TARGETING PKC AND NFkB BY
POLYUNSATURATED FATTY ACID MIMETICS
IN DIABETIC RETINOPATHY

ELAINE BING-AI THAM
M.B.,B.S., DCH

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Department of Immunopathology
Women’s and Children’s Hospital

Faculty of Health Sciences
Department of Paediatrics
The University of Adelaide
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SUMMARY

Hyperglycaemia-induced vascular complications of diabetes mellitus continues to cause significant morbidity despite recent advances in therapy. Protein kinase C (PKC) and nuclear factor κB (NFκB) are two key signaling molecules which contribute to the development of diabetic complications, nephropathy, retinopathy and cardiovascular disease. While the omega-3 fatty acids have some protective value in diabetes their use has remained limited. To improve this type of application, our department has undertaken studies to identify the relationship between specific structural elements of polyunsaturated fatty acids and their biological activities. The findings led to the synthesis of a group of novel polyunsaturated fatty acids (PUFAs), one of which (β-oxa 21:3n-3/MP5) inhibits PKCβ activation and another (β-oxa 23:4n-6/MP3) which inhibits NFκB activation. It was therefore of interest to determine the relevance of this technology to the treatment of retinopathy.

This research characterizes the effect of hyperglycaemic conditions on PKC and NFκB activation in bovine retinal endothelial cells (BREC) in culture, and assesses the ability of the novel PUFAs to inhibit PKC and NFκB activation.

The research first established conditions for optimal isolation procedures for preparation of highly pure BREC and culture conditions under which the cells retained the BREC characteristics. The cell preparations were Von Willebrand Factor positive and devoid of pericyte contamination.
A major finding was that BREC expressed PKC α, βI, δ and ε but not βII. This contrasts with previous findings which have reported the activation of the βII isozyme in BREC. Since we were unable to see the expression of PKC βII, the results suggest that previous work had been conducted with BREC contaminated with pericytes.

The data demonstrated that BREC exposed to hyperglycaemic conditions (25 mM glucose) showed preferential activation of PKC βI and δ. Hyperglycaemia-induced PKC activation is therefore not generalized to all isozymes, with PKC βI being the most significant.

The results showed that while high glucose alone failed to activate NFκB (measured as degradation of IκBα), it caused a more persistent activation of NFκB in response to tumour necrosis factor α (TNF) suggesting that the pathogenic effects of TNF are amplified by hyperglycaemic conditions. The results are consistent with the recent evidence that TNF is a cytokine involved in the pathogenesis of diabetes.

The findings from the research showed that β-oxa 21:3n-3 preferentially inhibited the activation of PKCβI in BREC cultured under hyperglycaemic conditions. Treatment of BREC with β-oxa 23:4n-6 significantly inhibited the activation of NFκB induced by TNF under hyperglycaemic conditions.

This research not only contributes to a better understanding of diabetic retinopathy but also demonstrates novel ways of targeting these signaling molecules (PKC and NFκB) with the PUFA mimetics, MP3 and MP5.
DECLARATION

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

I consent to the thesis being made available for photocopying and loan if accepted for the award of the degree.

Elaine Tham
ACKNOWLEDGEMENTS

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<td>BREC</td>
<td>bovine retinal endothelial cells</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
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<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DHA</td>
<td>docosahexaenoic acid (22:6n-3)</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<td>free fatty acid</td>
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<td>HBSS</td>
<td>Hanks balanced salt solution</td>
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<td>HDL</td>
<td>high density lipoprotein</td>
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<td>HETE</td>
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<tr>
<td>HPETE</td>
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</tr>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
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<td>ICAM</td>
<td>intracellular adhesion molecule</td>
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<tr>
<td>IkB</td>
<td>inhibitory kappa B</td>
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<tr>
<td>IKK</td>
<td>inhibitory kappa B kinase</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
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<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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LT          leukotriene
NFKB        nuclear factor kappa B
PBS          phosphate buffered saline
PDGF         platelet derived growth factor
PDK1         phosphoinositide-dependent kinase 1
PI3-kinase   phosphatidylinositol 3 kinase
PIP2         phosphatidylinositol 4,5 bisphosphate
PKC          protein kinase C
PMA          phorbol 12-myristate 13-acetate
PS           phosphatidylserine
PUFA         polyunsaturated fatty acid
TLC          thin layer chromatography
TNF          tumour necrosis factor $\alpha$
VEGF         vascular endothelial growth factor
VCAM         vascular cell adhesion molecule
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CHAPTER 1

INTRODUCTION
1.1 General Introduction

Diabetes mellitus is a chronic condition characterized by hyperglycaemia and depressed metabolism of carbohydrate, protein and fat due to impairment of insulin secretion or resistance to insulin action. Long term morbidity occurs due to microvascular and macrovascular disease including cardiovascular disease, nephropathy, retinopathy and neuropathy. It is classified into Type 1 and Type 2 diabetes, as well as other rarer forms of the disease (Table 1.1). It has been reported that 7.5% of Australian adults aged 25 years or older have diabetes mellitus with 10-15% having Type 1 diabetes and 85-90% having Type 2 diabetes (Dunstan et al, 2001).

Treatment involves the combination of insulin injections (type 1 diabetes), oral hypoglycaemic medications (type 2 diabetes), dietary modifications and exercise, in order to maintain near normal blood glucose levels and to minimise long term vascular complications. The diet requires close attention to carbohydrate intake to help regulate the interaction between exogenous insulin injections and carbohydrates so that glucose levels can be controlled (Sperling, 2002). Fat intake also needs to be monitored due to the increased risk of hyperlipidaemia and vascular disease. Dietary intake of saturated fats needs to be reduced and replaced with polyunsaturated fats, in particular, omega-3 fatty acids which have been shown to improve vascular function (Hu et al, 2003).

In order to maintain long-term health and to minimise the morbidity caused by the vascular complications of diabetes, intensive self-monitoring and significant lifestyle changes need to be instituted. Current approaches to improve quality of life for diabetics include the development of more effective insulin preparations, intensive insulin injection regimes, insulin pump therapy and stem cell transplants. Advancements in our knowledge of
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<td></td>
<td>Immune mediated</td>
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<td>Idiopathic</td>
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<td>2</td>
<td><strong>Type 2 diabetes</strong></td>
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<td></td>
<td>Typical</td>
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<td></td>
<td>Atypical</td>
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<td><strong>Genetic defects of β cell function</strong></td>
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appropriate nutrition have been made with focus not only on carbohydrate intake but the
glycaemic index of foods to allow a more steady release of glucose into the circulation.
The role of dietary omega-3 fatty acids is also being studied due to their known benefits in
cardiovascular disease (Abeywardena and Head, 2001) as they may also improve the
vascular function of diabetics and minimise vascular complications.

Research into the biochemical pathways of hyperglycaemia induced endothelial cell
damage is also extensive with newer approaches to target specific pathways being
developed. There has been interest in the role of protein kinase C (PKC) and nuclear factor
kappa B (NFκB) in endothelial cell damage with the potential to inhibit their effects at a
cellular level.

Novel approaches to target these biochemical pathways and to improve treatment of
hyperglycaemia and vascular sequelae are essential to improve the long term outcome and
quality of life for individuals with diabetes.

1.2 Type 1 diabetes

The incidence of type 1 diabetes in Australia is 20 per 10,000 (Broyda et al, 2003) with the
peak age of presentation between 5 and 7 years and at puberty. Males and females are
equally affected. Throughout the world there is variation of incidence within ethnic groups
suggesting a genetic influence. The coexistence of other autoimmune diseases such as
Hashimoto thyroiditis and Addison disease in some individuals with type 1 diabetes is
recognized to be associated with an increased frequency of certain HLA types (major
histocompatibility complex DR3 and DR4). Twin studies have shown that the concordance
rate in identical twins where one has type 1 diabetes is only 30%-50% suggesting environmental factors also play a role. There also is a seasonal variation with increased incidence during winter and autumn, supporting the role of viral infections as a trigger for the onset of diabetes. Viruses may directly destroy pancreatic beta cells or trigger a superantigen response which activates T cells (Sperling, 2002).

Autoimmune destruction of the beta cells of pancreatic islets is well recognized in type 1 diabetes. This is supported by the presence of auto-antibodies such as islet cell antibodies (ICA) in 80-90%, glutamic acid decarboxylase antibodies (GAD) in 80%, and anti-insulin antibodies in 30-40% of newly diagnosed individuals. The destruction of these cells results in the complete loss of insulin production, which is required for utilisation of glucose as energy (Sperling, 2002).

Subsequent hyperglycaemia causes polyuria and polydipsia which results in dehydration and physiologic stress with the elevation of stress hormones (adrenaline, cortisol, growth hormone, glucagon). These counter-regulatory hormones antagonise the action of insulin and promote glycogenolysis, gluconeogenesis, lipolysis and ketogenesis resulting in acceleration of metabolic decompensation and progression to ketoacidosis (Sperling, 2002).

Children with type 1 diabetes usually present with a short history (2-4 weeks) of polyuria, polydipsia and weight loss and the diagnosis is confirmed by an elevated blood glucose level (Table 1.2), glucosuria and ketonuria. Twenty-five percent present with ketoacidosis with vomiting, polyuria and dehydration. In severe cases an odour of acetone is present on the breath and Kussmaul respiration is present due to ketosis. This can lead to coma.
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<th>Random blood glucose</th>
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<td>Normal</td>
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<tr>
<td>Impaired glucose tolerance</td>
<td>6.1 - 6.9 mmol/L</td>
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<tr>
<td>Diabetes Mellitus</td>
<td>&gt; 7.0 mmol/L</td>
<td>&gt; 11.1 mmol/L</td>
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Table 1.2 Blood glucose levels in diabetes (Alberti, 1998)
Individuals with type 1 diabetes therefore require insulin injections to reduce their blood glucose levels. There are a variety of insulin regimes which aim to mimic the pancreas’ secretion of insulin in response to a carbohydrate load. This is achieved by combinations of short, intermediate and long acting insulin by subcutaneous injections 2-4 times per day.

More recent advances in insulin administration include the use of an insulin pump that provides continuous subcutaneous insulin with the ability to give boluses of insulin at meal times. Diabetics need to monitor their blood glucose levels frequently to ensure adequate doses of insulin are given so as to prevent hyperglycaemia and hypoglycaemia. In addition to this, the diet needs to contain adequate calories for growth and development and the carbohydrate content must consist of at least 70% complex carbohydrates. Complex carbohydrates and low glycaemic index foods are absorbed more slowly providing a more steady release of glucose into the circulation compared with the short acting carbohydrates. A diet low in saturated fats is also necessary to prevent cardiovascular complications of diabetes (Sperling, 2002).

Prolonged exposure of serum proteins to hyperglycaemic conditions results in the formation of advanced glycation end products. These include glycated haemoglobin (HbA1C) which provides the best indication of long term control. The Diabetes Control and Complications Trial (DCCT Research Group, 1993) showed that a HbA1C <8.1% (non diabetics 4-6%) decreased the risk of long term micro- and macro-vascular complications, but normo-glycaemia is difficult to maintain despite intensive therapy. Long term monitoring and screening for vascular complications is necessary so that medical therapies to reverse and treat conditions such as diabetic retinopathy, nephropathy, neuropathy, and cardiovascular disease can be commenced before irreversible damage occurs.
Advancements in insulin preparations have been made with the availability of ultra-short, short, intermediate, and long-acting insulins. There has also been the development of newer techniques to administer insulin. Insulin pump therapy allows continuous subcutaneous infusion of insulin with the capability of giving bolus insulin at meal times. This avoids the need for frequent injections as the catheter can be changed every 3 days. It also provides a more physiological method of insulin administration. Continuous subcutaneous insulin infusion (CSII) has been shown to lower HbA1C, while decreasing episodes of severe hypoglycaemia (Willi et al, 2003).

Newer therapies are being investigated to improve the outcome for diabetics. Pancreatic transplantation techniques have been developed where the pancreas is either transplanted alone, or in combination with a kidney. This provides up to 90% insulin independence after one year and decreases progression of diabetes-related complications (Larsen et al, 2002). Despite the early success of this treatment, surgical risks remain high.

Transplantation of pancreatic islet cells has shown promising results with 80% remaining insulin independent one year after transplant (Ryan et al, 2002). Limitations in this treatment are due to the inadequate numbers of islet cells available for transplantation and the need for at least two or three procedures to remain insulin independent. Side effects of immunosuppression are becoming less of a concern due to the development of newer immunosuppressive medications. Long term success is yet to be defined.

With the limited availability of islet cells for transplantation, interest has focused on the possibility of β-cell stem or progenitor cells as a source of insulin-producing tissue. There
is evidence that these cells exist but no clearly identifiable pancreatic stem cell has been isolated to date. Research in this area is continuing (Lechner and Habener, 2003).

1.3 Type 2 diabetes

Type 2 diabetes is characterised by insulin resistance rather than a lack of insulin, and insulin levels are found to be normal to high. The most common cause of type 2 diabetes is obesity and although type 2 diabetes was previously thought to be a disease of adulthood, there is an increasing incidence during childhood due to an increase in number of obese children. The mean annual incidence in childhood was found to be 2.3 per 10,000 with a mean age of 14.2±2y (Broyda et al, 2003).

In contrast to type 1 diabetes, the onset of type 2 diabetes does not usually occur until teenage or young adult years and there is no evidence of auto-antibodies and no increase in frequency of HLA-DR3, or DR4. There is often a family history of type 2 diabetes. The onset of the disease is more insipid with individuals having milder symptoms of hyperglycaemia with polyuria and polydipsia over a longer time period (Sperling, 2002).

Management of type 2 diabetes involves significant changes in diet and exercise with a focus on weight loss. It is difficult to institute these changes in lifestyle making it necessary to add oral hypoglycaemic medications and/or insulin injections to help achieve normoglycaemia (Tamborlane and Ahern, 2003).
### 1.4 Diabetes associated complications

Significant morbidity and premature mortality occurs due to the complications of diabetes. The degree of hyperglycaemia has been shown be significant in the development and progression of clinical complications (DCCT Research Group, 1993).

Hyperglycaemia results in biochemical changes which lead to microvascular and macrovascular dysfunction. Macrovascular complications include coronary artery disease, atherosclerosis and peripheral vascular disease which can lead to acute myocardial infarction, angina and poor wound healing. Microvascular complications include nephropathy, neuropathy, and retinopathy which can result in end-stage kidney disease, impaired nerve conduction and blindness (Sperling, 2002).

The Diabetes Control and Complications Trial (DCCT Research Group, 1993) compared the effect of intensive treatment (3 or more insulin injections a day or insulin pump treatment) with conventional treatment (1-2 daily insulin injections) on the development and progression of vascular complications. The trial demonstrated over a 6.5 year follow-up period, that intensive therapy significantly reduced the development and progression of retinopathy, nephropathy and neuropathy by 35% to more than 70%. The reduction in the risk of progression of these complications persisted in the intensive therapy group despite similar levels of glycaemic control in both groups in the four years after completion of the initial trial (DCCT/EDIC Research Group, 2001). This suggests that periods of poor glycaemic control in the group not receiving intensive therapy may trigger off processes leading to the development of vasculopathy.
Hyperglycaemia plays an important role in the development of diabetic vascular complications, ie cardiovascular disease, nephropathy, neuropathy and retinopathy. The biochemical and haemodynamic changes which occur result in pathological changes which will be described later in this chapter.

1.4.1 Cardiovascular disease

Individuals with diabetes have an increased risk of cardiovascular disease. There is an increased risk of coronary artery disease, in particular, myocardial infarction, as well as cerebrovascular disease, ie stroke. Limb ischaemia from peripheral arterial disease is also increased, contributing to the development of non-healing ulcers and amputation (Luscher et al, 2003).

Diabetics have abnormal lipid metabolism with elevated levels of free fatty acids and triglycerides. This lowers HDL (high density lipoprotein) levels. Both hypertriglyceridaemia and low HDL contribute to endothelial dysfunction. Elevated free fatty acid levels in individuals is believed to contribute to insulin resistance in type 2 diabetes (Creager et al, 2003).

Hyperglycaemia induced biochemical changes along with the abnormal lipid metabolism and insulin resistance results in endothelial cell damage, platelet activation and aggregation and increased coagulation factors which increases the risk of vascular thrombosis. This leads to coronary artery disease, cerebrovascular disease ie stroke and peripheral vascular disease (Creager et al, 2003).
Dietary therapy, weight loss and exercise are necessary to optimize glucose control and lipid levels to minimize the progression to angina and myocardial infarction. It is usually difficult to institute these lifestyle changes therefore pharmacological therapy is required (Florkowski, 2003).

Oral hypoglycaemic medications which also lower insulin resistance are in use to control glucose levels but their ability to lower the risk of cardiovascular events is not yet clear (Luscher et al, 2003). Lipid-lowering drugs (eg statins), control of hypertension (eg angiotensin converting enzyme inhibitors) and antiplatelet therapy (eg aspirin) have been shown to be effective in reducing the risk of cardiovascular disease and lowering LDL levels (Florkowski, 2003; Luscher et al, 2003).

1.4.2 Nephropathy

Diabetic nephropathy is the leading cause of death and disability in diabetes with 35% of type 1 diabetics and 15-60% of type 2 diabetics developing end-stage kidney disease (Foster, 1994). It is the major cause of end-stage renal disease (Sheetz and King, 2002).

Hyperglycaemia results in biochemical changes which alter blood vessel function, resulting in haemodynamic changes and hyperfiltration through the glomeruli. Pathological changes in the kidneys occur characterised by widening of the glomerular basement membrane and thickening of the mesangium (Sheetz and King, 2002). The earliest indication of nephropathy is microalbuminuria which has a prevalence of 3.7-30.6% in type 1 diabetics. Approximately 1.6% of diabetics develop microalbuminuria per year (The Microalbuminuria Collaborative Study Group, 1999). This progresses to overt proteinuria as glomerular function deteriorates and eventually results in end-stage renal failure. The
presence of hypertension can accelerate the progression of nephropathy (Sheetz and King, 2002).

Screening for microalbuminuria and hypertension enables early detection of renal disease so that pharmacological therapy can be instituted. Angiotensin-converting enzyme inhibitors have been shown to be effective in delaying the progression of nephropathy (Foster, 1994; Sheetz and King, 2002).

1.4.3 Neuropathy

Diabetic neuropathy affects approximately 50% of individuals with diabetes. It can manifest as a mono- or poly-neuropathy and is associated with significant morbidity. Peripheral neuropathy presents with varying degrees of numbness, paraesthesia, hyperaesthesia and severe neuropathic pain. Loss of peripheral sensation is often associated with impaired peripheral vascular function which contributes to the development of non-healing ulcers and the potential risk of limb amputation. Autonomic neuropathy can result in gastrointestinal dysfunction, orthostatic hypotension, syncope, bladder dysfunction and impotence (Foster, 1994; Sheetz and King, 2002).

Neuronal damage is due to direct hyperglycaemic-induced damage to nerve parenchyma. Hyperglycaemia-induced endothelial changes in the microvessels contribute to neuronal ischaemia and damage (Sheetz and King, 2002). Treatment of these conditions is difficult, with patients often requiring potent analgesia to control the pain (Foster, 1994).
1.4.4 Retinopathy

Diabetic retinopathy is the leading cause of blindness with the prevalence of retinopathy increasing with the duration of diabetes. Eighty to ninety percent will have some evidence of retinopathy after 20 years of the disease (Elkington, 1993) with 30-40% having proliferative retinopathy (Stefansson et al, 2000).

Blood vessel damage occurs due to hyperglycaemia induced metabolic and chemical changes. Background retinopathy is characterised by capillary vasodilation, increased permeability, and capillary occlusion resulting in retinal ischaemia. Neutrophils in diabetics are also less deformable than those in non-diabetics and neutrophils entering the microvessels become trapped in the vessels and adhere to the endothelium of choroidal and retinal capillaries. This contributes to retinal vasculature injury by increasing capillary dropout and blood vessel occlusion (Lutty et al, 1997). Microaneurysms, retinal haemorrhages and exudates are also seen. Proliferative retinopathy results from hypoxia which stimulates endothelial cell migration and proliferation. These new vessels are fragile and frequently rupture, causing retinal haemorrhages that can lead to scarring, retinal detachment and blindness. Macular oedema due to the increase in vascular permeability contributes to the loss of visual acuity (Foster, 1994).

There is currently no effective way to prevent diabetic retinopathy. Intensive glycaemic control can slow the progression of retinopathy (DCCT/EDIC Research Group, 2001) and lower the risk of blindness but normoglycaemia is difficult to maintain. Treatment by laser photocoagulation is effective if given in the early stages of disease to destroy new capillaries and microaneurysms, thus reducing the risk of blindness but requires adequate screening programs to detect early changes of the disease. Once visual loss has occurred,
laser treatment is less effective in improving visual acuity (Frank, 2002). Despite early
detection and treatment, diabetic retinopathy remains a significant cause of morbidity in
diabetes.

1.5 New approaches to treat diabetes-associated complications

Recently, focus has centred on identifying the hyperglycaemia induced biochemical
changes that are significant in causing vascular and neurological dysfunction. It has
become evident that the pathogenic effects of hyperglycaemia are mediated by at least two
intracellular signalling molecules, protein kinase C (PKC) and nuclear factor kappa B
(NFkB) in tissues at risk of developing diabetic complications (Koya and King, 1998).

Several pathways have been recognised which contribute to hyperglycaemic-induced
endothelial cell dysfunction (Figure 1.1). Hyperglycaemia results in activation of the
polyol pathway. The subsequent increase in the NADH/NAD± ratio increases free radical
formation and DAG synthesis. Auto-oxidation of excess glucose and non-enzymatic
glycation results in oxidative cellular damage (Chakrabarti et al, 2000). These pathways
have a complex interaction which results in the activation of PKC through DAG and
reactive oxygen species, and of NFkB through AGE and reactive oxygen species (Di

1.5.1 Protein kinase C

PKC is a serine/threonine protein kinase. Eleven isozymes have been identified which are
classified into classical (α, β1, β2, γ), novel (δ, ε, η, μ, θ), and atypical (ζ, λ, τ) forms
depending on their sensitivity to diacylglycerol (DAG) and calcium. Classical PKCs are
activated by calcium, DAG, phosphatidylserine (PS), unsaturated fatty acids and phorbol 12-myristate 13-acetate (PMA). Novel PKCs are activated by DAG, PS, unsaturated fatty acids and phorbol esters such as PMA. Atypical PKCs are activated by PS and phosphatidylinositolides (Srivastava, 2002).

PKC regulates a diverse range of cellular processes in an isozyme-specific manner. It plays a role in receptor desensitization, membrane structure, regulation of transcription, immune responses, and cell growth (Newton, 1995).

When PKC is in the inactive state, the kinase domain is masked by the folding back of a pseudosubstrate domain (Newton, 1995). Diacylglycerol (DAG) enhances PKC activity by removing the pseudosubstrate from the kinase domain and increasing the affinity of PKC for the phospholipid cofactors. DAG can be formed through the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP$_2$), or through the de novo synthesis (Figure 1.2) via glycerol-3-phosphate and phosphatidic acid. De novo synthesis is the major mechanism by which DAG is increased in response to hyperglycaemia (Koya and King, 1998; Lee et al, 1989) and it has been shown that this elevation remains despite a reduction in glucose concentration (Xia et al, 1994). In unstimulated cells, PKC is generally found in the soluble fractions and it translocates to the particulate fraction such as cellular membranes upon stimulation where it associates with proteins such as RACKs (receptor for activated C kinase) and STICKS (substrates that interact with C kinases) (Keenan and Kelleger, 1998).
Variations in PKC isozyme expression occur between species and in different cell types. For example, PKC α, βI, βII, δ, ε and ζ are expressed in bovine retinal endothelial cells (Park et al, 2000), PKC α, βI, βII, and ε in the rat retina (Shiba et al, 1993), PKC α, δ, ε and ζ are expressed in rat mesangial cells (Kikkawa et al, 1994), and PKC α and βII in rat aortic endothelial cells (Inoguchi et al, 1992).

It is now well established that hyperglycaemia in diabetes activates PKC (Koya and King, 1998). However, not all PKC isozymes are activated by hyperglycaemia. PKCβ is well recognised in mediating the action of glucose in the glomeruli, retina, aorta and heart of diabetic animals and cultured cells. PKC α, βI, βII and δ have been shown to be activated by high glucose conditions in bovine retinal endothelial cells (Park et al, 2000), PKC α, β, and ε in the rat retina (Shiba et al, 1993), and PKC α and ζ in rat mesangial cells (Kikkawa et al, 1994), and PKC βII in rat aorta (Inoguchi et al, 1992).

Glucose induced PKC activation promotes the production of endothelin-1 from retinal endothelial cells. Endothelin-1 is a potent vasoconstrictor which has been implicated in causing a reduction in blood flow in the early diabetic eye (Koya and King, 1998). The resultant hypoxia stimulates the production of vascular endothelial growth factor (VEGF) which increases vascular permeability and endothelial cell proliferation and migration (Chakrabarti et al, 2000). This ultimately leads to retinal neovascularisation (Figure 1.3).

### 1.5.2 Nuclear Factor kappa B

Nuclear factor kappa B (NFκB) is a cytoplasmic transcription protein and is an important mediator of the inflammatory response. It regulates the expression of interleukins, growth
Figure 1.1 Current concept of the role of hyperglycaemia in endothelial cell damage.

Hyperglycaemia results in activation of the polyol pathway, DAG synthesis, free radical formation and AGE. Oxidative cellular damage occurs along with activation of PKC and NFκB resulting in endothelial cell dysfunction/neovascularisation.
Figure 1.2 Synthesis of diacylglycerol (DAG). DAG can be formed through the hydrolysis of phosphatidylinositol bisphosphate (PIP$_2$) or phosphatidylcholine (PC). In response to hyperglycaemia DAG is largely produced by de novo synthesis through glycolysis and acylation of the 3-carbon metabolites. IP$_3$, inositol triphosphate; PLC, phospholipase C; PLD, phospholipase D.
Figure 1.3 Potential mechanism of hyperglycaemia-induced neovascularisation and capillary injury through PKC and NFkB activation. Activation of PKC by hyperglycaemia results in vasoconstriction of vessels through the production of endothelin-1. Vasoconstriction simulates production of VEGF which contributes to neovascularisation by increasing endothelial cell permeability, proliferation and migration. NFkB activation upregulates adhesion molecule expression which promotes neutrophil adhesion resulting in blood vessel occlusion and capillary dropout. ICAM: intercellular adhesion molecule; VCAM: vascular cell adhesion molecule; VEGF: vascular endothelial growth factor; TNF: tumour necrosis factor α.
factors and cytokines, cell adhesion receptors and genes which regulate apoptosis (Janssen-Heininger et al, 2000).

Although the role of NFκB in inflammatory and cardiovascular disease is well known, a role for NFκB in diabetic complications is less well characterised. However, the recent observations that both glucose and advanced glycation end-products (AGE) stimulate NFκB activation in a variety of cell-types strongly implicate NFκB as a mediator of macrovascular and microvascular complications (Pieper and Riaz-ul-Haq, 1997; Morigi et al, 1998; Yerneni et al, 1999).

NFκB exists as a heterodimer composed of 2 subunits, the most common being p50 (50kDa) and p65 (65kDa). Other family members include c-rel, RelB and p52. Within the cytoplasm it remains inactive due to its interaction with the inhibitory protein, Inhibitory kappa B (IκB-α, β or ε). Several factors have been recognised in activating NFκB such as the inflammatory mediators TNF, interleukin-1 (IL-1), bacterial lipopolysaccharide (LPS), advanced glycation end products (AGEs), and platelet-activating factor. It is also activated by conditions such as hyperglycaemia, shear stress, oxidized lipids, oxidant stress, and hypoxia/reperfusion (de Martin et al, 2000).

Upon activation, NFκB inducing kinase (NIK) is phosphorylated and activates IκB kinases (IKK1/IKKα and IKK2/IKKβ, NEMO/IKKγ) which then phosphorylates IκB at two serine residues, Ser 32 and 36. IκB is subsequently ubiquitinated and degraded by the 26S proteasome (deMartin et al, 2000) (Figure 1.4). MEKK1 (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1), transforming growth factor (TGF)-
**Figure 1.4. NFκB activation.** Mediators and cytokines activate kinases which in turn activate IkB kinases (IKK1, IKK2). These phosphorylate IkBα which dissociates from NFκB and is degraded leaving NFκB free to migrate to the nucleus. IL-1: interleukin-1; LPS: lipopolysaccharide; TNF: tumour necrosis factor α; AGE: advanced glycation end products; PDGF: platelet derived growth factor; oxLDL: oxidised low density lipoprotein; NIK: NFκB inducing kinase; MEKK1: mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1; Akt: protein kinase B; Pi: inorganic phosphate; NEMO: NFκB essential modulator.
β-inducible kinase (TAK1), Akt, and protein kinase C-ζ and θ are also recognised to interact with IKKs in the NFκB signalling pathway (deMartin et al, 2000). NFκB then translocates to the nucleus where it acts to regulate gene transcription (May and Ghosh, 1998).

NFκB activation is transient due to intracellular regulatory mechanisms. The resynthesis of IκBα after phosphorylation results in shuttling of NFκB back to the cytoplasm where it is re-coupled with IκB and inactivated (deMartin et al, 2000).

Hyperglycaemia and AGEs upregulate the expression of adhesion molecules such as ICAM-1 (intercellular adhesion molecule), VCAM-1 (vascular cell adhesion molecule) and E-selectin (Lutty et al, 1997; Barouch et al, 2000) and this is believed to play significant roles in increasing the adhesiveness of the endothelium. As discussed above, the role of NFκB has been implicated (Figure 1.3).

1.5.3 Targeting PKC and NFκB in diabetes

Several therapies have been investigated to reduce diabetic retinopathy but have shown limited success such as aldose reductase inhibitors, protein glycation inhibitors, and growth hormone receptor inhibitors (Frank, 2002).

The available evidence strongly indicates that preventing the activation of PKCβ and NFκB offers an approach to prevent/block diabetic retinopathy. For example, rotterlin inhibits PKCδ but also inhibits other important enzymes, and indolocarbazoles inhibit multiple PKC isozymes and other enzymes, thus limiting their use. PKC412 inhibits PKC
α, β and γ, VEGF, and PDGF and is currently undergoing phase I trials for its anti-tumour effects (Aiello, 2002).

In contrast, studies with PKCβ specific inhibitors show the most promising results in preventing diabetic vascular disease. The PKCβ specific inhibitor, LY333531 (Ruboxistaurin) has been shown to be highly selective for both the βI and βII isozymes with little effect on other enzymes and is currently undergoing phase III clinical trials. In diabetic rats it has been shown to improve retinal blood flow and block the ability of VEGF to increase permeability and proliferation (Aiello, 2002). It is also effective in reducing albuminuria and mesangial expansion through its effect on PKCβ in the diabetic kidney (Kelly et al., 2003) and in improving peripheral nerve conduction and blood flow (Cameron et al., 1999). Phase I studies of LY333531 in patients with type 1 or 2 diabetes showed the drug to be well tolerated with minimal side effects and early results suggest that it is effective in improving retinal blood flow (Aiello, 2002).

NFκB inhibitors which target different levels of the NFκB signalling pathway are currently being investigated. Anti-oxidants and oxygen-free radical scavengers may inhibit NFκB activation but the mechanism of action is not clear (Schreck et al., 1992). Proteasome inhibitors which inhibit IκBα degradation and have anti-inflammatory properties have been shown to inhibit NFκB (Meng et al., 1999). These, however, may become toxic when used for prolonged periods. Inhibitors of IκB kinases are also being studied for their anti-inflammatory activity (Rossi et al., 2000).
Some cardiovascular and anti-inflammatory drugs inhibit the activation of NFκB in addition to their other modes of action. The glucocorticoids which are widely used as anti-inflammatory agents have been shown bind to the RelA (p65) and NFκB1 (p50) subunits of NFκB, thus preventing activation of NFκB (Scheinman et al, 1995). Salicylates inhibit IKK2 (Yin et al, 1998) and atorvastatin (cholesterol lowering agent) stabilises IκBα (Ortego et al, 1999).

Despite several advances in the development of NFκB inhibitors, their use is limited due to the lack of specificity of their actions. In developing clinically useful therapeutic agents to inhibit PKC and NFκB, compounds need to be identified that inhibit the hyperglycaemia induced pathways without affecting other cellular responses.

1.6 Omega-3 polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFA) have become recognised as having important biological roles in the pathogenesis and prevention of diseases, in particular. They are important components of the cell membrane, regulate biochemical activities of the cell and are involved in physiological processes such as lipid metabolism and immune function. Their role in disease processes such as cardiovascular disease and carcinogenesis is also well recognised (Gill and Valivety, 1997).

Supplementation with fish oil (source of omega-3 fatty acids) has been shown to lower triglyceride levels in diabetics, decrease endothelial cell activation, improve endothelial dysfunction, inhibit platelet aggregation, suppress the expression of adhesion molecules, reduce the incidence of coronary heart disease and lower the risk of microalbuminuria (Hu
et al, 2003; Brown and Hu, 2001). One mechanism by which omega-3 fatty acids protect against atherosclerosis is by their ability to inhibit the activation of NFkB (Weber et al, 1995).

Omega-3 fatty acids therefore have a potential role in suppressing inflammatory pathways leading to diabetic vascular disease.

1.7 **Structure and synthesis of fatty acids**

Unsaturated fatty acids are composed of a hydrocarbon chain with a carboxyl group at one end and contain 1 or more double bonds which determine the degree of unsaturation. Polyunsaturated fatty acids contain multiple double bonds. Short chain fatty acids have <six carbon atoms, medium chain have 6-12 carbon atoms, long chain have 14-22 carbon atoms, and very long chain have >22 carbon atoms. One standard nomenclature of fatty acids is dictated by the number of carbon atoms followed by the number of double bonds and the position of the double bond from the ω end of the molecule (Ferrante et al, 1999). Figure 1.5 illustrates the structure and nomenclature of three common fatty acids.

*De novo* synthesis of fatty acids in the human body from acetyl coenzyme A produces fatty acids of up to 16 carbon atoms (palmitate). The carbon chain (16:0) is elongated by a series of steps to form stearate (18:0) which is desaturated to form oleate (18:1n-9). Palmitate (16:0) can also be desaturated to palmitoleate (16:1n-9) and elongated further to 18:1n-9. Further synthesis of essential longer chain fatty acids such as linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) cannot occur in mammalian cells due to absence of specific desaturases required to introduce double bonds beyond carbon-9. These essential fatty acids can be obtained through dietary sources and are required for the synthesis of longer
chain fatty acids which are important in the regulation of cellular processes (Wallis et al, 2002; Ferrante et al, 1999).

Sources of dietary fatty acids include animal meat, green vegetables, fish and oils. Green leafy vegetables and seeds provide the essential fatty acids. Marine oils and fish are rich sources of n-3 PUFAs such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). Animal fats are rich in arachidonic acid (AA, 20:4n-6) (Ferrante et al, 1999). Synthesis of omega-3 and omega-6 fatty acids is shown in Figure 1.6.

1.8 Transport of fatty acids

The absorption and transport of fatty acids is shown diagrammatically in Figure 1.7. Fatty acids of less than 10-12 carbon atoms in length are absorbed directly into the portal blood. Longer chain fatty acids of greater than 10-12 carbon atoms are re-esterified to triglycerides in the mucosa and transported to the tissues by chylomicrons. Chylomicrons are large lipoprotein complexes consisting of triglycerides, phospholipids, cholesterol and proteins that enter the circulation via the lymphatics. Lipoprotein lipase on the capillary endothelium catalyzes the breakdown of triglycerides within the chylomicrons to FFA and glycerol, which enter the adipose cells and are re-esterified (Ganong, 1993).

Very low density lipoproteins (VLDL) are formed in the liver and transport FFA which are synthesized in the liver. FFAs are transported to adipose tissue for storage or to various tissues for cell structure and metabolism. Released FFAs in the circulation are bound to binding proteins, eg albumin and are transported to various tissues (Ferrante et al, 1999).
FFAs bind to a fatty acid transporter protein in the plasma membrane for transport into the target cells. Once within the cell they are transported by a cytosolic fatty acid binding protein and mediate cellular responses (Ferrante et al, 1999). Within the mitochondria, fatty acids undergo β-oxidation where 2 carbon atoms are serially split off the fatty acid generating energy in the form of ATP (Ganong, 1993).

1.9 Metabolism of fatty acids

The n-6 PUFA, arachidonic acid (AA), is metabolised via the cyclooxygenase and lipoxygenase pathways (Figure 1.8). AA is essential to membrane phospholipid structure and its metabolism gives rise to active eicosanoids and prostagnoids which are mediators of inflammation. The cyclooxygenase pathway gives rise to the 2-series prostaglandins (PG) and thromboxanes (TX) which are implicated in the inflammatory processes of rheumatoid arthritis and psoriasis, as well as fever, pain and thrombosis (Simopoulos, 1991).

The metabolism of AA via the lipoxygenase pathway results in the formation of 5-, 12-, and 15-monohydroperoxy-eicosatetraenoic acids (HPETE) by the action of 5-, 12-, and 15-lipoxygenases. HPETE metabolism generates hydroxyeicosatetraenoic acids (HETE) and leukotrienes (LTA₄, LTB₄, LTC₄, LTD₄), some of which are pro-inflammatory and others showing anti-inflammatory activity. For example, LTB₄ activates neutrophils, enhances chemotaxis, and generates superoxide, while the hydroxy product, 15-HETE inhibits LTB₄-induced neutrophil activation (Ferrante et al, 1999).
The metabolites, 15-HETE and 15-HPETE, have been shown to possess greater anti-inflammatory properties than AA. In contrast to AA, these eicosanoids do not stimulate superoxide production and degranulation in neutrophils (Ferrante et al, 1999). They also inhibit adhesion molecule expression in endothelial cells (Huang et al, 1997), and inhibit LPS-induced TNF production in macrophages (Ferrante et al, 1997). Interestingly, 15HPETE has been shown to inhibit the activation of PKC (Ferrante et al, 1997; Huang et al, 1997).

The n-3 PUFAs (eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA) can also be metabolised by the lipoxygenase and cyclooxygenase pathways. They generate eicosanoids with lower inflammatory properties whilst decreasing the production of the pro-inflammatory AA metabolites. Omega-3 fatty acids have been used effectively in treating autoimmune and anti-inflammatory diseases and have been shown to suppress the cytokines TNF, IL-1, IL-2, and platelet activating factor (PAF) (Ferrante et al, 1999).

The use of omega-3 PUFAs as anti-inflammatory agents is limited by their ability to stimulate oxygen radical production in human neutrophils (Ferrante et al, 1996). There is a lack of specificity in their action and therefore other essential pathways are at risk of becoming suppressed leading to adverse effects (Gill and Valivety, 1997). Similarly, it is not feasible to use 15-HPETE therapeutically because of its instability and lack of distinction between PKC isozymes.

The need for more selective activity has led to the development of PUFA mimetics specific in their action against PKC and NFκB, the key intracellular mediators of glucose action
**Figure 1.5 Structure and nomenclature of fatty acids.** Palmitate, a saturated fatty acid has 16 carbon chains and no double bonds. Arachidonic acid has 20 carbon chains, 4 double bonds, with the 1st double bond starting at the 6th carbon from the ω end. DHA (docosahexaenoic acid) has 22 carbon chains with 6 double bonds, with the 1st double bond starting at the 3rd carbon from the ω end.
Figure 1.6 Synthesis of essential omega-6 and omega-3 PUFAs. Essential fatty acids such as linoleate and linolenate are obtained from dietary fats and are elongated and desaturated to form longer chain fatty acids. Key products are shown in bold print. The dotted arrows show metabolism to eicosanoids and esterification into membrane phospholipids. (Ferrante et al., 2004).
Figure 1.7. Absorption and transport of fatty acids. FA: fatty acid, TG: triacylglycerols, VLDL: very low density lipoprotein, FFA: free fatty acid, FATP: fatty acid transport protein, mFABP or cFABP: membrane or cytosolic fatty acid binding protein, PKC: protein kinase C, PLA₂: phospholipase A₂, FA CoA: fatty acyl coenzyme A. (McGarry, 1993)
Figure 1.8. Metabolism of omega-3 and omega-6 fatty acids through the cyclooxygenase and lipoxygenase pathways. PLA2: phospholipase A2, AA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, HDCHA: hydroxydocosahexaenoic acid, HEPE: hydroxyeicosapentaenoic acid, HETE: hydroxyeicosatetraenoic acid, PG: prostaglandin, TX: thromboxane, LT: leukotriene. (Ferrante et al, 1999)
(Koya and King, 1998; Aiello, 2002; Morigi et al, 1998)

1.10 Polyunsaturated fatty acid mimetics

Studies undertaken by our group have shown that the biological activity of fatty acids is related to specific structural elements. This includes the number of carbon atoms, degree of unsaturation, position of double bonds, methylation and esterification of the carboxyl group, and addition of either a hydroxyl or hydroperoxy group to the side chain.

Recently, a group of novel fatty acids was synthesised (Pitt et al, 1997; Pitt et al, 1998) which has similar properties to 15-HPETE but with greater stability. Nearing forty in total, some contain an oxygen or sulphur atom in the β or γ position of the carbon backbone of the PUFA. Others are conjugated with amino acids (Robinson et al, 1999). The third series contain a NO2 group. These novel compounds differ from natural PUFA in conformation and polarity (Figure 1.9). The β-substituted compounds are not readily β oxidized therefore improving their intracellular stability (Pitt et al, 1998).

When the engineered PUFA were examined for biological activity in human umbilical vein endothelial cells (HUVEC) and other cell types, several were found to display a more selective range of actions than their natural counterparts (Robinson et al, 1999; Costabile et al, 2001). One of these, MP5 (β-oxa-21:3n-3), inhibited PMA-stimulated translocation of PKCβ to the particulate fraction in HUVEC, without affecting other isozymes (unpublished results). This effect has also been demonstrated in T-lymphocytes (Costabile et al, 2001) and human mesangial cells (Mascolo, 2002).
Figure 1.9. Structure of engineered polyunsaturated fatty acids. Structures of $\beta$-oxa, $\beta$-thia, $\gamma$-thia, and nitro engineered fatty acids.
Thus, MP5 is a leading compound for blocking the actions of glucose. Data in rats showed that MP5 (100mg/kg) exerted no adverse effects on liver and kidney function and electrolyte levels (Robinson et al, 2003). Incubation of human neutrophils with MP5 also resulted in the accumulation of non-esterified MP5 in cells and the incorporation of MP5 into DAG and phospholipids (Robinson et al, 2003). The ratio of non-esterified MP5 to non-esterified 20:4n-6 reached 5:1.

Another novel PUFA, MP3 (β-oxa-23:4n-6), was more effective than the fish oil-derived docosahexaenoic acid (22:6n-3) at suppressing the upregulation of E-selectin, ICAM-1 and VCAM-1 expression on human umbilical vein endothelial cells (HUVEC) induced by tumour necrosis factor α (TNF), lipopolysaccharide (LPS) or PMA (Ferrante et al, submitted). This is likely to be due to the ability of MP3 to prevent agonist-induced degradation of IκBα and hence the activation of NFκB.

MP3 also suppressed TNF-stimulated adherence of neutrophils to HUVEC (Robinson et al, 1999) and was effective in vivo at suppressing LPS-stimulated upregulation of E-selectin expression in the aorta of mice and prevented the infiltration of leukocytes into sites of inflammation. Despite having these actions, MP3 was devoid of many of the actions of 22:6n-3, including stimulating the neutrophil respiratory burst. Given at 50 mg/kg, MP3 did not cause any observable adverse effects to mice for the duration of the experiments. MP3 does not affect PKC translocation and MP5 has minimal effects on NFκB activation. These factors indicate that MP5 and MP3 have the hallmarks of candidate molecules that may be effective at preventing diabetic retinopathy.
1.11 Significance

The hyperglycaemia-induced vascular complications of diabetes mellitus cause significant morbidity despite recent advances in therapy. Prevention of the progression of vascular disease would improve the quality of life for many affected individuals. This research focuses on the pathogenesis of diabetic retinopathy. Recent novel approaches have been initiated to develop ways to prevent the progression of endothelial cell damage in the retina by targeting pathways which are inappropriately activated by hyperglycaemia.

PKCβ and NFκB have been identified as playing important roles in the pathogenesis of diabetic retinopathy. The development of novel chemically engineered polyunsaturated fatty acid mimetics which target PKCβ and NFκB provides a strategy for developing new types of drugs to treat the disease.

1.12 Hypotheses

The novel polyunsaturated fatty acids MP5 and MP3, target PKCβ and NFκB respectively in bovine retinal endothelial cells and thereby inhibit glucose-induced activation of PKCβ and NFκB.

1.13 Aims

The aims of the present research are to assess in bovine retinal endothelial cells:

1. The effect of hyperglycaemic conditions on PKC isozyme translocation/activation and on NFκB activation.
2. The effect of MP5 and MP3 on high glucose-induced activation of PKC and NFκB.
CHAPTER 2

MATERIALS AND METHODS
2.1 Materials

2.1.1 General biochemicals

Trizma base (tris(Hydroxymethyl)aminomethane), EGTA, EDTA, Triton X-100, Ponceau S, Nonidet-P40, D-glucose, phorbol 12-myristate 13-acetate (PMA), Folin and Ciocalteu’s Phenol Reagent, sodium diatrizoate, DTT (DL-Dithiothreitol, Cleland’s reagent), sigma 104 (phosphatase substrate) were from Sigma-Aldrich, St Louis, MO, USA. Tween-20, sucrose, NaCl, Na₂CO₃ were from Asia Pacific Specialty Chemical Limited, Seven Hills, NSW. β-mercaptoethanol, acetic acid, Na/K-tartrate, NaOH, diethyl ether, hexane, acetic acid, and acetone were from AJAX Finechemicals, Auburn, NSW. CuSO₄ was from BDH Chemicals, Port Fairy, VIC. Dimethyl sulphoxide (DMSO) was from Merck, Darmstadt, Germany. Trypsin-EDTA was from Gibco, Grand Island, NY, USA. HEPES was from Paton Scientific, Stepney, SA.

Acrylamide (N,N’-Methylbisacrylamide 30%), ammonium persulfate (APS), sodium dodecyl sulfate (SDS), TEMED (N,N,N’,N’-Tetramethylethylenediamine) were from Bio-Rad Laboratories, Hercules, CA, USA. Chemiluminescence reagent was from Perkin Elmer Life Sciences, Boston, MA, USA. Western Blot recycling kit was from Alpha Diagnostic International, San Antonio, TX, USA.

Ficoll 400 was from Pharmacia Biotech, Uppsala, Sweden. Angiografin was from Schering, Munich, Germany. Lymphoprep gradient was from Nycomed, Oslo, Norway. ISOTON II was from Coulter electronics, Brookvale, NSW.
Collagenase type I, collagen type VI, and gelatin type B were from Sigma-Aldrich. Collagenase type II, deoxyribonuclease were from Worthington, Lakewood, NJ, USA.

Tumour Necrosis Factor α (TNF) was a gift from Dr. G.R. Adolf (Ernst-Boehringer Ingelheim Institut, Vienna, Austria) and was produced by Genentech, Inc (San Francisco, CA, USA). It had a specific activity of 6×10⁷ U/mg as assessed for cytotoxicity by the supplier on actinomycin-D-treated murine fibrosarcoma cell line (L929), and was >98% pure using the Limulus amoebocyte lysate assay. Lipopolysaccharide (LPS) contamination was < 0.125 LPS U/ml. TNF was stored at a concentration of 5×10⁷ U/ml in 5µL aliquots at -20°C and was prepared fresh daily in HBSS.

2.1.2 Serum, Albumin, Culture media and buffers

Bovine Serum Albumin was from Boehringer Manheim, W Germany. Fetal calf serum (FCS), Hanks balanced salt solution (HBSS), RPMI 1640, L-glutamine (200mM) were from JRH biosciences, Lenexa, KS, USA. Dulbecco’s Minimal Essential Media (DMEM) was from Sigma-Aldrich. Fungizone was from Bristol-Myers Squibb, Noble Pk, VIC. Penicillin (5000U/ml)/streptomycin (5000µg/ml) was from Commonwealth Serum Laboratories (CSL), Parkville, VIC. Phosphate buffered saline (PBS) and medium 199 were from Cell Image, Adelaide, SA.

2.1.3 Protease Inhibitors

Leupeptin, pepstatin A, benzamidine hydrochloride, PMSF (phenylmethylsulfonyl fluoride) were from Sigma-Aldrich. Aprotinin (bovine lung, 100,000U/5ml) was from Calbiochem, La Jolla, CA, USA.
2.1.4 Antibodies and Conjugates

Rabbit anti-human to Von Willebrand Factor, anti-mouse Ig, (HRP conjugated) was from DAKO Corporation, Carpinteria, CA, USA. Anti rabbit Ig (HRP conjugated) was from Chemicon, Boronia, VIC. FITC conjugated rabbit IgG, PKC βI, βII, α, δ, ε, θ antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA, USA. β-actin antibody was from Sigma-Aldrich.

2.1.5 Materials

Nitrocellulose paper (Optitran BA-S83, reinforced NC) was from Schleicher & Schuell, Dassel, Germany. Bio-Rad Minigel Apparatus, and low range molecular markers were from BioRad, Hercules, CA, USA. Tissue culture plates (60x15mm, 100x20mm) were from TPP, Trasadingen, Switzerland. Culture flasks (25cm², 75cm²) were from Corning, NY, USA. Thin Layer Chromatography (TLC) aluminium sheets silica gel was from Merck, Darmstadt, Germany. Sterile nylon wool was from Geneworks, Adelaide, SA.

2.2 Preparation of plasma

Human group AB serum and pooled plasma: Serum was collected from group AB healthy donors or pooled from several donors. It was heat inactivated for 30min at 56°C. Serum was stored at –20°C. Plasma was collected from healthy donors as described in section 2.5 and heat inactivated for 30min at 56°C. It was frozen at –20°C, thawed, then centrifuged at 10,000g for 20min. This process was repeated to remove excess fibrin and plasma was stored in aliquots of 10ml.
2.3 Preparation of culture media for BREC

Various growth factors have been used to sustain long-term culture of bovine retinal endothelial cells. Buzney (1983), showed the effectiveness of sarcoma conditioned media, Gitlin and D’Amore (1983), used retinal crude extract, and Wong et al (1987), used pericyte conditioned media. In this project, C6 glioma cell-conditioned media and retinal crude extract were used to support the growth of these cells as Wong (personal communication) subsequently found these conditions to be optimal for culturing BREC.

Culture flasks were coated with type VI collagen for a minimum of 1h, then the collagen was removed and flasks allowed to air-dry prior to culturing of cells. Endothelial cells seeded onto matrices such as collagen and fibrinogen have been shown to attach and proliferate more readily than those seeded onto laminin, fibronectin and gelatin (Ando et al, 1991).

C6 conditioned media: C6 glioma cells were obtained from American Type Culture Collection (ATCC), Rockville, MD, USA. They were grown in 75cm² culture flasks in 5.5mM DMEM supplemented with 10% plasma, 100U/ml penicillin/100μg/ml streptomycin, and 4mM L-glutamine. The media were collected after 3-4 days, and centrifuged at 1550g for 10min to remove cells. The media were then stored at −20°C to ensure death of any residual C6 glioma cells, and thawed for use in BREC culture media.

Retinal crude extract: After isolation of retinal tissue from fresh cow eyes, the retinas were incubated at room temperature in PBS (1 retina per ml) for 2h. They were centrifuged at
450g for 5min and the supernatant filtered through a 0.2µm filter and used as retinal crude extract.

**BREC culture media:** BREC were grown in culture media containing a combination of 20% FCS (heat inactivated in a 56°C water bath for 30min), 30% DMEM (5.5mM glucose supplemented with antibiotics and 4mM L-glutamine), 10% plasma, 40% C6 conditioned media, and 10µl/ml retinal crude extract.

### 2.4 Preparation of endothelial cells

#### 2.4.1 Primary cell culture of BREC

BREC were prepared from fresh eyes (Lobethal Abattoir, Adelaide, SA) using methods described by Wong *et al* (1987). The eyes were dissected removing excess fat and muscle from the surface and rinsed in ethanol and PBS. They were bisected and the retina removed and rinsed in DMEM/HEPES pH 7.4. Retinas were homogenised 3x at 200-300rpm (Multifix homogeniser, Orpington, Kent, UK) and centrifuged at 700g for 10min. The resultant pellet was resuspended in PBS and filtered through an 83µm filter. Blood vessel fragments were inverted onto collagenase type I (1mg/ml)/deoxyribonuclease (300µg/ml) enzyme mixture and incubated on a rotary shaker for 25min at 37°C until devoid of pericytes (Wong *et al*, 1987). This was then filtered through a 53µm filter and inverted onto DMEM supplemented with 20% FCS. Cells were centrifuged at 450g for 10min, resuspended in culture medium supplemented with fungizone (2.5µg/ml) and transferred to collagen-coated culture flasks. Cells were grown at 37°C in an atmosphere of 5% CO₂ in air and high humidity. Culture media was changed every 2-3 days.
2.4.2 Primary cell culture of HUVEC

HUVECs were prepared using methods described previously (Bates et al, 1993). Human umbilical cords were collected immediately after delivery and stored at 4°C for less than 36h. The umbilical vein was cannulated and washed with HBSS. They were then filled with collagenase type II (0.4mg/ml), clamped and incubated for 2min in a 37°C water bath. The contents of each vein were collected and the vein flushed through with HBSS to collect any remaining cells. Cells were centrifuged at 700g for 5min and the pellet resuspended in RPMI 1640 (supplemented with antibiotics and 4mM L-glutamine) and 20% pooled heat inactivated human group AB serum. Cells were grown in 0.2% gelatin-coated culture flasks at 37°C in an atmosphere of 5% CO₂ in air and high humidity. The medium was changed every 2-3 days.

2.4.3 Determination of BREC and HUVEC purity

BREC were identified by their growth as a monolayer and their spindle shaped appearance, whereas HUVEC display a cobblestone appearance when confluent. Purity was assessed by Von Willebrand Factor staining using methods by Jaffe et al (1973). For this, BREC or HUVEC were grown to confluence on collagen- or gelatin-coated coverslips and fixed with ethanol for 5min at 4°C. Coverslips were incubated for 45min at room temperature with rabbit anti-human Von Willebrand Factor (dilution 1:150 in azide/PBS 1:100), washed 3x with azide/PBS (1:100) before being incubated with FITC-conjugated antirabbit IgG for 45min (dilution 1:50 in azide/PBS 1:100). After washing to remove unbound secondary antibody, Von Willebrand Factor staining was assessed by fluorescent microscopy.
2.4.4 Trypsinisation of cells

For passaging, BREC and HUVEC were trypsinised by incubating for 3-5 min at 37°C in trypsin/EDTA diluted in PBS (1:5), and centrifuged at 560g for 5 min. Cells were resuspended in culture media and plated into matrix-coated tissue culture dishes. Cells were used in experiments up to passage 5.

2.4.5 Cryopreservation and thawing of cells

BREC were frozen and stored in liquid nitrogen at passage 3 for future use. Following trypsinisation and centrifugation of cells, BREC were resuspended in BREC culture media supplemented with 20% plasma and 10% DMSO to a concentration of 1x10⁶ cells/ml and kept on ice. 1.8 ml was transferred into cryovials and placed over liquid nitrogen for 1 h, then placed in liquid nitrogen and stored at -70°C until required.

BREC were thawed by placing cryovials briefly in a 37°C water bath and diluting in 10 ml BREC culture media. Cells were centrifuged for 5 min at 560g and resuspended in culture media and transferred to 75 cm² collagen-coated culture flasks.

2.5 Culture of T lymphocytes

Venous blood was collected from healthy donors and placed into tubes containing 25 IU/ml heparin. Blood (6 ml) was layered onto 4 ml Hypaque-Ficoll media (8% Ficoll 400, sodium diatrizoate and angiograffin, density 1.114) and centrifuged at 560g for 35 min (Ferrante and Thong, 1978). After centrifugation, the leukocytes were resolved into two discrete bands with the mononuclear leukocytes (monocytes, T and B lymphocytes) in the top band and neutrophils in the lower band. Red blood cells were at the bottom of the tube
and plasma on the top. The lymphocyte layer was gently aspirated and washed twice with Medium 199 for 5min at 560g. The viability of the cells was >99%, assessed by their ability to exclude trypan blue.

T lymphocytes were purified using a modification of the method of Zhang et al (1992). Tissue culture plates (10cm) were coated with 3ml autologous plasma for 30min at 37°C in a humidified atmosphere of 5% CO₂ in air. The isolated mononuclear cells were resuspended in 40ml RPMI 1640, supplemented with 20% heat inactivated FCS and 5ml added to each plate. After incubation at 37°C in the CO₂ incubator, monocytes adhered to the plastic tissue culture dishes and were therefore depleted. The plates were washed with 10ml RPMI 1640/10% FCS to remove non-adherent T and B lymphocytes and the media collected and centrifuged for 5min at 560g. The cell pellet was resuspended in 0.8ml RPMI 1640/10% FCS and applied to a 1ml syringe packed with nylon wool which was incubated at 37°C in the CO₂ incubator. (The column had been pre-equilibrated by passing RPMI 1640/10% FCS through the column at 37°C). Ten ml of RPMI 1640/10% FCS was flushed through the column to remove non-adherent T lymphocytes and residual red blood cells were removed by centrifugation over Lymphoprep gradient for 15min at 955g. T lymphocytes were removed and washed twice with serum-free RPMI 1640. The viability of purified T lymphocytes was >99%, assessed by their ability to exclude trypan blue. Cells were resuspended at a density of 4x10⁶ cells/ml.

2.6 Culture of HL 60 cells

HL-60 cells were obtained from American Type Culture Collection (ATCC), Rockville, MD, USA, and maintained in RPMI 1640 supplemented with 10% heat inactivated FCS,
antibiotics and 4mM L-glutamine. They were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air, and kept at a density of <1x10⁶ cells/ml.

For cryopreservation, cells were centrifuged at 560g and resuspended in RPMI 1640/10% FCS/10% DMSO to a concentration of 1x10⁶ cells/ml and kept on ice. 1.8ml was transferred to cryovials and placed over liquid nitrogen for 1h before storage in liquid nitrogen at -70°C. Cells were thawed as described in section 2.4.5.

2.7 PKC isozyme expression in different cell types

BREC and HUVEC were grown in 10cm collagen- or gelatin-coated tissue culture plates until confluent (3 x 10⁶ cells). At confluence, the culture medium was aspirated from the plate and the adhered cells were rinsed with PBS. After removal of PBS, cells were scraped from the plate using a rubber policeman. Samples were disrupted in sonication buffer as described below.

T lymphocytes and HL60 cells (1x10⁷ cells) were centrifuged to remove the media and disrupted in sonication buffer.

Total cell extracts were prepared by sonicating samples in 150ul of 2% TritonX-100/sonication buffer (20mM Tris-HCl, pH 7.4, 5mM EGTA, 2mM EDTA, 2mM dithiothreitol (DTT), 10µg/ml leupeptin, 10µg/ml aprotinin, 10µg/ml pepstatin A, 10mM benzamidine hydrochloride, and 10mM phenylmethylsulfonyl fluoride (PMSF)). Samples were sonicated (3 x 10 sec) at 4°C on setting 7, tune 2 using a Heat Systems sonicator (Lab Supply, Adelaide, SA) and left on ice for 30min to allow PKC to dissociate from the
membranous fraction of the cell. Samples were then centrifuged (100,000g) for 30min at 4°C. The supernatant was collected and protein content determined using the Lowry method. Laemmli buffer (3x strength of 60mM Tris-HCl, pH 6.8, 40% sucrose, 6% SDS, 10mM β-mercaptoethanol) was added to the supernatant in a ratio of Laemmli buffer:sample of 1:2. The samples were then boiled at 100°C for 5min and stored at -20°C. Samples were analysed using Western blotting for the presence of PKC isozymes α, βI, βII, δ, ε and θ, using isozyme specific antibodies (Table 2.1). Identification was facilitated by running brain PKC on the same gel.

2.8 PKC isozyme translocation in BREC

When PKC is activated it translocates from the cytosol to the membranous fraction of the cell (Nishizuka Y, 1995). The amount of PKC on the membranous fraction was determined using Western Blot analysis with PKC isozyme specific antibodies (Hii et al 1995).

BREC were grown in 6cm collagen-coated tissue culture plates in culture media. At 40-50% confluence, cells were treated with DMEM containing either 5.5mM or 25mM glucose and 10% plasma for 3 days until confluent (1x10⁶ cells/plate). Cells were then treated with PMA (100nM) or vehicle (DMSO, 0.1% v/v) for 5min before they were harvested. To each plate was added 300μl of sonication buffer (see above for composition) and the cells scraped and transferred into centrifuge tubes. After sonication (3 x 10 sec) and centrifugation (100,000g x 30min, at 4°C) the pellets were resuspended in sonication buffer (100μl) containing 2% TritonX-100, re-sonicated (3 x 10 secs) and the samples were left to stand on ice for 30min. The samples were centrifuged (100,000g x 30min, 4°C) and the supernatants were collected. An aliquot was used for protein estimation using
the Lowry method. Laemmli buffer was added to the remaining supernatants and the samples were boiled at 100°C for 5min and stored at -20°C until Western blotted.

2.9 IκBα degradation

BREC were grown to confluence (1x10⁶ cells/plate) in 6cm collagen-coated tissue culture plates. After treatment with various concentrations of glucose or TNF for different time periods, cells were lysed. To each plate was added 300μl of lysis buffer (NP40 0.5% v/v, 20mM HEPES pH 7.2, 100mM NaCl, 1mM EDTA, 2mM dithiothreitol (DTT), 10μg/ml leupeptin, 10μg/ml aprotinin, 10μg/ml Sigma 104, 10μg/ml pepstatin A, 10mM benzamidine hydrochloride, and 10mM PMSF) and cells scraped from the plate with a rubber policeman and transferred into eppendorf tubes. Samples were lysed on a rotary shaker at 4°C for 2h, then centrifuged. The supernatant was collected and protein content determined using the Lowry method. Laemmli buffer was added to the remaining supernatant. The samples were then boiled at 100°C for 5min and stored at -20°C until Western blotted for IκBα.

2.10 Lowry’s Protein determination

Protein content of samples was determined using the Lowry method (Harrington, 1990). Protein standards were obtained using serial dilutions of BSA (1mg/ml) with H₂O, resulting in 6 standards, 0μg, 3.125μg, 6.25μg, 12.5μg, and 50μg. Each unknown sample (5μl) was diluted in 45μl of H₂O, then 150μl of CuSO₄/Lowry’s solution (1:100) was added to all tubes and these were left to stand for 20min at room temperature. Lowry’s Solution (2% Na₂CO₃, 1% SDS, 0.4% NaOH, 0.16% Na/K-tartrate. Folin and Ciocalteu’s Phenol Reagent/H₂O (1:1)), 15μl, was then added and the tubes were left to stand for
10min. 180μl of each sample was transferred to a 96 well plate and the light absorption at 560nm was determined in a plate reader (Dynatech MR5000, Guernsey, Channel Islands, UK). The absorbance of the protein standards was plotted on a linear regression nomogram (using Instat: GraphPad Software Incorporated, San Diego, CA, USA) and the protein content of the treatment samples were calculated.

2.11 Western Blotting

Western blotting was used to detect the presence of PKC isozymes or IκBα. Bio-Rad minigel apparatus was used and a 10% SDS-PAGE gel (for PKC) or 12% SDS-PAGE gel (for IκBα) prepared. The samples were boiled for 5min at 100°C and 20μg (PKC) or 40μg (IκBα) protein were loaded to separate lanes. The volume required to load these amounts of protein was calculated using the protein concentration/5μl determined by the protein assay. A SDS-PAGE Low Range marker was loaded to provide a range of molecular weight markers. The gel was run at 200V for approximately 45min until the dye front migrated off the gel.

Protein was transferred onto a nitrocellulose membrane using electrophoresis at 100V for 1.5h. Ponceau S staining (0.1% in 5% acetic acid) was used to confirm equal protein load and even transfer of protein to the membrane. The membrane was blocked for 1h at 37°C in blocking buffer (5% skimmed milk, 25mM Tris/HCl pH 7.4, 100mM NaCl) then incubated with primary antibody (Table 2.1) diluted in Tris/HCl-Tween20 (pH 7.4) for 45min. The membrane was washed (3x5min) in blocking buffer + 0.1% Tween-20 before being incubated for 45min with horse radish peroxidase conjugated secondary antibody (Table 2.1) diluted in blocking buffer. The membrane was washed again (3x5min) with
<table>
<thead>
<tr>
<th>Protein</th>
<th>Raised in</th>
<th>Dilution for primary</th>
<th>Dilution for secondary</th>
<th>Approximate Mr in SDS PAGE (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCα</td>
<td>mouse</td>
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<td>1:2000</td>
<td>82</td>
</tr>
<tr>
<td>PKCβI</td>
<td>rabbit</td>
<td>1:5000</td>
<td>1:2000</td>
<td>82</td>
</tr>
<tr>
<td>PKCβII</td>
<td>rabbit</td>
<td>1:2000</td>
<td>1:2000</td>
<td>82</td>
</tr>
<tr>
<td>PKCδ</td>
<td>rabbit</td>
<td>1:5000</td>
<td>1:2000</td>
<td>76-80</td>
</tr>
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<td>1:2000</td>
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<td>43</td>
</tr>
</tbody>
</table>

Table 2.1. Antibody types and dilutions for primary and secondary antibodies.
blocking buffer. The protein bands were visualised using enhanced chemiluminescence. The relative density of each band was determined using Image Quant software, version 3.3 (Molecular Dynamics, Charlottesville, VA, USA).

Membranes which were probed for IκBα were stripped and reprobed (see 2.12) for β-actin as a protein loading control. Densitometry scanning facilitated the correction of any unequal protein loading and results were expressed as a ratio of IκBα:β-actin signal.

2.12 Western Blot recycling
Following Western blotting, nitrocellulose membranes were stored at -20°C. When required the blots were stripped using a Western blot recycling kit. The membrane was incubated in the stripping solution (1:10) for 10min, then blocked using the supplied blocking solution (1:20) (2x5min). The membrane was then re-probed with primary and secondary antibodies as previously described. Stripping and re-probing was possible up to 3 times.

2.13 Synthesis of Engineered Polyunsaturated Fatty Acids
Engineered polyunsaturated fatty acids were synthesized by a lipid biochemist using the method described by Pitt et al, 1997. This is outlined briefly below.

For synthesis of β-oxa 21:3n-3 (MP5), a solution of linolenyl alcohol (1.06g, 4.01mmol) and rhodium (II) acetate dimer (9mg, 0.5% mol equiv) was stirred in CH₂Cl₂ (15ml) at room temperature under dry nitrogen. A solution of tert-butyl diazoacetate (1.46g, 10.22mmol) in CH₂Cl₂ (5ml) was added dropwise and stirring continued at room
temperature for 2 h. The mixture was concentrated under a stream of dry nitrogen and the residue was purified by flash column chromatography, eluted with hexane/Et₂O (9:1), to afford tert-butyl (Z,Z,Z)-(Octadeca-9,12,15-triencyloxy) acetate. Trifluoroacetic acid (4 ml) was added to afford tert-butyl (Z,Z,Z)-(Octadeca-9,12,15-triencyloxy) acetate (728 mg, 1.92 mmol) in CH₂Cl₂ (10 ml) under N₂. The solution was stirred at room temperature for 2 h. Flash column chromatography on silica was used to purify the crude reaction mixture, eluted with hexane/Et₂O/acetic acid (40:60:2, v/v), affording (Z,Z,Z)-(Octadeca-9,12,15-triencyloxy) acetic acid as a colourless oil.

For synthesis of β-oxa:23:4n-6 (MP3), tert-butyl (all-Z)-(eicosa-5,8,11,14-tetraencyloxy) acetate was used in the above procedure and (all-Z)-(eicosa-5,8,11,14-tetraencyloxy) acetic acid obtained as a colourless oil.

The β-oxa fatty acids were identified using ¹H and ¹³C nuclear magnetic resonance spectroscopy (Pitt et al., 1997). Engineered fatty acid purity was assessed using thin layer chromatography (TLC). 50 μl of a 20 mM stock solution of the fatty acid was spotted onto a silica coated aluminium plate and allowed to dry. The plate was developed in a tank containing diethyl ether:hexane:acetic acid:H₂O (65:35:0.5:0.5, v/v). After the solvent front had travelled 16 cm, the plate was air dried and exposed to iodine vapour until the lipids could be visualised. There was no evidence of decomposition or degradation of the fatty acids.
2.14 Presentation of fatty acids to cells

Stocks of β-oxa 23:4n-6 (MP3) and β-oxa 21:3n-3 (MP5) were prepared in chloroform. Chloroform was evaporated with N₂ and the fatty acids were reconstituted with ethanol (100% purity) to the required concentration (20mM) and stored at -20°C in siliconized glassware.

The fatty acid was diluted in serum-free DMEM to the required concentration (5-30μM). The final concentration of ethanol was 0.01-0.1%. Control cells were treated with equivalent amounts of ethanol. Cells were pre-treated with the fatty acid for 1-2h prior to any other treatment and lysis of cells. After pre-incubation with the fatty acid, cells were maintained in culture media containing 2-10% serum.

2.15 Statistics

Statistics were calculated using Graph Pad InStat V2.02 (Graph Pad Software). Difference between treatments were compared to control values which were set at 100%, using a one-sample t test with a hypothetical mean of 100. In some experiments, differences were analysed by analysis of variance (ANOVA). Where appropriate, paired t-tests were also used. Results are considered statistically significant when p<0.05.
CHAPTER 3

PROTEIN KINASE C EXPRESSION

IN BOVINE RETINAL ENDOTHELIAL CELLS
3.1 Introduction

Diabetic retinopathy is the result of several different processes amongst which is the activation of endothelial cells of retinal capillaries (Koya and King, 1998; Ways and Sheetz, 2001). Endothelial cell activation most likely occurs through hyperglycaemia-induced activation of specific PKC isozymes (Park et al, 2000). This pathogenic process results from the production of diacylglycerol (DAG), through de novo synthesis, which then activates PKC (see Chapter 1.5.1). Activation also occurs through the NFκB pathway with increased adhesiveness of the endothelium which promotes binding of leukocytes and neovascularization.

There is past evidence that natural polyunsaturated fatty acids (PUFAs) or their products target PKC and NFκB and evidence that these intracellular signaling molecules can also be targeted by novel engineered PUFAs in a more selective manner (Robinson et al, 1999; Costabile et al, 2001). This will be discussed in more detail in later chapters which study the effects of engineered PUFAs on PKC and NFκB activation.

In this thesis, bovine retinal endothelial cells (BREC) were used as a model to examine the ability of novel polyunsaturated fatty acids to target PKC activation under hyperglycaemic conditions. It is known that different cell types express a different spectrum of PKC isozymes. Thus it was essential to firstly establish which PKC isozymes are expressed in BREC and the ability of classical agonists such as PMA to translocate (ie activate) these isozymes from the cytosol to the membrane. Comparisons of PKC isozyme expression were made with different cell types, ie, human umbilical vein endothelial cells (HUVEC), human T lymphocytes and HL60 cells.
3.2 Isolation of BREC

Bovine retinal endothelial cells were prepared from fresh bovine eyes using methods modified from Wong et al (1987). Cell culture and culture media preparation are described in Chapter 2. Isolated retinal endothelial cells were grown in collagen-coated culture flasks at 37°C with 5% CO₂ in air and high humidity in BREC culture media which was changed every 2-3 days. Stocks were stored for future use at −70°C and cells were used in experiments up to passage 5.

BREC were recognized by their characteristic spindle-shaped appearance and their growth as a monolayer (Figure 3.1). Von Willebrand Factor fluorescent staining was used as confirmation that the preparations were endothelial cells and cells were shown to be >95% pure and devoid of pericytes which do not stain positively for Von Willebrand Factor (Figure 3.2).

3.3 PKC isozyme expression in endothelial cells

BREC and HUVEC were cultured in 10cm tissue culture plates for approximately 3 days in culture medium. After adherence to the collagen-coated plates (BREC) and gelatin-coated plates (HUVEC) and reaching confluence (3×10⁶ cells), the media was aspirated and the cells were rinsed with PBS. Cells were scraped from the plate using a rubber policeman. Total cell extracts were prepared by sonicating in sonication buffer containing 2% Triton-X100 to extract PKC from the particulate fraction. The soluble fraction was analyzed by Western blotting for the presence of PKC isozymes. This was partly based on the availability of antibodies to these isozymes and analyses were conducted for PKC α,
Figure 3.1. Monolayer of bovine retinal endothelial cells prepared by primary cell culture at the 4th passage.
Figure 3.2. Fluorescent antibody staining with Von Willebrand Factor antibody.

Endothelial cells shown to be $>95\%$ pure.
βI, βII, δ, and ε. PKC θ was not assessed as this isozyme is known to be expressed mainly in haemopoietic and skeletal muscle cells.

The expression of PKC isozymes in BREC are shown in Figure 3.3. The data show that PKC α, βI, δ, and ε are expressed in BREC shown by the dense bands. In comparison, there was no evidence of PKC βII expression in these cell preparations.

Figure 3.4 shows the expression of PKC isozymes in HUVEC. The data demonstrate that HUVEC express PKC α, βI, βII, δ, and ε shown by the dense bands on Western blotting.

### 3.4 PKC isozyme expression in human T lymphocytes

Venous blood was collected from healthy donors and mononuclear cells were obtained by density gradient centrifugation as described in Chapter 2. Mononuclear cells were isolated and T lymphocytes purified by adherence to nylon wool. The cells were cultured in RPMI1640 supplemented with 10%FCS and antibiotics to a concentration of 4x10^6 cells/ml, then sonicated in buffer containing and 2% Triton-X100. The PKC expression in these preparations was determined by Western blotting.

The results presented in Figure 3.5 show the presence of PKC α, βI, βII, δ, ε, and θ in T-lymphocytes as demonstrated by dense bands on Western blotting.

### 3.5. PKC isozyme expression in myeloid HL60 cells

HL60 cells were cultured in tissue culture flasks in RPMI1640 supplemented with 10% FCS and antibiotics. The cells were maintained at <1x10^6 cells/ml. The cells (1x 10^7) were
Figure 3.3. Expression of PKC isozymes in bovine retinal endothelial cells (BREC). BRECs were grown to confluence (3 x 10^6 cells) in tissue culture plates and sonicated in buffer containing 2% Triton-X100 to extract PKC from the particulate fraction of the cells. The presence of PKC isozymes in BREC was analysed by Western blotting and stained with antibodies for PKC α, βI, βII, ε and δ. Representative bands derived from 3 separate experiments are shown. Each antibody is specific for the intended isozyme and the isozymes are identified by their Mr in SDS gels (Table 2.1) and their alignment with brain PKC. Each blot was stripped and reprobed with a different antibody for up to 2 times in a random order. This applies to all the blots shown in this thesis.
Figure 3.4. Expression of PKC isozymes in human umbilical vein endothelial cells (HUVEC). HUVECs were grown to confluence (3 x 10^6 cells) in tissue culture plates and sonicated in buffer containing 2% Triton-X100 to extract PKC from the particulate fraction of the cells. The presence of PKC isozymes was analysed by Western blotting and stained with antibodies for PKC α, βI, βII, ε and δ. Representative bands derived from 3 separate experiments are shown.
Figure 3.5. Expression of PKC isozymes in human T lymphocytes. Human T lymphocytes were prepared from healthy donors and adjusted to a concentration of $4 \times 10^6$ cells/ml. Cells ($1 \times 10^7$) were sonicated and the soluble fractions were analysed for the expression of PKC $\alpha$, $\beta$I, $\beta$II, $\epsilon$, $\theta$ and $\delta$ by Western blotting. Representative bands derived from 3 separate experiments are shown.
Figure 3.6. Expression of PKC isozymes in HL60 cells. HL60 cells were cultured at a density of \(<1 \times 10^6\) cells/ml. Cells \((1 \times 10^7)\) were sonicated and soluble fractions were analysed for the presence of PKC \(\alpha, \beta I, \beta II, \varepsilon, \theta\) and \(\delta\) by Western blotting. Representative bands derived from 3 separate experiments are shown.
sonicated in buffer containing 2% Triton-X100 and the expression of PKC α, βI, βII, δ, ε and θ was determined by Western blotting.

The results show that HL60 cells express PKC α, βII, δ, ε and θ (Figure 3.6). PKCβI was not detected.

### 3.6 Summary

PKC isozyme expression varies in different cell types which could account for differences in cellular responses to PKC activation. BREC were found to express PKC α, βI, δ and ε whereas HUVECs contain PKC α, βI, βII, δ and ε. T lymphocytes express the greatest number of PKC isozymes, including PKC α, βI, βII, δ, ε, and θ. HL60 cells express PKC α, βII, δ, ε, and θ.

The results presented in this chapter contrast with that of Park et al (2000) who reported that PKC βII was also expressed in BREC. In our studies, cell type and purity were confirmed by morphology and Von Willebrand factor staining, and our methods and reagents were validated by the demonstration that PKCβII could readily be detected in HUVEC, T cells and HL60 cells. These results imply that PKCβII is not expressed in BREC. It is possible that previous studies (Park et al, 2000) have had their endothelial cell cultures contaminated by pericytes which are known to contain PKCβII. Further work using bovine retinal endothelial cells as a model of diabetes must therefore focus on PKC isozymes α, βI, δ, and ε rather than PKC βII.
CHAPTER 4

INHIBITION OF

PROTEIN KINASE C ACTIVATION

IN BOVINE RETINAL ENDOTHELIAL CELLS

BY β-OXA 21:3n-3
4.1 Introduction

There has been a very limited amount of information on the effects of glucose on PKC activation in retinal endothelial cells and previous findings have not been independently confirmed. Although glucose concentrations of 20mM have been shown by Lee et al. (1989) to increase PKC activity in BREC, this study did not investigate which PKC isozymes were involved. Park et al. (2000) reported an elevation of PKC δ>βII>α>βI by hyperglycaemia in BREC. However, as discussed in the previous chapter, there is some doubt as to whether their cultures were contaminated by pericytes. The purpose of this chapter is to attempt to confirm the effect of glucose on PKC isozyme translocation/activation. The isozymes of interest are PKC α, βI, δ and ε as these have been shown in Chapter 3 to be expressed in BREC.

The main objective of this research was to examine the effect of the engineered PUFA, β-oxa 21:3n-3 (MP5) on PKC activation in BREC. MP5 has been shown to selectively inhibit PKCβ activation in other cells types such as T-lymphocytes (Costabile et al., 2001), supporting its potential therapeutic role in preventing vascular complications of diabetes.

4.2 Activation of PKC by PMA

The translocation of PKC from the cytosol to the cell membrane equates to PKC activation (Nishizuka, 1995). Thus, a convenient way to study PKC activation is to determine the degree of PKC translocation to the particulate fraction (containing cell membranes). To ensure that our experiments were conducted under optimal conditions for PKC translocation, 100nM PMA, a classical activator of PKC, was used to standardise the experimental conditions.
BREC were isolated from bovine eye retinal capillaries and cultured in tissue culture plates as described in Materials and Methods. They were grown to confluence (1 x 10^6 cells) and treated with either 100nM of PMA or the equivalent amount of diluent for 5 min at 37°C. The cells were detached from the plate and sonicated. The particulate (membranous) fraction was isolated from the soluble fraction by centrifugation at 100,000g for 30 min. PKC was extracted from the membranous fraction by sonication in 2% Triton-X100 and after centrifugation, the soluble fraction was subjected to Western blot analyses using a panel of PKC isozyme specific antibodies. The density of each band was quantified by Image Quant software (version 3.3).

PKC isozyme translocation is shown in Figures 4.1-4.4. Treatment of BREC with 100nM PMA for 5 min resulted in significant increases in PKC α (249 ± 22.3%, p < 0.05) and PKC βI (203 ± 21.5%, p < 0.01) in the particulate fraction. There was a modest increase in PKC δ (174 ± 29.3%, p = 0.054) and PKC ε (167 ± 38%, p = 0.2) but this was not found to be statistically significant from control. In all these blots, a certain amount of particulate fraction-associated PKC is seen in unstimulated BREC. This may reflect basal levels of PKC activation by serum in the medium.

4.3 Activation of PKC by hyperglycaemic conditions

BREC were grown in tissue culture plates (1 x 10^6 cells) and treated with high (25mM) glucose for 3 days and compared with low (5.5mM) glucose as a control (physiological glucose levels). Cells were scraped from the plate and sonicated. The membranous fraction was isolated and PKC extracted from the membranous fraction by sonication in 2% Triton-X100. Western blotting was used for detection of PKC α, βI, δ, and ε. The density of each
Figure 4.1 Translocation of PKC α in PMA-activated BREC. BREC were grown in tissue culture plates to confluence (1x10⁶ cells) supplemented with DMEM/plasma. After incubation with 100nM PMA for 5 min, cells were sonicated and PKC was extracted from the particulate fraction by sonication in the presence of 2% Triton-X100. The amount of PKCα in the particulate fraction was determined by Western blotting and a representative blot is shown. The densities of the bands were quantified with Image Quant software and are expressed as percentage of control (100%). The data are expressed as mean ± SEM derived from 3 separate experiments (*p<0.05).
Figure 4.2 Translocation of PKC βI in PMA-activated BREC. BREC were grown in tissue culture plates to confluence (1x10⁶ cells) supplemented with DMEM/plasma. After incubation with 100nM PMA for 5 min, cells were sonicated and PKC was extracted from the particulate fraction by sonication in the presence of 2% Triton-X100. The amount of PKCβI in the particulate fraction was determined by Western blotting and a representative blot is shown. The densities of the bands were quantified with Image Quant software and are expressed as percentage of control (100%). The data are expressed as mean ± SEM derived from 5 separate experiments. (*p<0.01)
Figure 4.3 Translocation of PKC δ in PMA-activated BREC. BREC were grown in tissue culture plates to confluence (1x10^6 cells) supplemented with DMEM/plasma. After incubation with 100nM PMA for 5 min, cells were sonicated and PKC was extracted from the particulate fraction by sonication in the presence of 2% Triton-X100. The amount of PKCδ in the particulate fraction was determined by Western blotting and a representative blot is shown. The densities of the bands were quantified with Image Quant software and are expressed as percentage of control (100%). The data are expressed as mean ± SEM derived from 6 separate experiments.
Figure 4.4 Translocation of PKCe in PMA-activated BREC. BREC were grown in tissue culture plates to confluence (1x10^6 cells) supplemented with DMEM/plasma. After incubation with 100nM PMA for 5 min, cells were sonicated and PKC was extracted from the particulate fraction by sonication in the presence of 2% Triton-X100. The amount of PKCe in the particulate fraction was determined by Western blotting and a representative blot is shown. The densities of the bands were quantified with Image Quant software and are expressed as percentage of control (100%). The data are expressed as mean ± SEM derived from 3 separate experiments.
band was quantified by Image Quant software (version 3.3).

Figure 4.5 shows that there is a 70% decrease in particulate fraction-associated PKCα after treatment with high (25mM) glucose for 3 days. In contrast, there is a significant increase in PKCβI translocation (201±27.8%, p<0.05) and PKC δ translocation (155±12.3%, p<0.05) after treatment with high glucose for 3 days (Figure 4.6, 4.7). PKC ε (Figure 4.8) translocation in response to high glucose was not significantly increased (133±13.5%, p=0.13).

4.4 Inhibition of PKCβI by β-oxa 21:3n-3 (MP5)

MP5 was reconstituted in 100% ethanol to a concentration of 20mM and diluted in serum-free DMEM to a concentration of 30μM. BREC were pre-treated with 30μM MP5 for 2h before exposure to high glucose (DMEM 25mM/2% plasma) for 3 days. Control samples were pre-treated with equivalent amounts of vehicle (ethanol) and maintained in high glucose media (DMEM 25mM/2% plasma). At confluence (1 x 10⁶ cells) cells were scraped from the plate with a rubber policeman and sonicated. The amount of particulate fraction-associated PKC was analysed by Western blotting. The density of each band was quantified.

Figure 4.9 shows that 30μM MP5 significantly inhibited the activation of PKCβI (37±11.8% reduction). No significant effects of MP5 were seen with respect to PKC α, δ, or ε.
Figure 4.5 Effect of high glucose on PKCα translocation in BREC. BREC were grown in tissue culture plates to confluence and treated with normal or high glucose DMEM/plasma for 3 days. Cells were sonicated and PKC was extracted from the particulate fraction by sonication in the presence of 2% Triton-X100. The amount of PKCα in the particulate fraction was determined by Western blotting and a representative blot is shown. The densities of the bands were quantified with Image Quant software and are expressed as percentage of control (100%). The data are expressed as mean ± SEM derived from 3 separate experiments. (*p<0.05)
Figure 4.6 Effect of high glucose on PKCβI translocation in BREC. BREC were grown in tissue culture plates to confluence and treated with normal or high glucose DMEM/plasma for 3 days. Cells were sonicated and PKC was extracted from the particulate fraction by sonication in the presence of 2% Triton-X100. The amount of PKCβI in the particulate fraction was determined by Western blotting and a representative blot is shown. The densities of the bands were quantified with Image Quant software and are expressed as percentage of control (100%). The data are expressed as mean ± SEM derived from 5 separate experiments. (*p<0.05)
Figure 4.7 Effect of high glucose on PKCδ translocation in BREC. BREC were grown in tissue culture plates to confluence and treated with normal or high glucose DMEM/plasma for 3 days. Cells were sonicated and PKC was extracted from the particulate fraction by sonication in the presence of 2% Triton-X100. The amount of PKCδ in the particulate fraction was determined by Western blotting and a representative blot is shown. The densities of the bands were quantified with Image Quant software and are expressed as percentage of control (100%). The data are expressed as mean ± SEM derived from 5 separate experiments. (*p<0.05)
Figure 4.8 Effect of high glucose on PKCε translocation in BREC. BREC were grown in tissue culture plates to confluence and treated with normal or high glucose DMEM/plasma for 3 days. Cells were sonicated and PKC was extracted from the particulate fraction by sonication in the presence of 2% Triton-X100. The amount of PKCε in the particulate fraction was determined by Western blotting and a representative blot is shown. The densities of the bands were quantified with Image Quant software and are expressed as percentage of control (100%). The data is expressed as mean ± SEM derived from 3 separate experiments.
Figure 4.9 Effect of MP5 on PKC βI activation. BREC were pre-treated with 30μM MP5 for 2h before exposure to high glucose (25mM/2%plasma) for 3 days and compared with control samples (25mM/2%plasma). At confluence (1x10^6 cells) cells were sonicated and PKC was extracted from the particulate fraction by sonication in the presence of 2% Triton-X100. The amount of PKCβI in the particulate fraction was determined by Western blotting and a representative blot is shown. The densities of the bands were quantified with Image Quant software and are expressed as percentage of control (100%). The data are expressed as mean±SEM derived from 3 separate experiments (*p<0.05)
4.5 Summary

PKC isozymes vary in their responsiveness to activation by PMA, a known activator. In BREC, PKC $\alpha$ appeared to be the most responsive to activation by PMA followed by PKC $\beta$I. PKC $\delta$ and $\varepsilon$ showed a trend towards activation but this was not statistically significant. Results show that PKC $\beta$I and $\delta$ are activated by hyperglycaemic conditions in BREC, demonstrated by an increase in PKC translocation after 3 days of treatment with 25mM glucose. PKC $\beta$I showed the greatest increase in activation, supporting its role as the main mediator of the effects of hyperglycaemia in these cells. PKC $\alpha$ and $\varepsilon$ were not activated by hyperglycaemic conditions in these cells.

Pre-exposure with $\beta$-oxa21:3n-3 (MP5) inhibited glucose-induced PKC$\beta$I activation in BREC. No significant effects of MP5 were seen with respect to PKC $\alpha$, $\delta$ or $\varepsilon$. This is consistent with previous results in mesangial cells where activation of PKC$\beta$I but not the other isozymes was inhibited by MP5 (Mascolo, 2002).
CHAPTER 5

INHIBITION OF
NUCLEAR FACTOR κB ACTIVATION
IN BOVINE RETINAL ENDOTHELIAL CELLS

BY β-OXA 23:4n-6
5.1 Introduction

Activation of the transcriptional factor, NFκB, is now recognised to be important in the processes leading to endothelial dysfunction. NFκB can be activated by both endogenous and exogenous mediators of inflammation including TNF, IL-1, LPS, AGE, and platelet-activating factor. It is also activated by conditions such as hyperglycaemia, oxidized lipids, oxidant stress and hypoxia/reperfusion (de Martin et al, 2000).

NFκB is associated with IκBα, β, or ε in its inactive state. Upon activation, IκB kinases are activated which phosphorylate IκBα. Phosphorylated IκBα dissociates from NFκB and is degraded in the cytosol (de Martin et al, 2000). NFκB is thus free to migrate to the nucleus where it exerts its action. Therefore, loss of IκBα corresponds to NFκB activation.

Activation of NFκB leads to the upregulation of cell adhesion molecules such as ICAM-1, VCAM-1 and E-selectin (Lutty et al, 1997). These changes lead to increased adhesiveness of the endothelium which promotes binding of leukocytes including neutrophils to the endothelium of choroidal and retinal capillaries. This, together with the observation that in diabetics, neutrophils entering the microvessels can become trapped within the vessels has been proposed to contribute to increased capillary dropout and blood vessel occlusion which are associated with pathological changes seen in diabetic retinopathy (Gardner et al, 2000; Lutty et al, 1997).

There is little known about NFκB activation in bovine retinal endothelial cells. We thus examined the effect of hyperglycaemic conditions on the IKK-NFκB system.
5.2 Activation of NFκB by hyperglycaemic conditions

BREC were grown in 6cm culture plates and treated with high (25mM) glucose for various time periods (10-30min, 1-4h, 3 days). This was compared to low (5.5mM) glucose as a control (physiological glucose level). At the appropriate time periods, the cells were lysed, the cytosol collected and analysed for IκBα levels by Western blotting. Assessing the loss of IκBα is a convenient and validated assay method for NFκB activation (Kim et al, 2004).

BREC incubated for 10 to 30min in the presence of 25mM glucose did not show any increase in IκBα degradation (data not shown). Similarly, when the incubation period with high glucose was increased to 4h, the level of IκBα was not significantly affected (Figure 5.1) (p>0.05). When the incubation period was further increased to 3 days, no significant change in IκBα levels were observed (Figure 5.2) (p>0.05). These results therefore demonstrate that high glucose concentrations alone do not activate NFκB.

5.3 Activation of NFκB by TNF in the presence of high ambient glucose levels

TNF has been shown to be involved in the pathogenesis of diabetic nephropathy (Nakamura et al, 1993). It was therefore of interest to examine the effect of TNF on NFκB activation in the presence of high glucose. To determine the optimum concentration of TNF which would activate NFκB, BREC were grown to confluence (1x10^6 cells) under low (5.5mM) or high (25mM) glucose conditions for 3 days. They were then exposed to 0, 1, 10 and 1000U/ml TNF for 15min. Cells were lysed and analysed by Western blotting for IκBα levels.
The results presented in Figure 5.3 show that under low glucose conditions, 1U/ml and 10U/ml TNF did not alter IkBα levels significantly but 1000U/ml caused a significant reduction (65%) indicating NFκB activation. Similar results were seen under high glucose conditions (Figure 5.4) with a greater reduction of IkBα levels (96%) than at 5.5mM glucose after treatment with 1000U/ml TNF (p<0.05). These data demonstrate that BREC respond to TNF in a manner similar to other cell types, including HUVEC (Ferrante et al, submitted) in that TNF caused the degradation of IkBα. The data also suggest that the inability of hyperglycaemic conditions to reduce the intracellular levels of IkBα (Figures 5.1 and 5.2) was not due to a technical problem. In fact, high ambient glucose levels amplified the effect of TNF.

To assess the optimum treatment time for TNF-induced activation of NFκB, BREC were cultured in 6cm culture plates until confluent (1x10⁶ cells). They were treated with TNF (1000U/ml), for 0, 5, 10, 15 and 30min under low (5.5mM) and high (25mM) glucose conditions for 3 days. Cells were lysed, the cytosol collected and analysed for the amount of IkBα by Western blotting.

Figure 5.5 shows that under low glucose conditions a dense band is seen in unstimulated BREC (0 time) indicating the presence of IkBα bound to NFκB in the cytosol. Treatment with 1000U/ml TNF caused a time dependent loss of IkBα which was reduced by 64.3±2.8% at 10min, 82.8±4.4% at 15min, with some recovery of levels by 30min (67.7±14.3% reduction). This reversal has also been seen previously in TNF-stimulated cells (Miller, 2000; Chen et al, 2000).
Figure 5.1. **Effect of hyperglycaemic conditions on IκBα degradation in short term culture.** BREC were grown to confluence and treated with 25mM glucose for 1, 2, 3, and 4h and compared to 5.5mM glucose as a control. Cells were lysed and lysates analysed by Western blotting for IκBα. The membrane was stripped and re-probed with β-actin. The density of each band was quantified and the ratio of IκBα:β-actin calculated. The ratios are expressed as percentage of control (100%). Representative blots are shown. The data are expressed as mean ± SEM of 3 experiments.
Figure 5.2. Effect of hyperglycaemic conditions on IκBα degradation in long term culture. BREC were grown to confluence and treated with 5.5mM or 25mM glucose for 3 days. Cells were lysed and lysates analysed by Western blotting for IκBα. The membrane was stripped and re-probed with β-actin. The density of each band was quantified and the ratio of IκBα:β-actin calculated. The ratios are expressed as percentage of control (100%). Representative blots are shown. The data are expressed as mean ± SEM of 7 experiments.
Figure 5.3. Concentration effect of TNFα on IκBα degradation under low (5.5mM) glucose conditions. BREC were grown to confluence in low glucose DMEM/10%plasma and treated with increasing concentrations of TNF (0, 1, 10, 1000U/ml) for 15min. Cytoplasmic extracts were analysed using Western blotting for IκBα. The membrane was stripped and re-probed with β-actin. The density of each band was quantified and the ratio of IκBα:β-actin calculated. The ratios are expressed as percentage of control (100%). Representative blots are shown. The data are expressed as mean ± SEM of 3 experiments. (*p<0.01)
**Figure 5.4. Concentration effect of TNF on IκBα degradation under high (25mM) glucose conditions.** BREC were exposed to 25mM glucose for 3 days until confluent then treated with increasing concentrations of TNF (0, 1, 10, 1000U/ml) for 15min. Cytoplasmic extracts were analysed using Western blotting for IκBα. The membrane was stripped and re-probed with β-actin. The density of each band was quantified and the ratio of IκBα:β-actin calculated. The ratios are expressed as percentage of control (100%). Representative blots are shown. The data are expressed as mean ± SEM of 3 experiments. (*p<0.001)
TNF exposure under hyperosmolar conditions (25mM glucose) showed a similar effect with a time dependent loss of IκBα (Figure 5.6). Density scanning showed a 61.5±7% reduction at 10min, a 95.7±0.7% reduction at 15min, with less recovery of IκBα levels by 30min (82.8±0.95% reduction) when compared to low glucose conditions. The loss of IκBα was greater at 15min than at 10min under low and high glucose conditions (p<0.01). This again demonstrates that high ambient glucose amplifies the effect of TNF on the NFKB pathway.

5.4 Inhibition of NFKB activation by β-oxa 23:4n-6 (MP3)

BREC were grown to confluence (1x10⁶ cells) and exposed to DMEM containing 25mM glucose and 10% plasma for 3 days. MP3 was reconstituted in 100% ethanol to a concentration of 20mM and diluted in serum-free DMEM to concentrations of 5, 10, 15 and 20μM. BREC were rinsed twice in serum-free DMEM and pre-treated with increasing concentrations of MP3 for 2h before exposure to 1000U/ml TNF for 10-15min. Control samples were pre-treated with equivalent amounts of vehicle (ethanol, maximum of 0.1%, v/v). Cells were scraped from the plate with a rubber policeman and lysed, the cytosol collected and analysed for IκBα levels by Western blotting.

Figure 5.7 shows the results from 3 individual experiments. MP3 dose-dependently inhibited TNF-induced IκBα degradation. Consistent with the above data, the effect of TNF per se was greater at 15min than at 10min.
Figure 5.5. Time course for TNF-induced IκBα degradation under low (5.5mM) glucose conditions. BREC were grown to confluence in 5.5mM glucose and treated with 1000U/ml TNF for 0, 5, 10, 15, 30min. The cells were then lysed and analysed by Western blotting for IκBα. The membrane was stripped and re-probed with β-actin. The density of each band was quantified and the ratio of IκBα:β-actin calculated. The ratios are expressed as percentage of control (100%). Representative blots are shown. The data are expressed as mean ± SEM of 2 (5 and 30min) to 5 experiments. Significance of difference between control and treated cells: p<0.0001 (ANOVA).
Figure 5.6. Time course for TNF-induced IκBα degradation under high (25mM) glucose conditions. BREC were exposed to 25mM glucose for 3 days until confluent and treated with 1000U/ml TNF for 0, 5, 10, 15, 30min. Cells were lysed and analysed by Western blotting for IκBα. The membrane was stripped and re-probed with β-actin. The density of each band was quantified and the ratio of IκBα:β-actin calculated. The ratios are expressed as percentage of control (100%). Representative blots are shown. The data are expressed as mean ± SEM of 2 (5 and 30min) to 5 experiments. Significance of difference between control and treated cells: p<0.0001 (ANOVA).
Figure 5.7. Suppression of TNF induced IκBα degradation by MP3. BREC were exposed to 25mM glucose for 3 days until confluent (1x10^6 cells). Cells were treated with increasing concentrations of MP3 for 2h before exposure to TNF 1000U/ml. Cells were lysed and cytoplasmic extracts analysed for IκBα levels by Western blotting. Blots were stripped and re-probed with β-actin. The density of each band was quantified and expressed as IκBα:β-actin as a percentage of control (100%). Results of 3 separate experiments are shown.
5.5 Summary

High glucose alone does not activate NFκB in BREC, demonstrated by the lack of IκBα degradation after treatment of cells from 10min to 3 days with 25mM glucose. These results support those of Romeo et al (2002) who showed that exposure of BREC to 25mM glucose for 2h to 1 week did not activate NFκB.

The results shown in this chapter characterize the effects of TNF on NFκB activation in bovine retinal endothelial cells. Stimulation of cells for 10-30min with 1000U/ml TNF under low and high glucose conditions results in a reduction of IκBα in the cytosol, ie NFκB activation. Under high glucose conditions there is a greater reduction of IκBα levels at 15min and a slower recovery of levels than under low glucose conditions. This suggests that TNF caused a greater and more persistent activation of NFκB under high glucose conditions than at low glucose conditions.

The β-oxa PUFA, MP3 (β-oxa 23:4n-6), suppressed the TNF-induced activation of NFκB under high glucose conditions in bovine retinal endothelial cells. A concentration effect is demonstrated with 15μM and 20μM of MP3 producing the greatest inhibitory effects of IκBα degradation.
CHAPTER 6

DISCUSSION
6.1 PKC activation in BREC by exposure to hyperglycaemic conditions

In contrast to Park et al (2000) who demonstrated the presence of PKC α, βI, βII, δ, ε, and ζ, in BREC, we were unable to detect the presence of PKC βII (PKC ζ was not assessed). Under the same assay conditions we were able to demonstrate the presence of PKC βII in HUVEC which are known to express PKC βII. This demonstrates that the inability to detect βII in BREC was not due to inappropriate assay conditions for this PKC isozyme.

The isolation of endothelial cells rather than pericytes was confirmed by morphology under light microscopy and staining with antibody to Von Willebrand factor. Endothelial cells are spindle-shaped in appearance and will maintain their growth as a monolayer even when post-confluent. They can be identified by fluorescent staining with Von Willebrand factor antibody. In contrast, pericytes are more polygonal in shape and post-confluent cultures show overlapping and piling up of cells. These cells are Von Willebrand factor negative (Wong et al, 1987).

It has been difficult to maintain BREC in long term cultures and studies performed to achieve optimal cell growth have used sarcoma cell-conditioned media (Buzney et al, 1983), pericyte conditioned media (Wong et al, 1987), and retinal crude extract with platelet-poor plasma (Gitlin and D'Amore, 1983). The use of various matrices such as collagen and fibrinogen to enhance growth of these adherent cells has also been demonstrated (Ando et al, 1991). A study by Capetandes and Gerritsen (1990) demonstrated that using fibronectin and hyaluronic acid as a growth matrix improved the selectivity of endothelial cell growth over pericytes, whereas gelatin-coated or uncoated plastic supported pericyte growth over endothelial cells.
Techniques for primary culture described by Wong et al (1987), compared growth rates of BREC using fresh media (FM) and pericyte conditioned media (PCM). Cells grown in FM rarely reached confluence and were contaminated by a large number of pericytes. A combination of FM and PCM (1:1) resulted in attachment of cells by two days, and confluence reached by 5-6 days. The addition of retinal crude extract also enhanced the growth rate of these cells. By the 4th or 5th passage, BREC become more cuboidal in appearance rather than spindle-shaped though still staining for Von Willebrand Factor up to the 5th passage. By the 7th or 8th passage, senescent cells were seen displaying enlargement and pleomorphism.

Pericyte contamination of endothelial cell cultures is common and techniques to minimize this include the use of optimal culture media and various matrices as described above. In addition, methods for primary culture of pericytes do not differ significantly from isolation of BREC. Enzyme digestion for 15-20min is used for pericyte isolation and 20-30min of enzyme digestion is used for BREC isolation, at which time capillaries are devoid of pericytes by at least 50% (Wong et al, 1987). Pericytes are also unable to proliferate in the presence of plasma (Gitlin and D’Amore, 1983). Park et al (2000) used growth media lacking plasma, gelatin-coated plates and cells up to the 10th passage. It is likely that their endothelial cell cultures were contaminated by pericytes which are known to contain PKCβII.

The use of C6-glioma cell conditioned media supplemented with fresh media (DMEM), FCS and human platelet-poor plasma, and attachment to collagen-coated tissue culture flasks/plates in this project provided optimal conditions for maintaining these cells in
culture. Cells attached by 1-2 days and reached confluence by 5-6 days which is comparable to that reported by Wong et al (1987), and were used only up to the 5th passage as Von Willebrand factor positive cells.

It is evident that PKC isozyme expression is tissue and species-specific. This is shown in the difference in PKC isozyme expression between T lymphocytes (PKC α, βI, βII, δ, ε, θ), HL60 cells (PKC α, βII, δ, ε, θ), HUVECs (PKC α, βI, βII, δ, ε) and BRECs (PKC α, βI, δ, ε). In addition, compared to bovine retinal cells, rat retinal cells express PKC α, βI, βII, and ε (Shiba et al, 1993) providing an example of species-specific PKC expression.

Different PKC isozymes are activated by different stimulants. The activation of PKC by hyperglycaemia is not generalized to all isozymes but rather to specific ‘diabetes-related’ isozymes. It is also thought to be tissue specific with increases in PKC activity seen in the retina, heart, aorta and glomeruli of diabetic animals, but not in the brain or peripheral nerves (Koya and King, 1998). Our data (Chapter 4) showed that PMA preferentially stimulated PKC α followed by βI, with no increase in PKC δ and ε. Hyperglycaemic conditions in BRECs stimulated PKC βI followed by δ with no increase in PKC α or ε. Once again these results contrast to that of Park et al (2000) who demonstrated that PKC δ and βII were preferentially activated by high glucose with smaller but significant increases in PKC βI and α activation. Possible reasons for this difference in results have been discussed previously.

Hyperglycaemia activates PKC βII in rat aorta and heart (Inoguchi et al, 1952), PKC α, β, and ε in rat retina (Shiba et al, 1993), and PKC α and ζ (Kikkawa et al, 1994) or PKC β
(Koya and King, 1998) in rat mesangial cells. From animal studies it appears that PKCβ is the main mediator of the effects of hyperglycaemia, with βI being significant in some species and βII being significant in others. This may reflect the differences in structure of PKC isozymes, differences in their sensitivity to DAG, differential increases in DAG within the cell, or variations in synthesis and degradation of the isozymes (Way et al, 2001).

Further work using bovine retinal endothelial cells as a model for diabetes must focus on PKC isozymes α, βI, δ, and ε rather than PKC βII with preventive strategies targeting PKC βI and δ which are preferentially activated by hyperglycaemic conditions.

6.2 NFκB activation in BREC by exposure to hyperglycaemic conditions

NFκB activation in retinal capillary endothelial cells has not been widely investigated. A study by Romeo et al (2002), demonstrated NFκB activation by high glucose in retinal pericytes but not in retinal endothelial cells in vivo (rat and human) and in vitro (bovine). Our results are consistent with this finding that high glucose alone does not increase NFκB activation in bovine retinal endothelial cells. Nevertheless our results show that hyperglycaemic conditions may indirectly control the activation of NFκB in the presence of the pro-inflammatory cytokine, TNF.

Levels of inflammatory cytokines such as TNF are known to be raised in diabetics and are likely to contribute to the pathogenesis of diabetic complications (Lechleitner et al, 1999). Raised levels of TNF have been demonstrated in the uterus of diabetic rats (Pampfer et al, 1997) and in macrophages and mesangial cells in the kidney of diabetic rats (Nakamura et al,
1993). TNF expression is increased in bovine retinal pericytes exposed to high glucose (Romeo et al, 2002). TNF up-regulates the expression of adhesion molecules on endothelial cells which promotes adhesion of neutrophils to the endothelium (Dixit et al, 1990). It stimulates the activation of NFκB through the promotion of IκBα degradation which allows NFκB to translocate into the nucleus (Selzman et al, 1999).

One method by which activation of NFκB can be examined is by studying the degradation of its inhibitory protein, IκBα. While high glucose alone had no effect on NFκB activation such conditions modulated the effects of TNF. Our findings demonstrate not only that TNF causes the activation of this transcriptional factor but that NFκB activation by TNF is much greater under high glucose conditions. This activation was not only greater but also more persistent than under low glucose conditions. This suggests that diabetics are more susceptible to the effects of TNF due to their hyperglycaemic environment. The consequences of this activation such as increased adherence of neutrophils and capillary occlusion may be potentiated, resulting in a greater risk of vascular complications.

The enhancing effect of glucose on TNF-induced NFκB activation may also have important implications in retinal endothelial cell survival and death. TNF has been demonstrated to be proapoptotic in rat cardiomyocytes (Krown et al, 1996) and bovine aortic endothelial cells (Clermont et al, 2003) and NFκB activation by high glucose is proapoptotic in bovine retinal pericytes but not in retinal endothelial cells (Romeo et al, 2002). In other cell types such as rat aortic smooth muscle cells, NFκB activation protects against apoptosis (Erl et al, 1999). It is known that proinflammatory changes and leukostasis occur early in the course of diabetes and this is followed by the development of
microthrombi and apoptosis (Boeri et al., 2001). Interestingly, loss of pericytes with the sparing of endothelial cells is an early event in diabetic retinopathy (Romeo et al., 2002; Li et al., 1996). Thus, it would be of interest to determine in future studies, whether activation of NFκB is proapoptotic or protective in BREC and whether inhibition of NFκB activation may affect retinal endothelial cell survival.

6.3 Inhibition of PKC and NFκB activation by β-oxa polyunsaturated fatty acids

Evidence is presented that hyperglycaemia-mediated activation of PKC βI could be significantly depressed by pre-exposing BREC to MP5. In contrast, no consistent effect of MP5 on PKC α, δ or ε was seen. The ability of the novel chemically engineered fatty acid to target PKC βI in mesangial cells cultured under hyperglycaemic conditions has been previously described (Mascolo, 2002). In addition, rats with drug-induced diabetes treated with MP5 showed reduced PKCβI activation in kidney extracts (Mascolo, 2002). These results show the consistency of MP5 to inhibit PKCβI activation.

The β-oxa PUFA, MP3 (β-oxa 23:4n-6), suppressed TNF-induced activation of NFκB under high glucose conditions in bovine retinal endothelial cells. It appears to exert its inhibitory effect at the level of or above IκBα since degradation of IκBα was dose-dependently inhibited by MP3 at concentrations of up to 20μM. The high levels of cytoplasmic IκBα maintains NFκB in the cytoplasm, thereby preventing its activation. These results support previous work in our laboratory which has shown similar effects in HL60 cells (Miller, 2000) and HUVEC (Ferrante et al., submitted). Pre-treatment of BREC with MP3 was sufficient for MP3 to be transported into the cytoplasm to exert its inhibitory effect. MP3 therefore has favourable properties which would support
consideration for its use as an effective inhibitor of NFκB-mediated diabetic vascular disease.

Here we have demonstrated that two types of β-oxa polyunsaturated fatty acids can inhibit two different intracellular signaling pathways, the NFκB pathway and PKC βI, which are likely to mediate the diabetic complications of retinopathy (Figure 6.1). Current evidence suggests that activation of these pathways contributes to retinopathy (Aiello, 2002; Romeo et al, 2002). Although the precise mechanisms by which the novel fatty acids inhibited NFκB and PKC βI activation are not known, it is likely that esterification of MP5 and MP3 into membrane phospholipids is required since we have found these fatty acids to be esterified (see below) and that the efficacy of the fatty acids is increased with increasing pretreatment periods (Costabile et al, 2001).

A model in which MP5 could inhibit hyperglycaemia-induced PKC translocation/activation is shown in Figure 6.2. When applied to BREC, MP5 is taken up by the cells, becomes esterified in DAG and membrane phospholipids. This incorporation is essential for the expression of the MP5 inhibitory properties. In control cells, glucose stimulates the translocation and activation of PKC βI and δ. In the presence of MP5-containing DAG, translocation of PKCβI is inhibited. This selective inhibitory effect on PKC βI is likely to result in the inhibition of glucose-stimulated production of vascular endothelial growth factor (VEGF). VEGF has been shown to increase endothelial cell permeability, migration, and proliferation and these actions are also regulated by PKC. Inhibition of PKCβ prevents VEGF production and action (Aiello et al, 1997).
Figure 6.1. Inhibition of PKC βI by MP5 and inhibition of NFκB by MP3. Inhibition of these 2 intracellular signalling pathways by β-oxa PUFAs, thus preventing neovascularisation, blood vessel occlusion and capillary dropout. ICAM: intercellular adhesion molecule; VCAM: vascular cell adhesion molecule; VEGF: vascular endothelial growth factor; TNF: tumour necrosis factor.
Figure 6.2. Inhibition of PKC βI activation by MP5. In control cells, glucose stimulates the activation of PKC βI and PKC δ. Esterification of MP5 into DAG which is formed by de novo synthesis from glucose prevents the translocation and activation of PKC βI. PIP₂: phosphatidylinositol 4,5 bisphosphate; PC: phosphatidylcholine; PE: phosphatidylethanolamine; SM: sphingomyelin; PM: plasma membrane
Figure 6.3 Suppression of IKK-NFκB signaling by MP3. In control cells, glucose amplifies the ability of TNF to activate NFκB through the PI3-kinase/PDK/Akt pathway. In this signalling pathway, PI3-kinase phosphorylates inositol containing lipids such as PIP2 on the 3' position of the inositol ring. The 3'-phosphorylated inositol lipid causes the recruitment and activation of PDK1, PDK2 on the plasma membrane, which in turn phosphorylates Akt. Esterification of MP3 into DAG formed by de novo synthesis from glucose and esterification into PI(4,5)P2 may interfere with or prevent the activation of PI3-kinase/PDK/Akt and hence the downstream IKK and NFκB. PI(4,5)P2: phosphatidylinositol4,5,bisphosphate; PI(3,4,5)P3: phosphatidylinositol 3,4,5 trisphosphate; PDK: phosphoinositide-dependent kinase; Akt: protein kinase B; IKK: IκB kinase; NFκB: nuclear factor κB; TNF: tumour necrosis factor α; PM: plasma membrane.
The ability of MP3 to inhibit NFκB activation is likely to depend on its esterification into membrane phospholipids such as phosphatidylinositol 4,5 bisphosphate (PIP₂) (Figure 6.3). This may affect the normal function of the phospholipid. For example, signaling by proteins and/or kinases which depend on PIP₂ may be altered or impaired. One such kinase is phosphatidylinositol 3-kinase (PI3-kinase). In general, ligation of appropriate surface receptors by their cognate ligands leads to the activation of PI3-kinase which phosphorylates inositol-containing phospholipids, particularly phosphatidylinositol 4,5 bisphosphate (PIP₂) on the 3' position of the inositol ring. The 3'-phosphorylated PIP₃ recruits and activates phosphoinositide-dependent kinase 1 (PDK1) which in turn phosphorylates Akt/protein kinase B (PKB) on the plasma membrane (Vanhaesebroeck and Alessi, 2000). Downstream signaling involves activation of the IκB kinase (IKK) complex which phosphorylates IκB species and hence its degradation, resulting in the activation of NFκB and the transcription of genes, including those which are required for cell adhesion molecule production in endothelial cells. Incubation of cells with MP3 results in the esterification of MP3 into membrane phospholipids, including PIP₂.

The presence of a more polar and conformationally different fatty acid in the sn2 position of the glycerol backbone is likely to interfere with signaling via the PI3-kinase - PDK1/PDK2 - Akt/PKB module which is an upstream regulator of NFκB (Vanhaesebroeck and Alessi, 2000). Consequently, this leads to inhibition of NFκB-dependent gene transcription. Indeed our previous studies have demonstrated that MP3 is incorporated into inositol lipids in HUVEC (Ferrante et al, submitted). This prevents the activation of downstream signaling molecules and the nuclear translocation and activation
of NFκB. Such inhibition of NFκB is associated with the inhibition of ligand-induced upregulation of adhesion molecule expression on the surface of the endothelial cells. We have thus previously shown that MP3 inhibits the up-regulation of VCAM-1, ICAM-1 and E-selectin (Ferrante et al, submitted). This prevented the adhesion of neutrophils to HUVEC. Although not examined in this thesis it is evident that MP3 is likely to prevent the neutrophil adhesion to BREC since it inhibited the activation of NFκB in these cells. Both TNF and high glucose are involved in the pathogenesis of diabetes (Lechleitner et al, 1999; Pampfer et al, 1997; Nakamura et al, 1998, Romeo et al, 2002) and MP3 inhibited the activation of this transcriptional factor by TNF in BREC exposed to hyperglycaemic conditions.

There is currently no effective way to prevent diabetic retinopathy. Intensive glycaemic control can slow the progression of retinopathy (DCCT/EDIC Research Group, 2001) and lower the risk of blindness but normoglycaemia is difficult to maintain. Treatment by laser photocoagulation is effective if given in the early stages of disease to destroy new capillaries and microaneurysms, thus reducing the risk of blindness but requires adequate screening programs to detect early changes of the disease. The results presented in this thesis provide a new approach to treating diabetic retinopathy by molecules which target PKCβ and NFκB which are involved in mediating diabetic microvasculopathy. The properties of β-oxa fatty acids surpass that of natural fatty acids in their ability to be developed into therapeutic agents due their selectivity of action and intracellular stability. Compared to the natural fats, these, while being poor stimulators of the neutrophil respiratory burst (Ferrante et al, 1999), have greater ability to reduce the up-regulation of
intracellular adhesion molecules (Huang et al, 1997), and are not readily \(\beta\)-oxidized, thus improving intracellular stability.

### 6.4 Concluding remarks

Diabetic retinopathy is the leading cause of blindness with the prevalence of retinopathy increasing with the duration of diabetes. Eighty to ninety percent will have some evidence of retinopathy after 20 years of the disease (Elkington, 1993) with 30-40% having proliferative retinopathy (Stefansson et al, 2000). Blood vessel damage occurs due to hyperglycaemia induced metabolic and chemical changes. The research outlined in this thesis has demonstrated a new approach to developing therapeutics with the potential to treat diabetic retinopathy. Novel chemically engineered polyunsaturated fatty acid mimetics have been shown to inhibit the pathways which have been shown to mediate processes leading to diabetic retinopathy. Characterization of the activation of PKC isozymes in bovine retinal endothelial cells enabled us to focus on PKC \(\beta1\) as the likely target for hyperglycemia-induced effects on blood vessels of the retina in diabetics. Activation of this PKC isozyme could be prevented by treating BREC with MP5. The other well-recognized target of interest for the inhibition of retinopathy is the activation of NF\(\kappa\)B under high glucose conditions mediated by the inflammatory cytokine, TNF. The activation of NF\(\kappa\)B in this manner was inhibited by another \(\beta\)-oxa polyunsaturated fatty acid, MP3. The findings provide further evidence for the role of these two intracellular signaling pathways in the pathogenesis of diabetic retinopathy and opens new avenues for the development of therapeutic agents to minimise the morbidity associated with hyperglycaemia-induced vascular disease.
6.5 Future research

The inhibitory effects of MP5 and MP3 on the activation of PKCβI and NFκB in human retinal endothelial cells needs to be related to effects on the downstream production of VEGF and adhesion molecules in retinal endothelial cells. The effect of MP5 on inhibiting VEGF-induced endothelial cell permeability, migration and proliferation would further support potential use of this novel PUFA in preventing the pathogenic processes leading to diabetic retinopathy.

Investigations should also be extended to in vivo models of diabetic retinopathy. The models use special equipment to examine changes in blood flow in retinal blood vessels as well as angiogenesis. These would provide proof of principle to gain confidence that toxicology studies and clinical trials should be attempted.

The mechanisms by which MP5 and MP3 inhibit PKCβ and NFκB activation will need to be explored since this may lead to a better understanding of how these molecules work and hence an ability to improve the technology if necessary.

An understanding of pericyte as well as endothelial cell responses to high glucose is important in our understanding of the pathogenesis of diabetic retinopathy. Pericytes lie in proximity to endothelial cells and regulate endothelial cell proliferation through the release of cytokines such as TGF-β (Martin et al, 1999) and TNF (Romeo et al, 2002). The importance of pericytes in the pathogenesis of retinopathy is highlighted by Romeo et al (2002), who demonstrated the increase in TNF expression and the pro-apoptotic effects of NFκB activation in retinal pericytes. This effect on apoptosis was not seen in BREC. The
role of NFκB and TNF in apoptosis of retinal endothelial cells needs to be clarified. Whether the inhibition of NFκB activation by MP3 in BREC will affect the balance of apoptotic and protective effects is yet to be determined. The effect of MP3 on NFκB in pericytes would also be of interest as these cells appear to be significant in the pathogenesis of diabetic retinopathy.
CHAPTER 7

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