function

In the current study, the dose-effect relationship of BQ-123 on coronary flow was not evaluated due to the limited supply of BQ-123. Other ET_A receptor antagonists (such as BMS-182874) could be used in future to investigate whether the vasoconstrictor effect is unique for BQ-123, or that all ET_A receptor antagonist have such effects and to study the dose-effect relationship of this class of antagonist.

Furthermore, higher doses of ET_A receptor antagonist (10^{-5} M, to match the dose used for bosentan) can be used to compare the effects of selective ET_A and non-selective ET receptor antagonists on cardiac performance. The role of ET_B receptors on cardiac force of contraction has not yet been studied *in vitro*. Studies *in vivo* can not separate direct inotropic effect of ET receptor antagonists or effect on cardiac function secondary from their vasoactive effects. The system used in the present study (with fixed coronary flow

reduction) can be used to study the direct effect of selective ET_B receptor antagonist (such as BQ788) on cardiac force of contraction.

9.2.4. Effect of ET receptor antagonists on ET-1 release

In the present study, ET_A receptor antagonism induced an increase in ET-1. Further experiment using isolated endothelial cells should be performed to investigate whether this effect is due to increased ET release or decreased ET binding. The endothelin-converting enzyme (ECE) inhibitors can be used to investigate if ET_A receptor antagonists affect big-ET-1 converting to ET-1 or only affect the matured ET-1 release from endothelium.

9.2.5. Effect of PTCA on plasma ANP and ET-1 concentrations

PTCA can be used as a model of transient ischaemia with variable atrial distension. The negative results of the current study are possibly due to Type II error. Further investigations on more patients with larger series of sampling and haemodynamic monitoring can advance current understanding of the pathophysiology of ANP and ET-1 in human subjects. The correlation of ANP or ET-1 concentrations with anti-ischaemic drug therapy can be potentially investigated in a large group of patients. More extensive time of sampling is required in any future study as it is possible that ANP/ET-1 “washout” may be delayed, and coronary sinus sample may show the small change in ANP and ET-1 release from the coronary bed if PTCA involved the left coronary arteries. However, routine utilization of coronary sinus sampling in peri-PTCA patients may prolong the procedure, and thus raises some ethical problems. Serial RAP, PCWP HR and ECG ST segment monitoring may delineate between atrial distension and PTCA-induced ANP release. The impact of predominant right atrial distension on ANP release can be observed in patients with proximal right coronary artery PTCA.

9.2.6. The acute interaction between organic nitrate and ANP/ET-1

The current study raises the issue of a possible role for the early increase in ET-1 release and ANP “withdrawal” during GTN therapy on early “pseudo-tolerance” to organic nitrates. ET receptor antagonists and endopeptidase inhibitors may therefore affect the “pseudo-tolerance”. Thus additional groups of patients receiving these therapies can be studied when the drugs are available. The findings of the current study also provide an additional basis for examination of ET receptor antagonists and endopeptidase inhibitors in therapy of unstable angina pectoris.

Bibliography

- AHLBORG, G., LUNDBERG, J.M. (1997) Nitric oxide-endothelin-1 interaction in humans. *J Appl Physiol*, **82**(5), 1593-1600.
- AHN, K., SISNEROS, A.M., HERMAN, S.B., PAN, S.M., HUPE, D., LEE, C., *et al.* (1998) Novel selective quinazoline inhibitors of endothelin converting enzyme-1. *Biochem. Biophys. Res. Commun.*, **243**, 184-190.
- AHREN, B. (1988). ANF inhibits glucose-stimulated insulin secretion in mouse and rat. *Am. J. Physiol*, **255**, E579-E582.
- ALBERT, T.S., TUCKER, V.L., RENKIN, E.M. (1997). Acute alveolar hypoxia increases blood-to-tissue albumin transport: role of atrial natriuretic peptide. *J. Appl. Physiol.*, **82**(1), 111-117.
- ALLEN, M.J., ANG, V.T., BENNETT, E.D., JENKINS, J.S. (1988). Atrial natriuretic peptide inhibits osmolality-induced arginine vasopressin release in man. *Clin. Sci.*, **75**, 35-39.
- ALLEN, M.J., GILMOUR, S.M., SINGER, M., BENNETT, E.D. (1989) Effects of atrial natriuretic peptide on systemic haemodynamics and cardiac function in normal man. *Cardiovas. Res.*, **23**, 70-75.
- ANDERSON, J.V., DONKIER, J., PAYNE, N.N., BEACHAM, J., SLATER, J.D. *et al.* (1987) Atrial natriuretic peptide: evidence of action as a natriuretic hormone at physiological plasma concentrations in man. *Clin. Sci* , **72**, 305-312.
- ANEND-SRIVASTAVA, M.B., GUTKOWSKA, J., CANTIN, M. (1991) The presence of atrial-natriuretic-factor receptors of ANF-R₂ subtype in rat platelets. *Biochem. J* , **278**, 211-217.
- ANEND-SRIVASTAVA, M.B., TRACHTE, G. (1993) Atrial natriuretic factor receptors and signal transduction mechanism. *Pharm Rev* , **45**, 455-497.

- ANEND-SRIVASTAVA, M.B., SAIRAM, M.R., CANTIN, M. (1990) Ring-deleted analogs of atrial natriuretic factor inhibit adenylyl cyclase/cAMP system. *J. Biol. Chem.*, **265**, 8566-8572.
- ANONYMOUS. (1988) Yin and Yang in vasomotor control. *Lancet*, **July 2**, 19-20.
- ANTUNES-RODRIGUES, J., PICANCO-DINIZ, D.W., FAVARETTO, A.L., GUTKOWSKA, J., M^cCANN, S.M. (1993). Brain atrial natriuretic peptide neurons play an essential role in volume expansion-induced release of atrial natriuretic peptide and natriuresis. *Neuroendocrinology*, **58**, 696-700.
- ARAD, M., ZAMIR, N., HOROWITZ, L., OXMAN, T., RABINOWITZ, B. (1994). Release of atrial natriuretic peptide in brief ischaemia-reperfusion in isolated rat hearts. *Am J Physiol.*, **266**, H-1971-H1978.
- ARNAL, J.F., AMRANI, A.I.E., MICHEL, J.B. (1993). Atrial natriuretic factor influences in vivo plasma, lung and aortic wall cGMP concentrations differently. *Eur J Pharmacol.*, **237**, 265-273.
- ARONSON, C.E. & SEROICK, E.R. (1976). Effect of prolonged perfusion time on the isolated perfused rat heart. *Toxicol. Appl. Pharmacol.*, **38**, 479-488.
- ARSTALL, M.A., YANG, J., STAFFORD, I., HOROWITZ, J.D. (1995). N-acetylcysteine in combination with nitroglycerin and streptokinase for the treatment of evolving acute myocardial infarction. Safety and biochemical effects. *Circulation*, **92(10)**, 2855-2862.
- ATLAS, S.A., CODY, R.J. LARAGH, J.H. (1992) Atrial natriuretic peptide in heart failure. *Heart Disease* (Braunwald) update **17**, 377.
- BAERTSCHI, A.J., HAUSMANINGER, C., WALSH, R.S., MENTZER, R.M., WYATT, D.A., PENCE, R.A. (1986) Hypoxia-induced release of atrial natriuretic factor (ANF) from the isolated rat and rabbit heart. *Biochem Biophys Res Commun*, **140**, 427-33.
- BAERTSCHI, A.J., JIAO, J.H., CARLSON, D.E., CAMPBELL, R.W., TEAGUE, W.G., WILLSON, D., GANN, D.S. (1990) Neural control of ANF in hypoxia and pulmonary hypertension. *Am J Physiol.*, **259**, H735-H744.

- BAERTSCHI, A.J., TEAGUE, W.G. (1989) Alveolar hypoxia is a powerful stimulus for ANF release in conscious lambs. *Am J Physiol.*, **256**, H990-H998.
- BALWIERCZAK, J.L. (1993) Two subtypes of the endothelin receptor (ET_A and ET_B) mediate vasoconstriction in the perfused rat heart. *J. Cardiovasc. Pharmacol.*, **22** (Suppl 8), S248-S251.
- BARCLAY, P.L., BENNETT, J.A., SAMUELS, G.M.R., SHEPPERSON, N.B. (1993) The atriopeptidase inhibitor (±)candoxatrilat reduces the clearance of atrial natriuretic factor in both intact and nephrectomized rats: evidence for an extra-renal site of action. *Biochem. Pharmacol.*, **41**, 841-844.
- BARCLAY, P.L., BENNETT, J.A., SAMUELS, G.M., SHEPPERSON, N.B. (1991) The atriopeptidase inhibitor (+/-) candoxatrilat reduces the clearance of atrial natriuretic factor in both intact and nephrectomized rats: evidence for an extrarenal site of action. *Biochem. Pharmacol.*, **41**, 841-844.
- BAUER, W.R., NEUBAUER, S., OBITZ, G., ERTL, G. (1994) Interrelation of coronary effects of atrial natriuretic peptide and renin-angiotensin system in the isolated perfused rat heart. *J. Mol. Cell. Cardiol.*, **256**, 527-537.
- BELLONE, P., DERCHI, G., TOMMASINI, G., BIORCI, M.L., RANDAZZO, M., PRIMAROLO, P., VECCHIO, C. (1993). Selective activation of atrial natriuretic peptide in patients with myocardial infarction and mild left ventricular dysfunction. *Int J Cardiol.*, **42**, 115-120.
- BENACERRAF, A., SCHOLL, J.M., ACHARD, F., TONNELIER, M., LAVERGNE, G. (1983). Coronary spasm and thrombosis associated with myocardial infarction in a patient with nearly normal coronary arteries. *Circulation*, **67**, 1147.
- BERG, J.A., HAYASHI, M., FUJII, Y., KATZ, A.I. (1988) Renal metabolism of atrial natriuretic peptide in the rat. *Am. J. Physiol.*, **255**, F466-F473.
- BEYER, M.E., SLESAK, G., NERZ, S., KAZMAIER, S., HOFFMEISTER, H.M. (1995) Effect of endothelin-1 and IRL 1620 on myocardial contractility and myocardial energy metabolism. *J Cardiovasc Pharmacol*, **26** (Suppl. 3), S150-S152.

- BIDIVILLE, J., WAEBER, G., PORCHET, M., NUSSBERGER, J., BIOLLAZ, J. *et al.* (1988) Hemodynamic, renal, and endocrine effects of 4-h infusions of atrial natriuretic peptide in normal volunteers. *Fundamental and Clinical Pharmacology*, **2**, 413-429.
- BILDER, G.E., SCHOFIELD, T.L., BLAINE, E.H. (1986) Release of atrial natriuretic factor. Effects of repetitive stretch and temperature. *Am. J. Physiol.*, **251**: F817-F821.
- BILDER, G.E., SWEET, C.S., EMMERT, S.E., SCHOFIELD, T.L., MEDINA, R., STABILITO, I.I., FRIEDMAN, P.A. (1989) Cardiac and Noncardiac atrial natriuretic factor (ANF) biosynthesis and storage and plasma ANF in the rat model of chronic ventricular dysfunction. *Endocrinology*, **124** (3): 1515.
- BLOCH, D.K., HONG, C.C., EDDY, R.L., SHOWS, T.B., QUERTERMOUS, T. (1991) cDNA cloning and chromosomal assignment of the endothelin-2 gene: vasoactive intestinal contractor peptide in rat endothelin-2. *Genomics*, **10**, 236-242.
- BOGOYEVITCH, M.A., GLENNON, P.E., ADERSSON, M.B., CLARK, A., LAZU, A., MARSHALL, C.J., PARKER, P.J., SUGDEN P.H. (1994) Endothelin-1 and fibroblast growth factors stimulate the mitogen-activated protein kinase signaling cascade in cardiac myocytes. The potential role of the cascade in the integration of two signaling pathways leading to myocyte hypertrophy. *J. Biol. Chem. Biophys.*, **269**, 1110-1119.
- BOLGER, G., LIARD, F., KROGSRUD, R., WELCHNER, E., JARAMILLO, J. (1991) Interactions between endothelin and atrial natriuretic factor in the rat aortic ring preparation. *Can J Physiol Pharmacol*, **69**: 1155.
- BOLLI, P., MULLER, F.B., LINDER, L., RAINE, A.E.G., RESINK, T.J., ERNE, P., KIWISKI, W., RITZ, R., BUHLER, F.R. (1987) The vasodilator potency of atrial natriuretic peptide in man. *Circulation*, **75**, 221-228.
- BONARJEE, V.V., OMLAND, T., NILSEN, D.W., CAIDAH, K., SUNDSFJORD, J.A., DICKSTEIN, K. (1995) Plasma proatrial natriuretic peptide (1-98)

concentration after myocardial infarction: relation to indices of cardiac and renal function. *Br. Heart J.*, **73**(6), 511-516.

BOTTING, M., VANE, J.R. (1990) Endothelins: potent releasers of prostacyclin and EDRF. *Pol. J. Pharmacol. Pharm.*, **42**, 203-218.

BOUISSOU, P., GALEN, F.X., RICHALET, J.P., LARTIGUE, M., DEVAUX, F., DUBRAY, C., ATLAN, G. (1989) Effects of propranolol and pindolol on plasma ANP levels in humans at rest and during exercise. *Am. J. Physiol.*, **257**, R259-R264.

BOULANGER, C., LUSCHER, T. (1990) Release of endothelin from the porcine aorta. *J. Clin. Invest.*, **85**, 587-590.

BOURDILLON, P.D., LORELL, B.H., MIRSKY, I., PAULUS, W.J., WYNNE, J., Grossman, W. (1983) Increased regional myocardial stiffness of the left ventricle during pacing-induced angina in man. *Circulation*, **67**, 316.

BOURNE, A., KENNY, A.J. (1990) The hydrolysis of brain and atrial natriuretic peptides by porcine choroid plexus is attributable to endopeptidase-24.11. *Biochem. J.*, **271**, 381-385.

BORZAK, S., MURPHY, S., MARSH, J.D. (1991) Mechanisms of rate staircase in rat ventricular cells. *Am J physiol*, **260**, H884-H892.

BRAUWALD, E. (1988) Heart Disease, a textbook of cardiovascular medicine (Third edition). W.B. Saunders Company.

BRAUWALD, E., KLONER, R.A. (1982) The stunned myocardium: Prolonged, post-ischaemic ventricular dysfunction. *Circulation*, **66**, 1146.

BRIGGS, J.P., MAINGREZ, M., STEIPE, B., SCHUBERT, G., SCHNERMANN, J. (1984) Inactivation of atrial natriuretic substance by kallikrein. *Am. J. Physiol.*, **247**, F480-F484.

BRUNEAU, B.G., PIAZZZA, L.A., de BOLD, A.J. (1984). BNP gene expression is specifically modulated by stretch and ET-1 in a new model of isolated rat atria. *Am. J. Physiol.*, **273**, H2678-H2686.

- BRUNNER, F. (1995) Tissue endothelin-1 levels in perfused rat heart following stimulation with agonists and in ischaemia and reperfusion. *J Mol Cell Cardiol*, **27**, 1953-1963.
- BRUNNER, F., DOHERTY, A.M. (1996) Role of ETB receptors in local clearance of endothelin-1 in rat heart: studies with the antagonists PD 155080 and BQ-788. *FEBS Lett*, **396**, 238-242.
- BRUNNER, F., DU TOIT, E.F. & OPIE L.H. (1992). Endothelin release during ischaemia and reperfusion of isolated perfused rat heart. *J. Mol. Cell. Cardiol.*, **24**, 1291-1305.
- BUCKLEY, M.G., SETHI, D., MARKANDU, N.D., SAGNELLA, G.A., SINGER, D.R.J., MACGREGOR G.A. (1992). Plasma concentrations and atrial natriuretic peptide in normal subjects, cardiac transplant recipients and patients with dialysis-independent or dialysis -dependent chronic renal failure. *Clin Sci.*, **83**, 437-444.
- CADE, C., ILOZU, C.V., RUBANYI, G.M., BOTELHO, L.H. (1991) Smooth muscle cells contain a factor responsible for decreasing big endothelin and endothelin-1 produced by cultured endothelial cells. *J. Cardiovas. Pharmacol.*, **17**, S71-S75.
- CAMICI, P., ARAUJO, L.I., SPINKS, T., LAMMERTSMA, A.A., KASKI, J.C., SHEA, M.J. *et al.* (1986) Increased uptake of ¹⁸F-fluorodeoxyglucose in postischaemic myocardium of patients with exercise-induced angina. *Circulation*, **74**, 81-88.
- CAO, W., ZENG, Z., ZHU, Y. (1995) Effect of NG-nitro-L-arginine methylester on the secretion of endothelin-1 in vivo and in cultured endothelial cells. *Chung Hua I Hsueh Tsa Chih*, **75(3)**, 164-166.
- CARGILL, R.I., MCFARLANE, L.C., COUTIE, W.J., LIPWORTH, B.J. (1996). Acute neurohormonal responses to hypoxaemia in man. *Eur J Appl Physiol.*, **72(3)**, 256-60.

- CARROLL, J.D., HESS, O. M., HIRZEL, H.O., KRAYENBUEHL, H.P. (1983). Exercise-induced ischaemia: The influence of altered relaxation on early diastolic pressures. *Circulation*, **67**, 521.
- CERASOLA, G., COTTONE, S., MANGANO, M.T., D'LGNOTO, G., PINTO, A., CARONE, M.B. (1988) Effect of felodipine on natriuresis, atrial natriuretic factor, the renin-angiotensin-aldosterone system, and blood pressure in essential hypertension. *Clin Therap*, **10(6)**: 694.
- CESARI, M., PAVAN, E., SACCHETTO, A., ROSSI, G.P. (1996) Endothelin-1: a scientists' curiosity, or a real player in ischaemic heart disease? *Am. Heart J.*, **132(6)**, 1236-1243.
- CHAN, J., GREENBERG, D.A. (1991) Endothelin and calcium signaling in NG 108-15 neuroblastoma X glioma cell. *J. Pharmacol. Exp. Ther.*, **258**, 524-530.
- CHANG, M.S., LOWE, D.G., LEWIS, M., HELLMISS, R., CHEN, E., GOEDDEL, D.V. (1989) Differential activation by atrial and brain natriuretic peptides of two different receptor guanylate cyclase. *Nature*, **341**, 68-72.
- CHEN, B.N., RAYNER, T.E., MENADUE, M.F., MCLENNAN, P.L. & OLIVER, J.R. (1993). Effect of ischaemia and role of eicosanoids in release of atrial natriuretic factor from rat heart. *Cardiovasc. Res.*, **27**, 1576-1579.
- CHEN, B.N. (1994). The role of eicosanoids in ischaemia or hypoxia stimulated atrial natriuretic factor release from the rat heart. MSc Thesis: Flinders University of South Australia.
- CHEN, Y.F., DURAND, J., CLAYCOMB, W.C. (1997). Hypoxia stimulates atrial natriuretic peptide gene expression in cultured atrial cardiocytes. *Hypertension*, **29**, 75-82.
- CHENU, P.C., DONCKIER, J.E., SCHROEDER, E., BERBINSCHI, A. KETELSLEGERS J.M., MARCHANDISE, B., KREMER, R. (1991). Atrial natriuretic factor during percutaneous transluminal coronary angioplasty. *Acta Cardiologica*, **46**, 595-603.

- CHEUNG, C.Y. (1994). Regulation of atrial natriuretic factor release by endothelin in ovine fetuses. *Am J Physiol.*, **267**, R380-R386.
- CHINKERS, M., GARBERS, D.L., CHANG, M.S., LOWE, D.G., CHIN, H., GOEDDEL, D.V., SCHULZ, S. (1989) A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature*, **338**, 78-83.
- CHIRKOV, Y.Y., NAUJALIS, J.I., SAGE, R.E., HOROWITZ, J.D. (1993). Antiplatelet effects of nitroglycerin in healthy subjects and in patients with stable angina pectoris. *J. Cardiovas. Pharmacol.*, **21**, 384-389.
- CHRISTOU, H., ADATIA, I., VAN MARTER, L.J., KANE, J.W., THOMPSON, J.E., STARK, A.R., WESSEL, D.L., KOUREMBANAS, S. (1997) Effect of inhaled nitric oxide on endothelin-1 and cyclic guanosine 5'-monophosphate plasma concentrations in newborn infants with persistent pulmonary hypertension. *J Pediatr*, **130**(4), 603-611.
- CHU, A., COBB, F.R. (1987) Effects of atrial natriuretic peptide on proximal epicardial coronary arteries and coronary blood flow in conscious dogs. *Circ Res*, **61**, 485.
- CHU, A., MORRIS, K.G., KUEHL, W.D., CUSMA, J., NAVETTA, F., COBB, F.R. (1989a) Effect of atrial natriuretic peptide on the coronary arterial vasculature in humans. *Circulation*, **80**, 1627.
- CHU, A., STAKELY, A., LIN, C.C., COBB, F.R. (1989b) Effects of atrial natriuretic peptide on transmural blood flow and reactive hyperemia in the presence of flow-limiting coronary stenosis in the awake dog: evidence for dilation of the intramural vasculature. *Circ Res*, **64**, 600.
- CHU, A., MURRY, J.J., LIN, C.C., RUSSELL, M., HAGEN, P.O., COBB, F.R. (1990) Preferential proximal coronary dilation by activation of guanylate cyclase in awake dogs. *Am J Physiol*, **259**, H340.
- CIRINO, M., BATTISTINI, B., YANO, M., RODGER, I.W. (1994) Dual cardiovascular effects of endothelin-1 dissociated by BQ-153, a novel ET_A receptor antagonist. *J Cardiovasc Pharmacol*, **24**, 587-594.

- CIRINO, M., MARTIN, E., YANO, M., RODGER, I.W. (1997) Cardiovascular and renal actions of the endothelin receptor in pigs. *J Cardiovasc Pharmacol*, **29**, 704-712.
- CLARKE, J.G., BENJAMIN, N., LARKIN, S.W., WEBB, D.J., DAVIES, G.J., MASERI, A. (1989). Endothelin is a potent long-lasting vasoconstrictor in men. *Am. J. Physiol.*, **257**, H2033-H2035.
- CLELAND, J.G., SWEDBERG, K. (1998) Lack of efficacy of neutral endopeptidase inhibitor ecadotril in heart failure. The International Ecadotril Multi-centre Dose-ranging Study Investigators *Lancet*, **351**, 1657-1658.
- CLINKENBEARD, C., SESSIONS, C., SHENKER, Y. (1990) The physiological role of atrial natriuretic hormone in the regulation of aldosterone and salt and water metabolism. *J. Clin. Endocrin. Metab.*, **70**, 582-589.
- CLOUTHEIER, D.E., HOSODA, K., RICHARDSON, J.A., WILLIAMS, S.C., YANAGISAWA, H., KUWAKI, T. *et al.* (1998) Cranial and cardiac neural crest defects in endothelin-A receptor-deficient mice. *Development*, **125**, 813-824.
- CLOZEL, M., GRAY, G.A., BREU, V., LOFFLER, B.M., OSTERWALDER, R. (1992) The ETB receptor mediates both vasodilation and vasoconstriction in vivo. *Biochem. Biophys. Res. Commun.*, **186**, 867-873.
- CLOZEL, M., LOFFLER, B.M., BREU, V., HILFIGER, L., MAIRE, J.P., BUTSCHA, B. (1993) Downregulation of endothelin receptors by autocrine production of endothelin 1. *Am. J. Physiol.*, **265**, C188-C192.
- COGAN, M.G. (1990) Renal effects of atrial natriuretic factor. *Annual Review of Physiology*, **52**, 699-708.
- COHN, J.N. (1990) Neuroendocrine activation after acute myocardial infarction. *Am. J. Cardiol.*, **65**, 28I-31I.
- CONTI, C.R. (1986). Coronary artery spasm: Pathophysiology, diagnosis, and treatment. New York, Marcel Dekker, 1.

- CURRIE, M.G., GELLER, D.M., COLE, B.R., BOYLAN, J.G., WU, Y.S., HOLMBERG, S.W., NEEDLEMAN, P. (1983) Bioactive cardiac substances: potent vasorelaxant activity in mammalian atria. *Science*, **221**: 71-73.
- DAGASSAN, P.H., BREU, V., CLOZEL, M. & CLOZEL, J.P. (1994). Role of endothelin during reperfusion after ischaemia in isolated perfused rat heart. *J. Cardiovasc. Pharmacol.*, **24**, 867-874.
- DAKAK, N., MAKHOUL, N., MERDLER, A., YECHIELY, H., KAHANA, L., HALON, D.A., SCHNEEWEISS, A., LEWIS, B.S. (1993). Haemodynamic and neurohumoral effects of flosequinan in severe heart failure: similarities and differences compared with intravenous nitroglycerin therapy. *Eur Heart J.*, **14**, 836-844.
- DANILEWICZ, J.C., BARCLAY, P.L., BARNISH, I.T., BROWN, D., CAMPELL, S.F., JAMES, K., SAMUELS, G.M.R., TERRETT, N.K., WYTHES, M.J. (1989) UK-69.578, a novel inhibitor of EC 3.4.24.11 which increases endogenous ANF and is natriuretic and diuretic. *Biochem. Biophys. Res. Commun.*, **164**, 58-65.
- DAVENPORT, A.P. & MAGUIRE, J.J. (1994). Is endothelin-induced vasoconstriction mediated only by ET_A receptors in humans? *TiPS*, **15**, 9-11.
- DAVIS, M., ESPINER, E., RICHARDS, G., BILLINGS, J., TOWN, I., NEILL, A., DRENNAN, C., RICHARDS, M., TURNER, J., YANDIE, T. (1994). Plasma brain natriuretic peptide in assessment of acute dyspnoea. *The Lancet*, **343**, 440-444.
- DE ANGELIS, C., FERRI, C., URBANI, L., FARRACE, S. (1996). Effect of acute exposure to hypoxia on electrolytes and water metabolism regulatory hormones. *Aviat Space Environ Med.*, **67**(8), 746-750.
- DE BOLD, A.J., BENCOSME, S.E (1975). Autoradiographic analysis of labelled distribution in mammalian atrial and ventricular cardiocytes after exposure to treated laucine. *The Cardiac Cytoplasm*, vol. **8**, 129-138, University Park Press, Baltimore, MD.

- DE BOLD, A.J.(1979). Heart atria granularity. Effects of changes in water and electrolyte balance. *Proc. Sec. Exp. Bio. Med.*, **161**, 508-511.
- DE BOLD, A.J., BENCOSME, S.E., VERESS, A.T., SONNENBERG, H (1981). A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci.*, **28**, 89-94.
- DE MARZIO, P., MORELLI, S. FERRI, C., TORREGROSSA, G., BELLINI, C., ANGELIS, C., PERRONE, A., AGUGLIA, F., SANTUCCI, A., BALSANO, F. (1992). Enhanced release of atrial natriuretic factor during exercise-induced myocardial ischemia in patients after acute myocardial infarction. *International J Cardiol.*, **34**, 179.
- DE WOOD, M.A., STIFTER, W.F., SIMPSON, C.S., SPORES, J., EUGSTER, G.S., JUDGE, T.P., HINNEN, M.L. (1986). Coronary arteriographic findings soon after non-Q wave myocardial infarction. *N. Engl. J.*, **315**, 417.
- DI NARDO, P., PAFI, M. BARTOLI, M., MINIERI, M., BELLEGRANDI, F., RAIMONDI, G., PERUZZI, G., TALLARIDA, G. (1993). Effects of α -human atrial natriuretic peptide in guinea-pig isolated heart. *International J Cardiol.*, **40**, 211-220.
- DOHI, Y., LUSCHER, T.F. (1991) Endothelin in hypertensive resistance arteries, intraluminal and extraluminal dysfunction. *Hypertension*, **18**, 543-549.
- DOMENECH, R., MACHO, P., GOZALEZ, R., HUIDOBRO-TOTO, J.P. (1991) Effect of endothelin on total and regional coronary resistance and on myocardial contractility. *Eur. J. Pharmacol.*, **192**, 409-416.
- DONCKIER, J., HANET, C., STOLERU, L., VAN MECHELEN, H., GALANTI, L., HAYASHIDA, W., KEYEUX, A., KETELSLEGER, S., POULEUR, H. (1994) Effects of endothelin-1 at pathophysiologic concentrations on coronary perfusion and mechanical function of normal and postischemic myocardium. *J Cardiovasc Pharmacol*, **23**: 212-219.
- DOUGLAS, S.A., JAMES, S., HILEY, C.R. (1991) Endothelial modulation and changes in endothelin pressor activity during hypoxia in the rat isolated perfused superior mesenteric arterial bed. *Br J Pharm* , **103**(2), 1441-1448.

- DOUGLAS, S.A., MEEK, T.D., OHLSTEIN, E.H. (1994) Novel receptor antagonists welcome a new era in endothelin biology. *TiPS*, **15**, 313-316.
- DUPUIS, J., GORESKEY, C.A., FOURNIER, A. (1996). Pulmonary clearance of circulating endothelin-1 in dogs in vivo: exclusive role of ET_B receptors. *J Appl Physiol*, **81**(4), 1510-1515.
- DWYER, E.M. (1970) Left ventricular pressure-volume alterations and regional disorders of contraction during myocardial ischemia induced by atrial pacing. *Circulation*, **42**, 1111.
- EDWARDS, B.S., ZIMMERMAN, R.S., SCHWAB, T.R., HEUBLEIN, D.M., BURNEET, J.C. (1988) Atrial stretch, not atrial pressure, is the principal determinant controlling the acute release of atrial natriuretic peptide. *Cir. Res.*, **62**, 191-195.
- EDWARDS, D.L., ARORA, C.P., BUI, D.T., CASTRO, L.C. (1996) Long-term nitric oxide blockade in the pregnant rat: effects on blood pressure and plasma levels of endothelin-1. *Am J Obstet Gynecol*, **175**(2), 484-488.
- ELZ, J.S., NAYLER, W.G. (1987) Quantification of calcium paradox in neonatal rat hearts. *Am J Physiol*, **253** (6): H1358-1364.
- EMORI, T., HIRATA, Y., IMAI, T., EGUCHI, S., KANNO, K., MARUMO, F. (1993) Cellular mechanism of natriuretic peptides-induced inhibition of endothelin-1 biosynthesis in rat endothelial cells. *Endocrinology*, **133**: 2474.
- ENDO, M., NOROTA, I., YANG, H.T., FUJITA, S., TAKANASHI, M. (1996) The positive inotropic effect and the hydrolysis of phosphoinositide induced by endothelin-3 in rabbit ventricular myocardium: Inhibition by a selective antagonist of ET_A receptors, FR139317. *J. Pharm. Exp. Ther*, **277**, 61-70.
- EPSTEIN, S.E., TALBOT, T.L. (1981) Dynamic coronary tone in precipitation, exacerbation and relief of angina pectoris. *Am. J. Cardiol.*, **27**, 392.
- ERDOS, E.G., SKIDGEL, R.A. (1989) Neutral endopeptidase 24.11 (enkephalinase) and related regulators of peptide hormones. *FASEB J.*, **3**, 145-151.

- ERTL, G., BAUER, B. (1990) Coronary arteriolar vasoconstriction in myocardial ischaemia. Vasopressin, renin-angiotensin system and ANF. *Eur Heart J*, **11**: 53.
- EVANS, H.G., LEWIS, M.J., SHAN, A.M. (1994) Modulation of myocardial relaxation by basal release of endothelin from endocardial endothelin. *Cardiovasc. Res*, **28**, 1694-1699.
- EZRA, D., GOLDSTEIN, R.E., CZAJA, J.F., FEUERSTEIN, G.Z. (1989) Lethal ischaemia due to intracoronary endothelin in pigs. *Am. J. Physiol.*, **257**, H339-H343.
- FELDMAN, M.D., AYERS, C.R., LEHMAN, M.R., *et al.* (1992) Improved detection of ischaemia-induced increases in coronary sinus adenosine in patients with coronary artery disease. *Clin. Chem.*, **38**, 256.
- FERRARI, R., CECONI, C., CURELLO, S., ALFIERI, O., VISIOLI, O. (1993). Myocardial damage during ischaemia and reperfusion. *Eur. Heart J.*, **14** (suppl G), 25-30.
- FERRARI, R., CECONI, C., RODELLA, A., HARRIS, P., VISIOLI, O. (1990). Hormonal response in untreated myocardial infarction. *Cardioscience*, **1**, 55-60.
- FERRI, C., BELLINI, C., DESIDERI, G., De MATTIA, G., SANTUCCI, A. (1996) Endogenous insulin modulates circulating endothelin-1 concentrations in humans. *Diabetes*, **19**(5), 504-506.
- FERRO, C.J. SPRATT, J.C., HAYNES, W.G., WEBB, D.J. (1998) Inhibition of neutral endopeptidase causes vasoconstriction of human resistance vessels *in vivo*. *Circulation.*, **97**, 2323-2330.
- FOCACCIO, A., AMBROSIO, G., ENEA, I., USSO, R., BALESTRIERI, P., CHIARIELLO, M., VOLPE, M. (1995) Influence of O₂ deprivation, reduced flow, and temperature on release of ANP from rabbit hearts. *Am. J. Physiol.*, **268**, H2352-H2357.

- FONTANA, F., BERNARDI, P., SPAGNOLO, N., CAPELLI, M. (1990). Plasma atrial natriuretic factor in patients with acute myocardial infarction. *Eur Heart J.*, **11**, 779-787.
- FOX, A.C., REED, G.E., GLASSMAN, E., KALTMAN, A.J., SILK, B.B. (1974) Release of adenosine from human hearts during angina induced by rapid atrial pacing. *J. Clin. Invest.*, **53**, 1447.
- FOY, S.G., CROZIER, I.G., RICHARDS, A.M., NICHOLLS, M.G., TURNER, J.G., FRAMPTON, C.M., IKRAM, H. (1995) Neurohormonal changes after acute myocardial infarction. Relationships with haemodynamic indices and effects of ACE inhibition. *Eur Heart J.*, **16**(6), 770-778.
- FOZZARD, H.A., JENNINGS, R.B., HABER, E., KATZ, A.M., MORGAN, H.E. (1986) The heart and cardiovascular system. New York, Raven Press, 1133-1202.
- FREIFELD, A.G., SCHUSTER, E.H., BULKLEY, B.H. (1983). Non-transmural versus transmural myocardial infarction. *Am. J. Med.*, **75**, 423.
- FUJIMURA, A., LOWRY, R.W., KEM, D.C. (1989) Calcium infusion increases plasma atrial natriuretic factor in spontaneous hypertensive rats. *Hypertension*, **14**: 98-103.
- FUJISAKI, H., ITO, H., HIRATA, Y., TANAKA, M., HATA, M., LIN, M., ADACHI, S., AKIMOTO, H., MARUMO, F., HIROE, M. (1995) Natriuretic peptides inhibit angiotensin II-induced proliferation of rat cardiac fibroblasts by blocking endothelin-1 gene expression. *J. Clin. Invest.*, **96**, 1059-1065.
- FUKURODA, T., FUJIKAWA, T., OZAKI, S., ISHIKAWA, K., YANO, M., NISHIKIBE, M (1994). Clearance of circulating endothelin-1 by ET_B receptors in rats. *Biochem Biophys Res Commun* , **199**(3), 1461-1465.
- FULLER, F., PORTER, J.G., ARFSTEN, A.E., MILLER, J., SCHILLING, J.W., SCARBOROUGH, R.M., LEWICKI, J.A., SCHENK, D.B. (1988) Atrial natriuretic peptide clearance receptor. *J. Biol. Chem.*, **263**, 9395-9410.

- FURCHGOTT, R.F. & VANHOUTTER, P.M. (1989) Endothelium-derived relaxing and contracting factors. *FASEB J.*, **3**, 2007-2018.
- GAMEL, A.E., YONAN, N.A., KEEVIL, B., WARBUTON, R., KAKADELLIS, J., WOODCOCK, A., CAMPBELL, C.S., RAHMAN, A.N., DEIRANIYA, A.K. (1997). Significance of raised natriuretic peptides after bicaval and standard cardiac transplantation. *Ann Thorac Surg.*, **63**, 1095-1100.
- GANZ, P., ABBEN, R.P., BARRY, W.H. (1987). Dynamic variations in resistance of coronary arterial narrowings in angina pectoris at rest. *Am. J. Cardiol.*, **59**, 66.
- GANZ, W., DONOSO, R., MARCUS, H.S., FORRESTER, J.S., SWAN, H.J.C. (1971). A new technique for measurement of cardiac output by thermodilution in man. *Am. J. Cardiol.*, **27**, 392.
- GARCIA, R., THIBAUT, G., CANTIN, M., GENEST, J. (1984) Effect of a purified atrial natriuretic factor on rat and rabbit vascular strips and vascular beds. *Am J Physiol*, **247**, R34-R39.
- GARDNER, D.G., HANE, S., TRACHEWSKY, D., SCHENK, D., BAXTER, J.D. (1986). Atrial natriuretic peptide mRNA is regulated by glucocorticoids in vivo. *Biochem. Biophys. Res. Commun.*, **139**, 1047-1054.
- GARDNER, D.G., NEWMAN, E.D., NAKAMURA, K.K., NGUYEN, K.P. (1991). Endothelin increases the synthesis and secretion of atrial natriuretic peptide in neonatal rat cardiocytes. *Am. J. Physiol.*, **261**, E177-E182.
- GARDNER, D.G., SCHULZ, H.D. (1990). Prostaglandins regulate the synthesis and secretion of atrial natriuretic peptide. *J. Clin. Invest.*, **86**, 52-59.
- GASSER, R., LUHA, O., DUSLEAG, J., EBER, B., ROTMAN, R., KLEIN, W., FUEGER, G.R. (1993). Observations on plasma ANP levels during short-term transient myocardial ischaemia produced by PTCA in patients with LAD stenosis. *Angiology-the journal of vascular diseases*, **April**, 279-288.
- GERBES, A.L., VOLLMAR, A.M. (1990) Degradation and clearance of atrial natriuretic factors (ANF). *life Sci.*, **47**, 1173-1180.

- GEWIRTZ, H., OHLEY, W., WALSH, J., SHEARER, D., SULLIVAN, M.J., MOST, A.S. (1983) Ischaemia-induced impairment of left ventricular relaxation: relation to reduced diastolic filling rates of the left ventricle. *Am.Heart J.*, **105**, 72.
- GILL, J.S., PRASAD, K., STEWART, J.T., PUMPHREY, C.W., HOLT, D.W., M^CKENNA, W.J., CAMM, A.J. (1995) Acute changes in atrial natriuretic peptide, insulin-like growth factor-1, and lactate levels during left anterior descending coronary artery angioplasty. *Am. Heart. J.*, **130**, 717-722.
- GLOVER, V., MEDVEDEV, A., SANDLER, M. (1995). Isatin is a potent endogenous antagonist of guanylate cyclase-coupled atrial natriuretic peptide receptors. *Life Sciences*, **57(22)**, 2073-2079.
- GOODWIN, G.W., TAEGTMEYER, H. (1994). Metabolic recovery of the isolated working rat heart after brief global ischaemia. *Am. J. Physiol.*, **267**, H462-H470.
- GRAY, D.A., DOWNING, C., SAYED, N. (1997). Endogenous plasma atrial natriuretic peptide and the control of salt gland function in the Pekin duck. *Am. J. Physiol.*, **273**, 1080-1085.
- GROVER, G.J., DZWONCZYK, S. & PARHAM, C.S. (1993). The endothelin-1 receptor antagonist BQ123 reduces infarct size in a canine model of coronary occlusion and reperfusion. *Cardiovasc. Res.*, **27**, 1613-1618.
- GROSSMAN, W., M^CLAURIN, L.P. (1976). Diastolic properties of left ventricle. *Ann. Intern. Med.*, **84**, 316.
- GULATI, A., KUMAR, A., SHAHANI, B.T. (1996) Cardiovascular effects of centrally administered endothelin-1 and its relationship to changes in cerebral blood flow. *Life Sci*, **58(5)**, 437-445.
- GUNNING, M.E., BALLERMANN, B.J., BRENNER, B.M., ZEIDEL, M.L. (1988) Characterization of ANP receptors in rabbit inner medullary collecting duct cells. *Am. J. Physiol.*, **255**, F324-F330.

- GUNNING, M.E., BALLERMANN, B.J., SILVA, P., BRENNER, B.M., ZEIDEL, M.L. (1990) Brain natriuretic peptide interaction with renal ANP system. *Am. J. Physiol.*, **258**, F467-F472.
- GUTIERREZ-MARCOS, F.M., FERNANDEZ-CRUZ, A., GUTKOWSKA, J., HERRERO, C., BLESÁ, A., ESTRADA, V., LOSCOS, C., LOPEZ-NOVOA, J.M. (1991). Atrial natriuretic peptide in patients with acute myocardial infarction without functional heart failure. *Eur Heart J.*, **12**, 503-507.
- HALL, C., CANNON, C.P., FORMAN, S., BRAUNWALD, E. (1995) Prognostic value of N-terminal pro-atrial natriuretic factor plasma levels measured within the first 12 hours after myocardial infarction. Thrombolysis in Myocardial Infarction (TIMI) II Investigators. *J. Am. Coll. Cardiol.*, **26**, 1452-1456.
- HALL, C., ROULEAU, J.L., MOYE, L., de CHAMPLAIN, J., BICHET, D., KLEIN, M. *et al.* (1994) N-terminal pro-atrial natriuretic factor, an independent predictor of long-term prognosis after myocardial infarction. *Circulation*, **89**, 1934-1942.
- HAN, H., NEUBAUER, S., BRAEKER, B. & ERTL, G. (1995). Endothelin-1 contributes to ischaemia/reperfusion injury in isolated rat heart-attenuation of ischaemic injury by the endothelin-1 antagonists BQ123 and BQ610. *J. Mol. Cell. Cardiol.*, **27** (2), 761-766.
- HARADA, M., SAITO, Y., KUWAHARA, K., OGAWA, E., ISHIKAWA, M., NAKAGAWA, O., *et al.* (1998). Interaction of myocytes and nonmyocytes is necessary for mechanical stretch to induce ANP/BNP production in cardiocyte culture. *J. Cardiovasc. Pharmacol.*, **31**, S357-S359.
- HASDAI, D., HOLMES, D.R., Jr, GARRATT, K.N., EDWARDS, W.D., LERMAN, A. (1997). Mechanical pressure and stretch release endothelin-1 from human atherosclerotic coronary arteries *in vivo*. *Circulation*, **95**, 357-362.
- HAUFE, M.C., GERZER, R., WEIL, J., ERNST, J.E., THEISEN, K. (1988) Verapamil impairs secretion of stimulated atrial natriuretic factor in humans. *J Am Coll Cardiol*, **11**, 1199.
- HAUG, C., VOISARD, R., BAUR, R., HANNEKUM, A., HOMBACH, V., GRUENERT, A. (1998) Effect of diltiazem and verapamil on endothelin release

by cultured human coronary smooth-muscle cells and endothelial cells. *J. Cardiovas. Pharmacol.*, **31**, S388-S391.

HAYNES, W.G., STRACHAN, F.E., WEBB, D.J. (1995) Endothelin ETA and ETB receptors cause vasoconstriction of human resistance and capacitance vessels *in vivo*. *Circulation*, **92**, 357-363.

HAYNES, W.G., WEBB, D.J. (1994) Contribution of endogenous generation of endothelin-1 to basal vascular tone. *Lancet*, **344**, 852-854.

HEIM, J.M., GOTTMANN, K., WEIL, J., HAUFE, M.C., GERZER, R. (1988) Is cyclic GMP a clinically useful marker for ANF action. *Kardiologie*, **77**, 41-46.

HELFANT, R.H., *et al.* (1970) Differential hemodynamic, metabolic, and electrocardiographic effects in subjects with and without angina pectoris during atrial pacing. *Circulation*, **42**, 601.

HILLIS, L.D., BRAUNWALD, E. (1978) Coronary artery spasm. *N. Engl. J. Med.*, **299**, 695.

HINSON, J.P., KAPAS, S., CAMERON, L.A. (1996) Differential effects of endogenous and exogenous nitric oxide on the release of endothelin-1 from the intact perfused rat adrenal gland *in situ*. *FEBS Lett*, **379**(1), 7-10.

HIRATA, Y. (1996) Endothelin peptides. *Curr. Opin. Nephrol. Hypertens.*, **5**, 12-15.

HIRATA, Y., YASHIMI, H., TAKAICHI, S., YANAGISAWA, M., MASAKI, T. (1988) Binding and receptor down regulation of a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells. *FEBS Lett*, **239**, 13-17.

HISHIKAWA, K., NAKAKI, T., MARUMO, T., SUZUKI, H., KATO, R., SARUTA, T. (1995). Pressure enhances endothelin-1 release from cultured human endothelial cells. *Hypertension*, **25**(3), 449-452.

HOFFMAN, A., HARAMATI, A., DALAL, I., SHURANYI, E., WINAVER, J. (1994) Diuretic-natriuretic actions and pressor effects of big-endothelin (1-39) in phosphoramidon-treated rats. *P.S.E.B.M.*, **205**, 168-173.

- HOLLER, G.V., *et al.* (1984a) The pacing stress test: A re-examination of the relation between coronary artery disease and pacing induced electrocardiographic changes. *Am. J. Cardiol.*, **54**, 50.
- HOLLER, G.V., *et al.* (1984b) The pacing stress test: Thallium 201 myocardial imaging after atrial pacing. *J. Am. Coll. Cardiol.*, **3**, 1496.
- HOLLIMAN, W.H., BUDZIK, G.P., DEVINE, E.M., POLLOCK, D.M., OPGENORTH, T.J., THOMAS, A.M., VON GELDERN, T.W., ROCKWAY, T.W. (1991) Truncated atrial natriuretic factor analogs retain full agonist activity. *Can. J. Physiol. Pharmacol.*, **69**, 1622-1627.
- HOLLISTER, A.S., TANAKA, I., IMADA, T., ONROT, T., BIAGGONI, J., *et al.* (1986) Sodium loading and posture modulate human atrial natriuretic factor plasma levels. *Hypertension*, **8** (suppl II), II106-II111.
- HOLMBERG, S., VARNAUSKAS, E. (1971) Coronary circulation during pacing-induced tachycardia. *Acta. Med. Scand.*, **190**, 491.
- HORIO, T., KOHNO, M., TAKEDA, T. (1993a) Co-secretion of atrial and brain natriuretic peptides stimulated by endothelin-1 from cultured rat atrial and ventricular cardiocytes. *Metabolism*, **42**: 94-96.
- HORIO, T., SHIMADA, K., KOHNO, M., YOSHIMURA, T., KAWARABAYASHI, T., YASUNARI, K., MURAKAWA, K., YOKOKAWA, K., IKEDA, M., FUKUI, T., TAKEDA, T. (1993b). Serial changes in atrial and brain natriuretic peptides in patients with acute myocardial infarction treated with early coronary angioplasty. *Am Heart J.*, **126**, 293-299.
- HOROWITZ, J.D., HENRY, P.J. (1987). Recent developments in nitrate therapy of ischaemic heart disease. *Med. J. Aust.*, **146**, 93-96.
- HOSODA, K., NAKAO, K., MUKOYAMA, M. *et al.* (1991) Expression of brain natriuretic peptide gene in human heart. Production in the ventricle. *Hypertension.*, **17**, 1152-1155.

- HU, D.C.K., WONG, E.F.C., WONG, N.L.M. (1988). The differential response in atrial natriuretic peptide release during exercise in patients with and without ischemic heart disease. *Am J Med Sci.*, **296** (2), 111.
- HU, J.R., BERNINGER, U.G., LANG, R.E. (1988). Endothelin stimulates atrial natriuretic peptide (ANP) release from rat atria. *Eur J Pharmacology.*, **158**, 177-178.
- HU, R.M., LEVIN, E.R., PEDRAM, A., FRANK, J.L. (1992). Atrial natriuretic peptide inhibits the production and secretion of endothelin from cultured endothelial cells. *J. Biol. Chem.*, **267**, 17384-17389.
- HUGHES, A., GOLDBERG, T.P., MARTIN, G., SEVER, P. (1988) Direct effect of α -human atrial natriuretic peptide in human vasculature *in vivo* and *in vitro*. *Clin. Science*, **74**, 207-211.
- HUTTL, S., NUSSBERGER, J., LEHMANN, K., HASFORD, J., BRUNNER, H.R., DELIUS, W. (1995) Beneficial clinical effect of very early enalapril treatment in patients with acute left ventricular failure complicating myocardial infarction. *Clin. Cardiol.*, **18**, 317-323.
- HUXLEY, V.H., TUCKER, V.L., VERBURG, K.M., FREEMAN, R.H. (1987) Increased capillary hydraulic conductivity induced by atrial natriuretic peptide. *Cir. Res.*, **60**, 304-307.
- IHARA, M., YAMANAKA, R., OHWAKI, K., OZAKI, S., FUKAMI, T., ISHIKAWA, K., TOWERS, P. & YANO, M. (1996). [³H]BQ123, a highly specific and reversible radioligand for the endothelin ET_A receptor subtype. *Eur. J. Pharmacol.*, **274**, 1-6.
- IIDA, H., PAGE, E. (1989) Determinants of atrial natriuretic peptide secretion in cultured atrial myocytes. *Am J Physiol.*, **256**: C608-C613.
- IIVANAINEN, A.M., TIKKANEN, I., TILVIS, R., HEIKKILA, J., HELENIUS, T., KUPARI, M. (1997) Associations between atrial natriuretic peptides, echocardiographic findings and mortality in an elderly population sample. *J. Intern. Med.*, **241**, 261-268.

- IKAHEIMO, M.J., RUSKOaho, H.J., AIRAKSINEN, K.E.J., HUIKURI, H.V., KORHONEN, U.R., LEPPALUOTO, J., TUOMINEN, M.O., TAKKUNE, J.T. (1989). Plasma levels of atrial natriuretic peptide during myocardial ischemia induced by percutaneous transluminal coronary angioplasty or dynamic exercise. *Am Heart J.*, **117**, 837.
- IKEDA, T., MATSUDA, K., ITOH, H., SHIRAKAMI, G., MIYAMOTO, Y., YOSHIMASA, T., NAKAO, K., BAN, T. (1997). Plasma levels of brain and atrial natriuretic peptides elevate in proportion to left ventricular end-systolic wall stress in patients with aortic stenosis. *Am. Heart J.*, **133**, 307-314.
- IMURA, R., SANO, T., GOTO, J., YAMADA, K., MATSUDA, Y. (1992) Inhibition by HS-142-1, a novel nonpeptide atrial natriuretic peptide antagonist of microbial origin, of atrial natriuretic peptide-induced relaxation of isolated rabbit aorta through the blockade of guanylyl cyclase-linked receptors. *Mol. Pharmacol.*, **42**, 982-990.
- IRONS, C.E., MURRAY, S.F., GLEMBOTSKI, C.C. (1993) Identification of the receptor subtype responsible for endothelin-mediated protein kinase C activation and atrial natriuretic factor secretion from atrial myocytes. *J. Biol. Chem.*, **268**, 23417-23412.
- IWASAKI, T., NIWA, A., SHINODA, T., ISHIHARA, M., AIZAWA, T., YAMADA, T. (1989) Effect of calcium antagonists and nitroglycerin on atrial natriuretic peptide in normal subjects and patients with essential hypertension. *Angiology*, **40**, 24.
- IWATA, T., INUI, K., NAKAO, K., IMURA, H., MATSUO, H., HORI, H. (1980) Effect of brain natriuretic peptide on cyclic GMP accumulation in a kidney epithelial cell line (LLC-PKI). *Eur. J. Pharmacol.*, **159**, 321-323.
- JACKSON, G. (1985) Laboratory diagnosis of myocardial ischaemia. *Invasive Cardiol.*, 45-65.
- JANSEN, T.L.Th.A., SMITS, P., TAN, A.C.I.T.L., THIEN, T. (1991) Attenuated forearm vasodilator response to atrial natriuretic factor in the elderly. *Hypertension*, **18**, 640-647.

- JENNINGS, R.B., REIMER, K.A., HILL, M.L., MAYER, S.E. (1981) Total ischaemia in dog in vitro. I. Comparison of high energy phosphate production, utilization and depletion, and of adenine nucleotide catabolism in total ischaemia *in vivo* vs. severe ischaemia *in vivo*. *Circ. Res.*, **49**, 892.
- JIN, H., CHEN, Y.F., YANG, R.H., OPARIL, S. (1990) Atrial natriuretic peptide clearance receptor agonist lowers pulmonary pressure in hypoxic rats. *J. Appl. Physiol.*, **68**, 2413-2418.
- JOHN, S.W.M., KREGGE, J.H., OLIVER, P.M. *et al.* (1995) Genetic decreases in atrial natriuretic peptide and salt-sensitive hypertension. *Science*, **16**, 301-307.
- JOHNSON, J., ARIK, L., PITTS, B., Jr., FOSTER, C.J. (1990). rapid receptor-mediated catabolism of ¹²⁵I-atrial natriuretic factor by vascular endothelial cells. *Biochem. J.*, **268**, 771-776.
- KAANGAWA, K., MATSUO, H. (1984) Purification and complete amino acid sequence of α -human atrial natriuretic polypeptide (α -ANP). *Biochem. Biophys. Res. Commun.*, **118**, 131-139.
- KAMBAYASHI, Y., NAKAO, K., ITOH, H., HOSODA, K., SAITO, Y., YAMADA, T., MUKOYAMA, M., ARAI, H., SHRIRAKAMI, G., SUGA, S., OGAWA, Y., JOUGASAKI, M., MINAMINO, N., KANGAWA, K., MATSUE, H., INOUE, K., IMURA, H. (1989) Isolation and sequence determination of rat brain natriuretic peptide. *Boichem. Biophys. Res. Commun.*, **163**, 233-240.
- KAMBAYASHI, Y., NAKAO, K., MUKOYAMA, M., SAITO, Y., OGAWA, Y., SHIONO, S., INOUE, K., YOSHIDA, N., IMURA, H. (1990) Isolation and sequence determination of human brain natriuretic peptide in human atrium. *FEBS lett.*, **259**, 341-345.
- KARNE, S., JAYAWICKREME, C.K., LERNER, M.R. (1993) Cloning and Characterization of an endothelin-3 specific receptor (ET_C receptor) from *Xenopus laevis* dermal melanophores. *J. Biol. Chem.*, **268**, 19126-19133.
- KARWATOWSKA PROKOPCZUK, E., WENNMALM, A. (1990) Effects of endothelin on coronary flow, mechanical performance, oxygen uptake, and

formation of purines and on outflow of prostacyclin in the isolated rabbit heart. *Circ. Res.*, **66**, 46-54.

KASAI, H., TAKANASHI, M., TAKASAKI, C., ENDOH, P.L. (1994) Pharmacological properties of endothelin receptor subtypes mediating positive inotropic effects in rabbit heart. *Am. J. Physiol*, **266**: H2220-H2228.

KATOH, S., TOYAMA, J., AOYAMA, M., MIYAMOTO, N., SEO, H., KODAMA, I., YAMADA, K. (1990) Mechanisms of atrial natriuretic peptide (ANP) secretion by rat hearts perfused in vitro--Ca²⁺(+)-dependent signal transduction for ANP release by mechanical stretch. *Jpn Circ J*, **54**(10), 1283-1294.

KAWABE, J.I., OHSAKI, Y., ONODERA, S. (1992) Down-regulation of protein kinase C potentiates atrial natriuretic peptide-stimulated cGMP accumulation in vascular smooth-muscle cells. *Biochimica et Biophysica Acta*, **1175**, 81-87.

KAWAI, M., NARUSE, M., YOSHIMOTO, T., NARUSE, K., SHIONOYA, K., TANAKA, M., MORISHITA, Y., MATSUDA, Y., DEMURA, R., DEMURA, H. (1996). C-type natriuretic peptide as a possible local modulator of aldosterone secretion in bovine adrenal zona glomerulosa. *Endocrinology*, **137**(1), 42-46.

KAWASHIMA, A., KUBO, K., MATSUZAWA, Y., KOBAYASHI, T., SEKIGUCHI, M. (1992) Hypoxia-induced ANP secretion in subjects susceptible to high-altitude pulmonary edema. *Respir Physiol*, **89**(3), 309-317.

KELLY, R.A., EID, H., KRAMER, B.K., O'NEILL, M., LIANG, B.T., REERS, M., SMITH, T.W. (1990) endothelin enhances the contractile responsiveness of adult rat ventricular myocytes to calcium by a pertussis toxin-sensitive pathway. *J. Clin. Invest*; **86**, 1164-1171.

KETTUNEN, R.V.J., VUOLTEENAHO, O., UKKOLA, O., LILJA, M., JOUNELA, A., KESANIEMI, Y.A., LEPPALUOTO, J., HEIKKILA, J. (1994) Effects of early administration of enalapril on radionuclide left ventricular ejection fraction and plasma N-terminal atrial natriuretic peptide after acute myocardial infarction. *Am. J. Cardiol.*, **73**, 865-867.

KIKUTA, K., YASUE, H., YOSHIMURA, M., MORITA, E., SUMIDA, H., KATO, H., KUGIYAMA, K., OGAWA, H., OKUMURA, K., OGAWA, Y., NAKAO,

- K. (1996) Increased plasma levels of B-type natriuretic peptide in patients with unstable angina. *Am.Heart J.*, **132**, 101-107.
- KING, M.S., BAERTSCHI, A.J. (1989) Physiological concentration of atrial natriuretic factors with intact N-terminal sequences inhibit corticotropin releasing factor-stimulated adrenocorticotropin secretion from cultured anterior pituitary cells. *Endocrinol*, **124** , 286-292.
- KIOWSKI, W., SUTSCH, G., HUNZIKER, P., MULLER, P., KIM, J., OECHSLIN, E., SCHMITT, R., JONES, R. & BERTEL, O. (1995). Evidence for endothelin-1 mediated vasoconstriction in severe chronic heart failure. *Lancet*. **346**, 732-736.
- KIRKLIN, J.W., BLACKSTONE, E.H., ROGERS, W.J. (1985). The plights of the invasive treatment of ischaemic heart disease. *J. Am. Coll. Cardiol.*, **5**, 158.
- KISCH, B.(1956) Electromicroscopy of the atrium of the heart. I Guinea pig. *Exp. Med. Surg.*, **14**, 99-112.
- KISHIMOTO, I., DUBOIS, S.K., GARBERS, D.L. (1996) The heart communicates with the kidney exclusively through the guanylyl cyclase-A receptor: acute handling of sodium and water in response to volume expansion. *Proc. Natl. Acad. Sci*, **93**, 6215-6219.
- KLEMM, P., WARNER, T.D., HOHLFELD, T., CORDER, R., VANE, J.R. (1992) Endothelin 1 mediates *ex vivo* coronary vasoconstriction caused by exogenous and endogenous cytokines. *Proc. Natl. Acad. Sci.*, **92**, 2691-2695.
- KLOOG, Y., SOKOLOVSKY, M. (1989) Similarities in mode and sites of action of sarafotoxins and endothelins. *Trends Pharmacol. Sci.*, **10**, 212-214.
- KNEPPER, M.A., LANKFORD, S.P., TERADA, Y. (1991) Renal tubular actions of ANF. *Can. J. Physiol. Pharmacol.*, **69**, 1537-1545.
- KNORR, A.M., LAGUNA, C., STASCH, J.P. (1988) Modulation by chronic nifedipine of plasma volume in rats with renal hypertension. *J Hypertens*, **6**: 839.

- KOEHN, J.A., NORMAN, J.A., JONES, B.N., LESUEUR, L., SAKANE, Y., GHAI, R.D. (1987) Degradation of atrial natriuretic factor by kidney cortex membranes. Isolation and characterization of the primary proteolytic product. *J. Biol. Chem.*, **262**, 11623-11627.
- KOEPKE, J.P., TYLER, L.D., TRAPANI, A.J., BOVY, P.R., SPEAR, K.L., OLINS, G.M., BLAINE, E.H. (1989) Interaction of non-guanylate cyclase-linked atriopeptin receptor ligand and endopeptidase inhibitor in conscious rats. *J. Pharmacol. Exp. Ther.*, **249**, 172-176.
- KOHNO, M., TAKAORI, K., MATSUURA, T., MURAKAWA, K., KANAYAMA, K., TAKEDA, T. (1986) Atrial natriuretic polypeptide in atria and plasma in experimental hyperthyroidism and hypothyroidism. *Biochem. Biophys. Res. Commun.*, **134**, 178-183.
- KOHNO, M., YOKOKAWA, K., HORIO, T., YASUNARI, K., MURAKAWA, K., TAKEDA, T. (1992) Atrial and brain natriuretic peptides inhibit the endothelin-1 secretory response to angiotensin II in porcine aorta. *Circ Res*, **70**, 241-247.
- KOHNO, M., HORIO, T., IKEDA, M., YOKOKAWA, K., KUKUI, T., YASUNARI, K., *et al.* (1993a) Natriuretic peptides inhibit mesangial cell production of endothelin induced by arginine vasopressin. *Am. J. Physiol.*, **264**, F678-F683.
- KOHNO, M., IKEDA, M., JOHCHI, M., HORIO, T., YASUNARI, K., KURIHARA, N., TAKEDA, T. (1993b) Interaction of PDGE and natriuretic peptides on mesangial cell proliferation and endothelin secretion. *Am. J. Physiol.*, **265**, E673-E679.
- KOJIMA, M., MINAMINO, N., KANGAWA, K., MATSUO, H. (1989) Cloning and sequence analysis of cDNA encoding a precursor for rat brain natriuretic peptide. *Biochem. Biophys. Res. Commun.*, **159**, 1420-1426.
- KOJIMA, M., MINAMINO, N., KANGAWA, K., MATSUO, H. (1990) Cloning and sequence analysis of cDNA encoding a precursor for rat C-type natriuretic peptide (CNP). *FEBS Lett.*, **276**, 209-213.

- KOMATSU, Y., NAKAO, K., SUGA, S., OGAWA, Y., MUKOYAMA, M., ARAI, H., SHIRAKAMI, G., HOSODA, K., KISHIMOTO, I., NAKAGAWA, O., HAMA, N., IMURA, H. (1991a) C-type natriuretic peptide-tissue distribution and molecular form in rats. *Circulation*, **84**, 1730.
- KOMATSU, Y., NAKAO, K., SUGA, S., OGAWA, Y., MUKOYAMA, M., ARAI, H., SHIRAKAMI, G., HOSODA, K., NAKAGAWA, O., HAMA, N. (1991b) C-type natriuretic peptide in rats and humans. *Endocrinology*, **129**, 1104-1106.
- KOUREMBANAS, S., MARSDEN, P.A., MCQUILLAN, L.P., FALLER, D.V. (1991). Hypoxia induces endothelin gene expression and secretion in cultured human endothelium. *J Clin Invest*, **88**(3), 1054-1057.
- KRAMER, B.K., NISHIDA, M., KELLY, R.A., SMITH, T.W. (1992). Endothelins: myocardial actions of a new class of cytelines. *Circulation*, **85**, 350-356.
- KRUGER, D., GIANNITSIS, E., SHEIKHZADEH, A., STIERLE, U. (1998). Cardiac release and kinetics of endothelin after uncomplicated percutaneous transluminal coronary angioplasty. *Am. J. Cardiol.*, **81**, 1421-1426.
- KUNO, T., ANDERESSEN, J.W., KAMISAKI, Y., WALDMAN, S.A., CHANG, L.Y., SAHEKI, S., LEITMAN, D.C., NAKANE, M., MURAD, F. (1986) Co-purification of an atrial natriuretic factor receptor and particulate guanylate cyclase from rat lung. *J. Biol. Chem.*, **261**, 5817-5823.
- KUSUMOTO, K., AWANE, Y., FUJIWARA, S., WATANABE, T. (1993) Role of endogenous endothelin in extension of rabbit myocardial infarction. *J. Cardiovas. Pharmacol.*, **22**, 339-342.
- KYRIAKIDIS, M., PETROPOULAKIS, P., ASPIOTIS, N., TSELIKA, C., ANTONOPOULOS, A., MARAKAS, S., TOUTOUZAS, P. (1993) Plasma atrial natriuretic factor in uncomplicated acute myocardial infarction. *Cardiology*, **83**, 173-182.
- KYRIAKIDES, Z.S., MARKIANOS, M., ILIODROMITIS, E.K., KREMASTINOS, D.T. (1995) Vein plasma endothelin-1 and cyclic GMP increase during coronary angioplasty is related to myocardial ischaemia. *Eur. Heart J.*, **16**(7), 894-898.

- KYRIAKIDES, Z.S., MARKIANOS, M., PARASKEVAIDIS, I.A., TOUSOULIS, D., KREMASTINOS, D.T. (1996) Decreased vasomotor effect of endothelin on coronary angioplasty in hypertensive patients. *Int. J. Cardiol.*, **55**(1), 41-48.
- LANG, C.C., COUTIE, W.J., KHONG, T.K., CHOY, A.M.J., STRUTHERS, A.D. (1991). Dietary sodium loading increases plasma brain natriuretic peptide levels in man. *J. Hypertens.*, **9**, 779-782.
- LANG, C.C., CHOY, A.M.J., HENDERSON, I.S., CHOY, A.M.J., STRUTHERS, A.D. (1992a). Effect of haemodialysis on plasma levels of brain natriuretic peptide patients with chronic renal failure. *Clin Science.*, **82**, 127-31.
- LANG, C.C., CHOY, A.M.J., STRUTHERS, A.D. (1992b). Atrial and brain natriuretic peptides: a dual natriuretic peptide system potentially involved in circulatory. *Clin Science.*, **83**, 519-527.
- LANG, R.E., THOLKEN, H., GANTEN, D., LUFT, F.C., RUSKOAHO, H., UNGER, T. (1985). Atrial natriuretic factor-a circulating hormone stimulated by volume loading. *Nature*, **314**, 264-266.
- LAGERQVIST, B., BYLUND, H., GOTELL, P., MANNTING, F., SANDHAGEN, B., WALDENSTROM, A. (1991). Coronary artery vasoregulation and left ventricular function in patients with angina pectoris-like pain and normal coronary angiograms. *J. Intern. Med.*, **230**, 55-65.
- LANGENDROFF, O. (1895). Untersuchungen am uberlebenden saugethierherzen. *Pfugers Arch. Ges. Physiol.* **61**, 291-332.
- LASSERS, B.W., CARLSON, L.A., KAIJSER, L., *et al.* (1972) The nature and control of myocardial substrate metabolism in healthy man. In Oliver, M.F. (ed): Effect of acute ischaemia myocardial infarction. Edinburgh, Churchill Livingstone, 200.
- LEITE, M.F., PAGE, E., AMBLER, S.K. (1994) Regulation of ANP secretion by endothelin-1 in cultured atrial myocytes: desensitization and receptor subtype. *Am J Physiol.*, **267**, H2193-H2203.

- LEITMAN, D.C., ANDRESON, J.W., KUNO, T., KAMISAKI, Y., CHANG, J.K., MURAD F. (1986) Identification of multiple binding sites for atrial natriuretic factor by affinity cross-linking in cultured endothelial cells. *J. Biol. Chem.*, **261**, 11650-11655.
- LESKINEN, H., VUOLTEENAHU, O., RUSKOAHU, H. (1997). Combined inhibition of endothelin and angiotension II receptors blocks volume load-induced cardiac hormone release. *Circ Res.* **80**, 114-123.
- LESKINEN, H., VUOLTEENAHU, O., LEPPALUOTO, J., RUSKOAHU, H. (1995). Role of nitric oxide on cardiac hormone secretion: effect of NG-nitro-L-arginine methyl ester on atrial natriuretic peptide and brain natriuretic peptide release. *Endocrinology*. **136**(3), 1241-1249.
- LEW, R.A., BAERTSCHI, A.J. (1989) Mechanisms of hypoxia-induced atrial natriuretic factor release from rat hearts. *Am J Physiol*, **255**, H295-H300.
- LEWIS, B.S., MAKHOUL, N., DAKAK, N., FLUGELMAN, M.Y., YECHIELY, H., HALON, D.A. KAHANA, L. (1992) Atrial natriuretic peptide in severe heart failure: Response to controlled changes in atrial pressures during intravenous nitroglycerin therapy. *Am Heart J*, **124**, 1009.
- LEVIN, E.R. (1995) Endothelins. *New Engl. J. Med.*, **333**, 356-363.
- LINZ, W., SCHOLKENS, B.A., ALBUS, U., PETRY, P., BREIPOHL, G., KNOLLE, J. (1988) Atrial natriuretic factor protects the isolated working ischaemic rat heart against the action of angiotensin II. *J Hypertension*, **6**: S339.
- LIU, J.J., CASLEY, D.J., NAYLER, W.G. (1989). Ischaemia causes externalisation of endothelin-1 binding sites in rat cardiac membranes. *Biochem. Biophys. Res. Commun.*, **164**(3), 1220-1225.
- LIU, Q., NAKAE, I., TSUTAMOTO, T., TAKAOKA A., KINOSHITA, M. (1996). In vivo vasodilatory action of atrial natriuretic peptides in canine coronary circulation. *Jpn. Cir. J.*, **60**(5), 300-310.

- LOOP, F.D., SHELDON, W.C., LYTLE, B.W., COSGROVE, D.M., III, PROUDFIT, W.L. (1981). The efficacy of coronary artery surgery. *Am. Heart J.*, **101**, 86.
- LOPASCHUK, G.D., SPAFFORD, M.A., DAVIES, N.J., WALL, S.R. (1990) Glucose and palmitate oxidation in isolated working rat hearts reperfused after a period of transient global ischaemia. *Circ. Res.*, **66**, 546-553.
- LOPEZ, M.J., WONG, S.K., KISHIMOTO, I., *et al.* (1995) Salt-resistant hypertension in mice lacking the guanylyl cyclase-A receptor for atrial natriuretic peptide. *Nature*, **378**, 65-68.
- LORDICK, F., HAUCK, R.W., SENEKOWITSCH, R., EMSLANDER, HP. (1995). Atrial natriuretic peptide in acute hypoxia-exposed healthy subjects and hypoxaemic patients. *Eur. Respir. J.*, **8**(2), 216-221.
- LUFT, F.C., LANG, R.E., ARONOFF, G.R., RUSKOAHO, H., TOTH, M., GANTEN, D., STERZEL, R.B., UNGER, T. (1986) Atriopetin III kinetics and pharmacodynamics in normal and anephric rats. *J. Pharmacol. Exp. Ther.*, **236**, 416-418.
- MAACK, T., SUZUKI, M., ALMEIDA, F.A., NUSSENZVEIG, D., SCARBOROUGH, R.M., MCENDROE, G.A., LEWIKI, J.A. (1987) Physiological role of silent receptors of atrial natriuretic factor. *Science*, **238**, 675-678.
- MACDONALD, P.S., KUDO, K., DUBBIN, P.N., DUSTING, G.J. (1989) Rat aortic endothelium antagonizes nitroprusside-induced relaxation by release of the peptide endothelin. *Clin. Exp. Pharmacol. Physiol.*, **16**, 223-227.
- MACPHERSON, L.J., BAYBURT, E.K., CAPPARELLI, M.P., BOHACEK, R.S., CLARKE, F.H., GHAI, R.G., *et al.* (1993) Design and synthesis of an orally active macrocyclic neutral endopeptidase 24.11 inhibitor. *J. Med. Chem.*, **36**, 3821-3828.
- MAGGA, J., VUOLTEENAHO, O., MARTTILA, M., RUSKOAHO, H. (1997). Endothelin-1 is involved in stretch-induced early activation of B-type natriuretic peptide gene expression in atrial but not in ventricular myocytes: acute effects of

- mixed ET_A/ET_B and AT1 receptor antagonists *in vivo* and *in vitro*. *Circulation*, **96**(9), 3053-3062.
- MAGUIRE, J.J., JOHNSON, C.M., MOCKRIDGE, J.W., DAVENPORT, A.P. (1997) Endothelin converting enzyme (ECE) activity in human vascular smooth muscle. *Br. J. Pharmacol*, **82**(5), 1593-1600.
- MAIR, P., MAIR, J., BLEIER, J., HORMANN, C., BALOGH, D., PUSCHENDORF, B. (1997) Augmented release of brain natriuretic peptide during reperfusion of the human heart after cardioplegic cardiac arrest. *Clin. Chem. Acta.*, **261**(1), 57-68.
- MALATINO, L.S., LEUNARDI, C., STANCANELLI, B., POLIZZI, G., GRASSI, R., TAMBURIO, C., TAMBURINO, G. (1992). Transient myocardial ischemia stimulates atrial natriuretic factor release. *Am Heart J.*, **123**, 693.
- MALATINO, L.S., GRASSI, R., STANCANELLI, B., POLIZZI, G., LEONARDI, C., TAMBURINO, C. & TAMBURINO, G. (1993). Release of immunoreactive endothelin from the heart during percutaneous transluminal coronary angioplasty. *Am. Heart J.*, **126**, 700-702.
- MANGIERI, E., TAZILLI, G., BARILLA, F., CIAVOLELLA, M., PUDDU, P.E., ANGELIS, C. *et al.* (1998) Handgrip increases endothelin-1 secretion in normotensive young male offspring of hypertensive parents. *J. Am. Coll. Cardiol.*, **31**(6), 1362-1366.
- MANTYMAA, L.S., LEPPALUOTO, J., RUSKOAHO, H. (1990). Endothelin stimulates basal and stretch-induced atrial natriuretic peptide secretion from perfused rat heart. *Endocrinology.*, **126**, 587-595.
- MANTYMAA, L.S., TASKINEN, T., RUSKOAHO, H. (1991). Effect of stretch on atrial natriuretic peptide gene expression in perfused rat heart. *Am J Hypertens.*, **4**, 91A.
- MARALA, R.B., DUDA, T., SHARMA, R.K. (1993) Interaction of atrial natriuretic factor and endothelin-1 signals through receptor guanylate cyclase in pulmonary artery endothelial cells. *Mol Cell Biochem*, **120**, 69-80.

- MARISHITA, Y., SANO, T., ANDO, K., SAITOH, Y., KASE, H., YAMADA, K., MATSUDA, Y. (1991a). Microbial polysaccharide, HS-142-1, competitively and selectively inhibits ANP binding to its guanylyl cyclase-containing receptor. *Biochem. Biophysiol. Res. Commun.*, **176**, 949-957.
- MARISHITA, Y., TAKAHASHI, M., SANO, T., KAWAMOTO, I., ANDO, K., SANO, H., SAITOH, Y., KASE, H., MATSUDA, Y. (1991b). Isolation and purification of HS-142-1, a novel nonpeptide antagonist for the atrial natriuretic peptide receptor, from *Aureobasidium* sp. *Agric. Biol. Chem.*, **55**, 3017-3025.
- MARRUMOTO, K., HAMADA, M., ABURAYA, M., HIWADA, K. (1995a) Augmented secretion of atrial and brain natriuretic peptides during dynamic exercise in patients with old myocardial infarction. *Jpn. Circ. J.*, **59**, 715-24.
- MARRUMOTO, K., HAMADA, M., HIWADA, K. (1995b) Increased secretion of atrial and brain natriuretic peptides during acute myocardial ischaemia induced by dynamic exercise in patients with angina pectoris. *Clin. Sci*, **88**, 551-556.
- MATTHEWS, M.B., JULIAN, D.G. (1985) Angina pectoris: Definition and description. In Julian, D.G.: Angina Pectoris. 2nd ed. New York, Churchill Livingstone, 2.
- MCCLELLAN, G., WEISBERG, A., WINEGRAD, S. (1994) Endothelial cell storage and release of endothelin as a cardioregulatory mechanism. *Circ. Res*, **75**, 85-96.
- MCCLELLAN, G., WEISBERG, A., WINEGRAD, S. (1996) Effect of endothelin-1 on actomyosin ATPase activity-Implications for the efficiency of contraction. *Circ. Res*, **78**, 1044-1050.
- MCCULLOCH, K.M., DOCHERTY, C.C., MORECROFT, I. & MACLEAN, M.R. (1996). Endothelin receptor-mediated contraction in human pulmonary resistance arteries. *Br. J. Pharmacol.*, **119**, 1125-1130.
- MCDONOUGH, P.M., STELLA, S.L., GLEMBOTSKI, C.C. (1994). Involvement of cytoplasmic calcium and protein kinases in the regulation of atrial natriuretic factor secretion by contraction rate and endothelin. *J. Biol. Chem.*, **269**, 9466-9472.

- MC^GOWAN, F.X., Jr, DAVIS, P.J., SIEWERS, R.D., del NIDO, P.J. (1995). Coronary vasoconstriction mediated by endothelin-1 in neonates. *J. Thorac. Cardiovasc. Surg.*, **109**, 88-98.
- MC^KAY, R.G. *et al.* (1984). The pacing stress test re-examined: Correlation of pacing-induced hemodynamic changes with the amount of myocardium at risk. *J. Am. Coll. Cardiol.*, **3**, 1469.
- MC^MAHON, E.G., PALOMO, M.A., MOORE, W.M., MC^DONALD, J.F. (1991) Phosphoramidon blocks the pressor activity of porcine big endothelin-1-(1-39) *in vivo* and conversion of big endothelin-1-(1-39) to endothelin-1-(1-21) *in vitro*. *Proc. Natl. Acad. Sci.*, **109**, 88-98.
- MIASIRO, N., PAIVA, A.C. (1990) Homologous desensitization of the effects of endothelin on rabbit aorta rings and cultured rat aorta smooth muscle cells. *Eur. J. Pharmacol.*, **179**, 151-158.
- MICHEL, J.B., NICOLLETTI, A., ARNAL, J.F. (1995) Left ventricular remodelling following experimental myocardial infarction. *Eur. Heart J.*, **16**(suppl I), 49-57.
- MIZUGUCHI, T., NISHIYAMA, M., MOROI, K., TANAKA, H., SAITO, T., MASUDA, Y., *et al.* (1997) Analysis of two pharmacologically predicted endothelin B receptor gene knockout mouse. *Br. J. Pharmacol.*, **120**, 1427-1430.
- MONTALESCOT, G., VIOSSAT, I., CHABRIER, P.E., SOTIROV, I., DETIENNE, J.P., DROBINSKI, G., FRANK, R., GROSGOGEAT, Y., THOMAS, D. (1994) Endothelin-1 in patients with coronary heart disease undergoing cardiac catheterization. *J. Am. Coll. Cardiol.*, **24**(5), 1236-1241.
- MORGANTIA., GIUSSANI, M., SALA, C., GAZZANO, G., MARANA, I., PIERINI, A. *et al.* (1995) Effect of exposure to high altitude on plasma endothelin-1 levels in normal subjects. *J. Hypertens.*, **13**(8), 859-865.

- MORICE, A., PEPKI-ZABA, J., LOYSEN, E., LAPWORTH, R., ASHBY, M. *et al.* (1988) Low dose infusion of atrial natriuretic peptide cause salt and water excretion in normal man. *Clin. Science*, **74**, 359-363.
- MORIMOTO, A., NISHIKIMI, T., TAKAKI, H., OKANO, Y., MATSUOKA, H., TAKISHITA, S., KITAMURA, K., MIYATA, A., KANGAWA, K., MATSUO, H. (1997) Effect of exercise on plasma adrenomedullin and natriuretic peptides levels in myocardial infarction. *Clin. Exp. Pharmacol. Physiol.*, **24**(5), 315-320.
- MORITA, E., YASUE, H., YOSHIMURA, M., OGAWA, H., JOUGASAKI, M., MATSUMURA, T., MUKOYAMA, M., NAKAO, K. (1993). Increased plasma levels of brain natriuretic peptide in patients with acute myocardial infarction. *Circulation*, **88**, 82-91.
- MUKOYAMA, M., NAKAO, K., HOSODA, K., *et al.* (1991a). Brain natriuretic peptide as a novel cardiac hormone in humans. *J. Clin. Invest.*, **87**, 1402-1412.
- MUKOYAMA, M., NAKAO, K., OBATA, K., JOUGASAKI, M., YOSHIMURA, M., MORITA, E., HOSODA, K., SUGA, S., OGAWA, Y., YASUE, H., IMURA, H. (1991b). Augmented secretion of brain natriuretic peptide in acute myocardial infarction. *Biochem Biophys Res Com.*, **189**, 431-437.
- MULLER, J.E. (1980). Prinzmetal's angina: A model for the role of spasm in ischaemic heart disease. *J. Cardiovas. Med.*, **5**, 19.
- MUNZEL, T., KURZ, S., HOLTZ, J., BUSSE, R., STEINHAEUER, H., JUST, H., DREXLER, H. (1992) Neurohormonal inhibition and hemodynamic unloading during prolonged inhibition of ANF degradation in patients with severe chronic heart failure. *Circulation.*, **86**, 1089-1098.
- MURRY, C.E., JENNINGS, R.B. (1986). Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation*, **74**, 1124-1136.
- MURTHY, L.J., CORDER, R., MALLET, A.I., TURNER, A.J. (1994). Generation by the phosphoramidon-sensitive peptidases, endopeptidase-24.11 and thermolysin, of endothelin-1 and C-terminal fragment from big endothelin-1. *Br. J. Pharmacol.*, **113**, 137-142.

- MURTHY, K.K., THIBAUT, G., CANTIN, M. (1989). Binding and intracellular degradation of atrial natriuretic factor by cultured vascular smooth muscle cells. *Mol. cell. Endocrinol.*, **67**, 195-206.
- NAKAGAWA, H., OKUMURA, K., HASHIMOTO, H., ITO, T., OGAWA, K. (1988) Effects of atrial natriuretic peptide and organic nitrates on levels of relaxation and cyclic nucleotide of canine coronary artery with and without endothelial injury. *Heart Vessels*, **4**, 19.
- NAKAMURA, M., ARAKAWA, N., KATO, M. (1990) Renal, hormonal and hemodynamic effects of low-dose infusion of atrial natriuretic peptide in acute myocardial infarction. *Am. Heart J.*, **120**, 1078-1085.
- NAKAMURA, M., ARAKAWA, N., YOSHIDA, H., FINAKOSHI, T., CHIBA, M., MAKITA, S., AOKI, H., HIRAMORI, K. (1993) Prognostic implications of plasma levels of atrial natriuretic factor in patients with acute myocardial infarction. *Internal Medicine.*, **32**, 112-115.
- NAKAO, K., ITOH, H., KAMBAYASHI, Y. *et al.* (1990) Rat brain natriuretic peptide. Isolation from rat heart and tissue distribution. *Hypertension*, **15**, 774-778.
- NAKAO, K., OGAWA, Y., SUGA, S.I., IMURA, H. (1992) Molecular biology and biochemistry of the natriuretic peptide system. II: Natriuretic peptide receptors. *J. Hypertens.*, **10**, 1111-1114.
- NAKAO, K., SUGAWARA, N., MORII, N., SAKAMOTO, M., YAMADA, T., ITOH, H., SHIONO, S., SAITO, Y., NISHIMURA, K., BAN, T., KANGAWA, K., MATSUO, H., IMURA, H. (1986) The pharmacokinetics of α -human atrial natriuretic polypeptide in healthy subjects. *Eur. J. Clin. Pharmacol.*, **31**, 101-103.
- NAKAOKA, H., KITAHARA, Y., AMANO, M., IMATAKA, K., FUJII, J., ISHIBASHI, M., YAMAJI, TOHRU. (1987) Effect of β -adrenergic receptor blockade on atrial natriuretic peptide in essential hypertension. *Hypertension*, **10**, 221-225.

- NARUSE, M., TAKEYAMA, Y., TANABE, M., KATAGIRI, T., DEMURA, H. (1994). Atrial and brain natriuretic peptides in cardiovascular diseases. *Hypertension*, **23**, I231-I234.
- NAYLER, W.G., OU, R.C., GU, X.H., CASLEY, D.J. (1992) Effect of amlodipine pretreatment on ischaemia-induced increase in cardiac endothelin-1 binding site density. *J Cardiovasc Pharm*, **20** (3), 416-420.
- NEUBAUER, S., ERTL, G., HASS, U., PULZER, F., KOCHSIEK, K. (1990). Effects of endothelin-1 in isolated perfused rat heart. *J. Cardiovas. Pharmacol.*, **16**, 1-8.
- NEUSER, D., KNORR, A., STASCH, J.-P., KAZDA, S. (1990) Mitogenic activity of endothelin-1 and -3 on vascular smooth muscle cells is inhibited by natriuretic peptides. *Artery*, **17**, 311-324.
- NGO, L., VESELY, D.L., BISSETT, J.K., MURPHY, M.L., DINH, H., SALLMAN, A.L., RICO, D.M., WINTERS, C.J., WYETH, R.P. (1991). Acute and sustained release of atrial natriuretic factor prohormone N-terminus with acute myocardial infarction. *Am. J. Med. Sci.*, **301**, 157-164.
- NGO, L., VESELY, D.L., BISSETT, J.K., MURPHY, M.L., DINH, H., SETH, R., SALLMAN, A.L., RICO, D.M., WINTERS, C.J., WYETH, R.P., NEWTON, M.T., HESTER, W.L. (1989). Acute and sustained release of atrial natriuretic factor with acute myocardial infarction. *Am Heart J.*, **118**, 893-900.
- NGUYEN, T.T., LAZURE, C., BABINSKI, K., CHRETIEN, M., ONG, H., DE LEAN, A. (1989). Aldosterone secretion inhibitory factor: a novel neuropeptide in bovine chromaffin cells. *Endocrinology.*, **124**, 1591-1593.
- NICHOLSON, S., RICHARD, M., ESPINER, E., NICHOLLS, G., YANDLE, T. (1993). Atrial and brain natriuretic peptide response to exercise in patients with ischemic heart disease. *Clin Exp Pharm Physio.*, **20**, 535.
- NIGRI, A., SARDELLA, G., TOSTI-CROCE, C., MARTUSCELLI, E., PIZZUTO, F., GRECO, C., BERNI, A. (1996). Atrial natriuretic peptide release during myocardial ischaemia induced by percutaneous transluminal coronary angioplasty. *Am. Heart J.*, **132**, 698-699.

- NISHIKIMI, T., KOHNO, M., ITAGANE, H., HIROTA, K., AKIOKA, K., TERAGAKI, M., YASUDA, M., OKU, H., TAKEUCHI, K., TAKEDA, T. (1988). Influence of exercise on plasma atrial natriuretic factor levels in patients with myocardial infarction. *Am Heart J.*, **115**, 753.
- NISHIMURA, J., AOKI, H., CHEN, X., SHIKASHO, T., KOBAYASHI, S. & KANAIDE, H. (1995) Evidence for the presence of endothelin ET_A receptors in endothelial cells *in situ* on the aortic side of porcine aortic valve. *Br J. Pharmacol.*, **115**, 1369-1376.
- NIITSUMA, K., SHIMIZU, M., KAWAGUCHI, T., NAKAGAMI, G., KIKAWADA, R. (1991). Time course of released atrial natriuretic peptide after acute myocardial infarction. *Jpn. Circ. J.*, **55**, 1181-1186.
- NORTHRIDGE, D.B., JAMIESON, M.P., JARDINE, A.G., MacARTHER, K.J.D., MacFARLANE, N., DARGIE, H.J. (1992). Pulmonary extraction and left atrial natriuretic factor during cardiopulmonary bypass surgery. *Am Heart J.*, **123**, 698-703.
- NUSSENZVEIG, D.R., FONTOURA, B.M., OWADA, A., MAACK, T. (1990a). Biological and clearance receptors of atrial natriuretic factor. *Diuretics*. **III**, 560-568.
- NUSSENZVEIG, D.R., LEWICKI, J.A., MAACK, T. (1990b) Cellular mechanisms of the clearance function of type C receptors of atrial natriuretic factor. *J. Biol. Chem.*, **265**, 20952-20958.
- O'CONNELL, J.E., JARDINE, A.G., DAVIDSON, G., CONNELL, J.M.C. (1992) Candoxatril, an orally active neutral endopeptidase inhibitor, raise plasma atrial natriuretic factor and is natriuretic in essential hypertension. *J. Hypertension*, **10**, 271-277.
- OELKERS, W., KLEINER, S., BAHR, V. (1988) Effects of incremental infusions of atrial natriuretic factor on aldosterone, renin, and blood pressure in humans. *Hypertension*, **12**, 462-467.

- OGAWA, Y., NAKAO, K., MUKOYAMA, M. *et al.* (1991) Natriuretic peptides as cardiac hormones in normotensive and spontaneously hypertensive rats: the ventricle is a major site of synthesis and secretion of brain natriuretic peptide. *Cir. Res.*, **69**, 491-500.
- OHLSTEIN, E.H., BERKOWITZ, B.A. (1985) Cyclic guanosine monophosphate mediates vascular relaxation induced by atrial natriuretic factor. *Hypertension*, **7**, 306-310.
- OHNISHI, A., YAMAGUCHI, K., KUSUHARA, M., ABE, K., KIMURA, S. (1989) Mobilization of interacellular calcium by ET in Swiss 3T3 cells. *Biochem Biophys. Res. Commun.*, **161**, 489-495.
- OHOI, I., SONE, K., TOBARI, H., KAWANO, E., NAKAMURA, K. (1993) A simple chemiluminescence method for measuring oxygen-derived free radicals generated in oxygenated rat myocardium. *Jpn. J. Pharmacol.*, **61**(2), 101-107.
- OHTA, K., HIRATA, Y., IMAI, T., KANNO, K., EMORI, T., SHIRCHIRI, M., MARUMO, F. (1990) Cytokine-induced release of endothelin-1 from porcine renal epithelial cell line. *Biochem. Biophysiol. Res. Commun.*, **169**, 578-584.
- OHYAMA, Y., MIYAMOTO, K., MORISHITA, Y., MATSUDA, Y., SAITO, Y., MINAMINO, N., KANGAWA, K., MATSUO, H. (1992) Stable expression of natriuretic peptide receptors: effects of HS-142-1, a non-peptide ANP antagonist. *Biochem. Biophysiol. Res. Commun.*, **189**, 336-342.
- OKUMURA, H., ASHIZAWA, N., KOBAYASHI, F., ARAI, K., ASAKURA, R., ASHIKAWA, N., MATSUURA, A. (1993) Comparison of haemoconcentration induced by big endothelin-1 and endothelin-1 in mice. *Br J Pharmacol*, **110**: 1395-1400.
- OLINS, G.M., SPEAR, K.L., SIEGEL, N.R., REINHARD, E.J., ZURCHER NEELY, H.A. (1987) Atrial peptide inactivation by rabbit-kidney brush-border membranes. *Eur. J. Biochem.*, **170**, 431-434.
- OLIVA, P.B., POTTS, D.E., PLUSS, R.G. (1973). Coronary artery spasm in Prinzmetal angina. Documentation by coronary arteriography. *N. Engl. J. Med.*, **288**, 745.

- OLIVER, J.R., TWIDALE, N., LAKIN, C., CAIN, M., TONKIN, A.M. (1988). Plasma atrial natriuretic polypeptide concentrations during and after reversion of paroxysmal supraventricular tachycardias. *Br. Heart J.*, **59**, 458-462.
- OMLAND, T., AARSLAND, T., AAKVAAG, A., DICKSTEIN, K. (1993a) The effect of early converting enzyme inhibition on neurohumoral activation in acute myocardial infarction. *Internat. J. Cardiol.*, **42**, 37-45.
- OMLAND, T., BONARJEE, V.V.S., AAKVAAG, A., AARSLAND, T., DICKSTEIN, K. (1993b) The relationship between early plasma atrial natriuretic factor levels and exercise performance after myocardial infarction. *Eur. Heart J.*, **14**, 1022-1026.
- OMLAND, T., BONARJEE, V.V.S., NILSEN, D.W., SUNDSFJORD, J.A., LIE, R.T., THIBAUT, G., DICKSTEIN, K. (1993c) Prognostic significance of N-terminal pro-atrial natriuretic factor (1-98) in acute myocardial infarction: comparison with atrial natriuretic factor (99-126) and clinical evaluation. *Br. Heart J.*, **70**, 409-414.
- OSAKADA, G., HESS, O.M., GALLATHER, K.P., KEMPER, W.S., ROSS, J. Jr. (1983) End systolic dimension-wall thickness relations during myocardial ischemia in conscious dogs. *Am. J. Cardiol.*, **51**, 1750.
- OSHIMA, T., CURRIE, M.G., GELLER, D.M., NEEDLEMAN, P. (1984) An atrial peptide is a potent renal vasodilator substance. *Circ. Res.*, **54**, 612-616.
- OTA, K., KIMURA, T., SHOJI, M., INOUE, M., SATO, K., OHTA, M., YAMAMOTO, T., TSUNODA, K., ABE, K., YOSHINAGA, K. (1992) Interaction of ANP with endothelin on cardiovascular, renal, and endocrine function. *Am J Physiol*, **262**, E135-E141.
- OWEN, P., THOMAS, M., YOUNG, V., *et al.* (1970) Comparison between metabolic changes in local venous and coronary sinus blood after acute experimental coronary artery occlusion. *Am. J. Cardiol.*, **25**, 562.
- OZAKI, S., OHWAKI, K., IHARA, M., FUKURODA, T., ISHIKAWA, K., YANO, M. (1995). ET_B-mediated regulation of extracellular levels of endothelin-1 in

cultured human endothelial cells. *Biochem. Bioph. Res. Com.*, **209**(2), 483-489.

PAKER, J.D., FARRELL, B., FENTON, T., COHANIM, M., PARKER, J.O. (1991) Counter-regulatory responses to continuous and intermittent therapy with nitroglycerin. *Circulation*, **84**, 2336-2345.

PARKER, J.O., CHIONG, M.A., WEST, R.O., CASE, R.B. (1969) Sequential alterations in myocardial lactate metabolism, S-T segments, and left ventricular function during angina induced by atrial pacing. *Circulation*, **40**, 113.

PARKER, T.G., CHOW, K.L., SCHWATZ, R., SCHNEIDER, M. (1990) Differential regulation of skeletal α -actin transcription in cardiac muscle by two fibroblast growth factors. *Proc. Natl. Acad. Sci* , **87**, 7066-7070.

PELLETIER, S., BATTISTINI, B., JENG, A.Y., SIROIS, P. (1998) Effects of dual endothelin-converting enzyme/neutral endopeptidase inhibitors, CGS 26303 and CGS 26393, on lipopolysaccharide or interleukin-1 beta-stimulated release of endothelin from guinea pig tracheal epithelial cells. *J. Cardiovas. Pharmacol.*, **31**, S10-S12.

PERALTA, C., HOTTER, G., CLOSA, D., PI, F., BADOSA, F., GELPI, E., ROSELLO-CATAFAU, J. (1997) Nitric oxide enhances endothelin production in pancreas transplantation. *Pancreas*, **14**(4), 369-372.

PERNOW, J., MODIN, A. (1993). Endothelial regulation of coronary vascular tone *in vitro*: contribution of endothelin receptor subtypes and nitric oxide. *Eur. J. Pharmacol.*, **243**, 281-286.

PHILLIPS, P.A., SASADEUS, J., HODSMAN, G.P., HOROWITZ, J.D., SALTUPS, A., JOHNSTON, C.I. (1989). Plasma atrial natriuretic peptide in patients with acute myocardial infarction: effects of streptokinase. *Br Heart J.*, **61**, 139-143.

POOLE, C.J., CARTER, D.A., VALLEJO, M., LIGHTMAN, S.L. (1987) Atrial natriuretic factor inhibits the stimulated in-vivo and in-vitro release of vasopressin and oxytocin in the rat. *J. Endocrinol.*, **112**, 97-102.

- PRACHAR, H., OGRIS, E., DITTEL, M., ENENKEL, W. (1991). Rapid changes of atrial natriuretic peptide concentration during percutaneous transluminal coronary angioplasty. *Am Heart J.*, **122**, 157.
- PRINZMETAL, M., KENNAMER, R., MERLISS, R., WADA, T., BOR, N. (1959). A variant form of angina pectoris. *Am. J. Med.*, **122**, 157.
- QIU, Y., NAKAHARA, Y., OGAWA, T. (1996). Synthetic studies on polysaccharide HS-142-1, a novel nonpeptide antagonist for the atrial natriuretic peptide receptor: syntheses of the gentiobisyl fragments. *Biosci. Biotech. Biochem.*, **60**(6), 986-993.
- RAPPELLI, A., DESSI-FULGHERI, P., MADEDDU, P., GLORIOSO, N. (1987) Studies on the natriuretic effect of nifedipine in hypertensive patients: increase in levels of plasma atrial natriuretic factor without participation of the renal kallikrein-kinin system. *J Hypertens*, **5**: S61.
- RAY, S.G., MCMURRAY, J.J., MORTON, J.J., DARGIE, H.J. (1992) Circulation endothelin in acute ischaemic syndromes. *Br Heart J*, **67**, 383-386.
- RAYNER, T.E. (1992). Regulation of atrial natriuretic factor secretion from the heart. PhD Thesis: Flinders University of South Australia.
- REIMER, K.A., JENNINGS, R.B., HILL, M.L. (1981) Total ischaemia in dog *in vitro*. II. High energy phosphate depletion and associated defects in energy metabolism, cell volume regulation, and sarcolemmal integrity. *Circ. Res.*, **49**, 901.
- RESINK, T., SCOTT-BURDEN, T., WEBER, E., BUHLER, F.R. (1990) Phorbol ester promotes a sustained down-regulation of endothelin receptors and cellular responses to endothelin in human vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **166**, 1213-1219.
- RICCI, D., ORLICK, A., ALDERMAN, E. (1979) Role of tachycardia as an inotropic stimulus in man. *J. Clin. Invest.*, **63**, 695.

- RIOS, J.C., HURWITZ, L.E. (1976) Electrocardiographic responses to atrial pacing and multistage treadmill exercise testing. Correlation with coronary anatomy. *Am. J. Cardiol.*, **34**, 986.
- ROBSON, R.H., PRIDIE, R., FLUCK, D.C. (1976) Evaluation of rapid atrial pacing in diagnosis of coronary artery disease, Evaluation of atrial pacing test. *Br. Heart J.*, **38**, 986.
- ROSS, J., Jr., GALLAGHER, K., MATZUSAKI, M., LEE, J.D., GUTH, B., GOLDFARB, R. (1986) Regional myocardial blood flow and function in experimental myocardial ischemia. *Can. J. Cardiol.*, **suppl A**, 9A.
- ROUBERT, P., GILLARD, V., PLAS, P., CHABRIER, P.E., BRAQUET, P. (1990) Down regulation of endothelin binding sites in rat vascular smooth muscle cells. *Am. J. Hypertens.*, **3**, 310-312.
- ROULEAU, J.L., MOYE, L.A., de CHAMPLAIN, J., KLEIN, M., BICHET, D., PACKER, M., *et al.* (1991) Activation of neurohumoral systems following acute myocardial infarction. *Am. J. Cardiol.*, **68**, 80D-86D.
- RUBANYI, G.M., POLOKOFF, M.A. (1994). Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol. Rev.*, **46**, 325-415.
- RUDD, M.A., PLAVIN, S., HIRSCH, A.T., INGELFINGER, J.R., DZAU, V.J. (1989). Atrial natriuretic factor-specific antibody as a tool for physiological studies. Evidence for role of atrial natriuretic factor in aldosterone and renal electrolyte regulation. *Circ. Res.*, **65**, 1324-1329.
- RUSKOAHO, H., TOTH, M., GANTEN, D., UNGER, T., LANG, R.E. (1985) Atrial natriuretic peptide secretion: synergistic effect of phorbol ester and A23187. *Biochem. Biophys. Res. Commun.*, **133**, 581-588.
- RUSKOAHO, H. (1992) Atrial natriuretic peptide: synthesis, release, and metabolism. *Pharmacol Rev*, **44** (4), 479.

- RUTLEN, D.L., CHRISTENSEN, G., HELGESEN, K.G., ILEBEKK, A. (1990)
Influence of atrial natriuretic factor on intravascular volume displacement in pigs.
Am J Physiol, **259**: H1595-H1600.
- RYAN, U.S., ZHONG, R., HAYES, B.A., VISNER, G., SAUTHER, M.L. (1993)
Regulation of endothelin-1 expression in normal and transfected endothelial cells.
J. Cardiovasc. Pharmacol., **22**(suppl 8), S38-41.
- SAGNELLA, G.A., MARKANDU, N.D., SHORE, A., MACGREGOR, G.A. (1985)
Effects of changes in dietary sodium intake and saline infusion on immunoreactive
atrial natriuretic peptide in human plasma. *The Lancet*, **30**, 1208-1210.
- SAIJINMAA, O., RISTIMAKI, A., FYHRQUIST, F. (1990) Atrial natriuretic peptide,
nitroglycerine, and nitroprusside reduce basal and stimulated endothelin
production from cultured endothelial cells. *Biochem. Biophys. Res. Commun.*,
173, 514-520.
- SAKURAI, T., YANAGISAWA, M., MASAKI, T. (1992) Molecular characterization of
endothelin receptors. *Trends Phaemacol. Sci.*, **13**, 103-108.
- SAMSON, W.K. (1985) Atrial natriuretic factor inhibits dehydration and hemorrhage-
induced vasopressin release. *Neuroendocriol*, **40**, 277-279.
- SANCHEZ-FERRER, C.F., BURNETT, J.C.Jr., LORENZ, R.R., VANHOUTTE,
P.M. (1990) Possible modulation of release of atrial natriuretic factor by
endothelium-derived relaxing factor. *Am. J. Physiol.*, **259**, H982-H986.
- SANO, T., IMURA, R., MORISHITA, Y., MATSUDA, Y., YAMADA, K. (1992) HS-
142-1, a novel polysaccharide of microbial origin, specifically recognizes
guanylyl cyclase-linked ANP receptor in rat glomeruli. *Life Sciences*, **51**, 1445-
1451.
- SATO, K., OKA, M., HASUNMA, K., OHNISHI, M., SATO, K., KIRA, S. (1995)
Effects of separate and combined ET_A and ET_B blockade on ET-1-induced
constriction in perfused rat lungs. *Am J Physiol*, **269**(5), L668-672.

- SCHENK, D.B., PHELPS, M.N., PORTER, J.G., SCARBOROUGH, R.M., MCENROE, G.A., LEWICKI, J.A. (1985) Identification of the receptor for atrial natriuretic factor on cultured vascular cells. *J. Biol. Chem.*, **260**, 14887-14890.
- SCHIEBINGER, R.J., LI, Y., CRAGOE JR, E.J. (1994) Calcium dependency of frequency-stimulated atrial natriuretic peptide secretion. *Hypertension*, **23**, 710-716.
- SCHINI, V., GRANT, N.J., MILLER, R.C., TAKEDA, K. (1988) Morphological characterization of cultured bovine aortic endothelial cells and the effects of atriopeptin II and sodium nitroprusside on cellular and extracellular accumulation of cyclic GMP. *Eur. J. Cell Biol.*, **47**, 53-61.
- SCHOFIELD, J.P., JONES, D.S.C., FORREST, J.N. JR (1991) Identification of C-type natriuretic peptide in the heart of spiny dogfish shark (*Squalus acanthias*). *Am. J. Physiol.*, **261**, F734-F739.
- SCHULZ, S., SINGH, S., BELLET, R.A., SINGH, G., TUBB, D.J., CHIN, H., GARBERS, D.L. (1989) The primary structure of a plasma membrane guanylate cyclase demonstrates diversity within this new receptor family. *Cell*, **58**, 1155-1162.
- SCHULZ-KNAPPE, P., FORSSMANN, K., HERBST, F., HOCK, D., PIPKORN, R., *et al* (1988) Isolation and structural analysis of 'urodilatin', a new peptide of the cardiodilatin-(ANP)-family, extracted from human urine. *Klinische Wochenschrift*, **66**, 752-759.
- SCHWARTZ, J.C., GROS, C., LECOMTE, J.M., BRALET, J. (1990) Enkephalinase (EC 3.4.24.11) inhibitors: protection of endogenous ANF against inactivation and potential therapeutic applications. *Life Sci.*, **47**, 1279-1297.
- SEO, B., BARRY, S.O., SIEBENMANN, R., VON SEGESSER, L. & LUSCHER, T.F. (1994). Both ET_A and ET_B receptors mediate contraction to endothelin-1 in human blood vessels. *Circulation*, **89**, 1203-1208.

- SERIZAWA, T., KOHMOTO, O., IIZUKA, M., HIRATA, Y., MATSUOKA, H., OHYA, T., KIMURA, Y., SATO, H., TAKAHASHI, T., MASUO, M., MOCHIZUKI, T., ISHII, M., SUGIMITO, T., KANGAWA, K., MATSUO, H. (1988) Effect of alpha human atrial natriuretic polypeptide on the systemic hemodynamics and coronary circulation in patients with ischemic heart disease. *Jap. Circ. J.*, **52**, 1107.
- SERRA, A., JIMENEZ, W., RIBAS, M., BOSCH, X., PARE, C., RIVERA, F., SANZ, G. (1991) Impaired response of atrial natriuretic factor to blood volume expansion in acute right ventricular infarction. *Am. J. Cardiol.*, **68**: 719-724.
- SEYMOUR, A.A., ASAAD, M.M., ADDOA-OFFEI, B.E., SMITH, P.L., ROGERS, W.L., DORSO, C.R. (1996) In vivo pharmacology of dual neutral endopeptidase/angiotensin-converting enzyme inhibitors. *J. Cardiovasc. Pharmacol.*, **28**, 672-678.
- SEYMOUR, A.A., NORMAN, J.A., ASAAD, M.M., FENNELL, S.A., SWERDEL, J.N., LITTLE, D.K., DORSO, C.R. (1990) Renal and depressor effects of SQ 29,072, a neutral endopeptidase inhibitor, in conscious hypertensive rats. *J. Cardiovasc. Pharmacol.*, **16**, 163-172.
- SEYMOUR, A.A., SWERDEL, J.N., FENNELL, S.A., DRUCKMAN, S.P., NEUBECK, R., DELANEY, N.G. (1989) Potentiation of the depressor responses to atrial natriuretic peptides in conscious SHR by an inhibitor of neutral endopeptidase. *J. Cardiovasc. Pharmacol.*, **14**, 194-204.
- SHEN, Y.T., GRAHAM, R.M., VATNER, S.F. (1991). Effects of atrial natriuretic factor on blood flow distribution and vascular resistance in conscious dogs. *Am. J. physiol.*, **260**, H1893-H1902.
- SHEN, Y.T., YOUNG, M.A., OHANIAN, J., GRAHAM, R.M., VATNER, S.F. (1990). Atrial natriuretic factor-induced systemic vasoconstriction in conscious dogs, rats, and monkeys. *Cir Res.*, **66**, 647-661.
- SHENKER, Y., SIDER, R.S., OSTAFIN, E.A., GGREKIN, R.J. (1985) Plasma levels of immunoreactive atrial natriuretic factor in healthy subjects and in patients with edema. *J Clin Invest.*, **76**: 1684.

- SHIMONAKA, H., SAHEKI, T., HAGIWARA, H., ISHIDO, M., NOGI, S., SCICLI, A., FUJITA, T., WAKITA, K., INADA, Y., KONODO, J., HIROSE, S. (1987) Purification of atrial natriuretic peptide receptor from bovine lung. *J. Biol. Chem.*, **262**, 5510-5514.
- SHIMOYAMA, H., SABBAH, H.N., BORZAK, S., TANIMURA, M., SHEVLYAGIN, S., SCICLI, G., GOLDSTEIN, S. (1996) Short-term hemodynamic effects of endothelin receptor blockade in dogs with chronic heart failure. *Circulation*, **94**, 779-784.
- SHIRAKAMI, G., NAKAO, K., SAITO, Y., MAGARIBUCHI, T., JOUGASAKI, M., MUKOYAMA, M., ARAI, H., HOSODA, K. SUGA, S., OGAWA, W. (1991). Acute pulmonary alveolar hypoxia increases lung and plasma endothelin-1 levels in conscious rats. *Life Sci.*, **48** (10), 969-976.
- SIGURDSSON, A., HELD, P., SWEDBERG, K. (1993). Short- and long-term neurohormonal activation following acute myocardial infarction. *Am. Heart J.*, **126**, 1068-1076.
- SIMONSON, M.S. (1993). Endothelins: multifunctional renal peptides. *Physiol. Rev.*, **73**, 375-411.
- SKVORAK, J.P., NAZIAN, S.J., DIETZ, J.R. (1993). Endothelin acts as a paracrine regulator for stretch-induced atrial natriuretic peptide release. *Am J. Physiol*, **269**, R1093-R1098.
- SMULDERS, R.A., STEHOUWER, C.D., OLTHOF, C.G, VAN KAMP, G.J., TEERLINK, T., DE VRIES, P.M., DONKER, A.J. (1994). Plasma endothelin levels and vascular effects of intravenous L-arginine infusion in subjects with uncomplicated insulin-dependent diabetes mellitus. *Clin Sci*, **87**(1), 37-43.
- SOKOLOVSKY, M., GALRON, R., KLOOG, Y., BDOLAH, A., INDIG, F.E., BLUMBERG, S., FLEMINGER, G. (1990). Endothelins are more sensitive than sarafotoxins to neutral endopeptidase: possible physiological significance. *Proc. Natl. Acad. Sci.*, **87**, 4702-4706.

- SOLOMON, R.L., ATHERTON, J.C., BOBINSKI, H., HILLIER, V., GREEN, R. (1988) Effect of low dose infusion of atrial natriuretic peptide on renal function in man. *Clin. Science*, **75**, 403-410.
- SONNENBERG, J.L., SAKANE, Y., KOEHN, J.A., ANSELL, J.A., WENNPGL, L.P., GHAI, R.D. (1988) Identification of protease 3.4.24.11 as the major atrial natriuretic factor degrading enzyme in the rat kidney. *Peptides*, **9**, 173-180.
- SOUQUE, A., GROS, C., SCHWARTZ, J.C. (1992) Binding of <I-125> atrial natriuretic factor to mouse lung membranes in vivo: characterization and effects of peptidase inhibitors. *J. Pharmacol. Exp. Ther.*, **260**, 1373-1378.
- SOWTON, G.E., BALCON, R., CROSS, D., FRICK, M.H. (1967) Measurement of the angina threshold using atrial pacing. *Cardiovas. Res.*, **1**, 301.
- SPINAL, F.G., WALKER, J.D., MUKHERJEE, R., IANNINI, J.P., KEEVER, A.T., GALLAGHER, K.P. (1997) Concomitant endothelin receptor subtype-A blockade during the progression of pacing-induced congestive heart failure in rabbits-Beneficial effects on left ventricular and myocyte function. *Circulation*, **95**, 1918-1929.
- STANTON, B.A. (1991) Molecular mechanisms of ANP inhibition of renal sodium transport. *Can. J. Physiol. Pharmacol.*, **69**, 1546-1552.
- STEENBERGER, C., HILL, M.L., JENNINGS, R.B. (1985) Volume regulation and plasma membrane injury in aerobic, anaerobic, and ischaemic myocardium in vitro. *Circ. Res.*, **57**, 864.
- STEINHELPER, M.E., COCHRANE, K.L., FIELD, L.J. (1990) Hypotension in transgenic mice expressing atrial natriuretic factor fusion gene. *Hypertension*, **16**, 301-307.
- STEPHENSON, S.L., KENNY, A.J. (1987) The hydrolysis of alpha-human atrial natriuretic peptide by pig kidney microvillar membranes is initiated by endopeptidase-24.11. *Biochem. J.*, **243**, 183-187.

- STEWART, D.J., LANGLEBEN, D., CERNACEK, P., CIANFLONE, K. (1990) Endothelin release is inhibited by coculture of endothelial cells of vascular media. *Am. J. Physiol.*, **259**, H1928-H1932.
- STRUTHERS, A.D. (1993). Plasma concentrations of brain natriuretic peptide: will this new test reduce the need for cardiac investigations? *Br. Heart J.*, **70**, 397-398.
- SUDOH, T., KANGAWA, K., MINAMINO, N., MATSUO, H.A. (1988) A new natriuretic peptide in porcine brain. *Nature*, **332**: 78-81.
- SUDOH, T., MAEKAWA, K., KOJIMA, M., MINAMINO, N., KANGAWA, K., MATSUO, H. (1989) Cloning and sequence analysis of cDNA encoding a precursor for human brain natriuretic peptide. *Biochem. Biophys. Res. Commun.*, **159**: 1427-1434.
- SUDOH, T., MINAMINO, N., KANGAWA, K., MATSUO, H.A. (1990) C-type natriuretic peptide (CNP): a new member of the natriuretic peptide family identified in porcine brain. *Biochem. Biophys. Res Commun*, **168**: 863-870.
- SUGA, S., ITOH, H., KOMATSU, Y., OGAWA, Y., HAMA, N., YOSHIMASA, T., NAKAO, K. (1993) Cytokine-induced C-type natriuretic peptide (CNP) secretion from vascular endothelial cells, evidence for CNP as a novel autocrine/paracrine regulator from endothelial cells. *Endocrinology*, **133**, 3038-3040.
- SUGA, S., NAKAO, K., HOSODA, K., MUKOYAMA, M., OGAWA, Y., SHIRAKAMI, G., ARAI, H., SAITO, Y., KAMBAYASHI, Y., INOUE, K., IMURA, H. (1992) Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. *Endocrinology*, **92**, 229-239.
- SUMIDA, H., YASUE, H., YOSHIMURA, M., OKUMURA, K., OGAWA, H., KUGIYAMA, K., KIKUTA, K., MORITA, E., NAKAO, K. (1995) Comparison of secretion pattern between A-type and B-type natriuretic peptides in patients with old myocardial infarction. *J. Am. Coll. Cardiol.*, **25**(5), 1105-1110.
- SUNG, C.P., ARLETH, A.J., STORER, B.L., OHLSTEIN, E.H. (1994) Angiotensin type 1 receptors mediate smooth muscle proliferation and endothelin biosynthesis in rat vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **271**, 429-437.

- SUTSCH, G., OSMUND, B., KIEWSKI, W. (1996) Acute and short-term effects of the nonpeptide endothelin-1 receptor antagonist bosentan in humans. *Cardiovasc Drugs Ther.*, **10**, 717-725.
- SUZUKI, R., TAKAHASHI, A., HAZON, N., TAKEI, Y. (1991) Isolation of high molecular weight C-type natriuretic peptide from the heart of a cartilaginous fish (European dogfish, *Scyliorhinus canicola*). *FEBS Lett.*, **282**, 321-325.
- SUZUKI, T., HOSHI, H., MITSUI, Y. (1990). Endothelin stimulates hypertrophy and contractility of neonatal rat cardiac myocytes in a serum-free medium. *FEBS lett.*, **268**, 149-151.
- SUZUKI, T., KUMAZAKI, T., MITSUI, Y. (1993). Endothelin 1 is produced and secreted by neonatal rat cardiac myocytes in vitro. *Biochem. Biophysiol. Res. Commun.*, **191**, 823-830.
- SVANEGAARD, J., ANGELO-NIELSEN, K., PINDBORG, T. (1989). Atrial natriuretic peptide and acute myocardial infarction. *Am Heart J.*, **117**, 194-195
- SVANEGAARD, J., ANGELO-NIELSEN, K., PINDBORG, T. (1992). Plasma concentration of atrial natriuretic peptide at admission and risk of cardiac death in patients with acute myocardial infarction. *Br Heart J.*, **68**, 38-42.
- SYBERTZ, E.J., CHIU, P.J., VEMULAPALLI, S., PITTS, B., FOSTER, C.J., WATKINS, R.W., BARNETT, A., HASLANGER, M.F. (1989) SCH 393770, a neutral metalloendopeptidase inhibitor, potentiates biological responses to atrial natriuretic factor and lowers blood pressure in desoxycorticosterone acetate-sodium hypertensive rats. *J. Pharmacol. Exp. Ther.*, **250**, 624-631.
- SYBERTZ, E.J. (1991) SCH 34826, a overview of its profile as a neutral metalloendopeptidase inhibitor and ANF potentiator. *Clin. Nephrol.*, **36**, 187-191.
- TAKESHITA, H., NISHIKIBE, M., YANO, M., IKEMOTO, F. (1991) Coronary vascular response to endothelin in isolated perfused hearts of spontaneously hypertensive rats. *Clin. Exp. Pharmacol. Physiol.*, **18**, 661-669.

- TAKUWA, N., TAKUWA, Y., YANAGISAWA, M., YAMASHITA, K., MASAKI, T. (1989) A novel vasoactive peptide stimulates mitogenesis through inositol lipid turnover in Swiss 3T3 fibroblasts. *J. Biol. Chem.*, **264**, 7856-61.
- TAMBURINI, P.P., KOEHN, J.A., GILLIGAN, J.P., CHARLES, D., PALMESINO, R.A., SHARIF, R., M^cMARTIN, C., ERION, M.D., MILLER, M.J. (1989) Rat vascular tissue contains a neutral endopeptidase capable of degrading atrial natriuretic peptide. *J. Pharmacol. Exp. Ther.*, **251**, 956-961.
- TAMIRISA, P., FRISHMAN, W.H., KUMAR, A. (1995) Endothelin and endothelin antagonism: roles in cardiovascular health and disease. *Am. Heart. J.*, **130**, 601-610.
- TAMURA, N., OGAWA, Y., ITOH, H., ARAI, H., SUGA, S., NAKAGAWA, O., KOMATSU, I., TAKAYA, K., YOSHIMASA T., *et al.* (1994) Molecular cloning of hamster brain and atrial natriuretic peptide cDNAs. Cardiomyopathic hamsters are useful models for brain and atrial natriuretic peptides. *J. Clin. Invest.*, **94**(3), 1059-1068.
- TAN, A.C.I.T.L., RUSSEL, F.G.M., THIEN, T., BENRAAD, T.J. (1993) Atrial natriuretic peptide. *Clin. Pharmacokinet.*, **24**(1), 28-45.
- TANABE, K., SUZUKI, N., OSADA, N., YAMAMOTO, A., NAKAYAMA, M., YOKOYAMA, Y., SAKAKIBARA, M., ITOH, H., MURAYAMA, M., YAMADA, S., YAMASAKI, H. (1996) Effects of cilazapril on exercise tolerance in the chronic phase of acute myocardial infarction. *Jpn. Circ. J.*, **60**, 831-840.
- TANG, J., WEBBER, R.J., CHANG, D., CHANG, J.K., KIANG, J., WEI, E.T. (1984) Depressor and natriuretic activities of several atrial peptides. *Regul. Pept.*, **9**, 53-59.
- TENNANT, R., WIGGERS, C.J. (1935) The effect of coronary occlusion on myocardial contractions. *Am. J. Physiol.*, **112**, 351.
- THAMSBORG, G., SYKULSKI, R., LARSEN, J., STORM, T., KELLER, N. (1987) Effect of β_1 -adrenoceptor blockade on plasma levels of atrial natriuretic peptide during exercise in normal man. *Clin. Physiol.*, **7**, 313-318.

- THAWARAGI, Y., FUCHIMURA, K., TANAKA, S., MINAMINO, N., KANGAWA, K., MATSUO, H. (1991) Gene and preconstructure of human C-type natriuretic peptide. *Boichem. Biophys. Res. Commun.*, **175**, 645-651.
- THIBAUT, G., CHARBONNEAU, C., BILODEAU, J., SCHIFFRIN, E.L., GARCIA, R. (1992) Rat brain natriuretic peptide is localized in atrial granules and released into the circulation. *Am. J. Physiol.*, **263**, R301-R309.
- THIBAUT, G., DOUBELL, A.F., GARCIA, R., LARIVIERE, R., SCHIFFRIN, E.L. (1994) Endothelin-stimulated secretion of natriuretic peptides by rat atrial myocytes is mediated by endothelin a receptors. *Circ. Res.*, **74**, 460-470.
- THOMASSEN, A.R., BAGGER, J.P., NIELSEN, T.T., PEDERSEN, E.B. (1987). Atrial natriuretic peptide during pacing in controls and patients with coronary arterial disease. *Int J Cardiol.*, **17**, 267-276.
- THOMPSON, M., WESTWICK, J., WOODWARD, B. (1995). Responses to endothelins-1, -2, and -3 and sarafotoxin 6c after ischaemia/reperfusion in isolated perfused rat heart: role of vasodilator loss. *J. Cardiovas. Pharmacol.*, **25**, 156-162.
- TIKKANEN, T., SVARTSTROM-FRASER, M., TIKKANEN, I., SARIOLA, H., FYHRQUIST, F. (1987). Haemodynamic effects of atrial natriuretic peptide in rats with heart failure. *Eur J Pharmacol.*, **140**, 187-193.
- TOMODA, H. (1988). Atrial natriuretic peptide in acute myocardial infarction. *Am J Cardiol.*, **62**, 1122.
- TOTH, M., VUORINEN, K.H., VUOLYEENAHU, O., HASSINEN, I.E., PAAVO, A., LEPPALUOTO, J., RUSKOAHO, H. (1994) Hypoxia stimulates release of ANP and BNP from perfused rat ventricular myocardium. *Am. J. Physiol.*, **266**, H1572-H1580.
- TRACHTE, G.J. (1993) Atrial natriuretic factor alters neurotransmission independently of guanylate cyclase-coupled receptors in the rabbit vas deferens. *J. Pharmacol. Exp.*, **264**, 1227-1233.

- TRAPANI, A.J., SMITS, G.J., M^CGRRAW, D.E., SPEAR, K.L., KOEPKE, S.J.P., OLINES, G.M., BLAINE, E.H. (1990) Thiorphan, an inhibitor of endopeptidase 24.11, potentiates the natriuretic activity of atrial natriuretic peptide. *J. Cardiovasc. Pharmacol.*, **14**, 419-424.
- TRIPPODO, N.C., COLE, F.C., FROHLICH, E.D., MacPHEE, A.A. (1986) Atrial natriuretic peptide decreases circulatory capacitance in areflexic rats. *Cir. Res.*, **59**, 291-296.
- TRIPPODO, N.C., GABEL, R.A., HARVEY, C.M., ASAAD, M.M., ROGERS, W.L. (1991) Heart failure augments the cardiovascular and renal effects of neutral endopeptidase inhibition in rats. *J. Cardiovasc. Pharmacol.*, **18**, 308-316.
- TSENG, Y.C., LAHIRI, S., SELLITTI, D.F., BURMAN, K.D., D'AVIS, J.C., WARTOFSKY, L. (1990) Characterization by affinity cross-linking of a receptor for atrial natriuretic peptide in cultured human thyroid cells associated with reduction in both adenosine 3', 5'-monophosphate production and thyroglobulin secretion. *J Clin. Endocrinol. Metab.*, **70**, 528-533.
- TSUTAMOTO, T., BITO, K., KINOSHITA, M. (1989) Plasma atrial natriuretic polypeptide as an index of left ventricular end-diastolic pressure in patients with chronic left-sided heart failure. *Am Heart J*, **117**, 599.
- TSUTAMOTO, T., KINOSHITA, M., NAKAE, I., MAEDA, Y., WADA, A., YABE, T., HORIE, H. (1994a) Absence of haemodynamic tolerance to nicorandil in patients with severe congestive heart failure. *Am Heart J*, **127**, 866-873.
- TSUTAMOTO, T., KINOSHITA, M., OHBAYASHI, Y., WADA, A., MAEDA, Y., YABE, T., ADACHI, T. (1994b) Plasma arterio-venous cGMP difference as a useful indicator of nitrate tolerance in patients with heart failure. *Circulation*, **90**, 823-829.
- TWIDALE, N., RAYNER, T.E., MENADUE, M.F., CAIN, M.D., TONKIN, A.M., OLIVER, J.R. (1993). Atrial natriuretic factor release during rapid ventricular pacing: Interplay between autonomic and haemodynamic stimulants. *Am Heart J.*, **125**, 1638-1644.

- TZIVONI, D., *et al.* (1981) Diagnosis of coronary artery disease by multigated radionuclide angiography during right atrial pacing. *Chest*, **80**, 562.
- UEHLINGER, D.E., ZAMAN, T., WEIDMANN, P., SHAW, S., GNADINGER, M.P. (1987) Pressure dependence of atrial natriuretic peptide during norepinephrine infusion in humans. *Hypertension*, **10**, 249-253.
- UUSIMAA, P.A., IKAHEIMO, M.J., RUSKOAHO, H., AIRAKSINEN, K.E.J., HUIKURI, H.V., LINNALUOTO, M.K., TAKKUNEN, J.T., PEUHKURINEN, K.J (1993) Release of atrial natriuretic peptide in relation to metabolic changes during myocardial ischaemia induced by coronary angioplasty. *J Mol Cell Cardiol*, **24**, 191-205.
- UUSIMAA, P.A., PEUHKURINEN, K.J., VUOLTEENAHO, O., RUSKOAHO, H., HASSINEN, I.E. (1992a) Role of myocardial redox and energy states in ischemia-stimulated release of atrial natriuretic peptide. *J Mol Cell Cardiol*, **24**, 191-205.
- UUSIMAA, P.A., PEUHKURINEN, K.J., HASSINEN, I.E., VUOLTEENAHO, O., RUSKOAHO, H. (1992b) Ischemia stimulates the release of atrial natriuretic peptide from rat cardiac ventricular myocardium in vitro. *life sciences*, **50**: 365-373.
- VALEN, G., LETTREM, I., SUNDSFJORD, J., VAAGE, J. (1993) Ischemia-reperfusion and toxic oxygen metabolites do not induce release of immunoreactive atrial natriuretic factor from isolated rat hearts. *Scand J Clin Lab Invest*, **53**, 373-381.
- VANNESTE, Y., PAUWELS, S., LAMBOTTE, L., DESCHODT, L.M. (1990a) In vivo metabolism of brain natriuretic peptide in the rat involves endopeptidase 24 and angiotensin converting enzyme. *Biochem. Biophys. Res. Commun.*, **173**, 265-271.
- VANNESTE, Y., PAUWELS, S., LAMBOTTE, L., MICHEL, A., DIMALINE, R., DESCHODT, L.M. (1990b) Respective roles of kallikrein and endopeptidase 24.11 in the metabolic pathway of atrial natriuretic peptide in the rat. *Biochem. J.*, **269**, 801-806.

- VATNER, S.F. (1980) Correlation between acute reductions in myocardial blood flow and function in conscious dog. *Circ Res*, **47**, 201.
- VATNER, S.F, HEYNDRICKX, G.R., FALLON, J.T. (1986) Effects of brief periods of myocardial ischaemia on regional myocardial function and creatine kinase release in conscious dogs and baboons. *Can. J. Cardiol.*, **suppl A**, 19A.
- VEDERNIKOV, Y.P., GOTO, K. & VANHOUTTE, P.M. (1993). The ET_A antagonist BQ-123 inhibits anoxic contractions of canine coronary arteries without endothelium. *J. Cardiovasc Pharmacol*, **26 (Suppl. 3)**, S252-S256.
- VIERHAPPER, H., WAGNER, O., NOWOTNY, P., WALDHAUSL, W. (1990). Effect of endothelin-1 in man. *Circulation*, **81**, 1415-1418.
- VIJAYARAGHAVAN, J., SCICLI, A.G., CARRETERO, O.A., SLAUGHTER, C., MOOMAW, C., HERSH, L.B. (1990). The hydrolysis of endothelins by neutral endopeptidase 24.11 (enkephalinase). *J. Bio. Chem.*, **265**, 14150-14155.
- VINCENT, G.M., ANDERSON, J.L., MARSHALL, H.W. (1983). Coronary spasm producing coronary thrombosis and myocardial infarction. *N. Engl. J. Med.*, **309**, 220.
- VISNER, M.S., ARENTZEN, C.E., PARRISH, G.D., LARSON, E.V., O'CONNER, M.J., CRUMBELEY, A.J., III, BACHE, R.J., ANDERSON, R.W. (1985) Effects of global ischaemia on the diastolic properties of the left ventricle in the conscious dog. *Circulation*, **71**, 161.
- VOGT-SCHADEN, M., GAGELMANN, M., HOCK, D., HERBST, F., FORSSMANN, W.G. (1989) Degradation of porcine brain natriuretic peptide (pBNP-26) by endopeptidase-24.11 from kidney cortical membranes. *Biochem. Biophys. Res. Commun.*, **161**, 1177-1183.
- VON GELDERN, T.W., BUDZIK, G.P., DILLON, T.P., HOLLEMAN, W.H., HOLST, M.A., KISO, Y., NOVOSAD, E.I., OPGENORTH, T.J., ROCKWAY, T.W., THOMAS, A.M., YEH, S. (1990) Atrial natriuretic peptide antagonists: biological evaluation and structural correlations. *Mol. Pharmacol.*, **38**, 771-778.

- WAGGONER, W.G., GENOVA, S.L., HASH, V.A. (1992) Kinetic analyses demonstrate that the equilibrium assumption does not apply to [^{125} I] endothelin-1 binding data. *Life Sci*, **51**, 1869-1876.
- WAALDMAN, S.A., RAPOPORT, R.M., MURAD, F. (1984) Atrial natriuretic factor selectively activates particulate guanylate cyclase and elevates cyclic GMP in rat tissues. *J. Biol Chem.*, **259**, 14332-14334.
- WALLACE, E.M., MOLITERNI, J.A., MOSKAL, M.A., NEUBERT, A.D., MARCOPULOS, N., STAMFORD, L.B., *et al.* (1998). Design and synthesis of potent, selective inhibitors of endothelin-converting enzyme. *J. Med. Chem.*, **41**, 1513-1523.
- WANG, Y.T., UEMATSU, T., SATO, R., HAYASHI, Y., NAKASHIMA, M. (1989). Changes in plasma level of alpha-atrial natriuretic polypeptide (alpha-ANP) and responsiveness of the aorta to exogenous alpha-ANP subsequent to myocardial infarction in rats. *Jpn. J. Pharmacol.*, **49**, 463-469.
- WATANABE, H., MIYAZAKI, H., KONDOH, M., MASUDA, Y., KIMURA, S., YANAGISAWA, M., MASAKI, T., MURAKAMI, K. (1989a) Two distinct types of endothelin receptors are present on chick cardiac membranes. *Biochem. Biophys. Res. Commun.*, **161**, 1252-1259.
- WATANABE, T., KUSUMOTO, K., KITAYOSHI, T., SHIMAMOTO, N. (1989b) Positive inotropic and vasoconstrictive effects of endothelin 1 in *in vivo* and *in vitro* experiments: characteristics and role of L type calcium channels. *J. Cardiovas. Pharmacol.*, **13**, S108-S111.
- WEBER, C., SCHMITT, R., BIRNBOECK, H., HOPFGARTNER, G., VAN-MARLE, S.P., PEETERS, P.A., JONKMAN, J.H. & JONES, C.R. (1996). Pharmacokinetics and pharmacodynamics of the endothelin-receptor antagonist bosentan in healthy human subjects. *Clin. Pharmacol. Ther.*, **60** (2), 124-137.
- WEBER, W., FISHCLI, W., HOCHULIE, KUPFER, E., WIEBER, E.K. (1991). Anantin-a peptide antagonist of the atrial natriuretic factor (ANF). *J. Antibiotics*, **44**, 164-171.

- WEI, C.M., AARHUS, L.L., MILLER, V.M., BURNETT, J.C. (1993) Action of C-type natriuretic peptide in isolated canine arteries and veins. *Am J Physiol*, 264: H71-H73.
- WIECZOREK, I., HAYNES, W.G., WEBB, D., LUDLAM, C.A. & FOX, K.A.A. (1994). Raised plasma endothelin in unstable angina and non-Q wave myocardial infarction: relation to cardiovascular outcome. *Br. Heart J.*, 72, 436-441.
- WIEDERMENN, C.J., WIEDERMENN, F.J., HEROLD, M., TILG, H., MOES, N., MUHIBERGER, V., BEIMPOLD, H., KNAPP, E. (1991). Plasma levels of atrial natriuretic peptide and tumor necrosis factor- α during transient myocardial ischemia in patients with stable angina. *Klinische Wochenschrift*, 69, 944.
- WIEDMANN, P., HASLER, L., GNADINGER, M.P., LANG, R.E., UEHLINGER, D.E. *et al.* (1986). Blood levels and renal effects of atrial natriuretic peptide in normal man. *J. Clin. Inves.*, 77, 734-742.
- WIENMANN, M. (1991). Coronary revascularization: impact on myocardial metabolism and ischaemia in man. MD Thesis. University of Melbourne.
- WEISS, A.T., *et al.* (1983) Atrial pacing thallium scintigraphy in the evaluation of coronary artery disease. *Isr. J. Med. Sci.*, 19, 495.
- WILKINS, M.R., REDONDO, J., BROWN, L.A. (1997) The natriuretic-peptide family. *Lancet*, 349, 1307-1310.
- WILLIAMS, T.D., WALSH, K.P., LIGHTMAN, S.L., SUTTON, R. (1988) Atrial natriuretic peptide inhibits postural release of renin and vasopressin in humans. *Am. J. Physiol.*, 255, R368-R372.
- WINQUIST, R.J., FAISAN, E.P., NUTT, R.P. (1984) Vasodilator profile of synthetic atrial natriuretic factor. *Eur. J. Pharmacol.*, 102, 169-173.
- WONG, N.L.M., WONG, E.F.C., AU, G.H., HU, D.C.K. (1988) Effect of α - and β -adrenergic stimulation on atrial natriuretic peptide release *in vitro*. *Am. J. Physiol.*, 255, E260-E264.

- WOOD, R.L., ANDERSON, W.P. (1990). Atrial natriuretic peptide causes vasoconstriction after autonomic blockade in conscious dogs, rats, and monkeys. *Cir Res.*, **66**, 647-661.
- WRIGHT, C.E., FOZARD, J.R. (1988). Regional vasodilation is a prominent feature of the haemodynamic response to endothelin in anaesthetized, spontaneously hypertensive rats. *Eur. J. Pharmacol.*, **155**, 201-203.
- YAMAMOTO, K., BURNETT, J.C.Jr., REDFIELD, M.M. (1997). Effect of endogenous natriuretic peptide system on ventricular and coronary function in failing heart. *Am. J. Physiol.*, **273**, H2406-2414.
- YAMAMOTO, T., KIMURA, T., OTA, K., SHOJI, M., INOUE, M., OHTA, M., FUNYU, T., ABE, K. (1994). Effects of a nitric oxide synthase inhibitor on vasopressin and atrial natriuretic hormone release, thermogenesis and cardiovascular functions in response to interleukin-1 beta in rats. *Tohoku J Exp Med*, **174**(1), 59-69.
- YAMANAKA, M., GREENBERG, B., JOHNSON, L., SEILHAMER, J., BREWER, M., FRIEDEMANN, T., MILLER, J., ATLAS, S., LARGH, J., LEWICKI, J. (1984). Cloning and sequence analysis of the cDNA for the rat atrial natriuretic factor precursor. *Nature*, **309**, 719-722.
- YAMAZAKI, T., KUMURO, I., KUDOH, S., ZOU, Y., SHIOJIMA, I., HIROI, Y., *et al.* (1996). Endothelin-1 is involved in mechanical stress-induced cardiomyocyte hypertrophy. *J. Biol. Chem.*, **271**(6), 3221-3228.
- YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K., MASAKI, T. (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332**, 411-415.
- YANG, Z., RICHARD, V., von SEGESSER, L., BAUER, E., STULZ, P., TURINA, M., LUSCHER, T.F. (1990) Threshold concentrations of endothelin-1 potentiate contractions to norepinephrine and serotonin in human arteries. *Circulation*, **82**, 188-195.

- YANDLE, T.G., BRENNAN, S.O., ESPINER, E.A., NICHOLLIS, M.G., RICHARDS, A.M. (1989) Endopeptidase-24.11 in human plasma degrades atrial natriuretic factor (ANF) to ANF(99-105/106-126). *Peptides*, **10**, 891-894.
- YASUDA, M., KOHNO, M., TAHARA, A., ITAGANE, H., TODA, I., AKIOKA, K., TEROGAKI, M., OKU, H., TAKEUCHI, K., TAKEDA, T. (1990) Circulating immunoreactive endothelin in ischaemic heart disease. *Am Heart J*, **119**: 801.
- YASUE, H., OBATA, K., OKUMURA, K., KUROSE, M., OGAWA, H., MATSUYAMA, K., JOUGASAKI, M., SAITO, Y., NAKAO, K., IMURA, H. (1989) Increased secretion of atrial natriuretic polypeptide from the left ventricle in patients with dilated cardiomyopathy. *J Clin Invest* **83**, 46.
- YOKOKAWA, K., KOHNO, M., TAHARA, H., MANDAL, A.K., YASUNARI, K., MURAKAWA, K., *et al.* (1993a) Effect of heparin on endothelin-1 production by cultured human endothelial cells. *J. Cardiovas. Pharmacol.*, **22**, S46-S48.
- YOKOKAWA, K., TAHARA, H., KOHNO, M., MANDAL, A.K., YANAGISAWA, M., TAKEDA, E. (1993b) Heparin regulates endothelin production through endothelium-derived nitric oxide in human endothelial cells. *J. Clin Invest.*, **92**, 2080-2085.
- YOSHIMOTO, S., ISHIZAKI, Y., SASAKI, T., MUROTA, S.I. (1991) Effect of carbon dioxide and oxygen on endothelin production by cultured porcine cerebral endothelial cells. *Stroke*, **22**, 378-383.
- YOSHIMURA, M., YASUE, H., MORITA, E., SAKAINO, N., JOUGASAKA, M., SAITO, Y., NAKAO, K., IMURA, H. (1991) Hemodynamic, renal, and hormonal responses to brain natriuretic peptide infusion in patients with congestive heart failure. *Circulation*, **84**: 1581.
- YOSHIMURA, M., YASUE, H., OKUMURA, K., OGAWA, H., JOUGASAKI, M., MUKOYAMA, M., NAKAO, K., IMURA, H. (1993) Different secretion patterns of atrial natriuretic peptide and brain natriuretic peptide in patients with congestive heart failure. *Circulation*, **87**: 464-469.

- YU, J.C.M. & DAVENPORT, A.P. (1995). Regulation of endothelin receptor expression in vascular smooth-muscle cells. *J. Cardiovasc. Pharmacol.*, **26** (Suppl. 3), S348-S350.
- ZHU, Y., YANG, H.T., ENDOH, M. (1997) Negative chronotropic and inotropic effects of endothelin isopeptides in mammalian cardiac muscle. *Am. J. Physiol.*, **273**, 119-127.
- ZIEGLER, T., BOUZOURENE, K., HARRISON, V.J., BRUNNER, H.R., HAYOZ, D. (1998). Influence of oscillatory and unidirectional flow environments on the expression of endothelin and nitric oxide synthase in cultured endothelial cells. *Arterioscler Thromb. Vasc. Biol.*, **18**(5), 686-692.
- ZIVIN, M.A. CONDRA, A., DIXON, R.A., SEIDAH, N.G., CHRETIEN, M., NEMER, M., CHAMBERLAND, M., DROUIN, J. (1984). Molecular cloning and characterization of DNA sequences encoding rat and human artial natriuretic factors. *Proc. Natl. Acad. Sci. USA* **81**, 6325-6329.
- ZWEIER, J.L., FLAHERTY, J.T., WEISFELDT, M.L. (1987) Direct measurement of free radical generation following reperfusion of ischaemic myocardium. *Proc. Natl. Acad. Sci USA.*, **84**(5), 1404-1407.

The composition of modified Eagles MEM (mg/L):

L-Alanine, 8.9; L-Arginine HCl, 126.4; L-Asparagine H₂O, 15; L-Aspartic acid, 13.3; L-Cystine disodium salt H₂O, 31.2; L-Glutamic acid, 14.7; L-Glutamine, 292; Glycine, 7.5; L-Histidine HCl H₂O, 41.9; L-Isoleucine, 52.5; Leucine, 52.5; L-Lysine HCl, 72.5; L-Methionine, 15; L-Phenylalanine, 32.5; L-Proline, 11.5; L-Serine, 10.5; L-Threonine, 47.6; L-Tryptophan, 10; L-Tyrosine disodium salt, 43.2; L-Valine, 46.8; D-Ca pantothenate, 1; Choline chloride, 1; Folic acid, 1; I-Inositol, 2; Nicotinamide, 1; Pyridoxal, HCl, 1; Riboflavin, 0.1; Thiamin HCl, 1; CaCl₂·2H₂O, 200; KCl, 400; MgSO₄·7H₂O, 97.7; NaCl, 6800; NaH₂PO₄·2H₂O, 140; D-glucose, 1000; and Phenol red sodium salt, 10. Eagles MEM medium was further supplemented by the addition of Bovine Serum Albumin (BSA) 0.1%, pyruvic acid 5 mM (both from Sigma Chemical Co., U.S.A), D-glucose 11 mM (BDH Chemicals, Australia) and insulin 10 mUnits/ml (Actrapid; monocomponent Human, CSL-NOVO Pty. Ltd., Australia). The final composition of salt in the perfusion medium was (mmol/L): NaCl, 116; KCl, 5.4; NaHCO₃, 24; MgSO₄, 0.8; NaH₂PO₄, 1.0; and CaCl₂, 2.5.

Endothelin B receptor-mediated vasoconstriction induced by endothelin A receptor antagonist.

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Abstract

Objective: The vasoconstrictor effect of endothelins (ET) is mediated by endothelin A (ET_A) and endothelin B (ET_B) receptors. Furthermore, ET_B receptor stimulation results in release of vasodilators. Hence, ET_A receptor antagonists should attenuate ET-mediated vasoconstriction. The aim of the present study was to evaluate and compare the effects of BQ-123, an ET_A receptor antagonist, and bosentan, an ET_A and ET_B receptor antagonist, on coronary vasomotor tone, left ventricular systolic function and ET-1 efflux in the presence or absence of myocardial ischaemia/reperfusion. **Methods:** Isolated rat hearts were perfused using a Langendorff preparation. Global ischaemia was induced on average by 68±2% (±standard error of the mean) reduction of a baseline perfusion flow-rate 10 min after introduction of ET antagonists. Thirty minutes of ischaemia was followed by 30 min reperfusion. ET-1 efflux in coronary perfusate was measured by radioimmunoassay. **Results:** In non-ischaemic hearts (n=7), BQ-123 (10⁻⁶ M) perfusion induced a progressive decrease in coronary flow-rate compare with control group. This flow reduction persisted after wash-out of BQ-123. In contrast, bosentan (10⁻⁵ M, n=7) induced no change in perfusion rate. In the absence of ET antagonists (n=16), there was a 22±6% post-ischaemic increase in perfusion flow-rate. BQ-123 (n=5) but not bosentan (n=12) abolished this post-ischaemic increase in flow-rate. Neither BQ-123 nor bosentan induced significant variation in force of contraction. In ischaemic hearts, ischaemia per se induced a transient decrease in force of contraction. Bosentan significantly (P<0.05) accentuated and BQ-123 tended to accentuate (P=0.06) this decrease in force of contraction during ischaemia. Bosentan but not BQ-123 significantly impaired the recovery of systolic function during reperfusion (P<0.05). Both BQ-123 and bosentan perfusion increased ET-1 efflux rate to 730±188% and 315±81% respectively. This effect was abolished during ischaemia for BQ-123, but not for bosentan. **Conclusions:** In isolated perfused rat hearts, both BQ-123 and bosentan increased ET-1 efflux, but only BQ-123 exerted vasoconstrictor effects. These results thus generated the hypothesis that: (1) ET-1 release within the coronary vascular bed may be physiologically subject to negative feedback regulation mediated via ET_A receptors; (2) ET_A receptor antagonists increase ET-1 efflux, which may lead to net vasoconstriction via unopposed ET_B stimulation. Furthermore, the negative inotropic effects observed during ischaemia suggest that ET is critical to the maintenance of systolic function during ischaemia. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Isolated rat heart; Ischaemia; Endothelin; Endothelin receptor antagonists; Cardiac force of contraction

1. Introduction

Endothelins (ET) are a family of vasoactive hormones secreted mainly from endothelium. Their vasoconstrictor and vasodilator effects are mediated by two types of receptors, ET_A and ET_B [1]. The ET_A receptor is classified by having greater affinity for ET-1 than ET-3 whereas the ET_B receptor is non-isopeptide-selective. ET_A receptor

stimulation has generally been found to mediate vasoconstriction. The ET_B receptor subtype located on smooth muscle also mediates vasoconstriction. It appears to be functionally distinct from the ET_B subtype located on the vascular endothelium which mediates vasorelaxation via the release of NO [2,3]. ET_B receptors play a role in clearance of ET from the circulation by endothelial cells [4].

ET's potent vasoactive effects may have pathogenic

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relevance during myocardial ischaemia. Increased plasma ET concentrations are found in patients with unstable angina, myocardial infarction and immediately following percutaneous transluminal coronary angioplasty [5,6]. Endogenous ET is also released into the coronary perfusate of isolated perfused rat hearts during ischaemia/reperfusion [7]. In cultured human endothelium, hypoxia induces ET gene expression and secretion [8]. On the other hand, ischaemia caused an increase in ET-1 binding site density [9,10]. Furthermore, hypoxia increased the maximal responses of ET-1 and ET-3 in the rat isolated perfusion mesenteric arterial bed [11].

Currently available methodologies for investigating the putative pathophysiological role of endogenous ET include the use of endothelin converting enzyme inhibitors, anti-endothelin antibodies and endothelin receptor antagonists. A specific ET_A receptor antagonist, BQ-123, has been shown to inhibit ET-induced vasoconstriction in porcine coronary artery [3], to attenuate the hypertensive response to ET-1 and big ET in rats [12], and to reduce infarct size in a canine model [13]. However, BQ-123 failed to inhibit ET-induced coronary vasoconstriction in a Langendorff perfused rat heart model [14]. Seo et al. using bosentan (Ro 47-0203), an ET_A/ET_B receptor antagonist, demonstrated that both ET_A and ET_B receptors mediate contraction to ET-1 in isolated human blood vessels [15]. In isolated rat hearts, bosentan (10^{-5} M) increased coronary flow but did not influence contractility [16]. However, to date there are only limited data on the effect of ET_A and $ET_{A/B}$ antagonists on the relationship between endothelin production and coronary vasomotor tone during ischaemia/reperfusion.

Exogenous ET-1 has also been shown to exert a positive inotropic effect on isolated cardiac myocytes and papillary muscles [17–21]. However, *in vivo* studies could not demonstrate the positive inotropic effect of exogenous ET-1, almost certainly because of concomitant induction of myocardial ischaemia via coronary vasoconstriction [22–27]. The development of ET receptor antagonists also offers a means of obtaining inferential information concerning the inotropic effects of ET *in vivo*. To date, studies with intravenous administration of bosentan in patients with severe chronic heart failure have demonstrated both an increase in cardiac output and a decrease in peripheral vascular resistance [28–31]; these findings suggest little fluctuation in underlying inotropic state. However, it is known that ET-1 is released during acute myocardial ischaemia [32]. Hence the inotropic effects of ET may fluctuate during ischaemia, with consequent variability in effects of ET antagonists in modifying contractile state during severe myocardial ischaemia.

The aim of the present study was to examine, in the perfused rat heart, the effects of BQ-123 and bosentan on coronary flow, release of ET-1 into coronary effluent and contractile state during, and following global myocardial ischaemia.

2. Methods

2.1. Isolated heart preparation

The protocol was approved by the institutional Animal Ethics Committee. Male Porton rats weighing 406 ± 9 g ($n=70$) were anaesthetised with an intraperitoneal injection of sodium pentobarbitone (50 mg/kg). The chest of the animal was opened, the heart rapidly excised and arrested in ice-cold saline. The aorta was cannulated, and the heart transferred to a modified non-recirculating Langendorff apparatus perfused with modified Eagle's minimum essential medium (contained NaCl 116 mM, KCl 5.4 mM, $NaHCO_3 \cdot H_2O$ 24 mM, $MgSO_4$ 0.8 mM, $NaH_2PO_4 \cdot 2H_2O$ 1.0 mM, $CaCl_2 \cdot 2H_2O$ 2.5 mM, pyruvic acid 5.0 mM, glucose 11 mM, bovine serum albumin 0.1%, insulin 10 U/l, pH 7.4) at a constant pressure of 70 cm H_2O . Medium was equilibrated with O_2 – CO_2 (95:5 v/v) at 37°C [33].

2.2. Protocol

Hearts were paced by placing electrodes against the right atrium in order to minimise rate-related effects on myocardial oxygen demand and coronary vasomotor tone. Pacing was performed at a rate $9 \pm 8\%$ above the spontaneous heart rate (264 ± 42 beats per min) with square wave pulses of 1 ms at a voltage 100% above threshold; the major purpose of achievement of constant heart rate was to avoid the emergence of contractile changes during ischaemia partially mediated via the Treppe effect.

After 40 min equilibration, baseline samples of coronary effluent were collected for 10 min. BQ-123 (Auspep, Australia) was dissolved directly in Eagle's minimum essential medium. Bosentan (Roche, Switzerland) was dissolved in 1 ml of distilled water at 37°C before addition to Eagle's minimum essential medium. Hearts were perfused with BQ-123 (10^{-6} M) or bosentan (10^{-5} M) for 40 min followed by 30 min with normal medium, minus ET receptor antagonist. In some hearts ($n=5$ for BQ-123; $n=12$ for bosentan), global ischaemia (perfusion flow reduced by $72.8 \pm 2.4\%$ for BQ-123; $59.4 \pm 1.9\%$ for bosentan) was induced 10 min after ET receptor antagonist perfusion had commenced. The basis for the disparate reductions in coronary perfusion rate with the two ET receptor antagonists was the development of bradyarrhythmias when bosentan-pretreated hearts were subjected to $>67\%$ flow reduction. While the basis for this phenomenon was not elucidated, the reductions in perfusion rate were maximal for maintenance of constant heart rate for both agents tested.

Volume of perfusate was measured by calibrated pipette as an index of coronary flow. Force of contraction and heart rate were monitored on a Grass SD 9 polygraph and dF/dt derived using MacLab v 3.5.2. via a force-displacement transducer. Coronary effluent was collected, snap

frozen on dry ice and stored at -80°C . ET-1 concentration in the perfusate was measured by radioimmunoassay.

2.3. ET-1 radioimmunoassay

Perfusate was collected in tubes containing 0.1% tri-fluoroacetic acid, snap frozen and stored at -80°C until assayed. ET-1 was extracted from perfusate samples using Sep-Pak C_{18} columns (Waters, USA). Recovery of ET-1 was $98 \pm 6\%$ ($n=40$). Samples or ET-1 standard (Auspep) were incubated with rabbit anti-ET-1 serum (Peninsula, USA) for 48 h then with ^{125}I ET-1 (Amersham, UK) for 16–24 h. Bound and free endothelin were separated using 17.6% polyethylene glycol 6000 and human γ globulin to precipitate the bound fraction. Supernatant was discarded and the pellet counted in a gamma counter. Intra- and inter-assay coefficients of variation were 12.0% ($n=5$) at 0.802 fmol and 17.0% ($n=11$) at 1.433 fmol, respectively. Neither BQ-123 nor bosentan interfered with the measurement of ET-1 in the radioimmunoassay.

2.4. Analysis of data

Results are expressed as mean $\pm$ standard error of the mean. ET-1 release rate was expressed as fg/min/unit wet weight of heart. Changes in perfusion flow, increase of ET-1 release and decrease of force of contraction during ischaemia were expressed as area under curves. Comparison of area under curves was performed using unpaired Student's *t*-test. All other comparisons were made utilising two factor analysis of variance (ANOVA), with Dunnett's test where appropriate for multiple comparisons. A *P*-value of <0.05 was considered statistically significant.

All studies conform with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication. No.85-23, revised 1985).

3. Results

3.1. Effect of BQ-123 and bosentan on coronary perfusion flow-rate:

3.1.1. Non-ischaemic hearts (Fig. 1)

Basal coronary perfusion rate was 7.9 ± 0.3 ml/min/g wet heart weight. In control hearts ($n=6$), there was no significant fluctuation in perfusion rate over the duration of the experiment. During perfusion with BQ-123 (10^{-6} M) for 40 min, there was a progressive ($P<0.0001$) decrease in perfusion rate. The resultant difference between perfusion rates for BQ-123 and control hearts persisted ($P=0.01$) after wash-out of BQ-123. In contrast, bosentan (10^{-5} M) induced no significant changes in perfusion rate, either during, or subsequent to it.

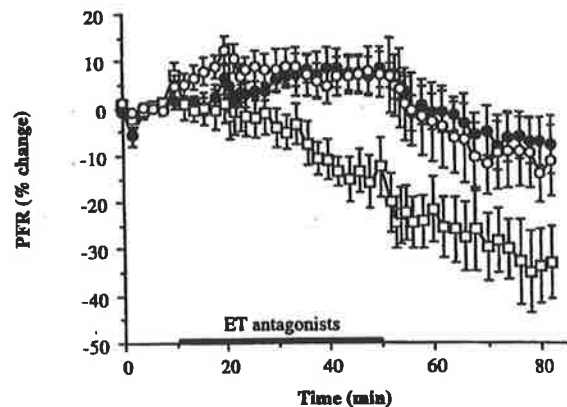


Fig. 1. Effects in non-ischaemic hearts of BQ-123 (10^{-6} M) ($\square$, $n=7$) and bosentan (10^{-5} M) ($\circ$, $n=7$) on perfusion flow-rate (PFR) compared with control ($\bullet$, $n=6$).

3.1.2. Ischaemic hearts

In the absence of BQ-123 or bosentan, the induced decrease in flow-rate was followed by a phase of post-reperfusion increase in perfusion flow-rate ($P<0.0001$) (Fig. 2a and b). After perfusion with BQ-123 (Fig. 2a),

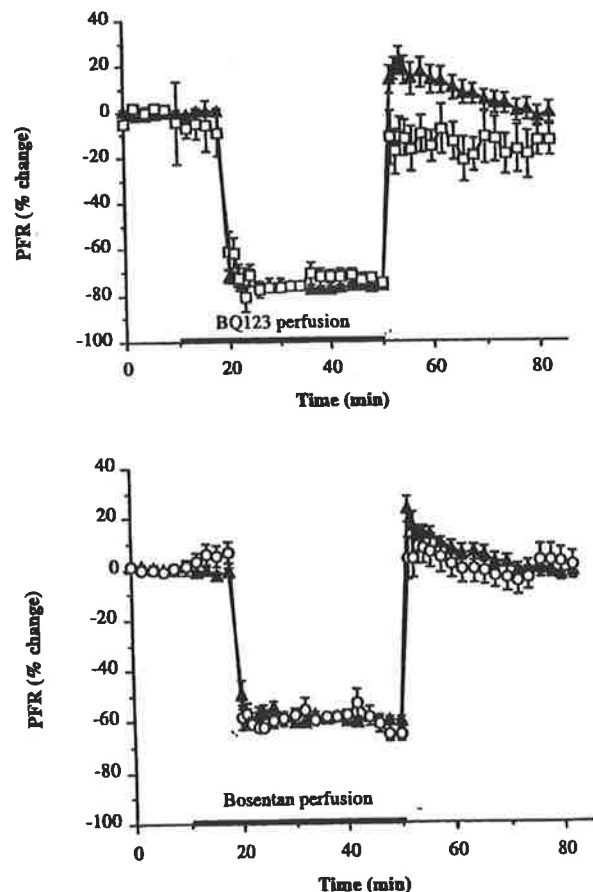


Fig. 2. Effect of BQ-123 (10^{-6} M) ($\square$, $n=5$) and bosentan (10^{-5} M) ($\circ$, $n=12$) on perfusion flow-rate (PFR) in ischaemic hearts compared with the same degree of ischaemia in absence of ET receptor antagonists ($\blacktriangle$, $n=16,17$).

post-ischaemic flow-rate was significantly ($P<0.05$) lower than for control hearts. Although bosentan tended to cause a mild increase in perfusion flow-rate during the pre-ischaemic phase, the effect was not significant. Bosentan did not significantly affect the post-ischaemic flow-rates (Fig. 2b).

3.2. Effect of BQ-123 and bosentan on ET-1 release:

3.2.1. Non-ischaemic hearts (Fig. 3)

Basal ET-1 release rate was 4.70 ± 0.024 fmol/s/g wet heart weight. In the absence of ischaemia and/or ET receptor antagonists, this basal rate did not fluctuate significantly. Almost immediately after BQ-123 perfusion had commenced, ET-1 efflux rate increased, the increase within 5 min being $730\pm188\%$ ($P<0.05$). This increase in ET-1 efflux was sustained throughout the BQ-123 perfusion period. Ten minutes post BQ-123 wash-out, the ET-1 efflux rate had returned to baseline. Bosentan caused a gradual but sustained increase ($P<0.0001$) in ET-1 efflux rate with a peak increase of $315\pm81\%$ after 30 min. The increase was significantly less than the increase in ET-1 efflux induced by BQ-123 ($P<0.05$). Following wash-out of either BQ-123 or bosentan, the ET-1 efflux rate returned towards baseline levels.

3.2.2. Ischaemic hearts

In the absence of BQ-123, ischaemia ($75.3\pm0.9\%$ perfusion flow reduction) did not alter ET-1 efflux, but there was a transient increase in ET-1 efflux during reperfusion. The increases in efflux induced by BQ-123 was less marked during ischaemia than in its absence ($P<0.05$) (Fig. 4a). In the absence of bosentan, moderate ischaemia ($58.2\pm0.9\%$ flow reduction) did not affect ET-1 efflux. However, with the same level of ischaemia, ET-1 efflux in the bosentan-treated group was significantly increased ($P<0.001$) (Fig. 4b), there was a secondary

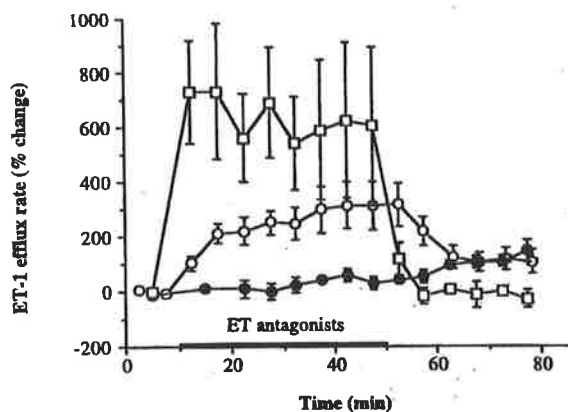


Fig. 3. Effect of BQ-123 (10^{-6} M) ($\square$, $n=7$) and bosentan (10^{-5} M) ($\circ$, $n=7$) compared with control ($\bullet$, $n=6$) on endothelin-1 (ET-1) efflux rate in non-ischaemic hearts.

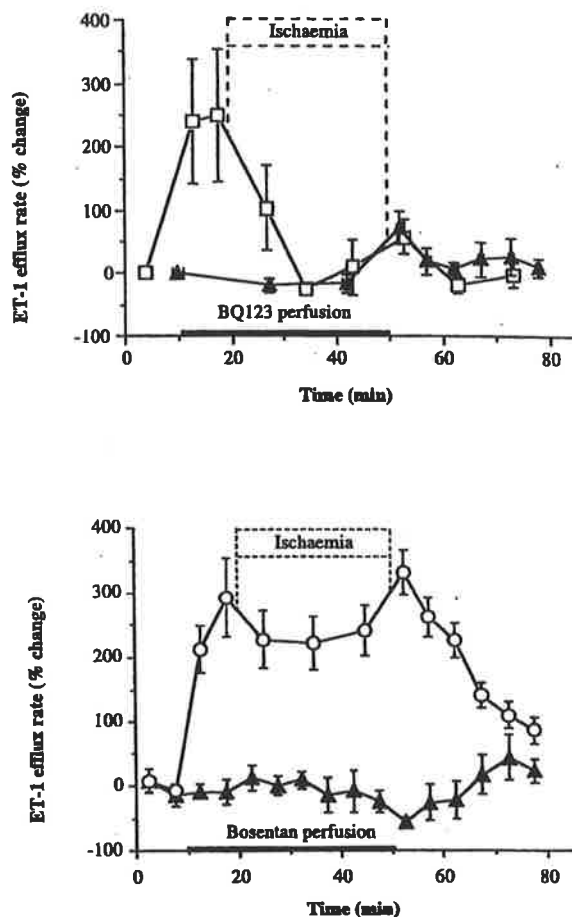


Fig. 4. Effects in ischaemic-reperfused hearts of BQ-123 (10^{-6} M) ($\square$, $n=5$) and bosentan (10^{-5} M) ($\circ$, $n=12$) on endothelin-1 (ET-1) efflux rate compared with control ischaemia/reperfusion ($\blacktriangle$, $n=16,17$).

increase ($P<0.0001$) in ET-1 efflux following reversal of ischaemia.

3.3. Effect of BQ-123 and bosentan on force of contraction

3.3.1. Non-ischaemic hearts

In the absence of ET-1 receptor antagonists, there was no significant change in force of contraction during the experimental period. BQ-123 or bosentan perfusion, started 10 min after the first baseline measurement, had no detectable effect on cardiac force of contraction. However, bosentan induced a significant change in the force-time interaction relative to that seen in control hearts (ANOVA; $F=2.977$, $P=0.008$). This implies a trend towards progressive reduction in force of contraction during bosentan infusion (Fig. 5).

3.3.2. Ischaemic hearts

Severe ischaemia ($n=16$) per se induced a transient decrease in force of contraction ($P<0.005$). BQ-123 perfusion for 10 min prior to ischaemia had no effect on

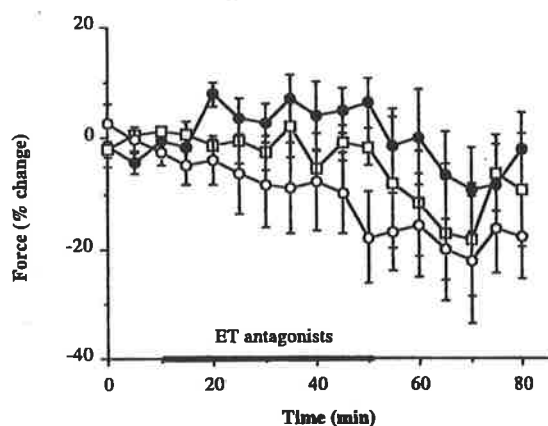


Fig. 5. Effects in non-ischaemic hearts of BQ-123 (10^{-6} M) ($\square$, $n=7$) and bosentan (10^{-5} M) ($\circ$, $n=7$) on force of contraction (Force) compared with control ($\bullet$, $n=6$).

cardiac force of contraction. However, during global ischaemia, BQ-123 induced a trend ($P=0.06$, $n=5$) towards accentuation of ischaemia-induced decrease in cardiac force of contraction (Fig. 6a). Fig. 6b illustrates the effect of bosentan on cardiac force of contraction in

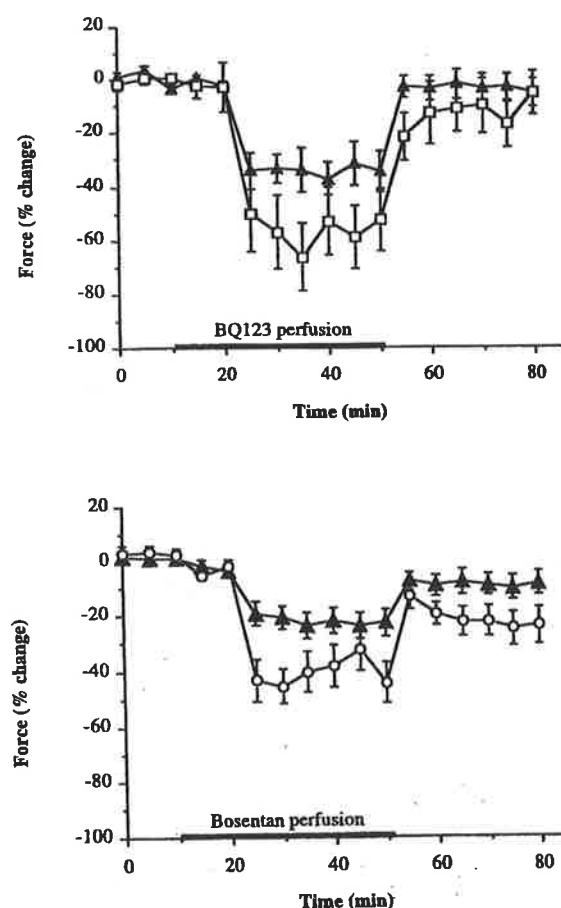


Fig. 6. Effects in ischaemic–reperfused hearts of BQ-123 (10^{-6} M) ($\square$, $n=5$) and bosentan (10^{-5} M) ($\circ$, $n=12$) on cardiac force of contraction compared with control ischaemia/reperfusion ($\blacktriangle$, $n=16,17$).

ischaemic hearts. In the absence of bosentan, coronary flow reduction induced a transient decrease in force of contraction ($P<0.05$ vs. control, $n=17$). Ten minute pre-ischaemic bosentan perfusion did not alter the cardiac force of contraction. However, bosentan induced a further decrease in force of contraction ($P<0.05$, $n=12$) during the ischaemic phase. During reperfusion, the recovery of cardiac systolic function was impaired in bosentan-treated groups ($P<0.05$).

4. Discussion

The results of the current study indicate that the effects of the selective ET_A receptor antagonist BQ-123 on coronary vasomotion and ET-1 efflux differ markedly from those of the ET_A/ET_B antagonist bosentan in the Langendorff-perfused rat heart. These differences are evident both in the presence and absence of transient myocardial ischaemia. Major differences were: (i) coronary vasoconstriction occurred during and after perfusion with BQ-123 but not bosentan; (ii) post-ischaemic perfusion flow-rate was significantly lower with BQ-123 than during control perfusion, while there were no significant differences between bosentan and control during this phase; and (iii) bosentan but not BQ-123 increased ET-1 efflux during ischaemia. Furthermore, while neither agent markedly affected contractile performance behaviour in the absence of ischaemia, bosentan significantly reduced force of cardiac contraction during myocardial ischaemia; BQ-123 tended to exert a similar effect ($P=0.06$). These findings have both physiological and pharmacological implications.

This is the first study demonstrating coronary vasoconstrictor effects of an ET_A antagonist. Previous studies with BQ-123 have primarily addressed its inhibition of *exogenous* ET-induced vasoconstriction which is usually evident in the 0.1 – 10 μ M range [3]. The concentration used in the present study (1 μ M) is therefore assumed to be an effective concentration for the ET_A receptors. Despite findings that BQ-123 reduces infarct size and inhibits anoxia-induced coronary vasoconstriction in canine models [13,34], specific investigations of the interaction between *endogenous* ET and ET_A receptor antagonists on vasomotor tone are relatively few. One investigation in isolated isovolumetric rat hearts by Han et al. [35] failed to demonstrate any effects of BQ-123 (estimated as 1 μ M) on coronary vasomotor tone. However, in their study, effects of BQ-123 infusion on coronary flow were examined only after 5 min of infusion: in the current experiment no vasoconstriction was detectable at that stage. Furthermore, they did not examine either recovery of coronary flow after reperfusion, or effects of BQ-123 on ET-1 efflux from the heart. Information related to coronary vasomotor effects of bosentan is also somewhat limited. However, Dagassan et al. demonstrated a coronary vasodilator effect (coronary flow increased by 14%) of bosentan in pre-ischaemic rat

heart, which did not persist during the immediate post-ischaemic phase [16]; these results are similar to those of the current study.

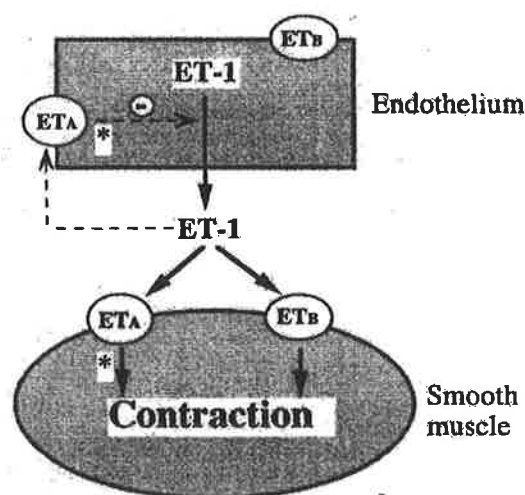
In this study, both BQ-123 and bosentan induced an increase in ET-1 efflux. The effect of BQ-123 accords with a previous finding in cultured human umbilical vein smooth-muscle cells where FR139 317, an ET_A receptor antagonist, caused an immediate, transient but marked increase in ET levels, with a decrease in levels of big ET-1 in the supernatant [36]. With the recent identification of ET_A receptors on endothelial cells [37,38], it is possible that ET antagonists might inhibit a negative feedback mechanism involving ET-1 regulating its own release. There are no previous studies which have specifically investigated this possibility. Nevertheless, the implication that endogenously released ET might in some way exert homeostatic control on tissue ET concentrations, presumably via interaction with cardiac ET_A receptors, is of considerable physiological interest.

In the specific case of bosentan, an additional mechanism might theoretically contribute to increased ET-1 efflux. There is considerable information showing that ET_B receptors mediate ET clearance from the circulation, especially into pulmonary endothelium [4,28,38–42]. Studies of clearance of ET-1 from plasma into pulmonary tissue during acute ET-1 administration in intact dogs [39] and rats [4] and also in isolated perfused rat lungs [40] demonstrate specific inhibition of the clearance process by the selective ET_B receptor antagonist BQ-788. These findings are also consistent with elevation of plasma ET-1 concentrations induced by bosentan [28,41]. As regards possible specific effects of ET_B receptor stimulation on ET-1 kinetics within the heart, Brunner and Doherty have demonstrated that BQ-788, but not the ET_A antagonist PD 155 080, increased ET-1 release using a perfused rat heart model [42]: these results are at variance with the findings in our current experiments, which suggest primary or sole modulation of ET-1 efflux via a ET_A receptor-dependent mechanism. While the basis for this discrepancy is not clear, there are a number of significant differences in experimental conditions. The utilization in Brunner and Doherty's experiments of (i) colloid-free Krebs solution (vs. albumin-containing medium), (ii) exclusion of potential effects of ventricular endocardial endothelium on ET-1 kinetics [42,43] (vs. ventricular perfusion utilizing current experimental design) and (iii) constant flow (vs. constant pressure) perfusion may contribute to this discrepancy. However, in their experiments, significant increases in ET-1 efflux with BQ-788 appeared progressively only after 75 min of perfusion, while the current experiments involved shorter duration of ET antagonist exposure. Irrespective of the reason, it must be emphasized that we have found no evidence of an ET_B-selective mechanism for increased ET-1 efflux in the current experiments: the observation that stimulated ET-1 efflux is more pronounced with BQ-123 than with bosentan implies that, at

least over the first 40 min of ET receptor antagonist perfusion, this phenomenon is ET_A receptor-mediated.

The finding of coronary vasoconstriction during and after ET_A receptor antagonist perfusion appears paradoxical since it implies activation of vasoconstrictor mechanisms both in the absence and presence of transient coronary flow reduction. It is conceivable that the increase in ET-1 efflux induced by BQ-123 mediated the observed vasoconstriction and that the total absence of vasoconstriction despite ET-1 release with bosentan is an indication that the increased ET-1 exerted its effects via the ET_B receptors alone. Although the selectivity can be attributed to blockade of the ET_A receptors mediating vasoconstriction, an additional factor may be the selectivity of the ET_B receptor for ET-1 in low concentrations [14,44]; which would render ET_A antagonists to be relatively ineffective.

After BQ-123 wash-out, in the non-ischaemic heart, despite return of the ET-1 efflux to baseline, the vasoconstrictor effect of BQ-123 persisted. This effect is likely to reflect persistent stimulation of ET_B receptors by ET-1, given the extremely slow dissociation rate of ET-1 from ET_B receptor [45]. This hypothesis is also consistent with the lack of "rebound" vasoconstriction with the ET_A/ET_B antagonist bosentan, since this agent, although accelerating ET release, would have prevented binding of ET with ET_B receptors. We are not aware of previous reports of "rebound" vasoconstriction after wash-out of an ET receptor antagonist. A model which takes into account the current observations is shown in Fig. 7. It is emphasised that this model is speculative and more detailed experiments exploring the source of the ET-1 will be required to assess its validity. Similarly, during post-ischaemic reperfusion,



* Inhibited in presence of BQ-123

Fig. 7. Schema of proposed mechanisms underlying incremental ET-1 efflux and resultant ET_B receptor-mediated coronary smooth muscle contraction in the presence of selective ET_A antagonist (BQ-123).

there was a significantly lower coronary flow for BQ-123 than control, while there was no such effect for bosentan (Fig. 2). The experimental design did not permit us to determine whether this difference reflected progressive differential effects of ET receptor antagonists on coronary vasomotor tone (i.e. continuation of the slight pre-ischaemic vasoconstriction with BQ-123 and vasodilation with bosentan), or whether it might represent a specific interaction with post-ischaemic vasodilator physiology.

The results of the current study as regards contractile performance of left ventricle also have implications regarding both the receptor mechanisms underlying positive inotropic effects of ET and also the potential homeostatic effects of these mechanisms during severe myocardial ischaemia. Furthermore, the effects observed with BQ-123 and bosentan are relevant to the use of ET receptor antagonists in patients prone to episodic myocardial ischaemia. In the current experiments, neither BQ-123 nor bosentan affected force of contraction during normal perfusion. However, during a moderate reduction in coronary flow, bosentan (10^{-5} M) tended to accentuate the ischaemia-induced reduction in force of contraction. These changes were not mediated via ET-induced ischaemia, as coronary flow was externally adjusted in order to eliminate variability between groups. Furthermore, heart rate was kept constant. The results therefore strongly imply a differential effect of endogenous ET on cardiac systolic function, consistent with incremental ET release [21] and/or effect during the ischaemic period.

The inconclusive results as regards BQ-123 may imply type 2 error; supplies of this agent were limited. No experiments with selective ET_B antagonists were performed in this series. Nevertheless, the magnitude of the observed changes with bosentan (10^{-5} M) was no greater than that seen with BQ-123. Therefore, the results remain consistent with previous observations in isolated ferret papillary muscle [8] that the positive inotropic effects of ET are entirely mediated by ET_A receptor stimulation. Surprisingly, in rabbit papillary (or ventricular) muscle, myocardial ET_A receptors appear to mediate inotropic responses to ET-3 [9,10], but not ET-1 [9]. A previous study in rat isolated right ventricular strips revealed that the ET_A receptor antagonist BQ-123 exerted a moderate negative inotropic effect in the absence of ischaemia [23]. This indicates that ET_A receptors mediate at least a component of the inotropic effect of endogenous ET in the rat. However, in the current study, no significant negative inotropic effects of BQ-123 or bosentan were detected during non-ischaemic perfusion.

The implied existence of an accentuated positive inotropic effect of endogenous ET during acute global ischaemia and/or early reperfusion period is a novel finding of this study. Such an effect would tend to be homeostatic, and its disruption is an area of potential concern regarding the future clinical use of ET receptor antagonists. However, currently available studies with whole animal models

of myocardial ischaemia/reperfusion do not reveal any deleterious effect of ET receptor antagonists [24,25]; indeed BQ-123 reduced infarct size in a canine model. [26] These results are not necessarily paradoxical. First, ET receptor antagonists may exert beneficial effects on coronary perfusion (direct or via collaterals) and on cardiac work (via afterload reduction); neither of these circumstances could be examined with the current experimental design. Furthermore, no studies to date have included detailed examination of ET receptor antagonist effects on haemodynamic status during myocardial ischaemia.

One of the unexpected results of the current study was the abolition of ET-1 efflux during ischaemia by BQ-123, with minimal changes during the ischaemic period in the presence of bosentan (Fig. 4). The mechanism of this effect was not elucidated in the current study. As the experimental design resulted in somewhat greater flow reductions during BQ-123 than during bosentan perfusion experiments, as discussed previously, it is possible that this small differential degree of ischaemia may have contributed to the effects observed. However, an alternative explanation would be an acute reduction in extent of ET_A receptor occupancy during ischaemia in the case of 10^{-6} M BQ-123 in particular. Previous investigations have demonstrated externalization of ET receptors in rat heart during ischaemia, with a consequent increase in B_{max} [9]. Hence, if ET-1 release in the presence of ET_A antagonists were modulated by an ET_A receptor-mediated negative "feedback" mechanism, an increase in ET_A receptor density would provide a basis for decreased extent of ET by low concentration of ET_A antagonists. The observed effects of BQ-123 in particular on contractile performance during ischaemia may reflect these mechanisms.

A possible limitation of the current study is inherent in the use of a colloid-perfused, rather than blood-perfused preparation. It is probable, for example, that extent of post-ischaemic increases in coronary flow would have been somewhat greater with blood-perfused preparations.

In conclusion, in isolated perfused rat hearts, both BQ-123 and bosentan increased ET-1 efflux, but only BQ-123 exerted vasoconstrictor effects. Furthermore, during ischaemia the effects of BQ-123 on ET-1 efflux were abolished, and bosentan induced a significant negative inotropic effect. These results offer new insights into possible local cardiac mechanisms of ET-1 release, and also indicate that transient exposure to the ET_A antagonist BQ-123 may induce coronary vasoconstriction which persists unchanged during a wash-out phase. The results also strongly suggest that the importance of ET-induced positive inotropic effects is markedly accentuated during ischaemia. Clinical implications are that selective ET_A antagonists may be disadvantageous relative to ET_A/ET_B receptor antagonists in the presence of myocardial ischaemia, and that if such agents were withdrawn, a gradual rather than an abrupt cessation would be preferable. Furthermore, the negative inotropic effects of ET receptor

antagonists (especially selective ET_A receptor antagonists) during ischaemia may limit their safety in patients with combined ischaemia and impaired left ventricular function.

Acknowledgements

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References

- [1] Rubanyi GM, Polokoff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol Rev* 1994;46:325–415.
- [2] Davenport AP, Maguire JJ. Is endothelin-induced vasoconstriction mediated only by ET_A receptors in humans? *Trends Pharmacol Sci* 1994;15:9–11.
- [3] Pernow J, Modin A. Endothelial regulation of coronary vascular tone in vitro: contribution of endothelin receptor subtypes and nitric oxide. *Eur J Pharmacol* 1993;243:281–286.
- [4] Fukuroda T, Fujikawa T, Ozaki S, et al. Clearance of circulating endothelin-1 by ET_B receptors in rats. *Biochem Biophys Res Commun* 1994;199(3):1461–1465.
- [5] Wiecek I, Haynes WG, Webb D, Ludlam CA, Fox KAA. Raised plasma endothelin in unstable angina and non-Q wave myocardial infarction: relation to cardiovascular outcome. *Br Heart J* 1994;72:436–441.
- [6] Malatino LS, Grassi R, Stancanelli B. Release of immunoreactive endothelin from the heart during percutaneous transluminal coronary angioplasty. *Am Heart J* 1993;126:700–702.
- [7] Brunner F, Du Toit EF, Opie LH. Endothelin release during ischaemia and reperfusion of isolated perfused rat heart. *J Mol Cell Cardiol* 1992;24:1291–1305.
- [8] Kourembanas S, Marsden PA, McQuillan LP, Faller DV. Hypoxia induces endothelin gene expression and secretion in cultured human endothelium. *J Clin Invest* 1991;88(3):1054–1057.
- [9] Liu JJ, Casley DJ, Nayler WG. Ischaemia causes externalisation of endothelin-1 binding sites in rat cardiac membranes. *Biochem Biophys Res Commun* 1989;164(3):1220–1225.
- [10] Nayler WG, Ou RC, Gu XH, Casley DJ. Effect of amiodipine pretreatment on ischaemia-induced increase in cardiac endothelin-1 binding site density. *J Cardiovasc Pharmacol* 1992;20(3):416–420.
- [11] Douglas SA, James S, Hiley CR. Endothelial modulation and changes in endothelin pressor activity during hypoxia in the rat isolated perfused superior mesenteric arterial bed. *Br J Pharmacol* 1991;103(2):1441–1448.
- [12] McMurdo L, Corder R, Thiemermann C, Vane JR. Incomplete inhibition of the pressor effects of endothelin-1 and related peptides in the anaesthetised rat with BQ-123 provides evidence for more than one vasoconstrictor receptor. *Br J Pharmacol* 1993;108:557–561.
- [13] Grover GJ, Dzwonczyk S, Parham CS. The endothelin-1 receptor antagonist BQ-123 reduces infarct size in a canine model of coronary occlusion and reperfusion. *Cardiovasc Res* 1993;27:1613–1618.
- [14] Balwierczak JL. Two subtypes of the endothelin receptor (ET_A and ET_B) mediate vasoconstriction in the perfused rat heart. *J Cardiovasc Pharmacol* 1993;22(Suppl 8):S248–S251.
- [15] Seo B, Barry SO, Siebenmann R, Von Segesser L, Luscher TF. Both ET_A and ET_B receptors mediate contraction to endothelin-1 in human blood vessels. *Circulation* 1994;89:1203–1208.
- [16] Dagassan PH, Breu V, Clozel M, Clozel JP. Role of endothelin during reperfusion after ischaemia in isolated perfused rat heart. *J Cardiovasc Pharmacol* 1994;24:867–874.
- [17] Kelly RA, Eid H, Kramer BK. Endothelin enhances the contractile responsiveness of adult rat ventricular myocytes to calcium by a pertussis toxin-sensitive pathway. *J Clin Invest* 1990;86:1164–1171.
- [18] McClellan G, Weisberg A, Winegrad S. Effect of endothelin-1 on actomyosin ATPase activity—implications for the efficiency of contraction. *Circ Res* 1996;78:1044–1050.
- [19] Evans HG, Lewis MJ, Shan AM. Modulation of myocardial relaxation by basal release of endothelin from endocardial endothelin. *Cardiovasc Res* 1994;28:1694–1699.
- [20] Kasai H, Takanashi M, Takasaki C, Endoh PL. Pharmacological properties of endothelin receptor subtypes mediating positive inotropic effects in rabbit heart. *Am J Physiol* 1994;266:H2220–H2228.
- [21] Endoh M, Norota I, Yang HT, Fujita S, Takanashi M. The positive inotropic effect and the hydrolysis of phosphoinositide induced by endothelin-3 in rabbit ventricular myocardium: inhibition by a selective antagonist of ET_A receptors, FR139 317. *J Pharmacol Exp Ther* 1996;277:61–70.
- [22] Beyer ME, Slesak G, Nerz S, Kazmaier S, Hoffmeister HM. Effect of endothelin-1 and IRL 1620 on myocardial contractility and myocardial energy metabolism. *J Cardiovasc Pharmacol* 1995;26(Suppl 3):S150–S152.
- [23] Domenech R, Macho P, Gozalez R, Huidobro-Toto JP. Effect of endothelin on total and regional coronary resistance and on myocardial contractility. *Eur J Pharmacol* 1991;192:409–416.
- [24] Gulati A, Kumar A, Shahani BT. Cardiovascular effects of centrally administered endothelin-1 and its relationship to changes in cerebral blood flow. *Life Sci* 1996;58(5):437–445.
- [25] Cirino M, Battistini B, Yano M, Rodger IW. Dual cardiovascular effects of endothelin-1 dissociated by BQ-153, a novel ET_A receptor antagonist. *J Cardiovasc Pharmacol* 1994;24:587–594.
- [26] Cirino M, Martin E, Yano M, Rodger IW. Cardiovascular and renal actions of the endothelin_B receptor in pigs. *J Cardiovasc Pharmacol* 1997;29:704–712.
- [27] Donckier J, Hanet C, Stoleru L. Effects of endothelin-1 at pathophysiologic concentrations on coronary perfusion and mechanical function of normal and postischemic myocardium. *J Cardiovasc Pharmacol* 1994;23:212–219.
- [28] Kiowski W, Sutsch G, Hunziker P. Evidence for endothelin-1 mediated vasoconstriction in severe chronic heart failure. *Lancet* 1995;346:732–736.
- [29] Sutsch G, Osmund B, Kiowski W. Acute and short-term effects of the nonpeptide endothelin-1 receptor antagonist bosentan in humans. *Cardiovasc Drugs Ther* 1996;10:717–725.
- [30] Spinal FG, Walker JD, Mukherjee R. Concomitant endothelin receptor subtype-A blockade during the progression of pacing-induced congestive heart failure in rabbits—beneficial effects on left ventricular and myocyte function. *Circulation* 1997;95:1918–1929.
- [31] Shimoyama H, Sabbah HN, Borzak S. Short-term hemodynamic effects of endothelin receptor blockade in dogs with chronic heart failure. *Circulation* 1996;94:779–784.
- [32] Ray SG, McMurray JJ, Morton JJ, Dargie HJ. Circulation endothelin in acute ischaemic syndromes. *Br Heart J* 1992;67:383–386.
- [33] Chen BN, Rayner TE, Menadue MF, McLennan PL, Oliver JR. Effect of ischaemia and role of eicosanoids in release of atrial natriuretic factor from rat heart. *Cardiovasc Res* 1993;27:1576–1579.
- [34] Vedernikov YP, Goto K, Vanhoutte PM. The ET_A antagonist BQ-123 inhibits anoxic contractions of canine coronary arteries without endothelium. *J Cardiovasc Pharmacol* 1993;26(Suppl 3):S252–S256.

- [35] Han H, Neubauer S, Bracker B, Ertl G. Endothelin-1 contributes to ischaemia/reperfusion injury in isolated rat heart—attenuation of ischaemic injury by the endothelin-1 antagonists BQ-123 and BQ-610. *J Mol Cell Cardiol* 1995;27(2):761–766.
- [36] Yu JCM, Davenport AP. Regulation of endothelin receptor expression in vascular smooth-muscle cells. *J Cardiovasc Pharmacol* 1995;26(Suppl 3):S348–S350.
- [37] Nishimura J, Aoki H, Chen X, et al. Evidence for the presence of endothelin ET_A receptors in endothelial cells in situ on the aortic side of porcine aortic valve. *Br J Pharmacol* 1995;115:1369–1376.
- [38] Ozaki S, Ohwalli K, Ihara M. ET_B-mediated regulation of extracellular levels of endothelin-1 in cultured human endothelial cells. *Biochem Biophys Res Commun* 1995;209(2):483–489.
- [39] Dupuis J, Goresky CA, Fournier A. Pulmonary clearance of circulating endothelin-1 in dogs in vivo: exclusive role of ET_B receptors. *J Appl Physiol* 1996;81(4):1510–1515.
- [40] Sato K, Oka M, Hasunuma K, et al. Effects of separate and combined ET_A and ET_B blockade on ET-1-induced constriction in perfused rat lungs. *Am J Physiol* 1995;269(5):L668–L672.
- [41] Weber C, Schmitt R, Birnboeck H. Pharmacokinetics and pharmacodynamics of the endothelin receptor antagonist bosentan in healthy human subjects. *Clin Pharmacol Ther* 1996;60(2):124–137.
- [42] Brunner F, Doherty AM. Role of ET_B receptors in local clearance of endothelin-1 in rat heart: studies with the antagonists PD 155 080 and BQ-788. *FEBS Lett* 1996;396:238–242.
- [43] Brunner F. Tissue endothelin-1 levels in perfused rat heart following stimulation with agonists and in ischaemia and reperfusion. *J Mol Cell Cardiol* 1995;27:1953–1963.
- [44] McClulloch KM, Docherty CC, Morecroft I, Maclean MR. Endothelin B receptor-mediated contraction in human pulmonary resistance arteries. *Br J Pharmacol* 1996;119:1125–1130.
- [45] Waggoner WG, Genova SL, Hash VA. Kinetic analyses demonstrate that the equilibrium assumption does not apply to [¹²⁵I] endothelin-1 binding data. *Life Sci* 1992;51:1869–1876.

Addenda and Corrigenda

page 2: (paragraph 1, line 2) "in the USA" should be added after "44% of all deaths"

page 6: (paragraph 2, line 1) "cirtical" should be "critical"

page 11: (paragraph 1, line 4) "redicals" should be "radicals"

page 19: (paragraph 1, line 6) "synthese" should be "synthesis"

page 32: (Section 1.2.5.2.2, last line) omit Ferro et al., 1998

page 38: (line 1) add "smooth muscle cells" prior to "endothelial cells"

page 46: (line 10) "sarafotoxn S6c" should be "sarafotoxin S6c"

page 51: (Section 1.6.1) Hypotheses 2) "modulate" should be "stimulate"

3) "regulates local vasomotion" should be
"stimulates vasoconstriction"

page 62: (paragraph 1, last line) "mesurement" should be "measurement"

page 63: (Section 2.1.3.1, last line) add " Note:- longer pre-perfusion periods were not used as they would have increased the length of the experiment to an impractical extent in the isolated heart model, particularly when the high cost of the antagonist BQ-123 is taken into account."

page 67: (Section 2.1.5.3), line 1) Following the first sentence add "The interference of other peptides was limited by using a specific antibody for ANP in the assay; refer to Section 2.1.5.4."

page 72: (Section 2.1.6.2.3, paragraph 2) Replace the last two sentences with " In order to minimize errors due to the high inter-assay coefficient of variation, samples from each experiment (each rat) were assayed within one assay and the results expressed as a percentage of baseline".

Page 76: (Section 2.2.1.1, paragraph 2: Exclusion criteria) add

"4) Haemodynamically significant left ventricular dysfunction"

"5) Arrhythmias"

page 90: (Section 3.3.2.3, After first sentence) add "The bradycardia seen during ischaemia in the isolated perfused rat heart is in contrast to the increase in heart rate associated with ischaemia in man and presumably reflects the much greater complexity of factors determining heart rate in vivo".

Page 97: (Paragraph 1, last sentence) Replace with "Therefore, reduction in ET-1 efflux rate (i.e. concentration multiplied by perfusion rate and divided by wet heart weight) reflected the reduced perfusion rate".

Page 103: (paragraph 2, line 12) "perfused rate" should be "perfused rat"

page 105: (last paragraph, line 5) "ischaemia is a stimulus" should be
"coronary flow reduction is a stimulus"



page 106: (last paragraph, line 2) "stimulated by moderate (or severe) ischaemia" should be "induced by decreased coronary flow".

page 109: (last paragraph, last line) "rate" should be "rat" and the following added "Two models of ischaemia (elevated and normal ET concentrations) were used in this study."

page 120: (Section 4.3.2.1.2, line 1) At the beginning of section add "The aim of this study is to induce coronary flow reduction by ET-1 perfusion and compare its effects (on ANP release) with those of the same degree of coronary flow reduction without ET-1 perfusion."

Page 120: (Section 4.3.2.1.3, line 3) add "ET is a positive inotrope in isolated myocytes or papillary muscles. However, in vivo studies have shown that exogenous ET induced ischaemia and secondary negative inotropic effects. In our study, these effects (primary positive and secondary negative inotropic effects) may balance."

Page 125: (paragraph 1) omit last sentence.

Page 126: (paragraph 1, line 2) insert "(it may be a Type II error)" after "release rate"

page 126: (paragraph 2, line 8) insert "(at concentrations and pre-exposure periods based on previous studies)" after "BQ-123 (10^{-6} M)"

page 127: (paragraph 2, last line) "(in theray)" should be "(in theory)"

page 128: (last paragraph) add "Another explanation for the increase in ANP release during reduced coronary flow rate is that the moderate and severe reductions in coronary blood flow led to significant reductions in coronary NO release due to reduced shear stress. Indeed, there is evidence that agents known to inhibit the release or effects of NO, caused an increase in ANP release (Sanchez-Ferrrer et al., 1990, Leskinen et al., 1995). Therefore a reduction in NO release during coronary flow reduction could have removed an inhibition of ANP release."

page 139: (Fig. 5.5 legend) add "BQ-123 (10^{-6} M) (open square, n=7) and" after "Effects in non- ischaemic hearts of"

page 151: (paragraph 2, line 3) "(Fig. 4)" should be "(Fig. 5.4)"

page 162: (Section 6.3.2, line 2) "decearses" should be "decreases"

page 166: (Section 6.3.3.2, second sentence) Replace second sentence with "During hypoxia, BQ-123 significantly increased ($P < 0.05$, Mann Whitney U on AUC) and bosentan tended to increase ET-1 efflux rate ($P = 0.069$, unpaired t-test on AUC)."

Page 174: (paragraph 3). Insert after first sentence "The effects of exogenous ET-1 and ET receptor antagonists on ANP release, independent of hypoxia /ischaemia are discussed in chapter 4 (Fig. 4.6 and Fig. 4.8)."

page 217: (paragraph 1, last line) "prervious" should be "previous"

page 234: omit the reference "Ferro et al., 1998"