The Effect of Hyperglycaemia on Aortic Endothelial and Smooth Muscle Cell Fate in Hypoxia

A dissertation submitted for the degree of

Doctor of Philosophy

By

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Declaration:

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from work of others save and to the extent that such work has been cited and acknowledged within the text of my own work.

Signature: _____

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留学异国多磨难，披荆斩棘勇登攀。父爱母慈遥寄语，手足情深暖心间。几度风雨几经年，几许感慨几重山。鱼跃龙门游四海，鹰击碧空傲长天。

Abstract

Diabetes, a metabolic disorder characterised by chronic hyperglycaemia due to relative insulin deficiency, insulin resistance or both, is associated with micro- and macro- vascular complications. Diabetes mellitus and impaired glucose tolerance are linked to increase cardiovascular morbidity and mortality. Endothelial and smooth muscle cells are the major factors in the development of cardiovascular diseases.

Both diabetes and hypertension have been shown to produce tissue hypoxia – a reduction in the normal level of tissue oxygen – that can occur from direct decreases in blood supply or from venous/arterial occlusion. Cellular responses to hypoxia include proliferation, angiogenesis, metabolism, apoptosis and migration. Therefore, we investigated the effects of hyperglycaemia on bovine aortic endothelial cell (BAEC) and bovine aortic smooth muscle cell (BASMC) growth (proliferation and apoptosis) under normoxic and hypoxic conditions.

Exposure of BAEC and BASMC to high glucose in media containing 10% fetal bovine serum (FBS) did not alter cell apoptosis and proliferation under normoxic conditions. Although serum deprivation （0.5% FBS containing media） significantly increased cell apoptosis and decreased cell proliferation under normoxic conditions, exposure to high glucose did not show any positive or negative effects. Mannitol, as controls for osmolarity, excluded the possibility of involvement of an osmotic effect on cells.

Hypoxia increased cell apoptosis and suppressed cell proliferation however; these effects on hypoxia-induced cell apoptosis and inhibition of proliferation were significantly reversed in the presence of high glucose.

The most evident response to hypoxia is *via* hypoxia-inducible factor 1 alpha (HIF-1α). To investigate if hyperglycaemia modulated cell apoptosis and proliferation under hypoxic conditions through HIF-1α; HIF-1α expression was silenced following siRNA knockdown. Under these conditions, the hypoxia induced response was significantly impaired.

Taken together, these studies suggest that hyperglycaemia has no effect on BAEC and BASMC cell fate under normoxic conditions; hyperglycaemia impairs hypoxia-induced apoptosis and hypoxia-induced inhibition of cell proliferation under hypoxic conditions which is *via* a HIF-1α-dependent mechanism.

Meeting Abstracts:

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Abbreviations:

Ang	Angiotensin
AIF	Apoptosis-inducing factor
AO	Acridine Orange
AP	Acid phosphatase
Apaf-1	Apoptosis protease-activating factor-1
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosine Tri-Phosphate
A/U	Arbitary units
AV	Annexin V
BAEC	Bovine Aortic Endothelial Cell
BASMC	Bovine Aortic Smooth Muscle Cell
BCA	Bicinchoninic Acid
β-gal	β-galactosidase
bHLH	basic Helix-Loop-Helix
BNIP3	Bcl2/adenovirus E1B 19kDa Interacting Protein 3
BSA	Bovine Serum Albumin
CAD	Caspase-activated DNase
CARD	Caspase recruitment domain
cDNA	Complimentary DNA
Ced	Cell death gene
CHAPSO	3[(3-cholamidopropyl)dimethylamm inonio]-2-hydroxypropanesulphonic acid
CFDA SE	Carboxy-fluorescein diacetate succinimidyl ester
CV	Cardiovascular

DKA	Diabetic ketoacidosis
DM	Diabetes Mellitus
DMEM	Dulbelco's modified Eagle's medium
DMSO	Dimethlysulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleoside tri-phosphate
ds	Double stranded
DTT	Dithiothreitol
EC	Endothelial cells
ECM	Extracellular matrix
EDTA	Ethyldiamine tetraacetic acid
EDRF	Endothelium-derived relaxing factor
EGF	Epidermal growth factor
EGTA	Ethyleneglycol-bis-(b-amino-ethyl ether) N,N,N'N'' tetra-acetic acid
eNOS	Endothelial nitric oxide synthase
EtBr	Ethidium Bromide
EU	European Union
ERK	Extracellular signal regulated kinase
ExC	Extracellular
FADD	Fas-associated death domain
Fas-L	Fas ligand
FBS	Foetal Bovine Serum
FIH	Factor inhibiting HIF-1α
FLICE	FADD-like Interleukin 1 beta converting enzyme
FLIP	FLICE-like inhibitory proteins
GLUT	Glucose transporter
H	Hypoxia

HBSS	Hanks balanced salt solution
HDL	High density lipoproteins
HIF-1α	Hypoxia inducible factor-1 alpha
HLA	Human leukocyte antigen
HRE	Hypoxia response element
HRT	Hairy related transcription factor
IC	Intracelluar
IDDM	Insulin-dependent diabetes mellitus
IGF	Insulin-like growth factor
IM	Inner membrane
LB	Luria-Bertani broth
LDL	Low density lipoproteins
Luc	Luciferase
MLCK	Myosin light chain kinase
N	Normoxia
NFκB	Nuclear factor-κB
NIDDM	Non insulin-dependent diabetes mellitus
NO	Nitric oxide
ODD	Oxygen-dependent degradation domain
OGTT	Oral Glucose Tolerance Test
OM	Outer membrane
PAGE	Polyacrylamide gel electrophoresis
PAK	p21-activated kinase
PAS	PER-ARNT-SIM
PBS	Phosphate buffered saline
pCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI	Propidium iodide

PKC	Protein kinase C
pNA	pNitroaniline
pVHL	Product of the von Hippel-Lindau
Raf	Receptor associated factors
RIP	Receptor interacting protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
RT	Reverse transcriptase
SDS	Sodium dodecyl sulphate
SSB	Sample solubilization buffer
ss	Single stranded
siRNA	Short interfering RNA
TAE	Tris acetate EDTA
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethlylenediamine
TGF-β	Transforming growth factor-β
TM	Transmenbrane domain
TNF	Tumour necrosis factor
t-PA	tissue plasminogen activator
TPR	Temperature, pulse, respiration
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoproteins
VSMC	Vascular smooth muscle cells

Units:

A/U	Arbitary units
bp	Base pairs
cm	Centimetre
cm^2	Centimetre squared
°C	Degree Celsius
kDa	KiloDaltons
µg	Microgram
µl	Microlitre
µM	Micromolar
g	Grams
h	Hours
kg	Kilogram
L	Litre
M	Molar
mA	Milliamperes
mg	Milligrams
mHz	Millihertz
min	Minutes
ml	Milliitres
mM	Millimolar
ng	Nanograms
nm	Nanometres
sec	Seconds
V	Volts

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Chapter 1

Introduction

1.1 Research Aims

Diabetes mellitus is a group of metabolic disease characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycaemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (Association 2004).

The long-term mortality and morbidity rate for diabetic patients with a diabetic vascular disease are significantly elevated relative to that of the nondiabetic patients (Aronson et al. 1997). Endothelial Cell (EC) and Smooth Muscle Cell (SMC) dysfunctions are the key factors which result in atherosclerosis, thrombosis and hypertension.

It has become increasingly evident that hypoxia plays an important role in all diabetes complications (Cameron et al. 2001). Atherosclerosis, a major complication of diabetes, highlights the fact that the more deeply situated parts of the arterial wall depend on diffusion to satisfy their need for oxygen and nutrients. When atherosclerosis lesions develop, the arterial wall thickness increases and diffusion capacity is impaired. Simultaneously, oxygen consumption is augmented (Bjornheden and Bondjers 1987) and an energy imbalance may occur.

In addition to deficient blood supply as a consequence of micro- and macrovascular disease, it has been postulated that hyperglycaemia induces a pseudohypoxia states. This theory is based on the finding that hyperglycaemic concentrations induce a high $NADH^+/NAD^+$ ratio in cells even when the oxygen tension is normal (Williamson et al. 1993).

Hyperglycaemia and hypoxia are suggested to play essential pathophysiological roles in the complications of diabetes, which may result from a defective response of the tissues to low oxygen tension. The possibility that hyperglycaemia and hypoxia may interact *via* a common metabolic imbalance to initiate and/or exacerbate complications of diabetes is suggested by the correspondence of several redox, metabolic, and pathophysiological changes evoked by either condition alone (Nyengaard et al. 2004).

Adaptive responses of cells to hypoxia are mediated by various hypoxia-responsive transcription factors. Hypoxia-inducible factor (HIF) is a master regulator during hypoxic stress which binds to the hypoxia response elements

(HRE) in the promoter or enhancer of the target genes such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), glucose transporter-1 (GLUT-1), p53 to induce angiogenesis, glycolysis, proliferation and apoptosis (Schofield and Ratcliffe 2004).

We are interested to study the interaction between these two denominators of the chronic complications of diabetes (hyperglycaemia and hypoxia) as well as the interactions between other factors suggested to play role in chronic complications of diabetes and hypoxia.

Aim 1: To investigate the effects of high glucose (HG 25mM) on Bovine Aortic Endothelial Cell (BAEC) and Bovine Aortic Smooth Muscle Cell (BASMC) growth (balance between proliferation and apoptosis).

Aim 2: To investigate the effects of hypoxia (2% O_2) on BAEC and BASMC growth (proliferation *vs* apoptosis) in the absence or presence of HG (25mM).

Aim 3: To investigate the role of HIF-1α as a target for hyperglycaemia (25mM HG) in hypoxia-induced apoptosis and proliferation.

1.2 Diabetes Mellitus (DM)

Diabetes Mellitus (DM) is a disease in which the body does not produce or properly use insulin. Insulin is a hormone that transports glucose, converts starches and other food into energy needed for daily life. The cause of diabetes continues to be a mystesry, although both genetics and environmental factors such as obesity and lack of exercise appear to play roles.

Diabetes was recognized, and its clinical features were recorded, over thousands of years ago and our understanding of the disease has progressed and advanced greatly, especially during the last two decades, but many aspects of its management are still enigmatic. The goal of preventing diabetes and thus, the long-term complications, has become evermore important as the disease threatens to explode to epidemic proportions.

The number of patients with diabetes is increasing due to population growth, aging, urbanization and the increasing prevalence of obesity and physical inactivity. In the year 2000, the number of diabetes cases worldwide among adults > 20 years of age was estimated to be approximately 171 million; 11% higher than the 154 million previously reported by the WHO in 1990. The International Diabetes Federation has subsequently released estimates of the number of people with diabetes for 2003 and 2025, at 194 million and 334 million respectively, indicating that DM will remain a global health threat.

In order to determine whether or not a patient has pre-diabetes or diabetes, there are several major symptoms which are clinically recognized namely: frequent urination; excessive thirst; extreme hunger; unusual weight loss; increased fatigue; irritability and blurred vision, in conjunction with any one of the following three criteria: a random plasma sugar level greater than 11 mmol/l; a fasting plasma sugar level greater than or equal to 7 mmol/l (or 6.1 mmol/l in whole blood); a plasma sugar level greater than 11 mmol/l two hours after drinking 75 grams of glucose dissolved in water in an oral glucose tolerance test (OGTT).

1.2.1 Classification of Diabetes Mellitus

The vast majority of cases of diabetes fall into two broad etiopathogenetic categories which were given names descriptive of their clinical presentation: "insulin-dependent diabetes mellitus" (IDDM) and "non insulin-dependent diabetes mellitus" (NIDDM).

(a) **Type 1 Diabetes Mellitus** - This form of diabetes is characterized by β-cell destruction, usually leading to absolute insulin deficiency.

The development of type 1 diabetes may be viewed as a two-step process. During the first step, presumed environmental triggers such as virus infection cause the destruction of beta cells. It is assumed that pancreatic antigen-presenting cells engulf dead beta cells. The antigen-presenting cells are thought to migrate to lymph nodes that drain the pancreas and in which islet-beta-cell-specific antigen presentation takes place (Kent et al. 2005). CD4+ T lymphocytes with T cell receptors that recognize beta-cell peptides lodged in a groove in the human leukocyte antigen (HLA) class II molecule may then be activated, which initiates an islet auto immune reaction. After auto-antibodies have developed, there is a second step, in which genetic as well as environmental factors may aggravate the islet autoimmunity. It is possible that CD8+ cytotoxic T cells are induced, leading to a rapid onslaught on beta cells (Pundziute-Lycka et al. 2002).

Onset is most common in childhood, thus the common term "juvenile-onset", but the onset up to age 40 is not uncommon and can even occur later. Patients are susceptible to diabetic ketoacidosis (DKA) which is a state of absolute or relative insulin deficiency aggravated by ensuing hyperglycaemia, dehydration, and acidosis-producing derangements in intermediary metabolism. This condition causes underlying infection, disruption of insulin treatment, and new onset of diabetes. DKA is typically characterized by hyperglycaemia over 300 mg/dL, low bicarbonate (<15 mEq/L), and acidosis (pH <7.30) with ketonemia (the presence of detectable levels of ketone bodies in the plasma) and ketonuria (an excessive concentration of ketone bodies in the urine).

(b) **Type 2 Diabetes Mellitus** - Type 2 diabetes is characterized by insulin resistance with relative insulin deficiency to predominatly an insulin secretory defect with insulin resistance.

Type 2 diabetics have a high (hyperinsulinemia) or normal basal insulin level and fewer insulin receptors on insulin-sensitive target tissues. The large majority of patients are overweight at onset. Most are over 40, hence the common terms "adult-onset", but onset can occur at any age. Patients are not susceptible to DKA (diabetic ketoacidosis).

There is a strong genetic tendency which is highly associated with a family history of diabetes, but not simple inheritance. Depending on the individual, treatment may be by diet, exercise weight loss, oral medications which stimulate the release of insulin, or the suppression of glucose production, or insulin injections. Patients are usually subjected to a combination of several of these treatments. Insulin resistance and hyperinsulinemia eventually lead to impaired glucose tolerance. Defective beta cells become exhausted, further fuelling the cycle of glucose intolerance and hyperglycaemia. The aetiology of type 2 diabetes mellitus is multifactorial and probably genetically based, but it also has strong behavioural components such as diet and exercise.

1.2.2 Clinical Complications of Diabetes Mellitus

The clinical complications of diabetes mellitus can be broadly classified into micro-vascular complications and macro-vascular complications.

Micro-vascular disease affects the retina which presents as diabetic retinopathy. Neuropathy presents with numbness and parasthesia, and often the formation of trophic ulceration on the feet secondary to reduced sensation. Diabetic nephropathy is the leading cause of kidney failure and presentation is determinant upon the stage of the disease ranging from mild proteinuria (protein in the urine) to renal failure or multi-system failure.

The macro-vascular disease is reflected in premature atherosclerosis, coronary heart disease and peripheral vascular disease. Abnormalities in vascular reactivity in both macro and micro-vascular disease are well established in diabetes (NDDG 1979; ECDM 1980; WHOTRS 1985; DCCT 1993). Macrovascular disease presents as premature atherosclerosis, stroke, coronary heart disease and poor perfusion to the distal limbs presenting as intermittent claudication secondary to ischaemia. Ulceration is also common.

1.2.3 Diabetic Effects on the Vasculature

Angiopathy is the generic term for a disease of the blood vessels (arteries, veins, and capillaries). The best known and most prevalent angiopathy is diabetic angiopathy; a complication that may occur in chronic diabetes.

There are two types of angiopathy: macroangiopathy and microangiopathy. In macroangiopathy, fat and blood clots build up in the large blood vessels, stick to the vessel walls, and block the flow of blood. In microangiopathy, the walls of the smaller blood vessels become so thick and weak that they bleed, leak protein, and slow the flow of blood through the body. The decrease of blood flow through stenosis or clot formation impairs the flow of oxygen to cells and biological tissues and leads to their death (necrosis) which in turn may require amputation. Thus, tissues which are very sensitive to oxygen levels develop microangiopathy and may cause blindness. Damage to nerve cells may cause peripheral neuropathy, and to kidney cells, diabetic nephropathy.

Macroangiopathy, on the other hand, may cause other complications such as ischemic heart disease, stroke and peripheral vascular disease which contribute to diabetic foot ulcers and the risk of amputation.

The term 'diabetic angiopathy' suggests that the pathological processes involved in the development of micro- and macro-angiopathies are uniform, irrespective of the type of diabetes. However, both micro- and macroangiopathy exhibit specific stages in their development and there is emerging evidence that the pathophysiology of microangiopathy may differ between type 1 and type 2 diabetes (Tooke et al. 1996).

Type 1 diabetes is characterized by an early increase in microvascular pressure and flow. It is thought that this leads to endothelial injury and resultant capillary basement membrane thickening and microvascular sclerosis. This in turn limits maximum hyperaemia and impairs the autoregulatory capacity of the vasculature (Parving et al. 1983). In contrast, in type 2 diabetes in the absence of hypertension, microvascular pressure appears normal (Shore et al. 1992). There is however a profound early reduction in vasodilatory reserve and increasing evidence that this abnormality, which probably represents endothelial dysfunction, precedes the development of diabetes (Tooke and Goh 1999). Whether or not endothelial dysfunction is itself a cause of insulin resistance through impaired insulin-mediated vasodilation of skeletal muscle remains debatable.

1.3 Vascular Disease

The metabolic abnormalities associated with DM result in vascular complications in multiple organ systems though it is the cardiovascular impact that accounts for the greatest morbidity and mortality associated with the disease. Cardiovascular diseases are any disease of the circulatory system, chief among which are myocardial infarction, cerebrovascular disease, and other peripheral vascular diseases. Cardiovascular diseases are the leading cause of death in the western world, accounting for 40% of all deaths in the United States. An estimated 17 million people die due to cardiovascular disease per annum, which translates into one death every 30 seconds. The incidence of cardiovascular disease in Ireland is the highest in the European Union (EU), with 53 deaths per 100,000 population compared to the EU average of 32. In addition, it is estimated that one quarter of people in the western world live with cardiovascular disease, resulting in a significant economic impact both in terms of health care expenditures and lost productivity (World Health Organization, American Heart Foundation). Therefore, an increased understanding of the mechanisms underlying the pathology of cardiovascular diseases is imperative both in the prevention and management of this condition.

A number of risk factors are associated with the development of cardiovascular diseases, including genetic pre-disposition, gender, age and race. Many factors, however, are lifestyle determined, such as poor nutrition, obesity, lack of exercise and smoking. A diet high in saturated fat and cholesterol can increase the risk of developing certain cardiovascular disorders. Whilst cholesterol has important functions in the body, such as the formation of cellular membranes, it is important that the correct ratio of cholesterols within the body is maintained. Cholesterol is insoluble in blood and is transported in the esterified form *via* plasma lipoproteins, namely high density lipoproteins (HDL), low density lipoproteins (LDL) and very low density lipoproteins (VLDL). The ratio of HDL to LDL is an important factor in determining the risk that cholesterol presents in the development of atherosclerosis.

1.3.1 Atherosclerosis

Atherosclerosis from the Greek athero (meaning gruel or paste) and sclerosis (hardness) is the process in which deposits of fatty substances, cholesterol, cellular waste products, calcium and other substances build up in the inner lining of an artery to form a plaque.

Atherosclerotic vascular disease, the primary cause of all cardiovascular diseases, is a systemic disease involving the intima of large and medium arteries including the aorta, carotid, coronary and peripheral arteries (Corti and Badimon 2002). Atherosclerosis is characterized by a dysfunctional endothelium; chronic inflammation; lipid accumulation; aberrant regulation of vascular smooth muscle cell (VSMC) fate decisions; and often calcification, which clinically manifests as an atherosclerotic plaque. Key risk factors associated with the development of atherosclerosis include: elevated LDL and low HDL levels (Sharrett et al. 1994); an increased level of homocysteine in the blood due to dietary deficiencies in vitamins B6, B12 and folic acid (Boers 2000); obesity; and smoking.

Obesity is a considerable problem in Ireland, where 57% of people are considered overweight or obese (Livingstone et al. 2001). Regular exercise can help combat or prevent obesity, in addition to increasing HDL levels in the circulation (Irish Heart Foundation). Smoking directly damages the endothelium; increases vascular tone; increases platelet activation; and promotes LDL oxidation (Muscat et al. 1991). Smoking increases the risk of developing cardiovascular disease by 50% and contributes to one fifth of all cardiovascular related deaths (American Heart Association).

The generation of obstructive plaques can result in additional cardiovascular disorders, for example, an obstructive coronary plaque can cause a critical reduction in coronary blood flow, and subsequent myocardial ischaemia (Schroeder and Falk 1995). Plaques can grow large enough to significantly reduce the blood's flow through an artery but most of the damage occurs when they become fragile and rupture. Ruptured plaques can result in the formation of a thrombus that can block blood flow or break off and travel to other parts of the body. If either happens and blocks a blood vessel that feed the heart, it causes a 'heart attack'. If it blocks a blood vessel that feeds the brain, it causes a stroke. Reduced blood supply to the arms or legs can cause difficulty walking and eventually result in cellular necrosis which may require amputation of the limb.

Atherosclerotic plaques occur preferentially at bifurcations and curvatures of arterial blood vessels due to the presence of disturbed blood flow at these sites (Glagov et al. 1988). Atherosclerotic lesions form at distinct sites in the arterial tree, especially at or near branch points, bifurcations, and curvatures. This distribution pattern suggests that local factors, such as hemodynamic forces (shear stress) that can be sensed by endothelial cells lining the artery wall, influence the initiation of atherogenesis. Blood flow is disturbed at branch points, bifurcations, and curvatures, unlike straight segments where it tends to be laminar. Alterations in shear stress can alter endothelial cell gene expression and modulates the expression of adhesion molecules. Hemodynamic forces may also influence topographic variations in gene expression by endothelial cells in arteries and may predispose regions to atherosclerotic lesion formation if appropriate systemic risk factors are present.

1.3.2 Hypertension (high blood pressure)

Blood is carried from the heart to all the body's tissues and organs *via* arteries. Blood pressure is the force of the blood pushing against the walls of those arteries. Hypertension (high blood pressure) is defined as either a systolic pressure consistently at 140/mmHg or higher or a diastolic pressure consistently at 90/mmHg or higher comparing to normal blood pressure which is considered to be 120/80 mmHg.

Hypertension can develop due to a genetic pre-disposition or may be environmentally induced due to poor diet, obesity, smoking and lack of exercise. In addition, the development of hypertension is itself a major risk factor in the development of cardiovascular diseases such as atherosclerosis, heart attack, stroke and enlarged heart. It also can result in VSMC hypertrophy and hyperplasia, and a subsequent increase in peripheral vascular resistance (Molloy et al. 1999).

The mechanisms behind the factors associated with secondary hypertension (hypertension that is secondary to another disease) are generally fully understood. However, those associated with essential hypertension are far less understood. What is known about inessential hypertension is that cardiac output is raised early in the disease course, with total peripheral resistance remaining normal; over time, cardiac output (the volume of blood being pumped by the heart in a minute; equal to the heart rate multiplied by the stroke volume) drops to normal levels but TPR (temperature, pulse, respiration) is increased. Three theories have been proposed to

explain this: (i) inability of the kidneys to excrete sodium, resulting in natriuretic factor being secreted to promote salt excretion with the side-effect of raising total peripheral resistance; (ii) an overactive renin / Angiotensin system leads to vasoconstriction and retention of sodium and water. The increase in blood volume leads to hypertension; (iii) an overactive sympathetic nervous system, leading to increased stress responses.

Malignant hypertension (or accelerated hypertension) which is a complication of hypertension characterized by very elevated blood pressure (>240/120 mm Hg) is distinct as a late phase in the condition, and may present with headaches, blurred vision and end-organ damage.

Elevated blood pressure is known to contribute to diabetic microvascular complications such as renal disease, autonomic neuropathy, retinopathy and macrovascular complications such as cardiac disease, cerebrovascular disease and peripheral vascular disease (UKPDS 1998; Fineberg 1999; Bakris et al. 2000).

Hypertension frequently coexists with diabetes. There is an increased prevalence of hypertension among diabetic patients (Sowers et al. 2001), but there is also a higher propensity among hypertensive patients to develop type 2 diabetes (Stolk et al. 1993; Gress et al. 2000). When occurring together, the two disease entities appear to aggravate one another, worsening both the diabetic and cardiovascular CV end points (Stamler et al. 1993). Twelve-year follow-up data from the large Multiple Risk Factor Intervention Trial (MRFIT, 1971) showed that absolute risk of death from cardiovascular disease approximately tripled for diabetic compared with nondiabetic patients. With a rise in systolic blood pressure, the absolute cardiovascular risk for diabetic patients increased beyond that of nondiabetic patients (Stamler et al. 1993). Hypertension also accelerates kidney damage and microvascular disease (Adler et al. 2000).

Dysregulated insulin production has further consequences. It promotes vascular smooth muscle cell proliferation and extracellular matrix deposition. Elevated blood glucose levels also mediate structural changes to the vasculature. It is this structurally compromised vasculature, found throughout the body, which appears to be particularly sensitive to damage by elevated blood pressure.

1.4 The Vasculature

Changes in the structure function and integrity of arterial blood vessels is central to the pathogenesis of many cardiovascular diseases. The arterial blood vessel is an active, integrated organ composed of endothelial cells (EC), smooth muscle cells (SMC) and fibroblasts divided into three structural layers, termed the tunicas intima, media and adventitia (Fig 1.1).

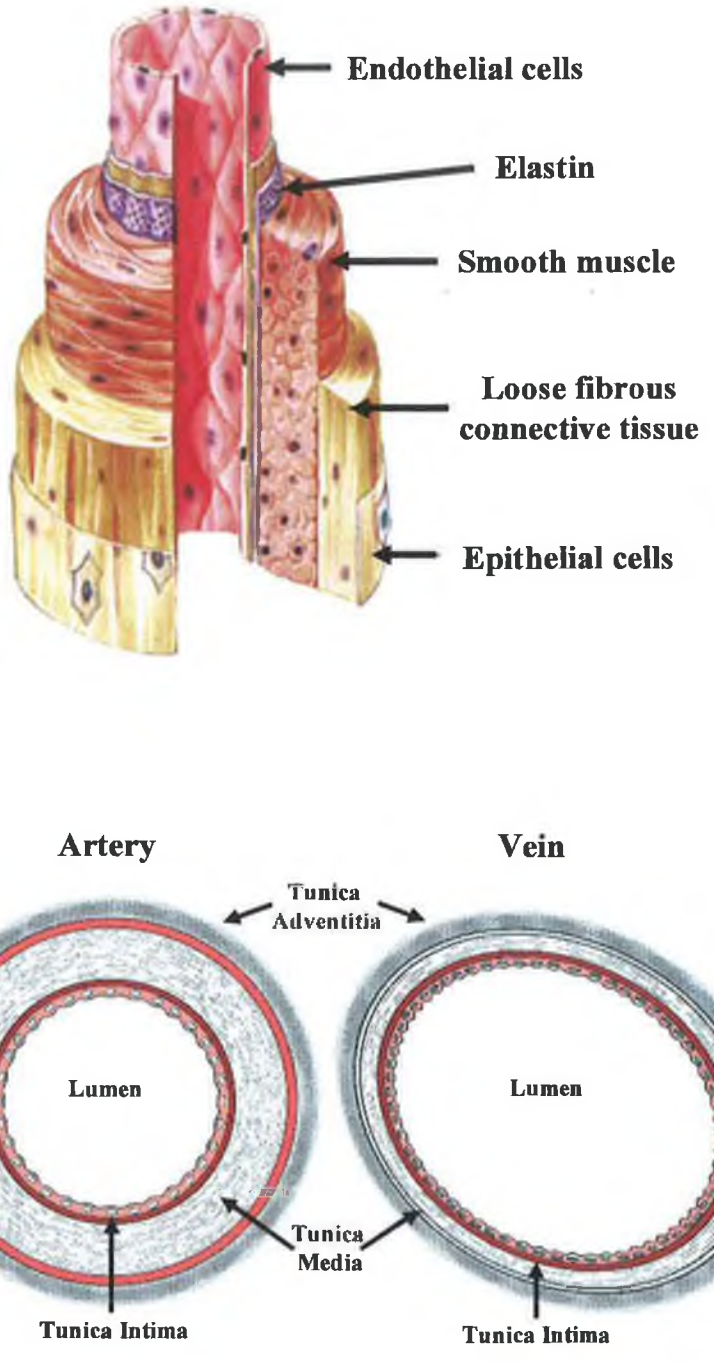


Fig. 1.1 Diagrammatic Representation of Macrovascular Vessel Structure

The innermost layer, the tunica intima, is a simple squamous epithelium surrounded by a connective tissue basement membrane with elastic fibres. The underlying tunica media is primarily comprised of smooth muscle cells, which play a key role in maintaining vascular tone and function. SMC are orientated circumferentially in the outer media, but closer to the lumen they present in a more random fashion (Gittenberger-de Groot et al. 1999). Several studies have demonstrated a marked heterogeneity of SMC phenotypes in the vessel wall of both human and animal models. These phenotypes are classified as synthetic and contractile, and are evident embryonically but become more prominent as the vessel matures. Whilst both the intimal and medial layers contain a mixture of both phenotypes, the synthetic phenotype is most commonly associated with the intimal layer and with vascular remodelling. The synthetic SMCs resemble immature, dedifferentiated SMC with lower levels of contractile proteins (α-actin, myosin, calponin, smoothelin) and fewer myofilaments than their medial counterparts (Shanahan et al. 1993; Bochaton-Piallat et al. 1995; Gittenberger-de Groot et al. 1999). These intimal cells are said to be similar in both morphology and gene expression to foetal or embryonic SMC, and have been shown to be more prone to both physiological and pathophysiological apoptosis than the medial SMC (Bochaton-Piallat et al. 1995; Rao and White 1997; Slomp et al. 1997). The contractile SMC, on the other hand, are most commonly associated with the tunica media. They express differentiated cell markers associated with contractile function and are involved in the synthesis and maintenance of extracellular components of the vessel wall. Smooth muscle contraction is caused by the sliding of myosin and actin fibres over each other. The energy for this to happen is provided by hydrolysis of ATP. Movement of the fibres over each other happens when heads on the myosin fibres form crossbridges with the actin fibre. These heads tilt and drag the actin fibre a small distance. The heads then release the actin fibre and adopt their original conformation. They can then re-bind to another part of the actin molecule and drag it along further. This process is called crossbridge cycling and is the same for all muscle.

Crossbridge cycling, though, cannot occur until the myosin heads have been activated to allow crossbridges to form. The myosin heads are made up of a "light" protein chain. When this is phosphorylated it becomes active and will allow contraction to occur. The enzyme that phosphorylates the light chain is called myosin

light chain kinase (MLCK). In order to control contraction, MLCK will only work when the muscle is stimulated to contract. Stimulation will increase the intracellular concentration of calcium ions. These bind to a molcule called calmodulin and form a calcium-calmodulin complex. It is this complex that will bind to MLCK to activate it, allowing the chain of reactions for contraction to occur.

MLCK can be inhibited to prevent crossbridge cycling: this causes relaxation of the muscle. It is by causing inhibition of MLCK those vasodilators such as endothelium-derived relaxing factor, or nitric oxide (NO), work to dilate blood vessels. Production of cAMP or cGMP is stimulated. This binds to MLCK and inhibits it, preventing it from activating the myosin light chains.

The outermost layer, which attaches the vessel to the surrounding tissue, is termed the tunica adventitia. This is a layer of connective tissue with varying amounts of elastic and collagenous fibres. The connective tissue is dense adjacent to the tunica media, but changes to loose connective tissue near the periphery of the vessel.

1.4.1 The Vascular Endothelium

Endothelial cells (EC) function as a semi-permeable barrier, and also as a dynamic paracrine and endocrine organ, exerting considerable influence on the underlying VSMC and circulating blood elements. EC regulate the maintenance of selective permeability; integration and transduction of blood-borne signals; regulation of inflammatory and immune reactions; regulation of vascular tone; maintenance of thromboresistance; modulation of leukocyte interactions with tissues and regulation of vascular growth. Normal endothelial function plays a pivotal role in vascular homeostasis, and limits the development of atherosclerosis. Endothelial dysfunction, therefore, is an initial pathological sign of atherosclerosis. EC are involved in maintaining the non-thrombogenic blood-tissue interface by regulating thrombosis, thrombolysis, platelet adherence, vascular tone and blood flow. This is achieved through the production of thrombogenic (thromboxane) and anti-thrombogenic (thrombomodulin) factors, and pro- and anti-inflammatory mediators, such as leukocyte adhesion molecules and nitric oxide respectively. In addition, the endothelium can release substances that act as SMC promoters and vasoconstrictors, such as angiotensin II, or as SMC inhibitors and vasodilators, such as prostacyclin (Corti and Badimon 2002). Therefore, endothelial dysfunction can

result in the accumulation of cellular components, lipids and extracellular matrix (ECM) to yield a fibrofatty plaque, which can ultimately narrow the arterial lumen (Geng and Libby 2002).

The normal healthy endothelium regulates vascular tone and structure and exerts anticoagulant, antiplatelet, and fibrinolytic properties. The maintenance of vascular tone is accomplished by the release of numerous dilator and constrictor substances. A major vasodilative substance released by the endothelium is nitric oxide (NO), originally identified as endothelium-derived relaxing factor (EDRF). Other endothelium-derived vasodilators include prostacyclin and bradykinin (Drexler 1998). Prostacyclin acts synergistically with NO to inhibit platelet aggregation (Luscher and Barton 1997). Bradykinin stimulates release of NO, prostacyclin, and endothelium-derived hyperpolarizing factor, another vasodilator, which contributes to inhibition of platelet aggregation (Drexler 1998). Bradykinin also stimulates production of tissue plasminogen activator (t-PA), and thus may play an important role in fibrinolysis.

The endothelium also produces vasoconstrictor substances, such as endothelin (the most potent endogenous vasoconstrictor identified to date) and angiotensin II (Ang II). Ang II not only acts as a vasoconstrictor but is also pro-oxidant (Sowers 2002) and stimulates production of endothelin. Endothelin and Ang II promote proliferation of smooth muscle cells and thereby contribute to the formation of plaques (Drexler 1998). Activated macrophages and vascular smooth muscle cells, characteristic cellular components of an atherosclerotic plaque, produce large amounts of endothelin (Kinlay et al. 2001).

Damage to the endothelium upsets the balance between vasoconstriction and vasodilation and initiates a number of events/processes that promote or exacerbate atherosclerosis; these include increased endothelial permeability, platelet aggregation, leukocyte adhesion, and generation of cytokines. Decreased production or activity of NO, manifested as impaired vasodilation, may be one of the earliest signs of atherosclerosis.

1.4.2 Cellular Proliferation

In a normal resting cell the intracellular signalling proteins and genes that are normally activated by extracellular growth factors are inactive. When the normal cell is stimulated by an extracellular growth factor, these signalling proteins and genes become active and the cell proliferates (Fig. 1.2).

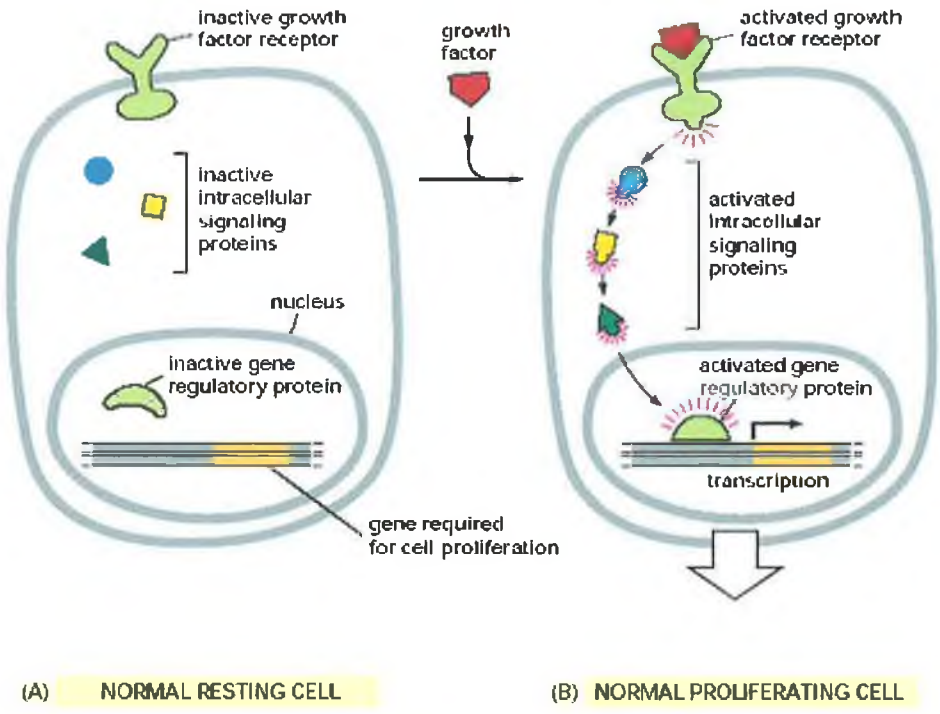


Fig. 1.2 Cell Proliferation

Molecular Biology of the Cell: Alberts, Johnson, Lewis, Raff, Roberts, Walter (1998)

Vascular proliferation contributes to the pathobiology of atherosclerosis and is linked to other cellular processes such as inflammation, apoptosis and matrix alterations. The contribution of vascular proliferation to the pathophysiology of in-stent restenosis, transplant vasculopathy and vein bypass graft failure is particularly important. Thus, an emerging strategy for the treatment of those conditions is to inhibit cellular proliferation by targeting cell cycle regulation. The understanding of the pathophysiology of atherosclerosis and related vascular diseases has changed over the last decade, providing new perspectives for preventive and therapeutic strategies (Dzau et al. 2002).

Recent studies have emphasized the involvement of inflammation in mediating all stages of atherosclerosis (Ross 1999; Libby et al. 2002). However, in

addition to inflammation, a key process of atherosclerosis involves the proliferation of vascular smooth muscle cells (VSMCs) (Ross and Glomset 1973; Ross 1995; Schwartz et al. 2000).

Understanding of the responses of growth factors and VSMC proliferation to vascular injury is derived mainly from studies involving animal models of arterial injury. Direct data are difficult to obtain from human disease; however, in the rat model, basic fibroblast growth factor, released from dying vascular cells, can initiate medial proliferation of VSMCs (Lindner and Reidy 1991), whereas platelet derived growth factor may induce subsequent migration of VSMCs toward the intima (Ferns et al. 1991). Intimal proliferation and matrix accumulation occur under the influence of platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), angiotensin II, epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1) (Dzau et al. 1991; Majesky et al. 1991; Nabel et al. 1993; Grant et al. 1994). Furthermore, loss of growth-inhibitory factors, occurring as a result of decreased endothelial cell secretion of nitric oxide (NO), inactivation of NO by reactive oxygen species or altered heparin sulfate proteoglycan synthesis, may also contribute to the migration and proliferation of VSMCs and to the increased inflammatory response (Kinsella and Wight 1986; Ignarro et al. 1987). With the recognition of the essential involvement of VSMC proliferation in the conditions described above and the improved understanding of the molecular and cellular mechanisms of cellular proliferation, antiproliferative therapeutic modalities have become a focus of research and development.

There is increasing evidence that connections exist between proliferation and other cellular processes that are important for the pathophysiology of vascular disease. The processes of proliferation and inflammation are also linked. Supporting this link of vascular proliferation and inflammation is the observation that impaired NO bioactivity in vascular disease is associated with VSMC proliferation and inflammation (Ross 1999). *In vivo* overexpression of NO synthase resulted in reduction of atherosclerotic or restenotic lesion formation in rabbits through both inhibition of VSMC proliferation and inhibition of adhesion molecule expression with subsequent reduction of vascular mononuclear cell infiltration (von der Leyen et al. 1995; Qian et al. 1999).

1.4.3 Vascular Remodelling

Remodelling is both a physiological and pathological process in the vasculature which can be defined as a change in the calibre of the vessel, and can be sub-divided into hypertrophic, hypotrophic or eutrophic remodelling, due to an increase, decrease or no change in the overall tissue mass respectively. It can result in a decrease or increase in both wall: lumen ratio and vessel luminal diameter, termed inward and outward remodelling respectively (Mulvany 1999). The biological processes that can affect vascular remodelling include VSMC proliferation, apoptosis and migration, adventitial fibrosis and migration of adventitial fibroblasts (Scott et al. 1996; Shi et al. 1996; Shi et al. 1997). These processes can exert major changes in arterial architecture either alone, or in co-ordination with each other (Bennett 1999) (Fig. 1.3).

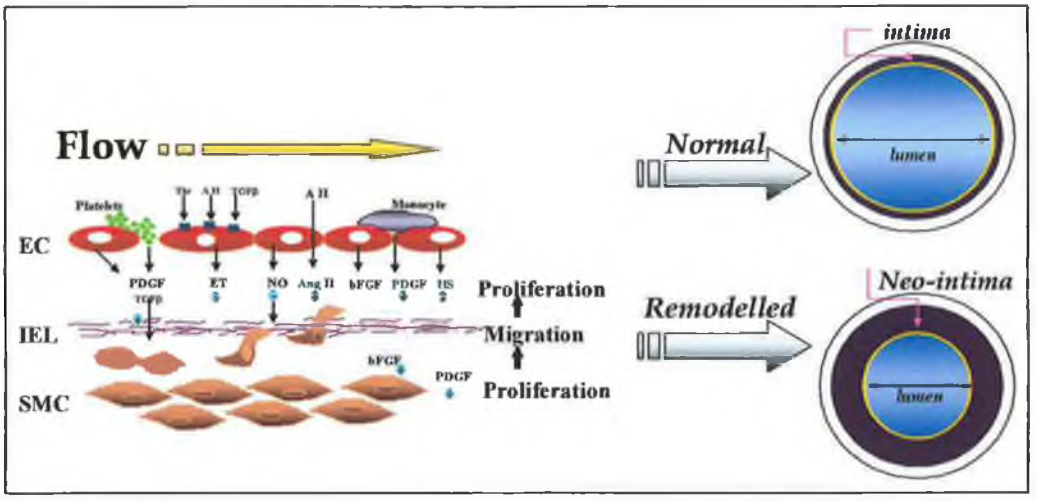


Fig. 1.3 Endothelial Control of Vascular Structure

Physiological remodelling is evident during vascular remodelling after birth. Closure of the ductus arteriosus, for example, is due to vascular remodelling accompanied by increased VSMC apoptosis, and concomitant changes in VSMC proliferation and matrix synthesis (Slomp et al. 1997). Inappropriate vascular remodelling, including its absence, is a prominent feature of the pathogenesis of many vascular diseases. Many studies have demonstrated that changes in intravascular forces that occur in disease states, such as hypertension, result in a decreased lumen and increased media: lumen ratios in arterial blood vessels, as seen in essential hypertensive as compared to normotensive patients (Nordborg et al. 1983).

1.4.4 Mechanical Forces

Shear stress is described as the dragging frictional force resulting from blood flow. Under normal physiological conditions, EC are primarily subjected to this haemodynamic shear stress. However, under conditions of endothelial dysfunction or denudation, shear stress can also exert its affect on the underlying VSMC. In arterial circulation, based on altering vessel wall diameters, the mean wall shear stress is 10 – 70 dyn/cm^2. Changes in shear stress can result from changes in pulse pressure, which is defined as the difference between peak systolic and diastolic blood pressure. EC, which contain shear stress response elements, respond to physiological or pathological alterations in shear stress by releasing vasoactive agents and pro- or anti-atherogenic substances (Davies 1995; Traub and Berk 1998).

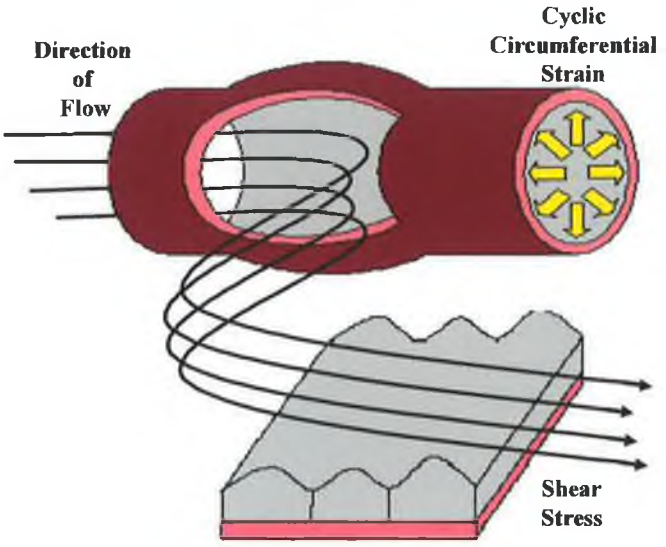


Fig. 1.4 Blood Vessels are Permanently Subjected to Mechanical Forces

Another well characterized haemodynamic force associated with the vasculature is cyclic circumferential strain (cyclic strain). Cyclic strain in arterial vessels is due to the repetitive pulsatile force on the vessel wall due to arterial blood pressure. Cyclic strain can be multi-dimensional as the pulsatile force acts perpendicular to the blood vessel, resulting in "stretching" of the vascular cells in multiple planes. All cells of the vessel wall experience cyclic strain under normal physiological conditions. VSMC, which constitute the major component of the vessel wall, together with elastin and collagenous components, absorb most of the pressure-induced cyclic strain. Normal blood pressure is considered to be 120/80

mmHg, whereas blood pressures of above 140/90 mmHg and below 90/60 mmHg are considered high and low respectively. Factors ranging from physical exertion to psychological stress can result in a transient rise in blood pressure, and a consequent transient increase in cyclic stress. Genetic predisposition to hypertension can lead to a chronic increase in cyclic stress, resulting in potentially serious clinical manifestations. Conversely, factors such as electrolyte imbalance, ischaemic heart disease and systemic sepsis can result in transient hypotension, and a consequent transient decrease in cyclic stress (Ikeda et al. 1999; Phillips et al. 2000; Lapinsky and Mehta 2005).

1.5 Atherosclerotic Plaque Formation

The damage to the normal endothelium results in the initiation of an inflammatory response. The endothelium expresses adhesion and chemoattractant molecules, that act to recruit inflammatory leukocytes such as monocytes and T-lymphocytes and, in addition, extra-cellular LDL begins to accumulate in the tunica intima, in part by binding to proteoglycans, and undergoes oxidative modification (Ananyeva et al. 1997; Williams and Tabas 1998). Accumulated monocytes in the arterial wall subsequently express scavenger receptors which bind to oxidized-LDL (ox-LDL), transforming them into lipid-laden foam cells, facilitated in part by macrophage colony-stimulating factor (MCSF) (Rohrer et al. 1990; Qiao et al. 1997). In addition, leukocytes and endogenous cells of the vascular wall can secrete inflammatory cytokines and growth factors that further amplify leukocyte recruitment and cause VSMC migration and proliferation (Fig. 1.5). The formation of this intimal macrophage-rich fatty streak, the precursor of an atherosclerotic lesion, appears to be ubiquitous in humans, and can develop over many years. In fact, these possible pre-cursors to atherosclerosis have been found in the intima of infants (Stary et al. 1994).

Lesion progression can occur due to the expression of tissue factor, a potent coagulant, and matrix-degrading proteinase that can act to weaken the integrity of the plaque. If the plaque subsequently ruptures, coagulation factors in the blood gain access to the thrombogenic, tissue factor-containing lipid core resulting in thrombosis (the formation of a clot or thrombus inside a blood vessel, obstructing the flow of blood through the circulatory system). Depending on the balance between

pro-thrombogenic and fibrinolytic mechanisms, an occlusive thrombus could occur at this stage with deleterious clinical consequences or death. Alternatively, re-absorption of the thrombus results in the release of thrombin and other mediators including PDGF and TGF-β from de-granulating platelets which results in collagen and SMC accumulation, and the progression of the lesion from a fibrofatty to an advanced fibrous and often calcified plaque, oftentimes causing significant stenosis of the vessel. In some cases, occlusive thrombi arise from superficial erosion of the endothelial layer and often complicate advanced stenotic lesions (Libby and Schonbeck 2001).

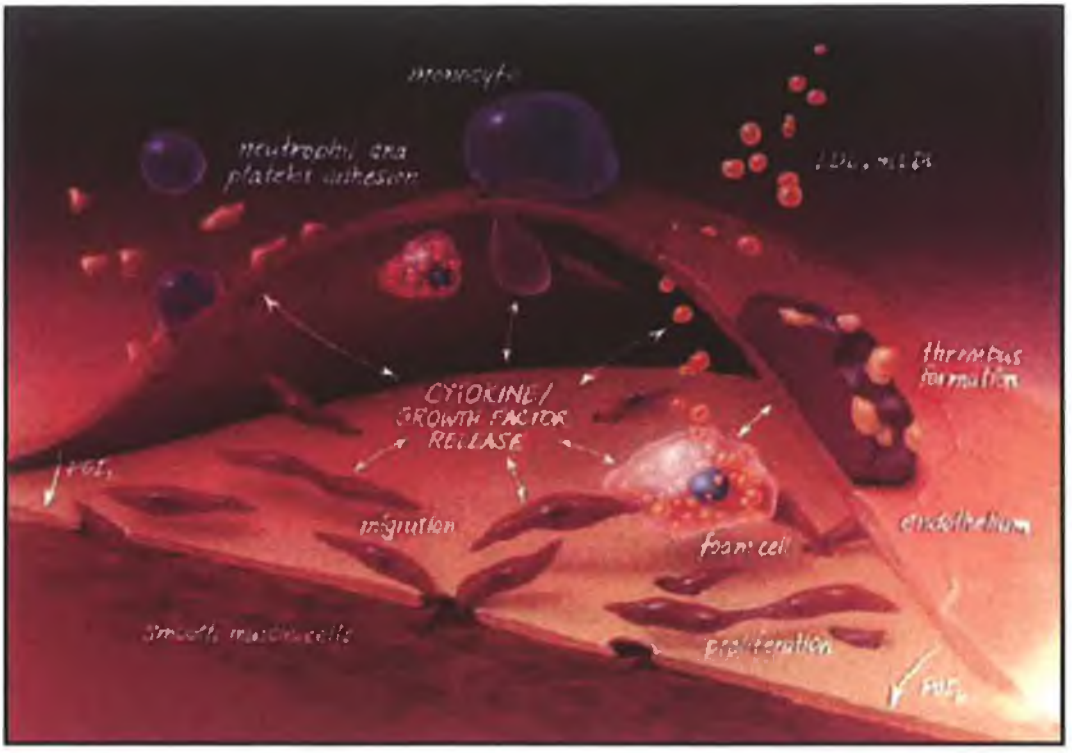


Fig. 1.5 Atherogenesis: Fibrous Plaque Formation

Tedgui A, Mallat Z: Atherosclerotic plaque formation; *Rev Prat* 49(19):2081-6; (1999)

However, not all fatty streaks evolve into advanced atherosclerotic lesions. The balance between pro- and anti-atherogenic factors determines whether a fatty streak progresses to form an atherosclerotic lesion or regresses. In addition to inflammatory cell infiltration, the progression of an atherosclerotic lesion requires the participation of VSMC, which are a principal source of the ECM that constitutes a large volume of an advanced atheroma (Geng and Libby 2002). Proliferation and migration of SMC plays an important role in the regulation of SMC number within an atherosclerotic plaque. However, the importance of SMC apoptosis in the

atherosclerotic plaque is being increasingly recognized. By counterbalancing proliferation, apoptosis may limit the cell accumulation in the intimal compartment, eliminate the lipid-laden foam cell, and decrease overall cell number, leading to regression of the fatty streak (Geng and Libby 2002).

1.6 Apoptosis

Apoptosis was first described in 1972 (Kerr et al. 1972) with the term originating from the Greek meaning falling (ptosis) off (apo). Apoptosis is described as a physiologically relevant and active form of cell death whose control and mediation is cell-specific and contextual, and is highly conserved throughout evolution (Hetts 1998; Bai et al. 1999). Apoptosis is recognized as an important physiological process, both during development and in the maintenance of homeostasis in the adult. This mode of cell death allows for the removal of damaged, injured, infected and incompetent cells from the body both quickly and efficiently.

Cells of multi-cellular organisms generally die in either one of two well-characterized ways, depending on the context and cause of death. These two forms of cell death, apoptosis and necrosis, can be defined and contrasted on the basis of their individual mechanisms, biochemistry, and altered cellular morphology (Hetts 1998).

Necrosis is an uncontrolled cell death characterized by cell swelling and mitochondrial damage leading to rapid depletion of energy levels; a breakdown of homeostatic control; cell membrane lysis and release of the intracellular contents, leading to an inflammatory response, with oedema and damage to the surrounding cells. A classic example of necrosis is ischemic necrosis of the cardiomyocyte during acute myocardial infarction (Yeh 1997). The release of extracellular components such as kinins incites localized inflammation, oedema, capillary dilation and macrophage aggregation (Yeh 1997; Hetts 1998; Kuan and Passaro 1998). The inflammatory response is lengthy and unpredictable in its timecourse, often taking hours to days to occur and subside (Kuan and Passaro 1998). Although necrosis may be important in acute injury and certain acute inflammatory responses, it is not the mechanism whereby cells normally die (Hetts 1998).

In contrast, apoptosis is an active, contained process resulting from either external or internal stimuli (Kuan and Passaro 1998). It is a controlled cell death that keeps the intracellular content of the dying cell sequestered and is defined by a series of cellular change. Firstly the cell shrinks, looses contact with neighbouring cells or surrounding matrix and starts to display intracellular proteins on its surface. The chromatin in the nucleus condenses and the DNA is cleaved into small fragments of 180 base pairs (Steller 1995; Yeh 1997), which lead to characteristic DNA laddering when subjected to gel electrophoresis. The plasma membrane then begins to show a bubbled appearance and small membrane bound bodies break off containing intracellular material which can include nuclear matter and cellular organelles, which are usually unaffected. The fragments are known as apoptotic bodies and they are quickly removed by phagocytes or by neighbouring cells. As no cytosolic components are released into the extracellular space, an inflammatory response is not initiated (Hetts 1998; Kuan and Passaro 1998). Unlike necrosis, this process is relatively rapid, reaching completion in approximately two hours (Kuan and Passaro 1998).

If this does not occur quickly enough the plasma membrane and intracellular organelles can breakdown resulting in lysis of the fragments; a process called secondary necrosis.

The genetics and molecular mechanisms of apoptosis were first characterized during studies in *C. elegans*. Programmed cell death during the development of this nematode is highly precise and predictable, of the 1090 cells produced during development, 131 are destined to die (Ellis et al. 1991). Such studies have identified four sequential steps during the process of apoptosis: (1) commitment to cell death induced by extracellular or intracellular triggers; (2) activation of intracellular proteases; (3) engulfment of the apoptotic bodies by other cells; and (4) degradation of the apoptotic bodies within the lysosomes of the phagoctyotic cells (Steller 1995). Genetic analysis of factors involved in apoptosis in *C. elegans* implicated three main genes, cell death defective 3 (ced 3), ced 4 and ced 9. As the genetic control of apoptosis is conserved throughout evolution, human homologues of these genes have been identified, these are caspase 8, Apaf-1, and Bcl-2 respectively (Hetts 1998).

1.6.1 Triggers of apoptosis

Apoptosis-inducing stimuli can be either extrinsic or intrinsic and can cause apoptosis through the activation of a number of different pathways. In most cases, however, these pathways converge on the caspase system of enzymes to execute their function (Fig. 1.6). Extrinsic triggers of apoptosis include activation of receptor-mediated death-signalling pathways, such as Fas ligand activation, exposure to substances that cause DNA damage including chemotheraputic agents and ionizing radiation (Hetts 1998; Rich et al. 2000). Apoptosis can also be induced due to the removal of death-inhibiting (or survival-promoting) ligands, for example, vascular smooth muscle cells undergo apoptosis due to the withdrawal of growth factors such as IGF and PDGF (Best et al. 1999). In addition, intrinsic signals such as increased intracellular oxidative stress can cause the initiation of apoptosis within the cell, in which the mitochondria play a pivotal role (Desagher and Martinou 2000)

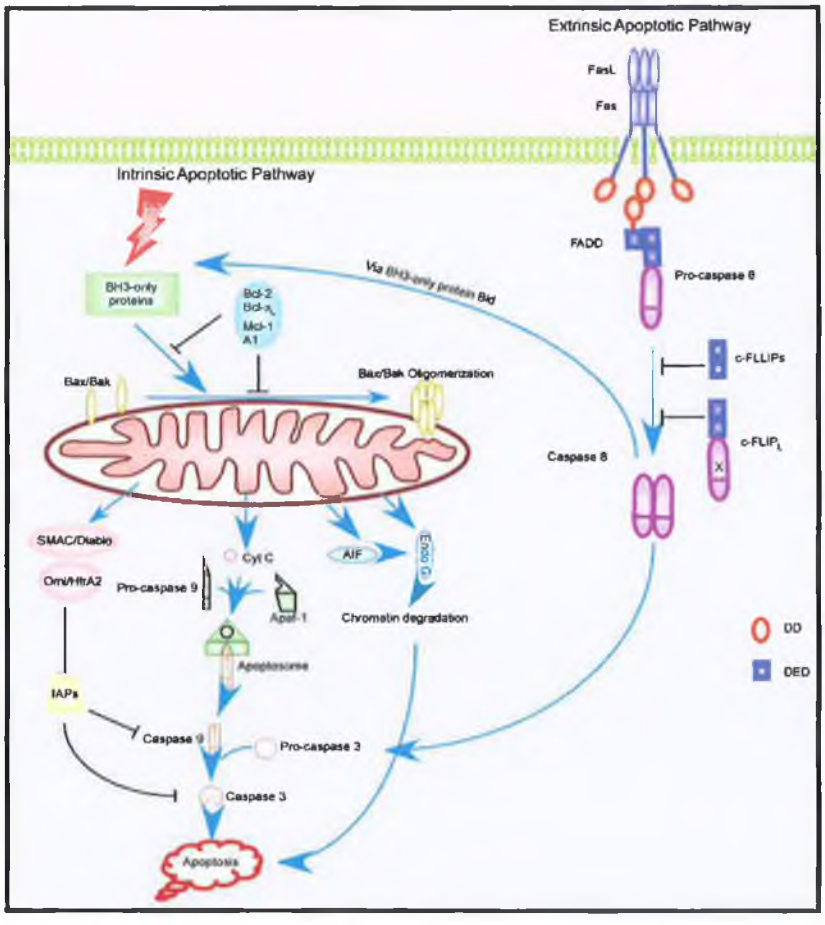


Fig. 1.6 Extrinsic and Intrinsic Activation of Apoptosis

Zang, N., Hartig, H., Dzhagalov, I., Draper, D. and He, Y-W.: The Role of Apoptosis in the Development and Function of T Lymphocytes; *Cell Res* 15: 749-769; (2005)

(a) Receptor-Mediated Death Signalling Pathways

Death receptors belong to the tumour necrosis factor (TNF) superfamily, and play a central role in instructive apoptosis (Ashkenazi and Dixit 1998). Members of the death receptor family contain one to five cysteine-rich repeats in their extracellular domain, and a death domain (DD) in their cytoplasmic tail (Fig. 1.7). This DD is essential for initiation of the apoptotic signal by these receptors. TNF receptor 1 and Fas (CD95) are two such receptors that initiate apoptosis when activated either by their respective ligands, TNF-α and Fas-L, or by agonist-like antibodies (MacLellan and Schneider 1997; Gupta 2003). Following receptor-ligand interaction, the receptor oligomerizes, recruits adaptor molecules forming a death inducing signalling complex (DISC), which recruits and activates the caspase cascade and can culminate in apoptosis of the cell (Yeh 1997; Gupta 2003).

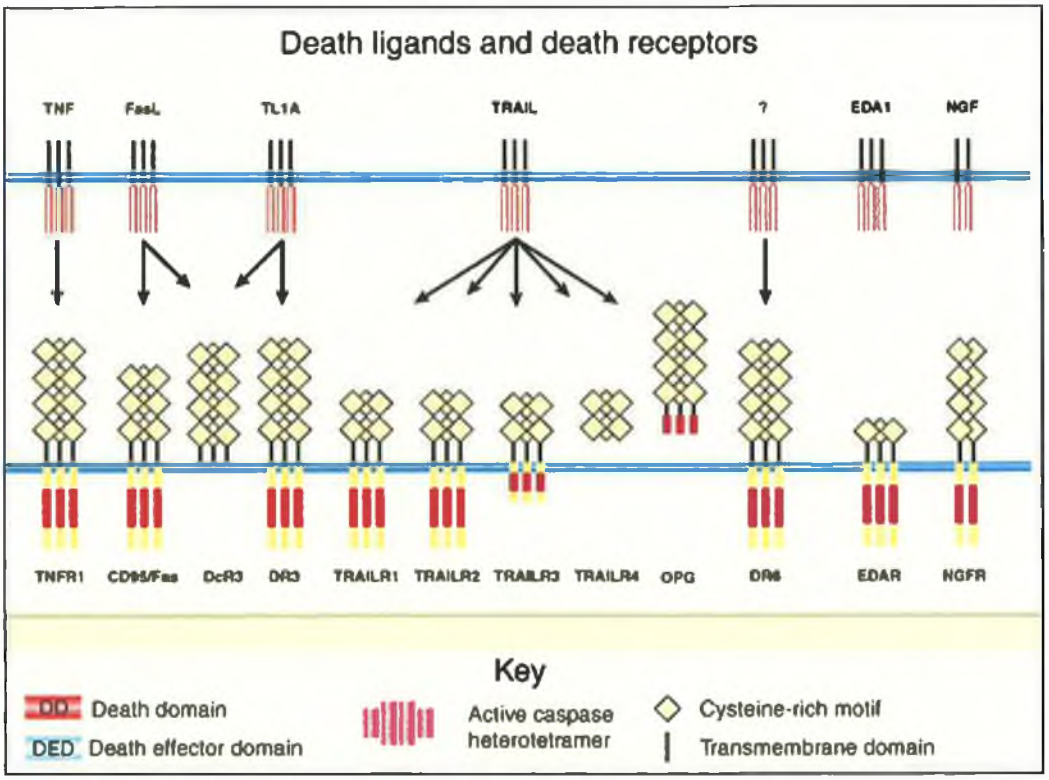


Fig. 1.7 Death Receptor Family Members
Lavrik. I, Golks A. and Krammer PH: Death Receptor Signalling; *J Cell Sci* 118: 265-267 (2005)

(b) Fas and Fas Ligand

Apoptosis following a cellular immune response is primarily mediated by Fas-Fas ligand (Fas-L) interaction (Krammer 2000). Fas is a type I transmembrane receptor, that is abundantly expressed in many tissues, including the heart, EC and VSMC (Gibbons and Pollman 2000). Fas-L however, displays a more restricted expression. Fas-L is a type II membrane protein, but is capable of undergoing cleavage by a metalloproteinase to generate a soluble but less biologically active form of the receptor (Tanaka et al. 1995). Both Fas and Fas-L expression can be upregulated by cytokines and stressful stimuli *via* nuclear factor-κB (NFκB) dependent mechanisms (Nagata 1999).

Fas receptor activation results in the trimerization of the receptor, and the formation of a signalling complex of molecules linked to the cytoplasmic portion of the receptor, as the cytoplasmic domain of the receptor does not have intrinsic enzymatic activity. The adaptor protein Fas-associated *via* death domain (FADD) is recruited to Fas through interactions between their respective death domains. In addition, FADD also contains a death effector domain (DED) at its N-terminus, which is responsible for binding to pro-caspase 8 (FLICE (FADD-like Interleukin 1 beta converting enzyme)) to form a DISC. Subsequently, pro-caspase 8 is auto-proteolytically cleaved, triggering the cellular apoptosis cascade (Ashkenazi and Dixit 1998; Gibbons and Pollman 2000; Gupta 2003). In some cells, the levels of pro-caspase 8 is low, therefore the caspase cascade in this case must be amplified by the mitochondria to result in apoptosis (Li et al. 1998) (Fig. 1.8).

Fas-mediated apoptosis is regulated by FLICE-like inhibitory proteins (FLIP), a protein containing two DEDs. FLIP is present within the cell in two alternatively spliced isoforms, the long form ($FLIP_L$) and the short form ($FLIP_S$), and stable over-expression of these results in resistance to receptor-mediated apoptosis. $FLIP_L$ resembles caspase 8 and caspase 10, but lacks protease activity (Irmler et al. 1997), therefore effectively functioning as an endogenous inhibitor of apoptosis. In addition, FLIP also promotes the activation of the NFκB and extracellular signal regulated kinase (ERK) signalling pathways through the recruitment of adaptor proteins including receptor interacting protein (RIP) and receptor associated factors (Raf) (Kataoka et al. 2000).

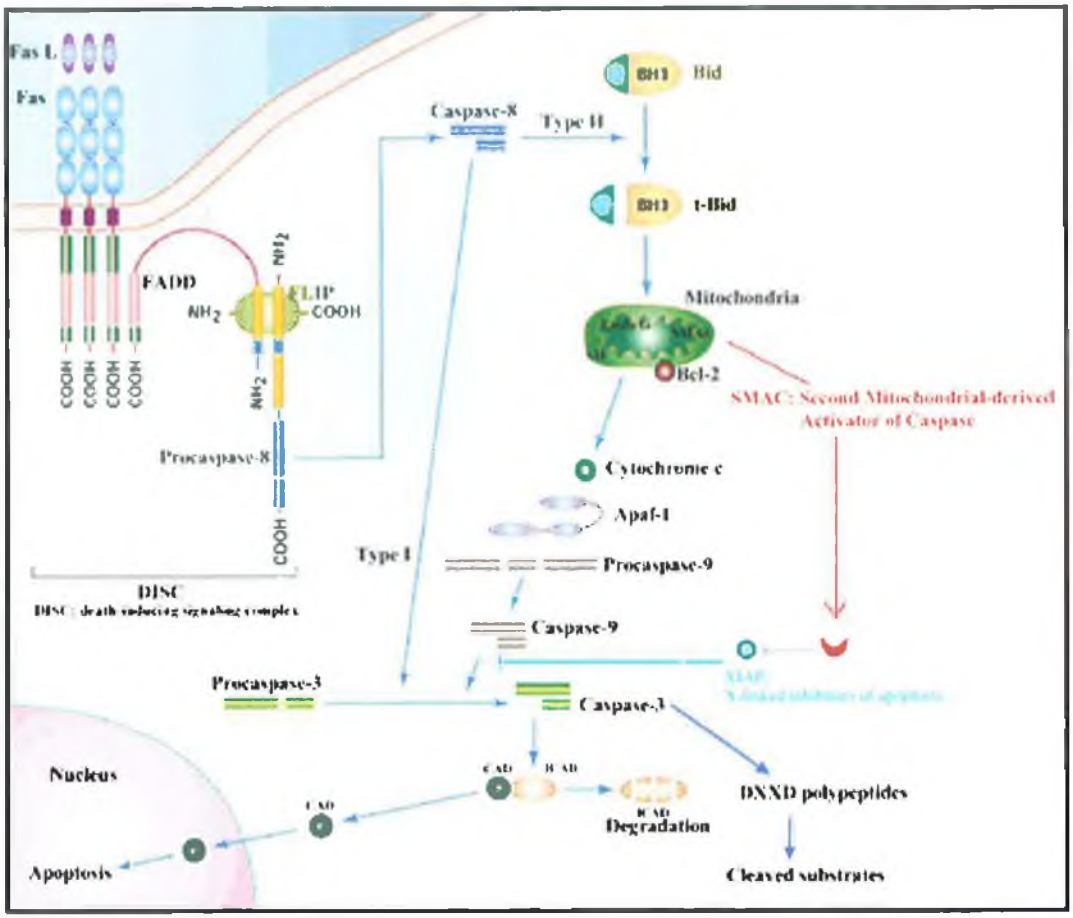


Fig. 1.8 The Fas-Fas Ligand Pathway of Apoptosis

Bobé, P.: The Fas-Fas Ligand Apoptotic Pathway
http://www.infobiogen.fr/services/chromcancer/Deep/Fas-FasLigandID20039.html

The induction of Fas-mediated apoptosis in VSMC has been the subject of a number of conflicting reports. Sata *et al.,* (2000) report that soluble Fas-L and agonistic Fas antibodies fail to induce VSMC apoptosis. In contrast, however, viral over-expression of membrane-tethered Fas-L has been shown to promote VSMC apoptosis (Gibbons and Pollman 2000; Sata et al. 2000). This discrepancy in VSMC response to Fas may be a consequence of differing VSMC phenotype. Chan *et al.,* (2000) has shown that the Fas-L resistant VSMC exhibit normal levels of receptor expression and receptor engagement mechanisms. However, these cells display decreased expression of FADD, caspase 8 and caspase 3, and increased expression of FLIP when compared to Fas-L susceptible VSMC (Chan et al. 2000). This highlights the fact that the VSMC population is heterogeneous and as such, may respond differently to stimuli governing cell fate decisions.

(c) Apoptosis due to DNA Damage

Growth arrest, repair and apoptosis are all legitimate cellular responses to DNA damage. The choice of cell fate in each instance will depend on cell type, location, environment, and extent of damage.

p53 is a transcription factor that has been implicated in cell cycle arrest and in some, but not all, forms of apoptosis (MacLellan and Schneider 1997). The level of p53 activity within the cell is maintained at a low level, under normal conditions, due to interaction with the Mdm-2 protein, which marks it for ubiquitin-mediated destruction (Mayo et al. 1997). DNA damage-induced phosphorylation of either p53 or Mdm-2 prevents these two proteins from interacting, thus stabilizing and activating p53 (Evan and Littlewood 1998). p53 levels are reported to increase within minutes of DNA damage, resulting in growth arrest or apoptosis of the cell (Lundberg and Weinberg 1999).

(d) Mitochondrial Pathway of Apoptosis

A number of stimuli, including UV radiation, stress molecules (reactive oxygen and reactive nitrogen species), and growth factor withdrawal mediate apoptosis *via* the mitochondrial pathway (Gupta 2003). During the process of apoptosis the mitochondria undergo morphological and cellular re-distribution changes including a reduction in size and an increase in matrix density (mitochondrial pyknosis). In addition, the mitochondria which are normally dispersed throughout the entire cell, display perinuclear clustering (Desagher and Martinou 2000).

Mitochondria are organelles comprising of a matrix surrounded by an inner membrane (IM), an inter-membrane space, and an outer membrane (OM). The IM contains molecules that contribute to the formation of an electrochemical gradient or membrane potential, including adenosine tri-phosphate (ATP) synthase and adenine nucleotide translocator. The OM contains a voltage-dependent anion channel, whilst the inter-membrane space contains proteins that, when activated, contribute to apoptosis, including holocytochrome c, some pro-caspases, and apoptosis-inducing factor (AIF) (Gupta 2003). At least three mechanisms contribute to mitochondrial-mediated apoptosis. These include disruption of electron transport, oxidative phosphorylation and ATP production, alteration of the cellular redox potential, and release of proteins such as cytochrome c, that trigger activation of the caspase family of proteases (Green and Reed 1998).

1.6.2 The Caspase Cascade

Caspases are an evolutionally conserved family of cysteine proteases, which are viewed as the "central executioners" in apoptotic cell death. Caspases are synthesized as enzymatically inert zymogens, requiring proteolytic cleavage at an internal aspartate residue to induce their activation (Gibbons and Pollman 2000; Hengartner 2000). These zymogens are composed of three domains, an N-terminal pro-domain, and two domains termed p10 and p20. The activation of caspases generally results in a serial sequence of caspase activation known as the caspase cascade, which is a common end pathway in apoptosis induced by many different stimuli. Three general mechanisms of caspase activation have been described to date, these are proximity-induced activation, processing by an upstream caspase, and association with a regulatory subunit (Hengartner 2000) (Fig. 1.9).

Proximity-induced activation occurs in the case of caspases 2 and 8. The aggregation of a number of caspase proteins *via* adaptor proteins renders the caspases capable of auto-proteolytic cleavage, and subsequent activation (Hengartner 2000). Most caspases are activated by cleavage between the p10 and p20 domains, and between the p20 and N-terminal pro-domain. Activation of caspase 8 and caspase 9, known as initiator caspases, results in subsequent cleavage and activation of downstream effector caspases such as caspase 3, caspase 6 and caspase 7. The effector caspases are responsible for the induction of the biochemical and morphological changes associated with apoptosis, and are usually more abundant and active than the initiator caspases (Gibbons and Pollman 2000; Hengartner 2000).

Caspase 9 is activated through association with a regulatory subunit known as an apoptosome. The apoptosome consists of cytochrome c, an adapter molecule Apaf-1 (apoptosis protease-activating factor), and pro-caspase 9 (Hengartner 2000; Gupta 2003). Cytochrome c is a nuclear DNA encoded protein, its precursor, apocytochrome c, is synthesized on free ribosomes within the cytoplasm and can spontaneously insert into the mitochondrial outer membrane (Gonzales and Neupert 1990; Stuart and Neupert 1990). This protein then incorporates a heme group, the protein re-folds, and is inserted into the inter-membrane space. The release of functional cytochrome c is reported to be an essential component for the formation of the apoptosome, and subsequent activation of caspases 9 and 3 (Liu et al. 1996). Apaf-1 is another essential component of the apoptosome, and appears to be activated by p53 and adenoviral early region 1A (E1A) (Fearnhead et al. 1998;

Moroni et al. 2001). Apaf-1 has an N-terminal caspase recruitment domain (CARD), adjacent Walker's A- and B-box sequences and C-terminal to this, twelve WD-40 repeats (Cai et al. 1998). The binding of cytochrome c to the WD-40 repeats allows a conformational change of Apaf-1, thus exposing the CARD domain. This conformational change is stabilized with the binding of ATP or dATP to the Walkers boxes. Pro-caspase 9 subsequently binds to the CARD domain, resulting in its activation. As Apaf-1 does not have caspase activity, it is proposed that it facilitates caspase 9 auto-catalysis (Cai et al. 1998).

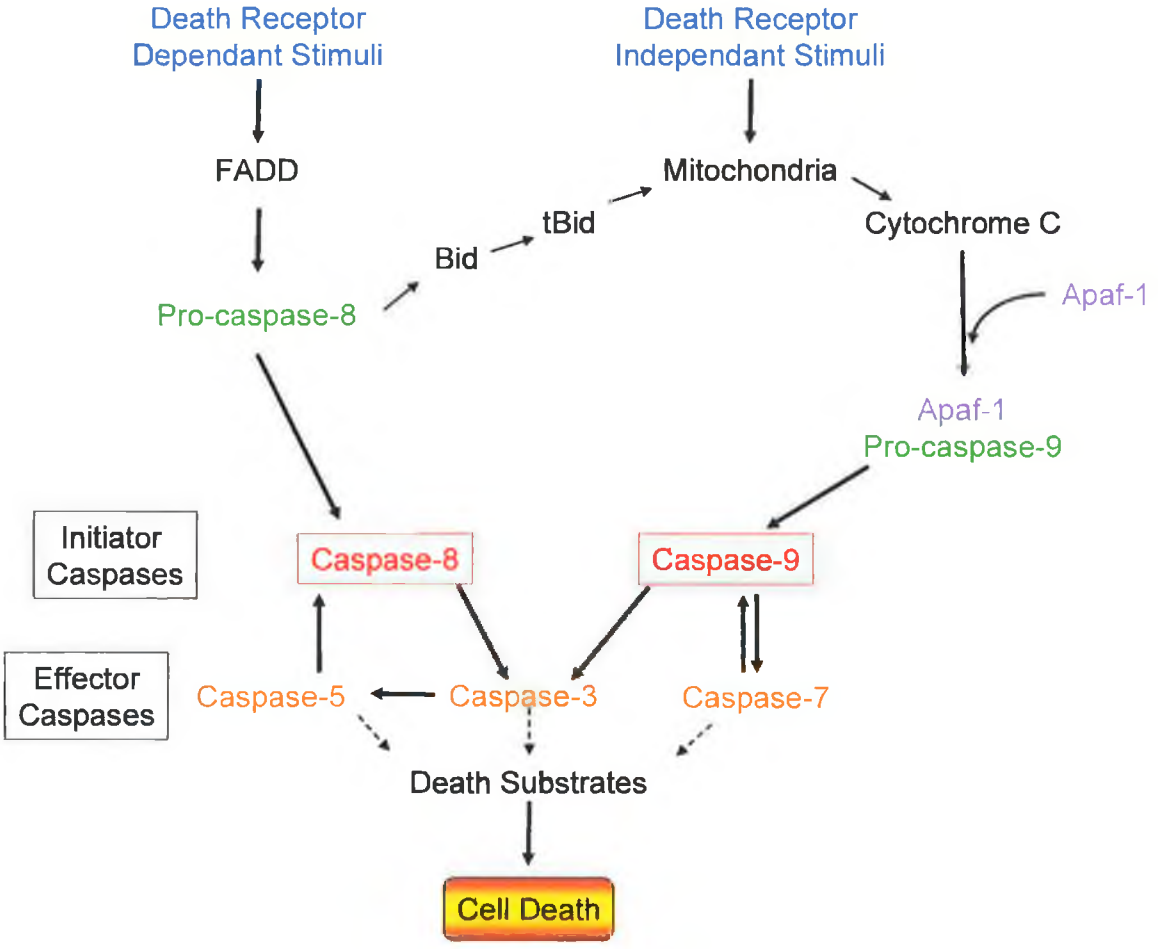


Fig. 1.9 Activation of the Caspase Cascade

Caspase 9 subsequently cleaves and activates caspase 3, caspase 6 and a number of other substrates resulting in the biochemical and morphological

characteristics of an apoptotic cell. These substrates include caspase-activated DNase (CAD), nuclear laminins, cytoskeletal proteins, and p21-activated kinase 2 (PAK) among others. Activation of CAD within the cell results from the caspase 3-mediated cleavage of the CAD inhibitory subunit. This active nuclease is subsequently responsible for the characteristic "DNA laddering" of apoptosis. Cleavage of cytoskeletal proteins, such as fodrin and gelsolin, results in an overall loss of cellular shape (Kothakota et al. 1997) whereas nuclear laminin cleavage is responsible for the characteristic nuclear shrinkage and budding seen in apoptosis (Rao et al. 1996). In addition, caspase-mediated cleavage of PAK 2, a member of the p21-activated kinase family, appears to mediate the distinctive blebbing of apoptotic cells (Rudel and Bokoch 1997).

Whilst caspase activation undoubtedly plays an important role in the initiation and execution of apoptosis, a number of caspase-independent inducers of apoptosis have also been identified. Reactive oxygen species (ROS) are associated with apoptosis (Suzuki et al. 1997). The generation of oxidants is involved in changes in mitochondrial permeability and release of molecules, other than cytochrome c, involved in the execution of apoptosis. Apoptosis inducing factor (AIF) is one such molecule that is released from the mitochondria and can induce caspase-independent apoptosis. AIF is transported to the nucleus where it causes ATP-independent large DNA fragmentation and chromatin condensation (Susin et al. 1996; Gupta 2003). In addition, the release of Endo G nuclease from the mitochondrial inter-membrane space is thought to mediate nuclear DNA fragmentation (Li et al. 2001).

1.6.3 The Bcl-2 Family

Bcl-2 was initially identified as a frequent translocation occurring in human lymphoma and was found to function by promoting cell survival (Kirshenbaum 2000). Bcl-2 is now recognized as being part of a large family of homologous proteins that can either promote or suppress apoptosis, known as the Bcl-2 family. The Bcl-2 family are considered the primary regulators of mitochondria-induced apoptosis, controlling mitochondrial membrane permeabilisation and cytochrome c release (Thompson 1995; Desagher and Martinou 2000; Marsden et al. 2002).

At least fifteen members of the Bcl-2 family have been identified, and these can be divided into two functional groups, pro-apoptotic and anti-apoptotic Bcl-2

family members. Examples of family members that prevent apoptosis are Bcl-2, Bcl-x_L and Bfl-1 among others, whilst Bcl-2 family members that promote apoptosis include Bad, Bax, Bid and Bik (Reed 1994; Sedlak et al. 1995; Green and Reed 1998).

Structural analysis of the Bcl-2 family of proteins has identified four conserved regions within the family, known as the Bcl-2 homology domains (BH1-BH4) (Muchmore et al. 1996). All members of the Bcl-2 family contain at least one of these domains, which are formed by α-helices and thus enable different members of the family to form either homo- or heterodimers and regulate each other (Oltvai et al. 1993; Kelekar and Thompson 1998) (Table 1.1). The majority of Bcl-2 family members share sequence homology at the C-terminal region, with a ~20-residue hydrophobic domain, which targets the Bcl-2 family of proteins to intracellular membranes. The principal membrane to which the Bcl-2 family members are directed is the outer mitochondrial membrane, therefore this C-terminal region is critical for the function of both the pro- and anti-apoptotic Bcl-2 family members (Goping et al. 1998; Kirshenbaum 2000). Variable sequence homology, however, exists between the BH1 to BH4 domains which implies that this variation in homology may determine whether the given Bcl-2 family member acts to promote or prevent cell death (Kirshenbaum 2000). The anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-x_L, contain at least three BH domains and all contain the N-terminal BH4 domain. The BH4 domain is restricted to Bcl-2 family members with anti-apoptotic properties, therefore it is postulated that this domain is critical in preventing apoptosis. This is supported in a number of studies in which the deletion of the BH4 domain rendered the anti-apoptotic Bcl-2 protein defective in suppressing apoptosis (Hunter et al. 1996; Huang et al. 1998). Pro-apoptotic Bcl-2 family members, such as Bax and Bak, have been identified as closely resembling Bcl-2, containing BH1-BH3 domains. Other pro-apoptotic members of this family are described as "BH3 only" as they contain the BH3 domain alone, which is therefore sufficient for the pro-apoptotic activity of these proteins (Kelekar and Thompson 1998) (Fig. 1.10). Cell fate is determined by the ratio of pro- and anti-apoptotic members of the Bcl-2 family within any given cell (Sedlak et al. 1995; Reed 1997).

Inhibition and Promotion of Apoptosis by Bcl-2 Family Proteins

Protein	Effect on Apoptosis	Protein-Protein Interactions
Bcl-2	↓	Bax, Bak
Bcl-x_L*	↓	Bax, Bak
Bcl-W	↓	
Bax	↑	Bcl-2, Bcl-x_L
Bad	↑	Bcl-2, Bcl-x_L
Bak	↑	Bcl-2, Bcl-x
Bcl-x_S*	↑	Bax, Bak

* Alternatively spliced

Table 1.1 Pro- and Anti-apoptotic Members of the Bcl-2 Family of Proteins
Table adapted from McLellan and Schneider, 1997

The Bcl-2 family can delay or prevent apoptosis by a diverse number of death signals thus suggesting that it influences a number of signalling factors that can lead to cell death. Bcl-2 has been shown to increase the anti-apoptotic NF-κB transactivation which can in turn, up-regulate anti-apoptotic Bfl-1 and Bcl-x_L expression (Lee et al. 1999; Kirshenbaum 2000).

However, members of the Bcl-2 family primarily exert their pro- or anti-apoptotic influence through regulation of mitochondrial membrane potential and corresponding cytochrome c release. Upon stimulation of apoptosis, many members of the pro-apoptotic Bcl-2 family translocate from the cytoplasm to the mitochondria. Following a conformational change, these proteins can insert into the mitochondrial membranes, disrupting membrane integrity and increasing mitochondrial membrane potential. This results in the release of several mitochondrial proteins involved in caspase activation and other apoptotic events (Goping et al. 1998; Zamzami and Kroemer 2001). The pro-apoptotic protein, Bax, is normally present in the cell cytoplasm. Following stimulation of apoptosis, Bax migrates to the mitochondria where it inserts into the mitochondrial membrane and forms a homodimer, resulting in an increase in mitochondrial membrane potential, thus facilitating apoptosis. Similarly, the pro-apoptotic protein Bid is cleaved by caspase 8 and the resulting C-terminal fragment, tBid, translocates to the mitochondria.

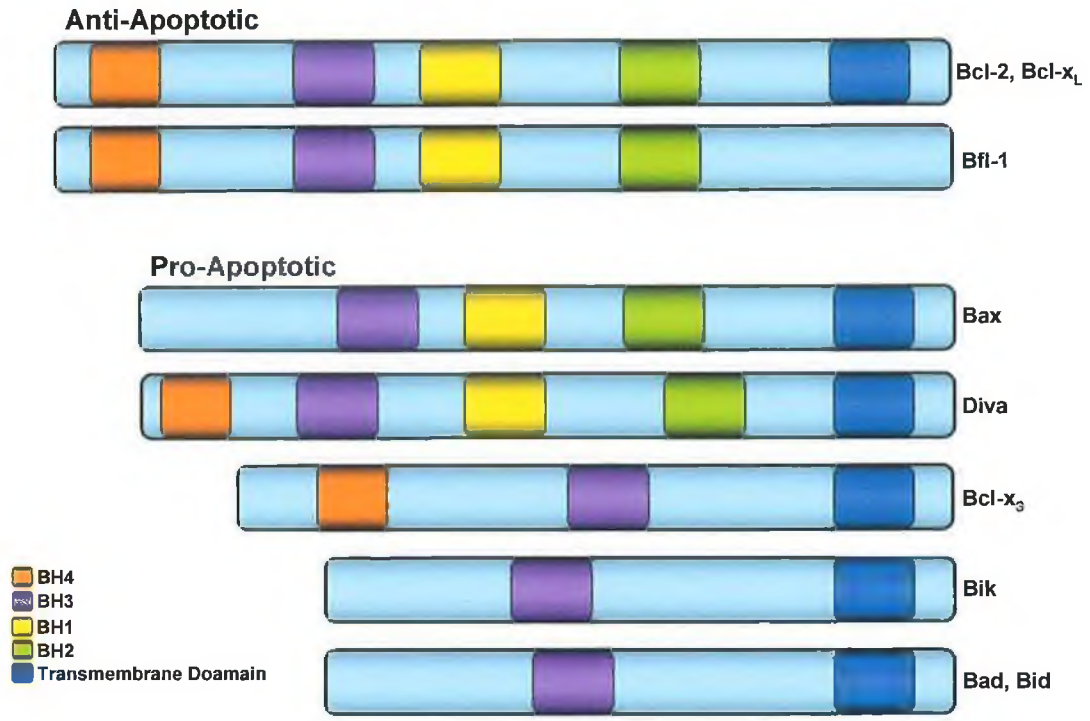


Fig. 1.10 Conserved Regions of Bcl-2 Family Proteins

Bid therefore mediates crosstalk between the death receptor and mitochondrial pathways of apoptosis. tBid facilitates insertion of other pro-apoptotic proteins into the mitochondrial membrane and promotes Bax dimerization (Eskes et al. 1998; Jurgensmeier et al. 1998; Ferri and Kroemer 2001). Conversely, many of the anti-apoptotic Bcl-2 family proteins are associated with the mitochondrial membrane where they act to inhibit increases in mitochondrial membrane potential, and prevent apoptosis by maintaining membrane integrity. Both the pro- and anti-apoptotic members of the Bcl-2 family appear, at least in part, to regulate each other. Bcl-2 can form a heterodimer with Bax, thus inhibiting the ability of Bax to increase mitochondrial membrane potential. Similarly, pro-apoptotic members can exert their effect by binding to their anti-apoptotic counterparts. Bad, binds to Bcl-x_L thus inhibiting its anti-apoptotic function (Desagher and Martinou 2000; Ferri and Kroemer 2001; Zamzami and Kroemer 2001).

Bcl-2 family members also appear to modulate other cellular processes in addition to apoptosis however; this appears to be restricted to certain family members. Bfl-1 is an anti-apoptotic member of the Bcl-2 family that also exhibits

proliferative and potent oncogene transforming activities (D'Sa-Eipper and Chinnadurai 1998). Therefore Bfl-1 communicates with both the apoptotic and proliferation cell machineries, suggesting a link between these two cellular processes.

The importance of the Bcl-2 family of proteins in normal physiology is highlighted by the fact that Bcl-2- and Bcl-x_L-deficient mice die either at an embryonic stage or immediately post-natal due to increased apoptosis in multiple organs and tissues of the body (Veis et al. 1993; Motoyama et al. 1995).

The adenovirus E1B 19K protein is functionally similar to Bcl-2 as a survival factor (White 1996). A two hybrid screen of proteins that interact with E1B 19K in the yeast *Saccharomyces cerevisiae* identified several unique cDNAs named NIP 1 (nineteen kD interacting protein-1), NIP 2 and NIP 3 (Boyd et al. 1994). All three proteins interact with discrete conserved domains of E1B 19K protein and Bcl-2 that are involved in suppression of cell death, although a function was not identified (Chen et al. 1997).

Bcl-2/E1B 19 kDa interacting protein (BNIP3) is a unique member of the Bcl-2 family members that is upregulated under hypoxic conditions (Chen et al. 1997; Cizeau et al. 2000). Forced overexpression of BNIP3 induces cell death characterized by localization at the mitochondria, opening of the permeability transition pore, loss of membrane potential and reactive oxygen species (ROS) production (Vande Velde et al. 2000). BNIP3 is also implicated in ischemia-induced apoptosis in rat cardiomyocytes (Kubasiak et al. 2002; Regula et al. 2002).

BNIP3 structure is similar to other Bcl-2 family members since it contains a C-terminal transmembrane domain that targets the protein to mitochondria and is required to induce cell death (Vande Velde et al. 2000). The BNIP3 protein also contains a BH3 domain as determined by sequence consensus with others BH3 domains in the Bcl-2 family members, BNIP3-induced cell death is blocked by overexpression of Bcl-2, however, Bcl-2 fails to associate with the BH3 domain of BNIP3 instead Bcl-2 associates with the transmembrane domain of BNIP3 (Vande Velde et al. 2000).

1.6.4 Role of Apoptosis in the Vasculature

Apoptosis is a normal physiological process, playing important roles in both development and maintenance of a wide variety of tissues, including the vasculature. It is also an essential component in the vascular response to tissue insult or injury. The critical role of apoptosis is evident throughout life and consequently, dysfunctions in apoptosis manifest themselves not only in developmental abnormalities, but also in a wide range of adult pathologies.

Apoptosis plays a critical role in normal vasculogenesis and aberrations in this process can result in embryonic lethality. Decreased apoptosis can result in increased development of endocardial cushions, and subsequently increased cardiac valve formation, whilst overabundance of apoptosis in the heart of developing mice has been shown to result in embryonic lethality (White et al. 2004). Apoptosis is also one of the mechanisms of neonatal vascular remodelling during the transition from foetal to neonatal circulation. Apoptosis is involved in the regression of the human umbilical vessels and the ductus arteriosus, and in the remodelling of the branching great arteries during the neonatal period (Kim et al. 2000). In addition, apoptosis has been shown to be involved in the post-natal morphogenesis of the atrioventricular node and Bundle of His, and aberrations in this process could predispose to tachyarrhythmias or bradyarrythmias in adulthood (James 1994).

The pathogenesis of various forms of vascular diseases involves dysregulation of both apoptosis and proliferation within the cells of the vasculature. Many vascular diseases involve an accumulation of cells within the intimal space. This was initially accredited to perturbations in cellular proliferation however, the importance of apoptosis in vascular remodelling and lesion formation is now recognized (Bai et al. 1999; Pollman et al. 1999).

Apoptosis can contribute to the pathogenesis of vascular disease through several potential mechanisms. The transition of a fatty streak to an atherosclerotic plaque is characterized by the appearance of focal and diffuse regions of cell death. The SMC of these fatty acid streaks express increased levels of the pro-apoptotic protein Bax, which increases the susceptibility of the cells to undergo apoptosis (Kockx et al. 1998). Apoptosis also occurs in advanced atherosclerotic lesions resulting in the formation of hypocellular fibrous zones and a lipid-rich core (Geng et al. 1996). It has also been extensively documented that SMC isolated from atherosclerotic arteries undergo apoptosis more frequently than their counterparts

isolated from healthy vessels (Bennett et al. 1995). Apoptosis can influence the structure of the atherosclerotic plaque, causing thinning of the fibrous cap, rendering the plaque more prone to rupture (Walsh et al. 2000). In addition, increased apoptosis can also cause activation of tissue factor, which may increase the thrombogenicity of the lesion (Mallat et al. 1999). It is postulated that the development of atherosclerotic lesions are regulated by forces governing cellular proliferation and migration, in addition to plaque remodelling due to apoptotic cell death. Similarly, the development of restenotic lesions following balloon angioplasty or atherectomy of diseased arteries is thought to be due, at least in part, to impaired apoptotic signalling (Kraemer 2002).

Many studies have documented increased VSMC apoptosis following balloon injury. This increase in apoptosis occurs both early after injury, with a 70% increase in apoptosis evident 30 minutes post-injury, and can also occur in the neointima from 7 – 30 days post-injury (Han et al. 1995; Bochaton-Piallat et al. 1996; Perlman et al. 1997). It is also postulated that apoptosis plays an important role in the development of vascular lesions, as exuberant balloon injury-induced apoptosis has been found to result in enhanced neointimal formation (Rivard et al. 1999). However, the mechanisms that regulate neointimal SMC apoptosis remain incompletely understood.

Cardiac myocyte apoptosis is a feature of many pathological disorders including myocardial infarction and congestive heart failure (Cook et al. 1999; Kang and Izumo 2000), with Bcl-2 family proteins becoming increasingly recognized as important modulators of cardiac myocyte apoptosis. Bcl-2 is expressed in both the developing and adult hearts, is up-regulated following coronary occlusion in rat hearts and myocardial infarction in human hearts (Kajstura et al. 1995; Misao et al. 1996; Liu et al. 1998). The pro-apoptotic protein Bax is also up-regulated following coronary occlusion and is over-expressed in spontaneously hypertensive rats, which may contribute to increased apoptosis (Fortuno et al. 1998; Liu et al. 1998). Therefore, the possibility of limiting cardiac myocyte loss by inhibiting apoptosis, possibly through interference with the level of expression of members of the Bcl-2 family, may have important therapeutic implications in the treatment of heart failure.

1.6.5 Apoptosis in the atherosclerotic plaque

Apoptosis has been observed at many stages of the development of an advanced atherosclerotic plaque (Kockx et al. 1998). Atherosclerotic plaques typically consist of a lipid-rich core in the central portion of a thickened tunica intima, containing lipids, connective tissue, and dead cells or cell debris. The lipid-core is bound on its luminal aspect by a fibrous cap, at its edges by what is termed the "shoulder" region, and on its abluminal side by the base of the plaque (Libby 1995). Apoptosis is important in the progression of atherosclerotic lesions, resulting in the formation of a mature lesion containing a dense ECM and a relatively sparse cell population. Apoptosis is concentrated in the lipid-rich core of the plaque and occurs in both macrophage/foam cells and VSMC. However, the high percentage of apoptosis in the lesion does not translate to a decrease in tissue volume, therefore it is postulated that the system of phagocytosis of apoptotic cells operates poorly in an atherosclerotic plaque. Several mechanisms may be responsible for this intracellular accumulation of lipids which may decrease the ability of macrophages and SMC to phagocytose apoptotic cells. Increased apoptosis of macrophages in the lesion decreases the population of apoptotic scavenging cells and cross-linking of macromolecules can result in the stabilization of apoptotic cells within the lesion (Aeschlimann and Thomazy 2000). The presence of apoptotic cells or bodies has recently been shown to increase calcification and fibrosis of atherosclerotic lesions (Geng and Libby 2002).

Atherosclerosis is considered to be a relatively benign disease as long as complicating thrombosis can be prevented. Thrombosis underlies the most acute complication of atherosclerosis, notably unstable angina and acute myocardial infarction in the coronary circulation. The lipid-rich core of an atherosclerotic lesion contains large amounts of tissue factor, which is a powerful coagulant that stimulates thrombus formation when in contact with the blood (Wilcox et al. 1989; Schroeder and Falk 1995; Libby 2001; Libby and Schonbeck 2001). Most thrombus formations are due to a fracture in the protective fibrous cap of the atherosclerotic plaque, which usually occurs at the "shoulder" region. Therefore the integrity of the fibrous cap fundamentally determines the stability of a plaque, and its clinical implications.

In 1995, Libby *et al.*, dubbed VSMC the "guardians of the integrity of the fibrous cap". As its name implies, the fibrous cap is made up of a dense fibrous

ECM, primarily composed of collagens and elastin. It is now well established that the fibrous cap can undergo continuous remodelling, which is largely influenced by VSMC. VSMC synthesize and assemble the interstitial collagen, which accounts for the bulk of the ECM of the fibrous cap. Therefore, apoptosis of VSMC and a subsequent reduction in cell number can seriously compromise the integrity of the fibrous cap. In addition, the integrity of the fibrous cap can be weakened by the breakdown of existing ECM proteins through induction of proteolytic enzymes, such as matrix metalloproteinases, by inflammatory cells (Libby 2001). SMC of the arterial plaque also produce matrix metalloproteinases, however, these enzymes primarily effect SMC migration and vascular remodelling (Plutzky 1999).

The realization that the probability of the atherosclerotic plaque rupture, rather than the severity of plaque stenosis, determines its clinical implications led to the classification of atherosclerotic plaques as stable or vulnerable plaques. Most myocardial infarctions, for example, occur in lesions of less than 70% stenosis, with plaque rupture being the underlying pathological event (Falk et al. 1995)

The stable atherosclerotic plaque is not considered likely to rupture, and as such, is clinically not as serious as its "vulnerable" counterpart. The stable plaques are described as hard, sclerotic plaques, with a high VSMC and collagen content, a subsequently thick fibrous cap, and a limited amount of lipid-laden inflammatory cells. In contrast, the vulnerable plaque usually has a large lipid-core, a thin fibrous cap, a high density of inflammatory cells, particularly at the "shoulder region" where rupture often occurs and a paucity of VSMC. An atherosclerotic plaque is considered vulnerable when its lipid-rich core accounts for more than 40% of the total plaque volume (Schroeder and Falk 1995; Plutzky 1999; Corti et al. 2002).

Three major factors determine the vulnerability of the fibrous cap; these are lesion characteristics (location, size and consistency), blood flow characteristics, and consequent vessel wall stress or "cap fatigue". Inflammation is also a key factor in plaque disruption. Inflammatory cells are a source of plaque tissue factor, and lesion thrombogenicity correlates with its tissue factor content (Moreno et al. 1996). In addition, inflammatory cells can secrete cytotoxic substances and proteolytic enzymes that induce VSMC apoptosis and degradation of the fibrous ECM. Mechanical forces experienced by blood vessels are critical in determining both plaque formation and disruption (Schroeder and Falk 1995).

The pathogenesis of atherosclerosis is a complex multifactorial process of vascular wall injury and atheroma formation due to dynamic local and systemic factors. Shear stress, especially when blood flow is disturbed, plays an important role in the pathogenesis of the atherosclerotic plaque especially where flow conditions are disturbed with low or oscillatory shear stress. The combination of altered arterial haemodynamics are around curvatures, arterial branch ostia and bifurcations (Resnick et al. 2003) where secondary flows occur. Systemic risk factors promote atherosclerotic lesion initiation, progression and ultimately, development of complicated plaques. Atherogenesis is promoted by decreased shear stress which is associated with reduction in several vascular wall functions including endothelial nitric oxide synthase (eNOS) production, vasodilatation and endothelial cell repair. These are coupled with increases in reactive oxygen species (ROS), endothelial permeability to lipoproteins, leukocyte adhesion, apoptosis, smooth muscle cell proliferation and collagen deposition (Gimbrone et al. 2000).

1.7 Oxygen Levels *in vivo* and in Cell Culture

Oxygen is an excellent electron acceptor and acts in this capacity as an essential component of multiple biologic redox processes, in particular cellular respiration (Gnaiger 2001; Gnaiger 2003; Nohl et al. 2003). Knowledge about the exact levels of extra- and intracellular oxygen tensions that occur in organisms is not comprehensive which is largely due to technical challenges in making meaningful measurement (Dewhirst et al. 1994). There is great heterogeneity both between and within tissues. Extreme examples of the latter are the upper respiratory tract where cells are directly in contact with air containing 21% oxygen, and the medulla of the kidney, where extracellular levels approximate to 1% oxygen or below (Maxwell 2003). Also rather striking is that tissues in the body are not equally resistant to the potentially harmful effects of sustained low oxygenation (hypoxia); for example, synaptically active neurons are relatively easily damaged by minor changes in oxygen supply (Olson and McKeon 2004), while neutrophils (at least *ex vivo*) have an increased lifespan in hypoxia (Walmsley et al. 2005)

Cells tolerate a broad range of oxygen tensions in vitro yet very little is known about how the oxygen levels they experience relate to physiologic conditions. However, it has recently become evident that varying oxygen tension in the gas phase over the range from 21 to approximately 0.5% has very potent effects on gene

expression in many different cell types (Liu and Simon 2004). Many of these changes occur at the level of gene transcription and involve the transcription factor hypoxia-inducible factor (HIF) as a master regulator (Semenza 2000). Although variable from one cell type to another, HIF activation is usually just detectable and has some effect on gene expression under standard culture conditions. However, below approximately 5% oxygen, it is progressively activated, reaching a maximum at 0.5%-1%.

To date, there are over 50 well-characterized HIF target genes, and the list is growing rapidly. It has been estimated that over 1% of all mammalian genes are regulated by HIF (Semenza 2000; Liu and Simon 2004). Target genes that have already been identified influence almost all aspects of cell behaviour. Accordingly, HIF is implicated in many processes relevant to human health such as embryonic development, inflammatory responses, wound healing, stroke and cancer (Semenza et al. 2000; Maxwell et al. 2001; Cramer et al. 2003; Melillo 2004). The ability to mount major adaptive changes to alterations in oxygen supply *via* HIF is conserved as far back as the nematode *Caenorhabditis elegans* (Jiang et al. 2001), which is entirely consistent with this pathway being a cornerstone of animal physiology.

Hypoxia-responsive transcription factors

Transcription factors	Functions
HIF (Hypoxia-inducible factor)	Vasodilatation Glycolysis Angiogenesis
NF κ B (Nuclear factor kappa-B)	Innate immunity Stress responses Cell survival and development
CREB (Cyclic AMP response element binding protein)	Inflammation Metabolism Signal transduction Stress responses
AP-1 Activating protein-1	Inflammation Metabolism Signal transduction Stress responses
AP-1 Activating protein-1	Apoptosis Proliferation Differentiation Inflammation Angiogenesis
p53	Apoptosis Tumours growth Interact with HIF-1 α

Table 1.2 Hypoxia-responsive transcription factors
Table adapted from Eoin P. Cummins and Cormac T. Taylor, 2005

1.7.1 Hypoxia-responsive transcription factors

There are various transcription factors that have been demonstrated to be hypoxia-responsive and contribute to the complex transcriptional profile activated by this important physiological and pathophysiological stimulus (Table 1.2).

1.7.2 Multiple Mechanisms of Hypoxia Response Acting through HIF-1α

HIF-1 is a heterodimeric transcription factor, composed of a HIF-1α subunit and a protein known as the aryl hydrocarbon receptor nuclear translocator (ARNT) which is also named as HIF-1β(Wang and Semenza 1995). Both HIF-1 subunits are members of the basic helix-loop-helix (bHLH)/PER-ARNT-SIM (PAS) domain family of transcription factors (Wang et al. 1995). The activity of HIF-1 is primarily regulated by a two-step mechanism of post-translational modification of HIF-1α, increasing both protein stability and transactivation capacity. By contrast, ARNT is insensitive to changes in oxygen availability.

Regulation of HIF-1α stability is mediated by a region that is referred to as the oxygen-dependent degradation domain (ODDD) (Huang et al. 1998). At normoxic condition (oxygen supply to cells *in vitro* corresponds to the atmospheric pressure), the degradation domain of HIF-1α interacts with the product of the von Hippel-Lindau tumour suppressor gene (pVHL), which functions as a multisubunit ubiquitin-protein ligase, promoting HIF-1α degradation by the proteasome (Maxwell et al. 1999; Cockman et al. 2000; Kamura et al. 2000; Ohh et al. 2000; Tanimoto et al. 2000). Interaction between pVHL and HIF-1α is dependent on hydroxylation of two conserved proline residues, Pro 402 and Pro 564, within the ODDD. Hydroxylation and subsequent pVHL-HIF-1α interaction and ubiquitination of HIF-1α, are inhibited under hypoxic conditions, resulting in accumulation of the HIF-1α protein (Fig. 1.11).

Under hypoxic conditions (a reduction in the normal oxygen supply to cells either in the tissue or in culture where cells are being exposed to oxygen concentrations below the ambient level in the atmosphere), the O_2-dependent hydroxylation of HIF-1α is decreased, which prevents its degradation. A further level of O_2-dependent regulation exists: the hydroxylation of an asparagine residue by factor inhibiting HIF-1α (FIH) blocks the interaction of HIF-1α with p300/CBP transcriptional co-activator proteins, thereby decreasing transcription of HIF-1α-regulated gene at normoxia.

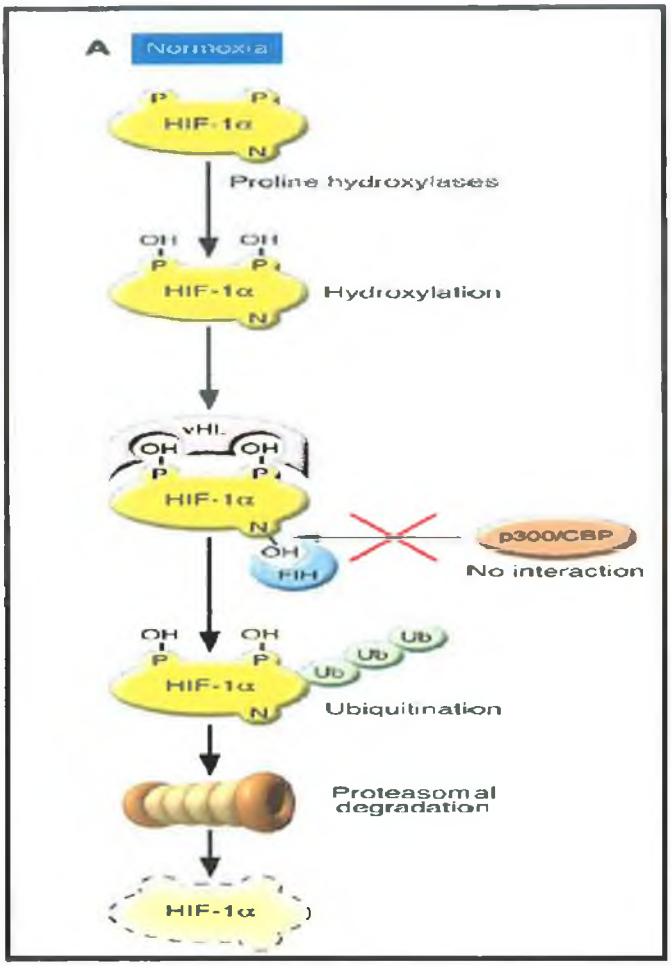


Fig. 1.11 HIF-1α Regulatory Pathway

Kol A. Zarember and harry L.Malech; *J Clin Invest* 115: 1702-1704 (2005)

P: proline residues 402 & 564; vHL: von Hippel Lindau protein; FIH: factor inhibiting HIF-1α; Ub:ubiquitination.

When HIF-1α levels increase in response to hypoxia in tissues, functional HIF-1 regulates transcription at HREs (hypoxia response elements) of target gene regulatory sequences, which results in the transcription of genes such as vascular endothelial growth factor (VEGF); endothelial nitric oxide synthases (eNOS); platelet derived growth factor (PDGF); and glucose transporter GLUT-1 and thereby enhances local vascularization and systemic oxygen transport.

Among the bHLH-PAS protein family, the other members were identified by data base searches for cDNA sequences encoding structurally related protein. The amino acid important functional domains in HIF-1α are highly conserved in HIF-2α (Tian et al. 1997; Wiesener et al. 1998; O'Rourke et al. 1999). In contrast to HIF-2α, HIF-3α also exhibits conservation with HIF-1α and HIF-2α in the HLH and PAS

domains, and an oxygen-dependent degradation domain, but does not possess a similar hypoxia-inducible transactivation domain (Gu et al. 1998). However, although HIF-3α is homologous to HIF-1α, it might be a negative regulator of hypoxia-inducible gene expression (Shimo et al. 2001). As for all the class I subunits, HIF-1α, -2α and -3α, each heterodimerize is with one of the class II subunits, ARNT (HIF-1β), ARNT 2 or ARNT 3.

HIF-1α and HIF-1β mRNA are expressed in most human and rodent tissues (Wenger et al. 1996; Wiener et al. 1996). In contrast, HIF-2α, HIF-3α, ARNT 2 and ARNT 3 show a more restricted pattern of expression to specific cell types (Jain et al. 1998; Tian et al. 1998). It appears that HIF-1α plays a very general role by signalling the existence of hypoxia to the transcriptional machinery in the nucleus of all cells, whereas HIF-2α and HIF-3α play more limited or specialized role in O_2 homeostasis which is still unclear (Wenger 2000; Fedele et al. 2002).

1.7.3 Protein hydroxylation as an oxygen-sensing mechanism

Recent major advances have shown that prolyl hydroxylation and acetylation, by controlling HIF-1α-pVHL physical interaction, are critical in the regulation of HIF-1α steady-state levels (Ivan et al. 2001). The proline residues subjected to hydroxylation reside in the HIF-1α ODDD which is strongly conserved between the HIF-1α isoforms (Jeong et al. 2002).

In mammalian cells, three isoforms, prolyl-hydroxylase 1(PHD1), PHD2 and PHD3, have been identified and shown to hydroxylate *in vitro* the key proline residues (Pro402 and Pro564) of HIF-1α (Epstein et al. 2001). PHDs are dioxygenases that utilize oxygen as co-substrate providing the molecular basis for the oxygen-sensing functions of these enzymes. In addition, the prolyl hydroxylation reaction requires 2-oxoglutarate and iron as cofactors, thereby accounting for the well known 'hypoxia-mimic' effects of iron chelators and transition metals on HIF-1α induction. Each PHD isoform differs in the relative abundance of their mRNA, howerer, all three show a ubiquitous pattern of expression (Lieb et al. 2002; Cioffi et al. 2003).

A separate hydroxylation reaction targets an asparagine residue in the carboxy terminal part of HIF (termed CTAD), preventing the recruitment of transcriptional coactivators (Hewitson et al. 2002; Stolze et al. 2004). This asparagynil hydroxylation is carried out by the enzyme FIH, which, like the PHD enzymes, is an

iron- and 2-oxoglutarate-dependent oxygenase. Both the FIH and PHD reaction rates are exquisitely influence by oxygen concentration, many circumstances may influence the HIF response in one way or another such as altering the transcription of the hydroxylase enzymes themselves (del Peso et al. 2003), the PHD/FIH enzymes act as the oxygen sensors, thus regulating HIF stability and activity (Fig. 1.12).

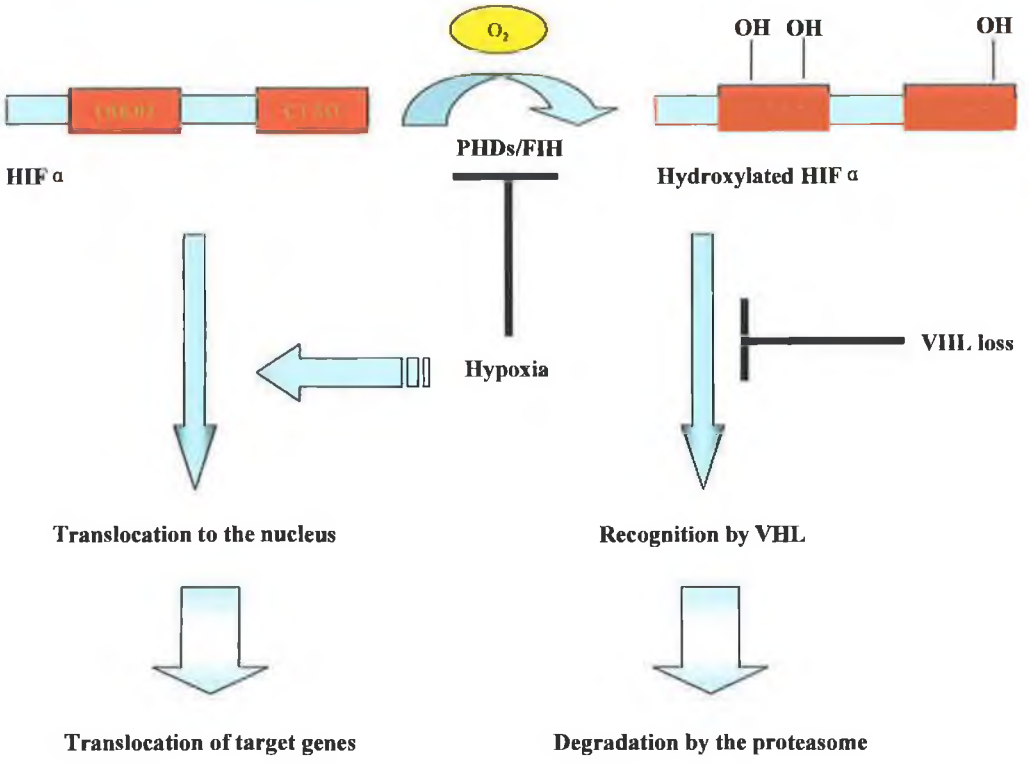


Fig. 1.12 Regulation of HIF by the level of oxygenation

Miguel A Esteban and Patrick H Maxwell; *Expert Rev. Proteomics* 2: 307-314 (2005)

PHD: prolyl hydroxylase domain; CTAD: C-terminal portion of HIF

1.7.4 HIF-1α Plays a Complex Role in Mediating Hypoxia-Induced Apoptosis.

HIF-1α can induce apoptosis *via* two mechanisms: firstly, it can increase the stability of product of the tumour suppressor gene p53 which regulates proteins such pro-apoptotic Bax and p21 causing growth arrest (Chen et al. 2003); and secondly, up-regulation of transcription of Nip3/BNIP3 (Bcl2/adenovirus E1B 19kDa Interacting Protein 3) a pro-apoptotic member of the Bcl2 family of cell death factors, induces apoptosis by binding to and inhibiting the anti-apoptotic proteins Bcl-2 and

Bcl-$_{XL}$ (Bruick 2000). However, while severe and prolonged hypoxia may result in reduced cell proliferation and increased apoptosis, cells can often adapt to acute and mild hypoxia and survive.

Hypoxic pulmonary vasoconstriction is an important mechanism for matching ventilation and perfusion (Gerritsen and Bloor 1993). The ability of hypoxia to induce the transcription and production of vasoconstrictions such platelet-derived growth factor-B (PDGF-B) and ET-1 may represent one molecular mechanism whereby low oxygen tension mediates vasoconstriction regionally (Kourembanas et al. 1990; Kourembanas et al. 1991; Kourembanas et al. 1993). It is also possible that the decrease in eNOS transcripts and enzyme by hypoxia may also contribute to the vasoconstrictor response to hypoxia, by inhibiting production of the counteracting vasodilator NO which itself can prevent the induction of vasoconstrictor gene transcription by hypoxia (Kourembanas et al. 1993).

Chronic hypoxia elicits a unique tissue vascular pathology. PDGF-B and ET-1 induction by hypoxia may mediate much of the structural remodelling that characterizes the tissue response to chronic hypoxia. Both are potent vasoconstrictors, strong mitogens for fibroblast and smooth muscle cells and also serve as fibroblast chemoattractants (Seppa et al. 1982; Berk et al. 1986; Komuro et al. 1988; Takuwa et al. 1989; Bobik et al. 1990; Dzau and Gibbons 1991; Peacock et al. 1992). Media conditioned by hypoxic endothelial cells has been show recently to inhibit chemoattractant and mitoegenic activity towards fibroblast and this activity was due to secreted ET-1 and PDGF (Dawes et al. 1994). Reciprocally, NO, the production of which is inhibited by hypoxia, inhibits fibroblast and smooth muscle cell mitogenesis, as well as endothelial cell migration, mitogenesis and proliferation (Garg and Hassid 1989; Garg and Hassid 1990; Nakaki et al. 1990; Nunokawa and Tanaka 1992; Scott-Burden et al. 1992; Sarkar et al. 1995; Lau and Ma 1996).

1.7.5 Hypoxia and Diabetic Vascular Disease

Hyperglycaemia and hypoxia are suggested to play essential pathophysiological roles in the complications of diabetes, which may result from a defective response of the tissues to low oxygen tension.

Exposure to acute/mild hypoxia can up-regulate HIF-1α expression to increase proliferation which helps normal tissues and tumours to survive under hypoxic conditions. Exposure to severe and prolonged hypoxia may result in reduced

cell proliferation and increased apoptosis leading to increased cell death.

Chronic complications of diabetes are a major health problem and it has become a priority to characterize further, their pathophysiological mechanisms to develop novel, rational therapeutic strategies. Even though prolonged exposure of the tissues to hyperglycaemia seems to be primary causative factor, some other factors may play a role, as intensive blood glucose control reduces chronic complications but does not prevent them altogether (DCCT 1993; UKPDS 1998). It has become increasingly evident that hypoxia plays an important role in all diabetes complications (Cameron et al. 2001).

The possibility that hyperglycaemia and hypoxia may interact *via* a common metabolic imbalance to initiate and/or exacerbate complications of diabetes is suggested by the correspondence of several redox, metabolic, and pathophysiological changes evoked by either condition alone (Nyengaard et al. 2004).

A biochemical event with diabetes is the formation of glycosylated products (Brownlee et al. 1988). Proteins constitute the principal substrate of this reaction which generates glycoproteins in the extracellular compartment, plasma membrane, cytoplasm, and ultimately, in the nucleus (Haltiwanger et al. 1997; Ramamurthy et al. 1999). Glycosylation and phosphorylation activate several transcription factors (Jackson and Tjian 1988) including p53 (Shaw et al. 1996). The processes of glycosylation and phosphorylation are tightly and dynamically regulated which affect the activation and stability of the p53 protein (Hupp and Lane 1994; Shaw et al. 1996; Ashcroft et al. 1999). There is direct competition between glucose and phosphate at a single amino acid residue on p53 which results in a decrease in the level of phosphorylation when glycosylation occurs (Hart et al. 1995; Haltiwanger et al. 1997). Moreover, p53 reduces the expression of genes opposing cell death, such as Bcl-2, and up-regulates genes promoting apoptosis such as Bax (Miyashita and Reed 1995).

It is well known that the major complication of diabetes is atherosclerosis; a disease of large and medium-sized muscular arteries characterized by endothelial dysfunction, vascular inflammation, and the build up of lipids, cholesterol, calcium, and cellular debris within the intima of the vessel wall. This build up results in plaque formation, vascular remodelling, acute and chronic luminal obstruction, abnormalities of blood flow and diminished oxygen supply to target organs.

Atherosclerosis highlights the fact that the more deeply situated parts of the arterial wall depend on diffusion to satisfy their need for oxygen and nutrients. When atherosclerotic lesions develop, the arterial wall thickness increases and diffusion capacity is impaired. At the same time, oxygen consumption is augmented (Morrison et al. 1972; Bjornheden and Bondjers 1987) and an energy imbalance may occur. Local metabolic disturbances may be envisioned that may endanger regression or even result in progression of the atherosclerotic process, with the formation of a necrotic core.

Risk factors for atherosclerosis impair erythrocyte deformability, leading to a non-ischemic microcirculatory derangement involving shunting of erythrocytes through large capillaries, metarterioles, and arteriovenous anastomoses. The result is a non-homogenous microcirculatory blood flow and a consequently altered tissue level oxygen tension which leads to focal areas of tissue-level hypoxia within the avascular media worsened by a variety of other effects which diminish trans-intimal and trans-adventitial oxygen delivery.

Arterial medial hypoxia initiates a pathophysiological cycle with release of growth factors and cytokines. These growth factors and cytokines stimulate macrophage migration and activation, intimal and adventitial proliferation, endothelial permeability, and platelet adherence and degranulation resulting in atheroma formation. Because intimal proliferation further worsens medial hypoxia by decreasing transintimal oxygen delivery, a pathophysiological positive feedback loop is completed. Disruption of the normal intimal connective tissue architecture and underlying necrosis diminish the artery's capacity for endothelial support, resulting in endothelial ulceration with consequent luminal thrombosis. These endpoints manifest clinically as myocardial infarction and non-hemorrhagic stroke. Medial hypoxia also causes medial necrosis with consequent aneurysm formation or rupture which results in hemorrhagic stroke and ruptured aortic aneurysms (Simanonok 1996).

In response to this decrease in oxygen availability, atherosclerotic plaques undergo angiogenesis in an attempt to re-perfuse the plaque and maintain cell viability. Formation of these new vessels however, can be detrimental to the plaque as: (i) it can lead to increased entry of leukocytes and macrophages resulting in weakening and breakdown of the fibrous cap and (ii) due to the weak and unstable nature of the new vessels, neovascularisation may result in plaque haemorrhage and

thrombosis (Tenaglia et al. 1997). Both diabetes and hypertension have been shown to produce tissue hypoxia.

Vascular homeostasis is subject to adaptation upon exposure of the endothelium to hypoxia and for this; it must be able to detect either the lack of oxygen itself or metabolic consequences of oxygen deprivation. During hypoxia, the transcription factor HIF-1α binds to hypoxic response elements (HRE) in the promoter or enhancer regions of various hypoxia-inducible genes including vascular endothelial growth factor (VEGF), eNOS, platelet derived growth factor (PDGF) and the glucose transporter GLUT-1 (Semenza 2002), promoting angiogenesis, glycolysis and growth factor signalling.

Chapter 2

Materials & Methods

2.1 Materials

All reagents used were of the highest grade commercially available and obtained by the following suppliers:

AGB Scientific (Dublin, Ireland)

Whatmann Chromatography paper

Amersham Pharmacia Biotech (Buckinghamshire, UK)

ECL Hybond nitrocellulose membrane, ECL Hyperfilm, Rainbow molecular weight marker, broad range (6-175kDa)

AXXORA (UK) Ltd.

Bnip3L antibody

BD Bioscience (USA)

Vybrant™ CFDA SE Cell Tracer Kit (V-12883), Vybrant™ Apoptosis Assay Kit #2, HIF-1α antibody, Bcl-xl antibody, PS Round Bottom Tube W/Cap, FACS Flow

Bio Sciences Ltd (Dun Laoghaire, Ireland)

DMEM, dNTP's, DEPC-treated water, Trizol® reagent

Cell Signalling TECHNOLOGY®

Cleaved Caspase-3 antibody, Bax antibody

PALL Corporation (Dun Laoghaire, Ireland)

Biotrace nitrocellulose membrane

Promega (UK)

Taq DNA Polymerase, MLV-RT, Rnase H, Oligo dT, Luciferase Reporter Reagents, Wizard® Plus Midipreps DNA purification kit, 100 bp DNA ladder.

Sigma Chemical Company (Poole, Dorset, England)

β-glycerophosphate, β-mercaptoethanol, Acetic Acid, Acetone, Agarose, Ammonium Persulphate, Acrylaminde/bis-Acrylamine, Bovine Serum Albumin, Brefeldin A,

Brightline Haemocytometer, Bromophenol blue, Calcium Chloride, CHAPSO, Chloroform, DMEM, DMSO, DTT, EDTA, EGTA, Ethidium Bromide, Foetal Bovine Serum, Glycerol, Glycine, Hanks Balanced Salt Solution, Hydrochloric acid, Isopropanol, Lauryl Sulphate, Leupeptin, N-Acetyl-Asp-Glu-Val-Asp-pNitroanilide, Methanol, Mineral oil (molecular grade), Penicillin-Streptomycin (100X), Ponceau S, Potassium Chloride, Potassium Iodide, Potassium Phosphate (Dibasic), p-Nitroaniline, Phosphatase inhibitor cocktail 1, Protease inhibitor cocktail, Sodium Acetate, Sodium Chloride, Sodium Doecly Sulphate, Sodium Hydroxide, Sodium Nitrite, Sodium Orthovanadate, Sodium Phosphate, Sodium Pyrophosphate, Sulphuric Acid, Tetracyline, TEMED, Tris Acetate, Tris Base, Tris Chloride, Triton X-100, Trypsin-EDTA solution (10X), Tween 20

Qiagen (West Sussex, UK)

SYBR Green® PCR Kit

2.2 Cell Culture and Transfections

2.2.1 Cell Maintenance

Bovine Aortic Endothelial Cells (BAEC) and Bovine Aortic Smooth Muscle Cells (BASMC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin sulfates, and 1% L-glutamine in a 37°C humidified atmosphere containing 5% CO_2. When confluent, cell monolayers were washed with Hanks Balanced Salt Solution (HBSS), 1ml of 1 x trypsin diluted with HBSS added and the flasks returned to the incubator to allow cells to detach, 7 mls of media was then added to the flasks and the cells pipetted gently to resuspend. Cells were either passaged 1:4 into flasks to maintain the cell line or seeded into dishes for experimental analysis.

2.2.2 DNA Transfections using LipofectAMINE™

On the day prior to transfection, 75cm^2 flasks of BAEC or BASMC were passaged 1:8 into 6 well plates and cells left overnight to plate down. For each well to be transfected, 2µg DNA and 5µl of LipofectAMINE™ reagent were mixed gently with 0.24ml serum/antibiotic free DMEM and incubated at room temperature for 15-45 mins in the dark. During the incubation, cell monolayers were washed with serum/antibiotic free DMEM and the medium replace with 0.76ml/well serum/antibiotic free DMEM. The DNA-LipofectAMINE™ mix was added dropwise to the cells and the plates returned to the incubator for 3hrs. Following incubation, the DNA-DMEM (serum/antibiotic free) were removed and replaced with fresh medium. Cells were analysed 24 hrs post-transfection.

2.2.3 siRNA Transfections using LipofectAMINE™ 2000

On the day prior to transfection, 75cm^2 flasks of BAEC and BASMC were passaged 1:8 into 6 well plates and cells left overnight to plate down. For each well to be transfected, 5µl of 20 pmol siRNA and 5µl of LipofectAMINE™ 2000 reagent were mixed gently with 0.99ml serum/antibiotic free DMEM and incubated at room temperature for 20-30 mins. Prior to the addition of siRNA to cells, cells were washed with serum/antibiotic free DMEM. The siRNA-LipofectAMINE™2000 mix

was then added to the cells and incubated for 3hrs. Following incubation the siRNA-LipofectAMINE™2000 mix was removed and replaced with fresh medium. Cells were analysed 5 days post-transfection.

2.3 Molecular Biology

2.3.1 Transformation of DNA

30-50ng of DNA was incubated with 50µl of competent XL1-blue cells on ice for 10 minutes. The cells were then placed for 5 minutes in a 37°C shaking waterbath before the addition of 0.5mls LB broth (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl). The mix was then returned to the waterbath and incubated for 45 minutes. 200µl from each transformation was spread onto agar plates containing the appropriate antibiotic (Amp 50µg/ml). Plates were incubated overnight at 37°C and transformed colonies selected the next day.

2.3.2 Preparation of Plasmid DNA

Transformed colonies were picked from agar plates and grown overnight in 5-10 mls of LB broth containing appropriate antibiotic (Amp 50µg/ml). DNA purification was by the Qiagen plasmid midi kit system as per the manufactures instructions. The concentration of DNA obtained was determined by measuring the absorbance at 260nm (A_{260}) of a 1:50 dilution in DNase/ RNase free H_2O, assuming that 1 absorbance unit was equivalent to 50µg/ml of double stranded DNA.

2.3.3 Preparation of Total RNA

Total RNA was isolated from BAECs and BASMCs using Trizol® reagent according to the manufacturer instructions. The pellet was air-dried and the RNA re-suspended in 30-50µl of DNase/RNase free water. All total RNA preparations were stored at -80 °C.

2.3.4 Quantification of Total RNA in Samples

To determine the amount of total RNA, the sample was diluted 1:500 in DNase/RNase free water and spectrophotometric analysis carried out using the

Shimadzu UV-160A dual spectrophotometer, blanked with DNase/RNase free water. The sample was measured, using a quartz cuvette, at wavelengths of 260 and 280 nm, and the concentration of the RNA in the sample carried out as follows;

Abs_{260nm} x dilution factor x 40 = concentration of RNA (μg/ml)

The purity of the RNA was determined by calculating the ratio of absorbance at 260nm to 280nm. A ratio of 1.9 to 2.0 was indicative of a highly purified preparation of RNA.

2.3.5 Reverse-Transcription (RT) of mRNA

mRNA was reverse transcribed from total RNA, prepared by the Trizol® method, using iScript™ cDNA Synthesis Kit according to the manufacturer instructions. All RT samples were stored at -80°C until needed for PCR.

2.3.6 Design of PCR Primer Sets

A number of web based programs, "Primer 3 Output" and "NCBI/BLAST" were utilized to design the primer sets used in this study (**Appendix 1**). The Primer 3 program designs primers from the sequence input by the user. Primer pairs are then entered into the BLAST program to determine multiple-species sequence alignment, which allows primers to be designed from highly conserved areas. Primers were designed with ~50% GC content; subsequently the annealing temperature for all sets was ~55°C.

2.3.7 Polymerase Chain Reaction (PCR)

Regions of the appropriate cDNA were amplified by polymerase chain reaction (PCR). A typical PCR contained 45.5μl Mastermix (36.5μl DNase/RNase free water, 5μl PCR Buffer, 3μl $MgCl_2$, 1μl DNTPs), 1μl 10pmol forward primers and 1μl 10pmol reverse primers, 0.5μl Taq, 2μl sample cDNA from reverse transcription, overlaid with 50μl emersion oil. The reaction was initiated by a denaturing phase of 94°C for 1min, followed by 40 cycles of annealing (55°C for 2 mins) and elongation (72°C for 3 mins). Reactions were placed on hold at 4°C until required.

2.3.8 Quantitative Real Time PCR (QRTPCR)

Quantitative PCR was carried out using a Real time Rotor-GeneRG-3000™ lightcycler (Corbett Research). The principle of real time amplification detection is that the amount of fluorescence is proportional to the concentration of product in a reaction. Higher fluorescence indicates a higher concentration of a product. Each PCR reaction was set up as follows; SYBR-Green (12.5µl), RNAse free water (8.5µl), cDNA (2.0µl), forward/reverse primer (1.0µl each from 10µM stocks).

Each sample was assayed in triplicate, and the program used for the primers was as follows; denaturing phase: 94°C; 20secs, annealing phase: 55°C; 30secs, elongation phase: 72°C; 30secs and run for 40 cycles.

2.3.9 Agarose Gel Electrophoresis

RT and QRTPCR products were analysed by agarose gel electrophoresis in which samples were prepared by the addition of a 1:3 dilution of loading buffer (15% Ficoll® 400, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.4% orange G, 10mM Tris-Hcl pH 7.5, 50mM EDTA). Electrophoresis took place on a 2% (w/v) gel containing 0.5µg/ml ethidium bromide at 80 V, 300 mA and 150 W for 20-30 minutes in 1 x TAE buffer (40mM Tris-acetate, 1mM EDTA, glacial acetic acid). When finished the gel was placed on an Ultra Violet Products UV transilluminator for visualization. A picture was taken using a Kodak DC290 digital camera for documentation.

2.4 Experimental Techniques

2.4.1 Preparation of Whole Cell Lysates

Confluent monolayers in 6-well dishes were washed three times with HBSS and solubilised by the addition of 0.25ml / well immunoprecipitation buffer (RIPA buffer [49.92mM HEPES (pH 7.5), 149.76mM NaCl, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate and 0.1% (w/v) SDS] supplemented with 0.1M NaF, 5mM EDTA (pH8), 0.01M $NaPO_4$, 1.04mM AEBSF, 0.08µM Aprotinin, 0.02mM Leupeptin, 0.04mM Bestatin, 0.015mM PepstatinA, 0.014mM E-64) and incubation at 4°C for 1 hour on a rotating wheel. Insoluble material was pelleted by

centrifugation for 15 minutes at 13,000rpm and the supernatant removed to fresh microfuge tubes.

BSA standards ranging from 0-2mg/ml were used to obtain a best-fit straight line of A_{562} in a bichinchonic acid (BCA) based assay using the graph package "Prism 4.0". Protein concentrations of 10µl samples of each unknown extract were calculated by comparison to the BSA standards.

10-30µg of each sample was incubated at 95°C for 5 mins in sample solubilization buffer (SSB) (2%SDS, 10%glycerol, 0.8% β-marcaptoethanol, 0.02% Bromophenol blue, 12% 0.25M TrisCl, pH 6.8) and separated by SDS-PAGE.

2.4.2 Preparation of Cell Lysates for Immunoprecipitation

For immunoprecipitation assays, following determination of protein concentration, 30-40µg of lysate were added to microfuge tubes containing 20µl of protein A-Sepharose beads with 10% (w/v) IgG free BSA in the presence of the appropriate antibody at a concentration of 0.025µg Ab/µg protein. Total volume in the tube was brought up to 500µl using RIPA buffer to allow sufficient volume for mixing and samples were rotated at 4°C overnight. The following day, immune complexes were isolated by centrifugation at 6,000 rpm for 2 mins and washed twice (1ml/wash) with immunoprecipitation buffer supplemented with 0.01% Triton X-100 and twice with immunoprecipitaiton buffer alone. Immune complexes were eluted from the protein A-Sepharose by a 10 min incubation at 90°C with 25µl electrophoresis sample buffer (50mM Tris (pH 6.8), 10% (v/v) glycerol, 12% SDS, bromophenol blue). 20µl of each sample was separated by SDS-PAGE.

2.4.3 SDS-PAGE Electrophoresis

Samples were separated by SDS-PAGE using a 6% or 12% acrylamide resolving gel ([6% or 12% (v/v) from 40% acrylamide/bis acrylamide stock], 0.4M Tris (pH 8.8), 0.1% (w/v) SDS, 3% (v/v) glycerol, 0.01% (w/v), ammonium persulphate and 0.001% (v/v) TEMED) and 3% acrylamide stacking gel (3% (v/v) acrylamide [from 40% acrylamide/bis acrylamide stock], 0.1% (v/v) bisacrylamide, 0.1M Tris (pH 6.8), 0.1% (w/v) SDS, 0.01% (w/v) ammonium persulphate and 0.001% (v/v) TEMED). Electrophoresis of the samples was carried out alongside prestained Rainbow molecular weight markers (10-250 kDa) in a running buffer (27.4mM Tris, 0.19M glycine, 0.1% (w/v) SDS) at 150 Volts until the dye front

reached the end of the gel, or if looking for larger proteins, until all but the 3 top markers remained on the gel only (250, 160, 105 kDa). At this point electrophoresis was stopped and the proteins transferred to nitrocellulose.

2.4.4 Transfer of Proteins to Nitrocellulose Membrane

For small sized proteins (under 100 kDa), the transfer procedure was carried out using an Atto Ae-6657 semi-dry transfer apparatus in semi-dry transfer buffer (25mM Tris, 102mM Glycine, 20% Methanol, 0.1% SDS) and run at 90 V, 500 mA for 45mins. For large size protein (upon 100kDa), a biorad system transfer apparatus was used in wet transfer buffer (25mM Tris, 192 mM Glycine, 20% Methanol, 0.5% SDS) and run at 100V, 500mA for 1hr and 20 mins.

Upon completion of the transfer, the nitrocellulose membrane was removed and stained in Ponceau S solution for 5 min. The membrane was then rinsed briefly in ultra pure water to remove any background staining, and the image scanned using an Epson perfection 1200S scanner. The image was saved and subsequently used to assess equality of protein loading onto gel, and quality of protein transfer onto nitrocellulose membrane.

2.4.5 Immunoblotting

Nitrocellulose membranes were washed twice briefly in PBS and non-specific protein binding sites blocked by a 60 min incubation in Blotto (5%(w/v) skimmed milk powder, 0.2% (v/v) Triton X-100, in PBS) at room temperature. Following blocking, membranes were washed briefly in 1 X PBS and incubated with the appropriate dilution of primary antibody (**Appendix 2**) in fresh Blotto overnight at 4°C. The following day, membranes were washed three times with Blotto and HRP-conjugated secondary antibody added in fresh Blotto for 1-3 hours at room temperature. Visualisation was detected by an enhanced chemiluminescent procedure (ECL).

2.4.6 Densitometric Analysis of Blot

Bands on a developed film were photographed using a Kodak DC290 digital camera. The image generated was then analysed using Kodak 1D (version 3.5.4) densitometry imaging software. A mean densitometric value was generated for each band; these values were then corrected using ponceau controls for each lane. The

corrected values were then expressed as fold increase over control, and graphically expressed using "Prism 4.0"

2.4.7 Detection of Apoptotic Cell Death

(a) Caspase Assay

A colourimetric caspase 3 assay was carried out on cell lysates (prepared as described in 2.4.1) to determine the level of caspase 3 activation in the sample, and therefore the level of apoptosis. Briefly, 10µl of caspase 3 substrate (2mM Ac-DEVD-pNA containing 10% DMSO in 20mM HEPES, 0.1% CHAPSO, 5mM DTT and 2mM EDTA) was added to 50µl cell lysate, and diluted in assay buffer (20mM HEPES, 0.1% CHAPS, 5mM DTT and 2mM EDTA) to a final volume of 100µl. Samples were incubated for 90 min and the absorbance measured at 405nm using a Tecan Spectra plate reader. Appropriate negative controls and blanks were included in the assay. A pNitroanilide (pNA) standard curve (0-200µg/µl) allowed for the specific activity of caspase 3 to be calculated for each sample.

(b) FACS analysis using the Vybrant® Apoptosis Assay Kit #2

Following treatment, BAEC and BASMC monolayers were washed once in HBSS and harvested by trypsinisation. Cells were pelleted by centrifugation at 1000 rpm for 5 mins and the supernatant removed. Cell pellets were washed by the addition of 1ml ice-cold 1 x PBS with 0.1% BSA and resuspended by gentle pipetting; followed by centrifugation at 1000 rpm for 5 mins. Following this wash, the supernatant was discarded and the cells resuspended in 100µl of 1 x Annexin-Binding Buffer (10mM HEPES, 140mM NaCl, 2.5 mM $CaCl_2$, PH 7.4). 0.4µl Propidium Iodide (from 100µg/ml working solution) and 1µl AlexaFluor 488 AnnexinV (in 25mM HEPES, 140mM NaCl, 1mM EDTA, PH 7.4, plus 0.1% BSA) were added to the cell suspension and incubated at room temperature for 15 mins. After the incubation period, a further 100µl of 1 x Annexin-Binding Buffer was added to the cells and mixed gently by pipetting. Samples were placed on ice and analysed by flow cytometry using a Becton Dickinson FACSCAN flow cytometer. Annexin V binds to phosphatidylserine located on the extracellular surface of apoptotic cells while PI is impermeable to live cells and apoptotic cells but stains necrotic cells with red fluorescence. Populations of cells are distinguished using flow cytometry where they are designated as viable (V), early apoptotic (EA), late

apoptotic (LA) or necrotic (N) depending on the AnnexinV / PI staining.

2.4.8 Determination of Cell Proliferation

(a) Cell Counting

Cell counts were carried out using a Sigma bright line haemocytometer. Following trypsinisation and resuspension in DMEM, a drop of cell suspension was used to fill the haemocytometer counting chamber. Following visualization under 10X magnification, the number of cells were counted in each of the four outer quadrants of the haemocytometer. The average of these four counts was equal to the number of cells x 10^4/ml of cell suspension.

(b) FACS analysis using the Vybrant® CFDA SE Cell Tracer Kit

BAECs and BSAMCs were seeded into 6-well-plates so as to reach 50% confluency 24hrs after plating. Having reached the desired confluency, the growth media was removed and cells washed x 1 with HBSS. A working solution of 5μM CFDA SE (carboxy-fluorescein diacetate succinimidyl ester) was prepared in an appropriate volume of HBSS to allow the addition of 1ml CFDA-HBSS per well. CFDA-HBSS was added to the wells and cells incubated for 15 minutes at 37°C. Following incubation, the CFDA-HBSS was removed and replaced with fresh, pre-warmed medium. Cells were incubated overnight, prior to treatment. CFDA SE passively diffuses into cells. It is colourless and non-fluorescent until its acetate groups are cleaved by intracellular esterases to yield highly fluorescent, amine-reactive carboxyfluorescein succinimidyl ester. The succinimidyl ester group reacts with intracellular amines, forming fluorescent conjugates that are well-retained. Upon cell division, the label is inherited by daughter cells causing sequential halving of the CFDA SE fluorescence, resulting in a cellular fluorescence histogram in which the peaks represent successive generations.

(c) Luciferase Assay

To analyse transactivation of luciferase tagged (Luc) reporter genes, cells were co-transfected with Luc (1.5μg) and β-Galactosidase (β-Gal; 0.5μg) (section 2.2.2), and harvested 24 hrs post transfection. Cells were washed twice in HBSS, and incubated with 300μl/well of 1 X reporter Lysis Buffer (Promega) for 10 min at 37°C in a humidified atmosphere. Cells were then scraped from the wells, transferred to

eppendorf tubes, and lysed by freeze-thawing once. The lysates were clarified by centrifugation at 3,000 rpm for 2 mins, and the supernatant saved in a fresh tube for analysis. Transactiviton of the luciferase tagged reporter genes was then analysed by luciferase assay as follows: 40µl sample and 50µl luciferase assay buffer (Promega) were incubated at room temperature for 30 mins and light emission measured over a period of 60 sec, after a lag period of 10 sec. To determine transfection efficiency, samples were normalized to β-Gal expression.

(d) β-Galactosidase Assay

A β-Galactosidase assay (High Sensitivity β-Galactosidase Assay, Stratagene) was carried out according to the manufacturers' instructions to normalise for differing transfection efficiencies. β-Gal, which is expressed due to transfection with pCMV-LacZ, catalyzes the hydrolysis of chlorophenol red-β-D-galactopyranoside (CRPG) into galactose and the chromophore chlorophenol red, yielding a dark red solution, which can be quantified using a spectrophotometer at 595nm. 20µl of cell lysate was analysed in triplicate in a 96 well plate. 130µl of 1 X CRPG substrate was added per well and the time recorded. The plate was then covered and incubated at 37°C until the sample turned a dark red up to 72 hrs. The incubation time was recorded and the reactions terminated by the addition of 80µl Stop solution (0.5M Na_2CO_3) to each well. The absorbance was then analysed at 595nm using a microplate spectrophotometer. A blank was set up for each experiment by substituting the 20µl of sample for 20µl of 1X report lysis buffer.

Luciferase assay was corrected for both β-Galactosidase activity and differing protein concentrations, and expressed as fold activation over the empty vector.

2.4.9 Statistical Analysis

All treatments were normalised to quiesced cells (serum starved control: C (t=0) and expressed as a fold increase or decrease compared to this value. Results are expressed as mean ± SEM. Statistical significance was assessed by Students paired and unpaired t-test or 2-way ANOVA, with significance values of $p \leq 0.01$ and $p \leq 0.05$.

Chapter 3

Effect of High Glucose on BAEC and BASMC Cell Fate (proliferation and apoptosis)

3.1 Introduction

Diabetes mellitus can cause a wide variety of vascular complications and cardiovascular dysfunction (DCCT 1993). The normal range of blood sugar level is between 80-110mg/dL (4.4-6.1 mM/L), when this level goes above 160mg/dL (8.9mM/L) which results in hyperglycaemia. This viewpoint was supported by the results of the large-scale study (DCCT 1993). Hyperglycaemia is an important etiologic factor in the development of vascular complications (Wilson et al. 1991), however the mechanisms of hyperglycaemia-related tissue damage and clinical complications still remain unclear.

It is widely accepted that atherosclerosis is accelerated by the coexistence of diabetes mellitus (Garcia et al. 1974; Melton et al. 1980) which could alter cell fate such as apoptosis, proliferation, differentiation, tube formation and migration. In this study, we focus on the effects on cell apoptosis and proliferation under high glucose conditions.

Apoptosis is particularly prominent in models of hyperglycaemic injury, affecting a significant proportion of vascular endothelium in tissue damage (Nakagami et al. 2001; Ceriello et al. 2002; Zou et al. 2002). Increased proliferation of vascular smooth muscle cells (VSMC) is a key feature in the atherosclerotic lesion (Hanke et al. 1990; O'Brien et al. 1993; Pickering et al. 1993; Ross 1999). It is well established that cell growth is a fundamental feature of intimal hyperplasia (Newby and Zaltsman 2000), and it is becoming clear that perturbation in the regulation of apoptosis is equally important (Haunstetter and Izumo 1998; Kockx and Knaapen 2000). Furthermore, apoptosis of VSMC is critically involved in the formation of the fibrous cap and fatty streak that is the lipid-rich core of the atheroma and may therefore contribute to the instability of advanced atherosclerotic plaques (Bennett et al. 1995; Geng and Libby 1995; Han et al. 1995; Isner et al. 1995; Crisby et al. 1997; Kockx et al. 1998). Excessive accumulation of VSMC in atherosclerosis suggests reduced apoptosis and excessive cell proliferation in the lesions, as apoptosis and cell proliferation are intimately coupled (Evan and Littlewood 1998). Although many studies have focused on the mechanisms of VSMC proliferation (Newby and Zaltsman 2000), the regulatory mechanisms of VSMC apoptosis have not been fully elucidated (McCarthy and Bennett 2000).

The role of hyperglycaemia in atherosclerosis has been investigated in recent studies. Several findings support the concept that hyperglycaemia accelerates the development of atherosclerosis (West et al. 1983; Wilson et al. 1991). Although high glucose concentrations enhance proliferation in cultured VSMC, little is known about the effect of glucose on the regulation of apoptosis in VSMC.

Aim 1: To investigate the effects of high glucose (HG 25mM) on Bovine Aortic Endothelial Cell (BAEC) and Bovine Aortic Smooth Muscle Cell (BASMC) growth (proliferation *vs* apoptosis)

3.2 Results

High glucose, a situation that mimics the hyperglycaemia of diabetes can influence macrovascular cell fate. Therefore, we initially examined the effect of high glucose (25mM) on bovine aortic endothelial cell (BAEC) and bovine aortic smooth muscle cell (BASMC) growth (proliferation vs apoptosis).

BAEC and BASMC were treated in normal growth media (10% FBS, NG 5.5mmol/L glucose) and high glucose (10% FBS, HG 25mmol/L glucose) containing media for up to 7 days. Mannitol (10% FBS, 19.5 mmol/L) was used as an osmotic control in all experiments. Exposure of BAEC and BASMC to normal growth media resulted in an increase in cell number after the 7 day period as determined by cell counting. Concurrent treatment of BAEC and BASMC with HG media did not alter the growth curve of the cells. Mannitol osmotic control does not alter the growth curve in either cell type (Figure 3.1).

Due to the subjective nature of manual cell counting, we confirmed our proliferative findings by FACS analysis. BAEC and BASMC were stained with a fluorescent nuclear marker carboxyfluorescein diacetate, succinimidyl ester (CFDA SE) as described in the methods (2.4.8) prior to treatment in the absence or presence of HG (Figure 3.2). Proliferation was determined by a reduction in the fluorescent intensity of the dye following cell division and subsequent halving of the dye to daughter cells (Fig. 3.3).

BAEC and BASMC were exposed to normal growth media or high glucose containing media for 24, 48 or 72 hrs. In normal glucose media, BAEC and BASMC showed a significant increase of proliferation during the time period. However, treatment of BAEC and BASMC with HG was not seen to alter cell proliferation (Figs. 3.4 & 3.5).

Further analysis of cell proliferation was carried out by western blotting for Proliferating cell nuclear antigen (pCNA) which is a co-factor of DNA polymerase delta and is involved in the control of eukaryotic DNA replication by increasing the polymerase processibility during elongation of the leading strand. BAEC and BASMC were treated with either normal growth media or high glucose containing media for 24, 48, or 72 hours prior to harvesting in RIPA buffer. pCNA expression was determined by western blot as described in the methods (2.4.3). pCNA expression increased in normal culture media up to 72 hours in BAEC and BASMC.

However, cells which were treated with high glucose showed no changes in pCNA expression, a marker of cell proliferation (Figs. 3.6 & 3.7).

Treatment of cells in the present of HG up to 72 hrs did not appear to affect the proliferation status of the cells. Therefore, we increased the exposure time to a period of 3, 5, or 7 days. Cell proliferation was measured by FACS analysis and western blot as previously described. BAEC and BASMC proliferation significantly increased over the 7 day period; however, exposure to HG was not seen to alter proliferation in any way (Figs. 3.8, 3.9, & 3.10).

These results, which correlate with cell counts, suggest that BAEC and BASMC proliferation were not affected by the presence of HG in 10% FBS during either short-term or long-term exposure.

Fetal bovine serum (FBS) is a common component of animal cell culture media which contains basic components, such as hormones and growth factors without cells, platelets and clotting factors. Since no change in proliferation was observed in normal serum containing media, we hypothesised that the effect of high glucose on BAEC and BASMC may be impaired by the presence of fetal bovine serum.

Therefore, BAEC and BASMC were treated with normal growth media containing 10% FBS and low serum media contained 0.5% FBS for 24 hours and the rate of cell proliferation determined by FACS analysis. A significant increase in proliferation was observed in cells treated with 10% FBS compared to 0.5% FBS. However, the exposure of BAEC and BASMC to HG in the presence of 0.5% FBS, failed to alter the proliferation profile of the cells (Figs. 3.11 & 3.12).

Concurrent western blot analysis for pCNA expression also showed no change in proliferation of cells cultured in 0.5% FBS in the presence of HG (Fig. 3.13).

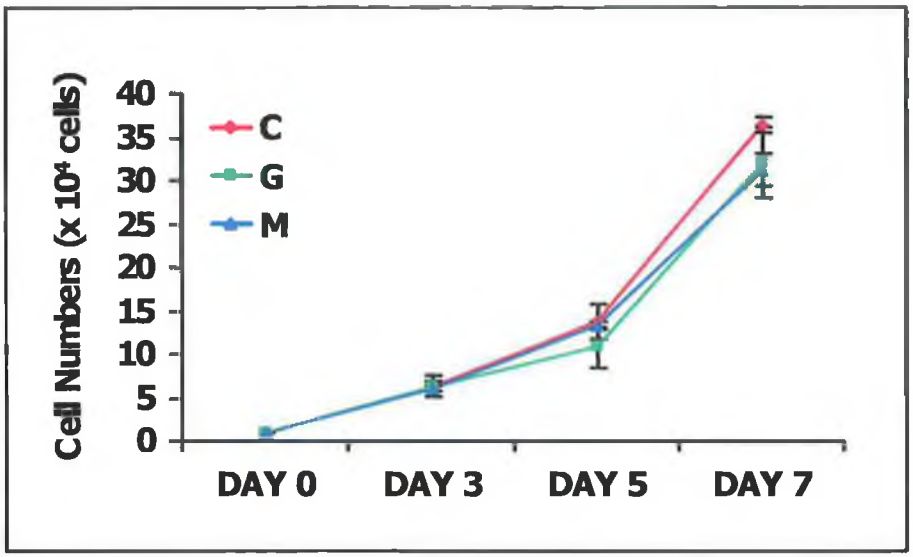
To determine the effects of high glucose on BAEC and BASMC apoptosis, cells were cultured in 10% or 0.5% FBS in the absence or presence of HG for 24, 48, or 72 hrs. Subsequent FACS analysis using a PI/Annexin V-based dual staining technique (2.4.7) distinguished a population of cells depending on their uptake of the two dyes (Fig. 3.14).

A basal range of apoptotic cells in normal growth media was approximately 0.5% ~ 2% in both cell types. During the time course, the percentage of apoptotic cells in 10% FBS conditions was not significant different compared to t=0 control.

High glucose does not alter this effect either in BAEC or on BASMC over the 72 hr treatment period. However, the percentage of apoptotic cells significantly increased when cultured in 0.5% FBS compared to t=0 control (BAEC 2.36-fold ± 0.26-fold t=24, 4.04-fold ± 0.27-fold t=48, 10.38-fold ± 0.76-fold t=72 *vs* 1.00 *p≤ 0.01; BASMC 3.74-fold ± 0.40-fold t=24, 6.28-fold ± 0.64-fold t=48, 11.05-fold ± 0.84-fold t=72 *vs* 1.00 *p≤ 0.01) and 10%FBS treated cells (BAEC 2.36-fold ± 0.26-fold t=24, 4.04-fold ± 0.27-fold t=48, 10.38-fold ± 0.76-fold t=72 *vs* 1.18-fold ± 0.12-fold #p≤ 0.01; BASMC 3.74-fold ± 0.40-fold t=24, 6.28-fold ± 0.64-fold t=48, 11.05-fold ± 0.84-fold t=72 *vs* 1.18-fold ± 0.12-fold #p≤ 0.01) This increase in apoptotic cells was not altered by the presence of HG (Figs. 3.15 & 3.16).

Regulation of caspase-3 activity (methods 2.4.7) was used to confirm the effects of serum starvation and HG on BAEC and BASMC apoptosis. BAEC and BASMC cultured in 10% FBS media containing normal glucose (5.5 mmol/L) or high glucose (25 mmol/L) for 24, 48 or 72 hours showed no significant change in caspase-3 activity compared to t=0 control. However, a significant increase in caspase-3 activity was observed in BAEC and BASMC cultured in 0.5% FBS when compared 10%FBS treated control cells. The addition of high glucose to cells cultured in 0.5% FBS showed no effect on the activity of caspase-3 when compared to cells cultured in alone 0.5% FBS (Figs. 3.17 & 3.18).

(A)



(B)

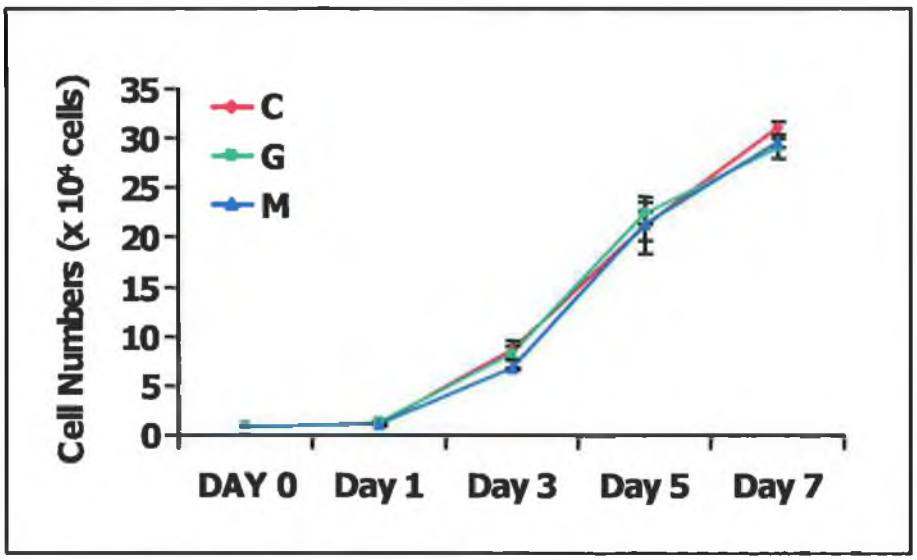


Fig. 3.1 Effect of high glucose on macrovascular cell proliferation

Bovine aortic endothelial cells **(A)** and smooth muscle cells **(B)** cultured in 10% FBS were plated in 6-well-plates ($5x10^3$ cells/well) and exposed to high glucose concentrations (G: 25mM) for up to 7 days. Cells were counted at the indicated times and the cumulative data plotted. Cells cultured in normal glucose (5mM: C) and those treated with 25mM mannitol (M) were used as controls. Data represents mean values from three independent experiments performed in triplicate.

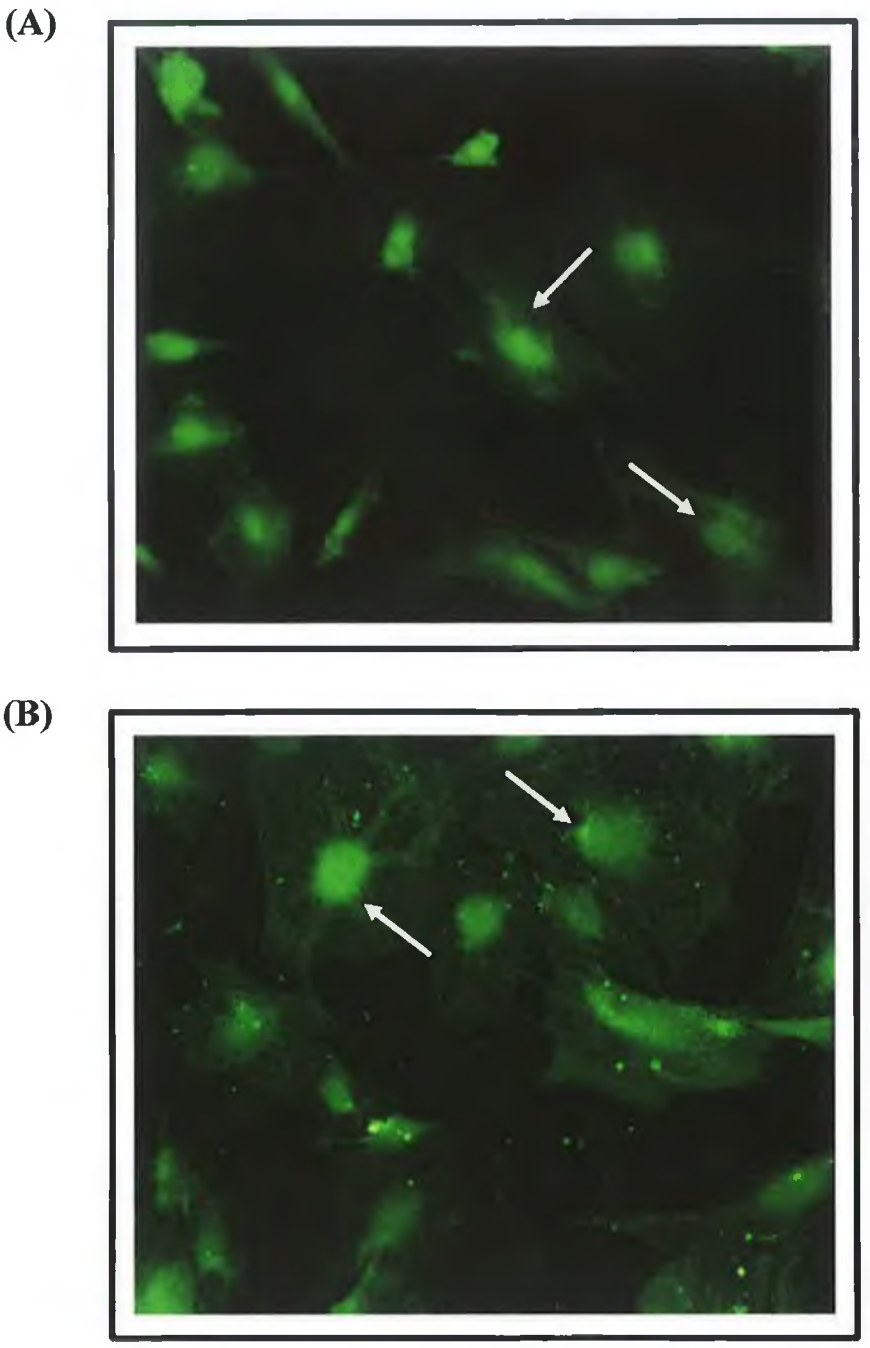


Fig 3.2 CFDA SE staining of BAEC and BASMCs

Cells were labelled with the fluorescent marker CFDA SE as described in the Methods section 2.4.8. The nuclear localisation of the marker (arrows) was captured by fluorescence microscopy (20x). **(A)** BAEC **(B)** BASMC.

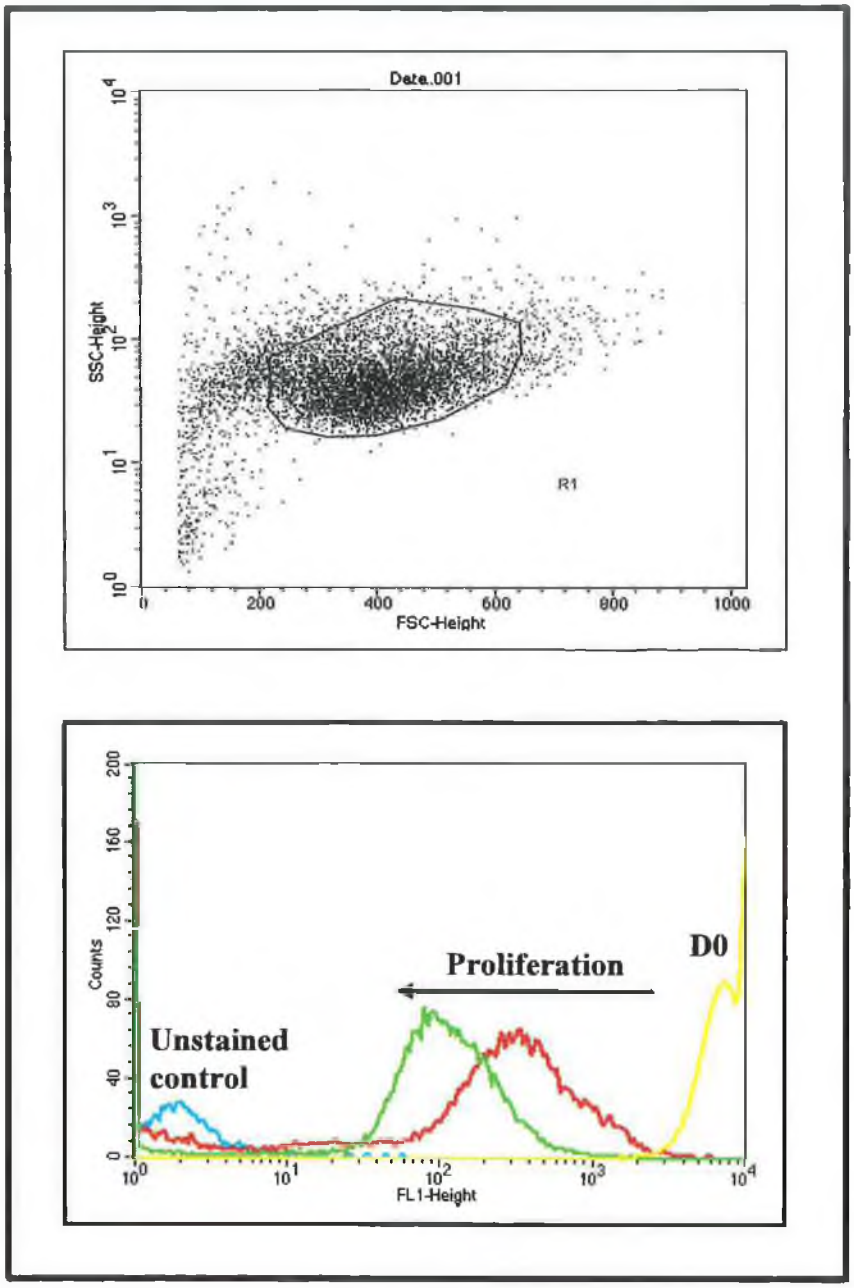


Fig 3.3 **Tracking of cell proliferation using CFDA SE labelling and flow cytometry**

Representative example of control and CFDA SE stained macrovascular cells obtained by flow cytometry. Cell division results in sequential halving of CFDA SE fluorescence, resulting in a cellular fluorescence histogram in which the peaks represent successive generations. Unstained control represents unlabelled cells.

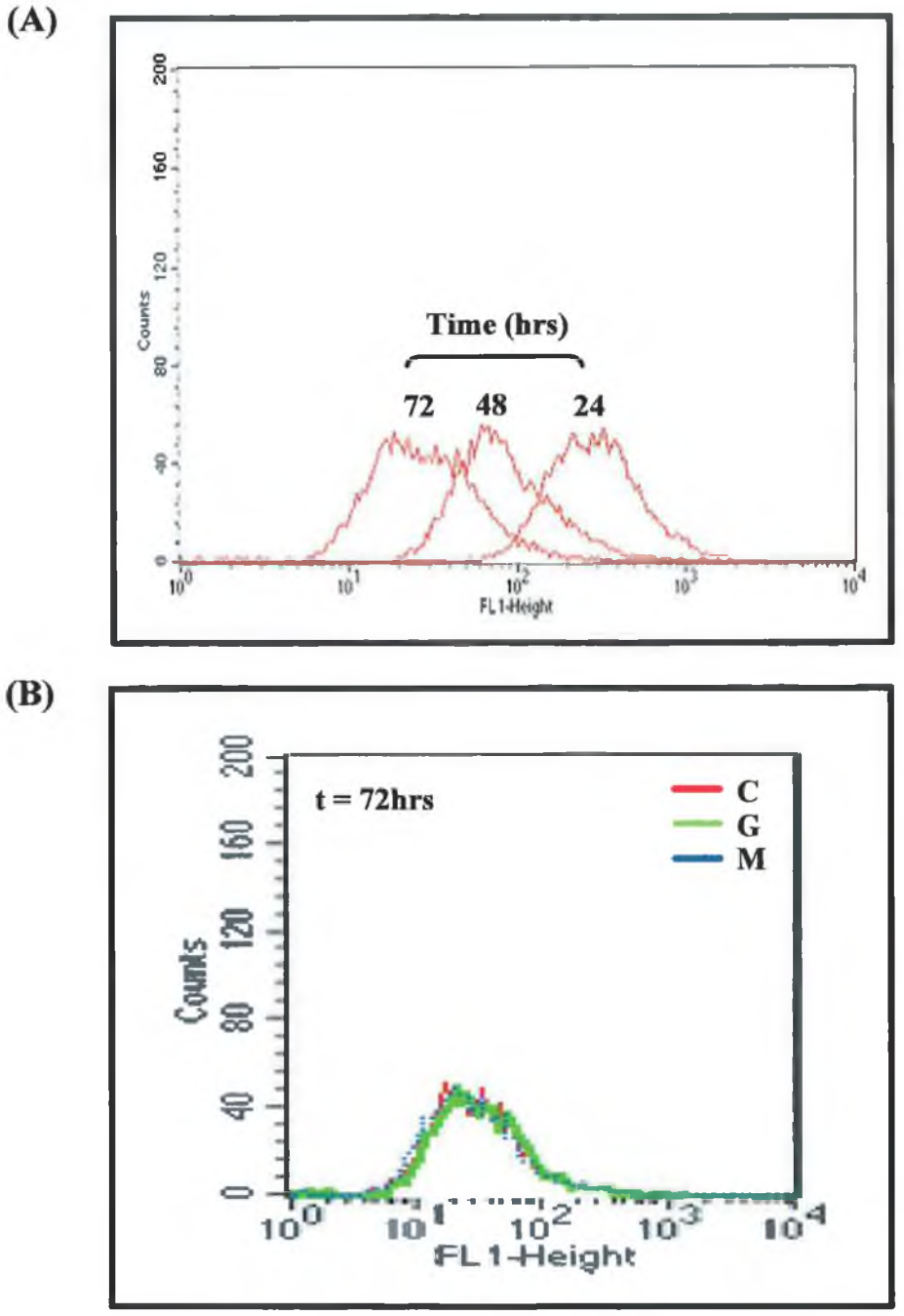


Fig. 3.4 Time course of BAEC Proliferation by FACS Analysis

BAECs were cultured in 10% FBS for 24hr, 48hr and 72hrs in the absence or presence of high glucose (25mM). After these time points, cells were collected and analysed by FACS as described in the Methods section 2.4.8. **(A)** Comparison of cell proliferation over the 72hr period **(B)** comparison of proliferation of normal glucose (5.5mM:C), high glucose (25mM:G) and mannitol (25mM:M) treated cells at 72hrs. Data are representative of at least 3 independent experiments.

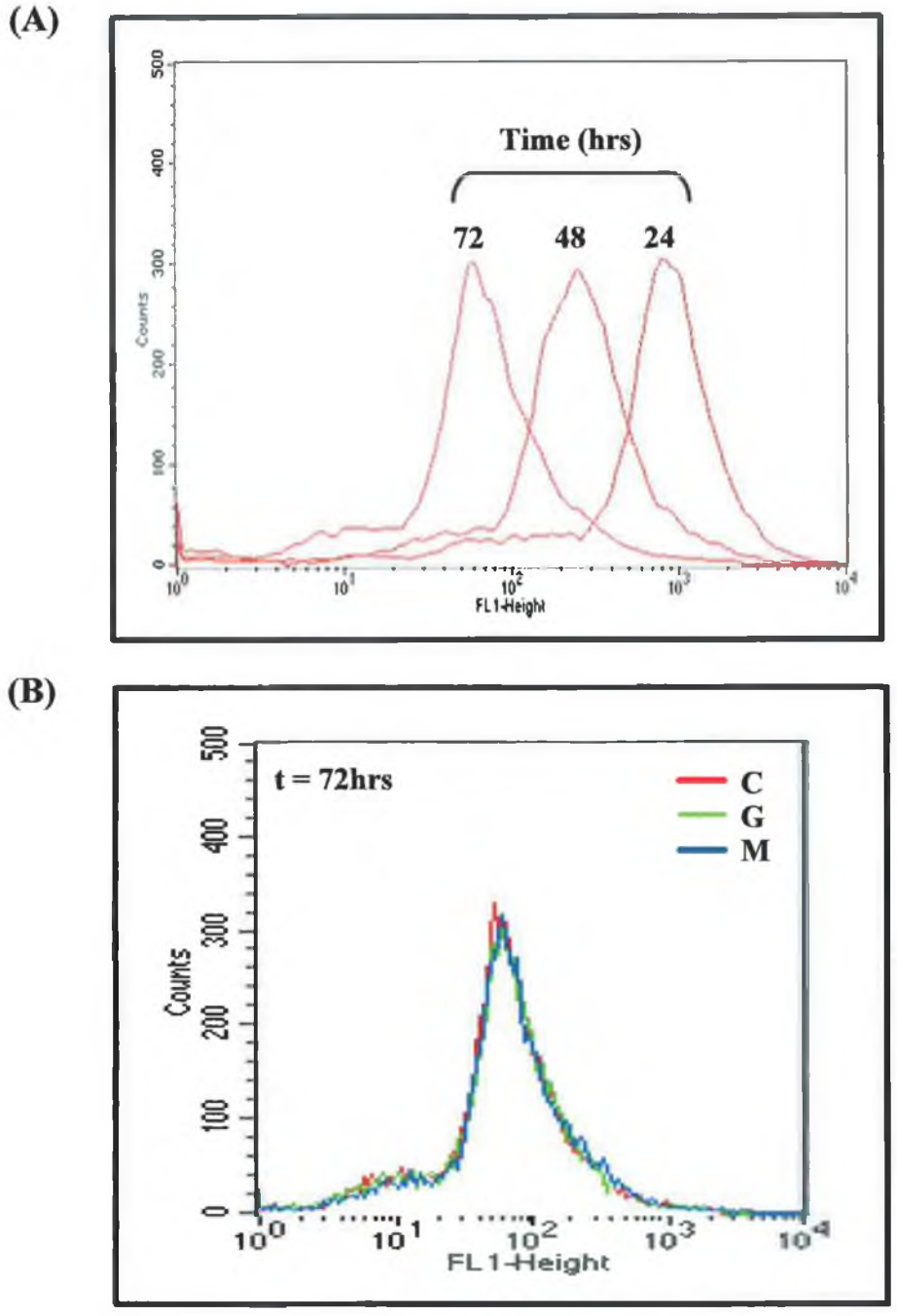


Fig. 3.5 Time course of BASMC Proliferation by FACS Analysis

BASMCs were cultured in 10% FBS for 24hr, 48hr and 72hrs in the absence or presence of high glucose (25mM). After these time points, cells were collected and analysed by FACS as described in the Methods section 2.4.8. **(A)** Comparison of cell proliferation over the 72hr period **(B)** comparison of proliferation of normal glucose (5.5mM:C), high glucose (25mM:G) and mannitol (25mM:M) treated cells at 72hrs. Data are representative of at least 3 independent experiments.

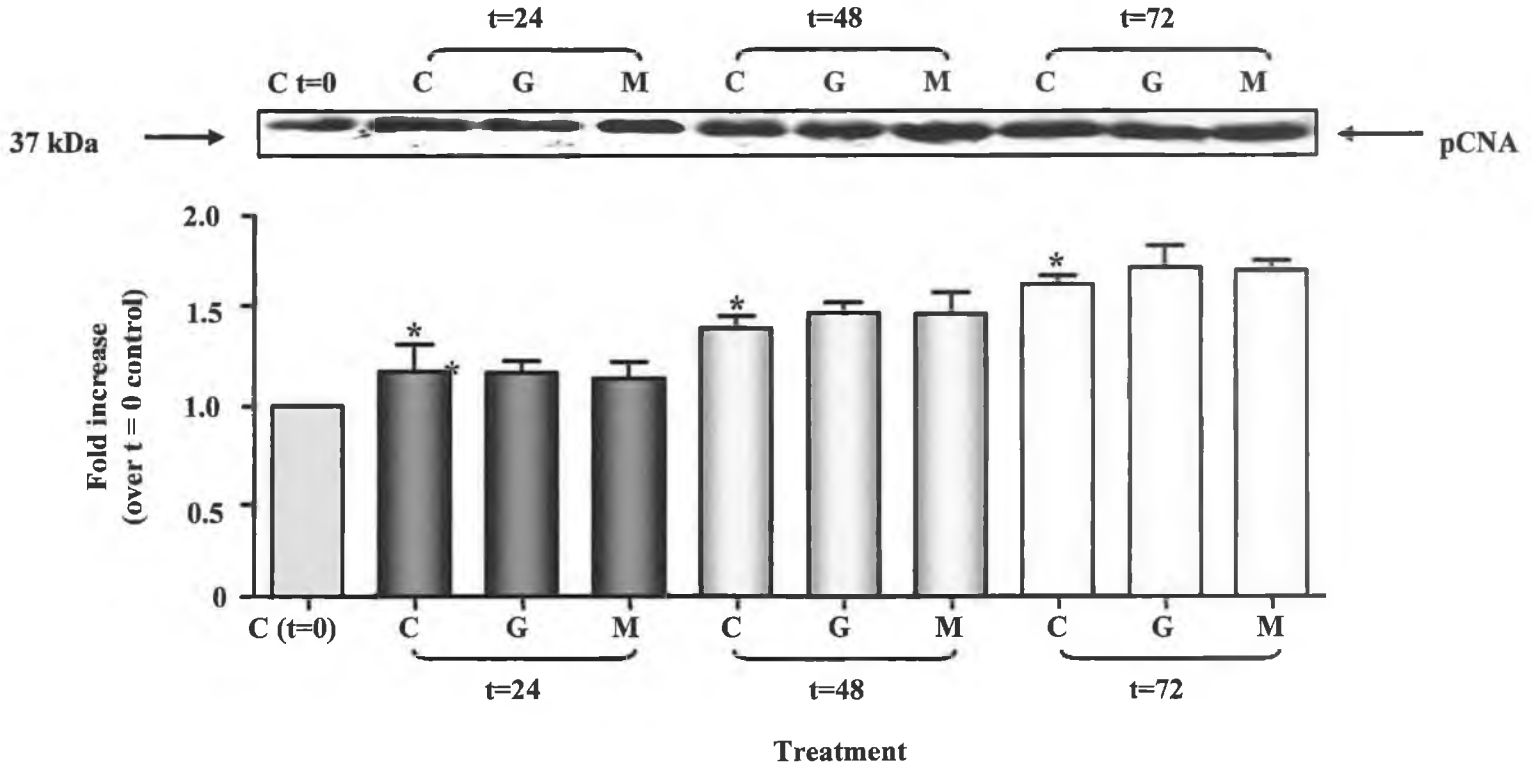


Fig. 3.6 **Effects of high glucose concentrations on pCNA expression in BAECs**

BAECs cultured in 10% FBS were exposed to high glucose concentrations over a period of 72hrs. Cells were harvested and cell lysates resolved by SDS-PAGE. Proteins were transferred to nitrocellulose and visualised by incubation with an anti-pCNA-specific antibody followed by HRP-conjugated anti-mouse secondary antibody. Blots were quantified by densitometric scanning and values represent mean ± SEM for three experiments. *$p \leq 0.05$ vs t=0 control.

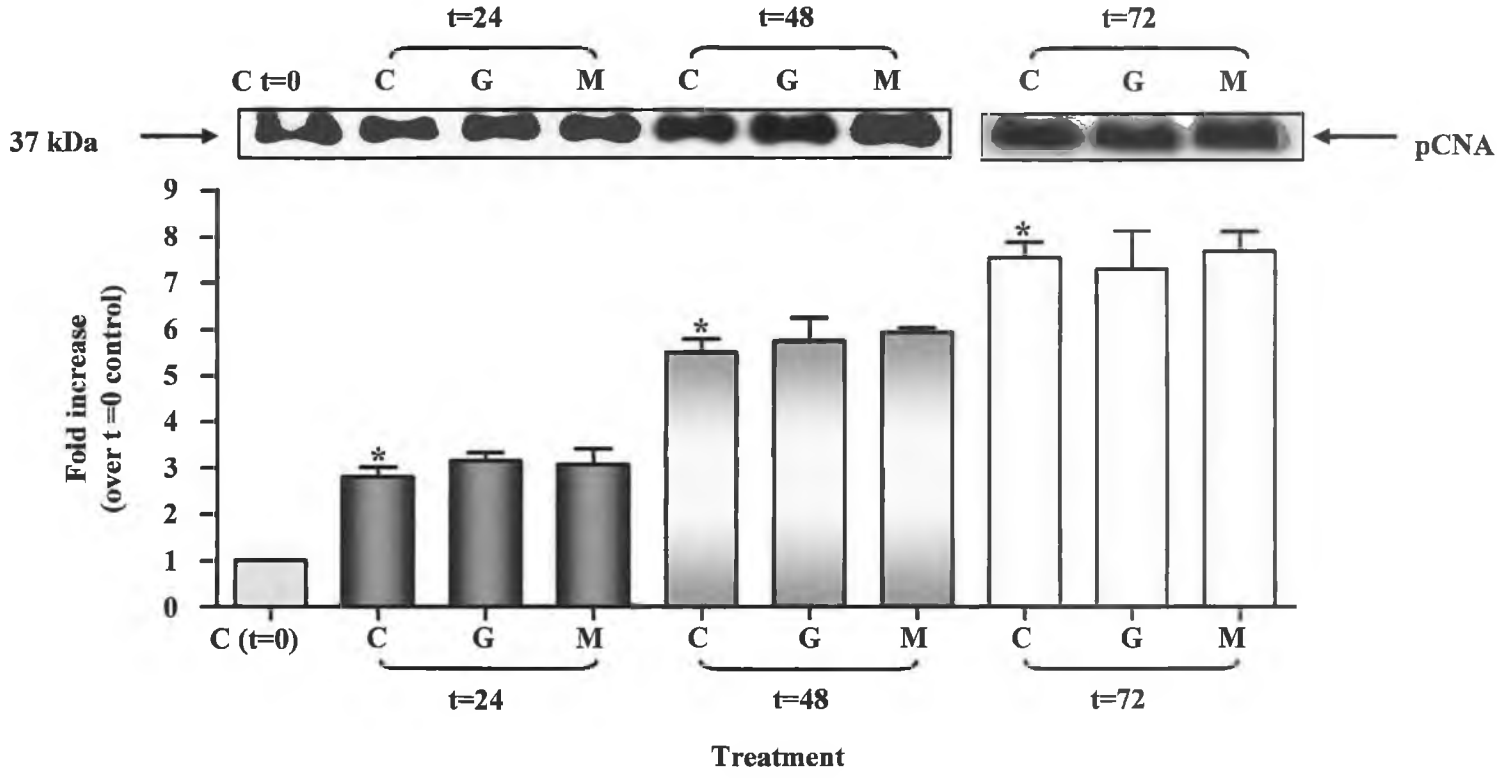


Fig. 3.7 Effects of high glucose concentrations on pCNA expression in BASMCs

BASMCs cultured in 10% FBS were exposed to high glucose concentrations over a period of 72hrs. Cells were harvested and cell lysates resolved by SDS-PAGE. Proteins were transferred to nitrocellulose and visualised by incubation with an anti-pCNA-specific antibody followed by HRP-conjugated anti-mouse secondary antibody. Blots were quantified by densitometric scanning and values represent mean ± SEM for three experiments. *$p \leq 0.01$ vs t=0 control.

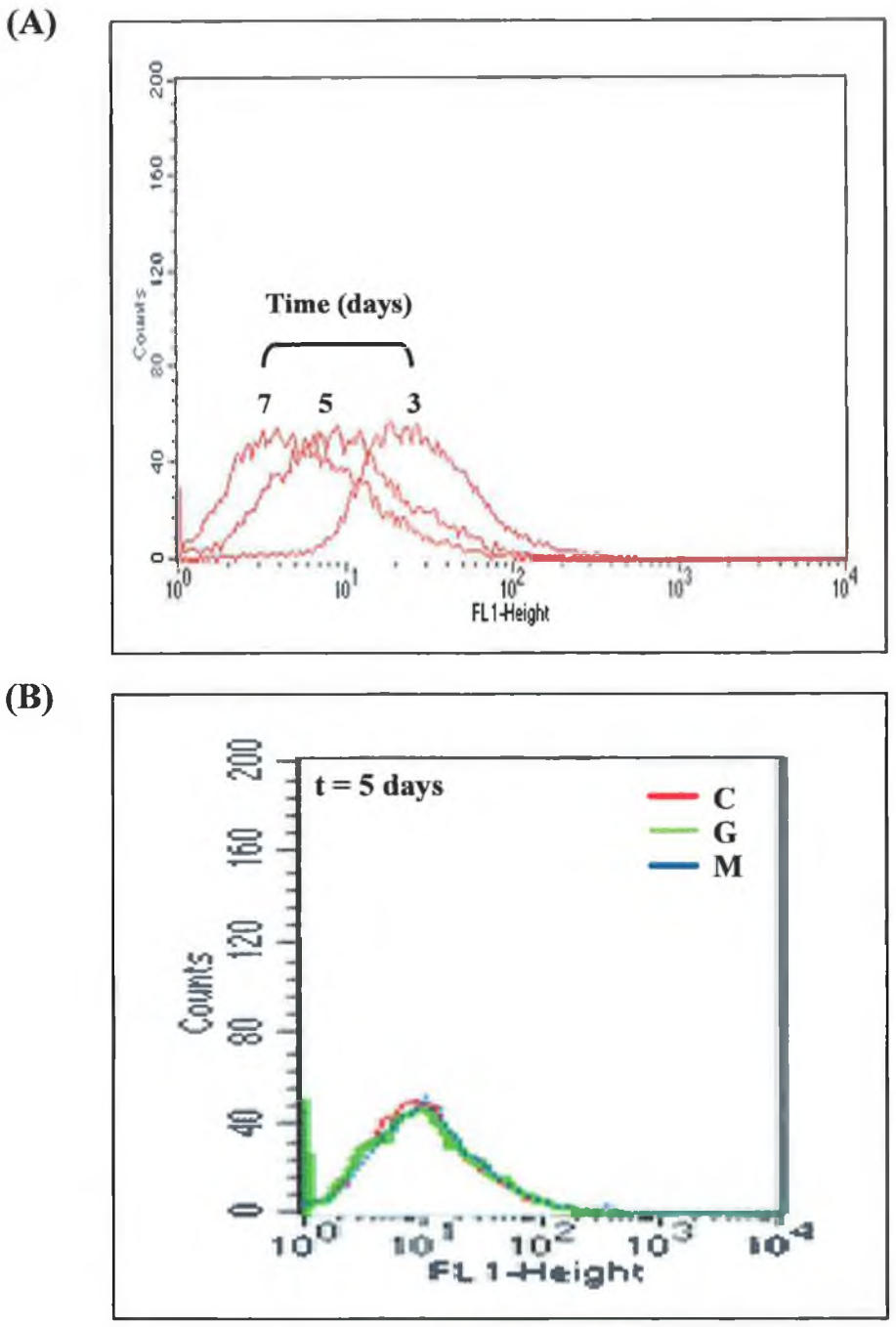


Fig. 3.8 Extended time course of BAEC Proliferation by FACS Analysis

BAECs were cultured in 10% FBS for 3, 5 and 7 days in the absence or presence of high glucose (25mM). After these time points, cells were collected and analysed by FACS as described in the Methods section 2.4.8. **(A)** Comparison of cell proliferation over the 7 day period **(B)** comparison of proliferation of normal glucose (5.5mM: C), high glucose (25mM: G) and mannitol (25mM: M) treated cells at 5 days. Data are representative of at least 3 independent experiments

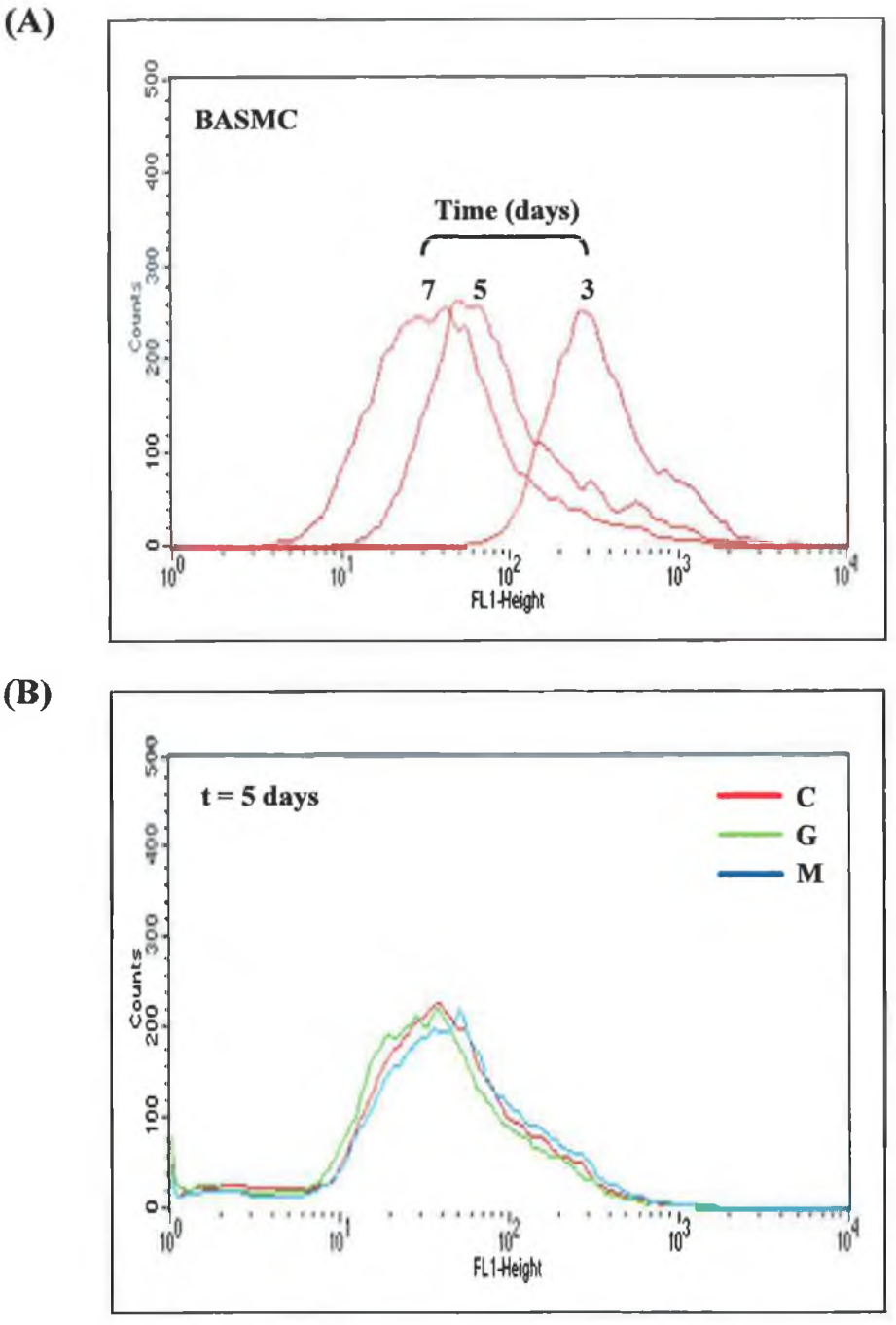


Fig. 3.9 Extended time course of BASMC Proliferation by FACS Analysis

BASMCs were cultured in 10% FBS for 3, 5 and 7 days in the absence or presence of high glucose (25mM). After these time points, cells were collected and analysed by FACS as described in the Methods section 2.4.8. **(A)** Comparison of cell proliferation over the 7 day period **(B)** comparison of proliferation of normal glucose (5.5mM: C), high glucose (25mM: G) and mannitol (25mM: M) treated cells at 5 days. Data are representative of at least 3 independent experiments.

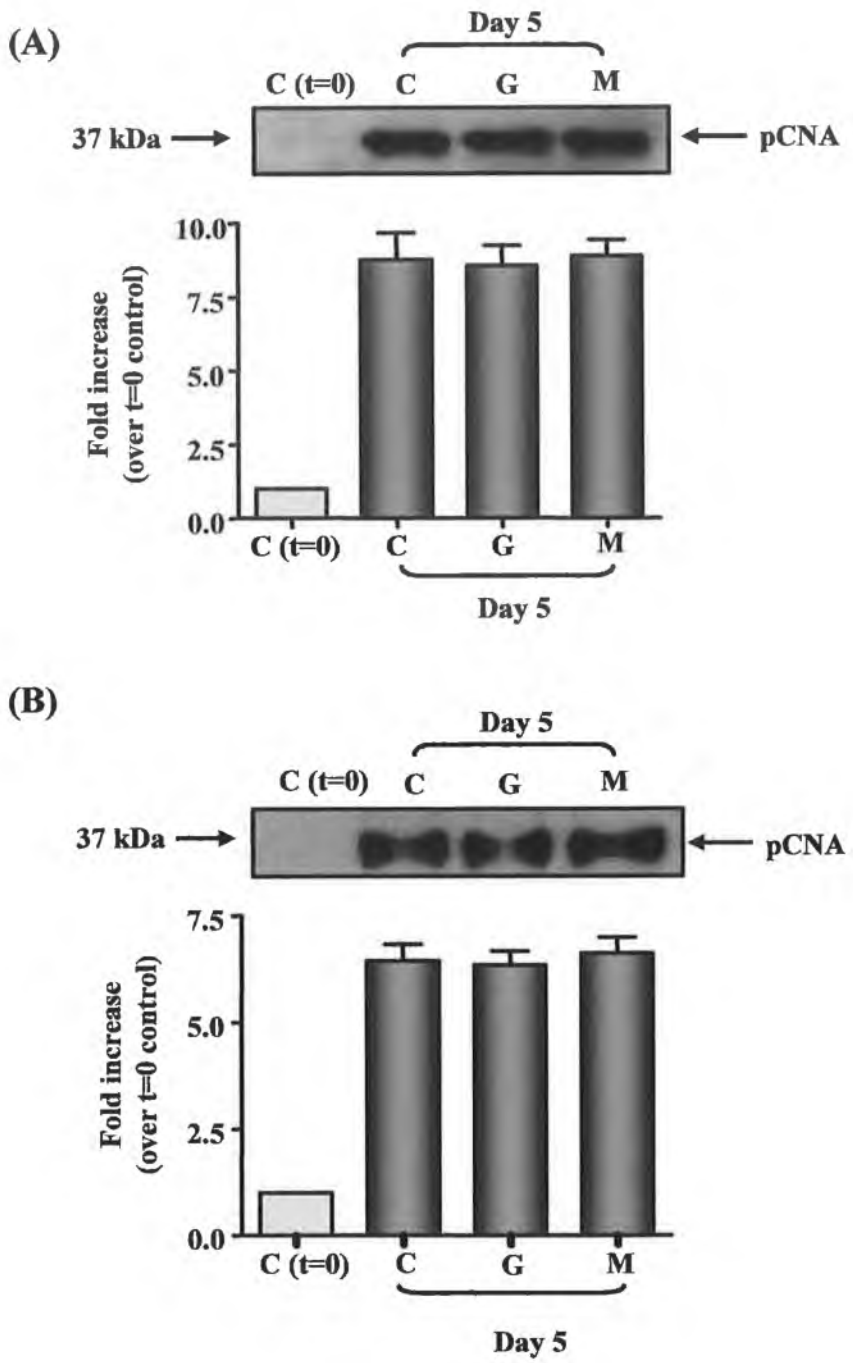


Fig. 3.10 Effects of high glucose concentrations on pCNA expression after 5 days treatment

(A) BAECs and **(B)** BASMCs cultured in 10% FBS were exposed to high glucose concentrations over a period of 5 days. Cells were harvested and cell lysates resolved by SDS-PAGE. Proteins were transferred to nitrocellulose and visualised by incubation with an anti-pCNA-specific antibody followed by HRP-conjugated anti-mouse secondary antibody. Blots were quantified by densitometric scanning and values represent mean ± SEM for three experiments.

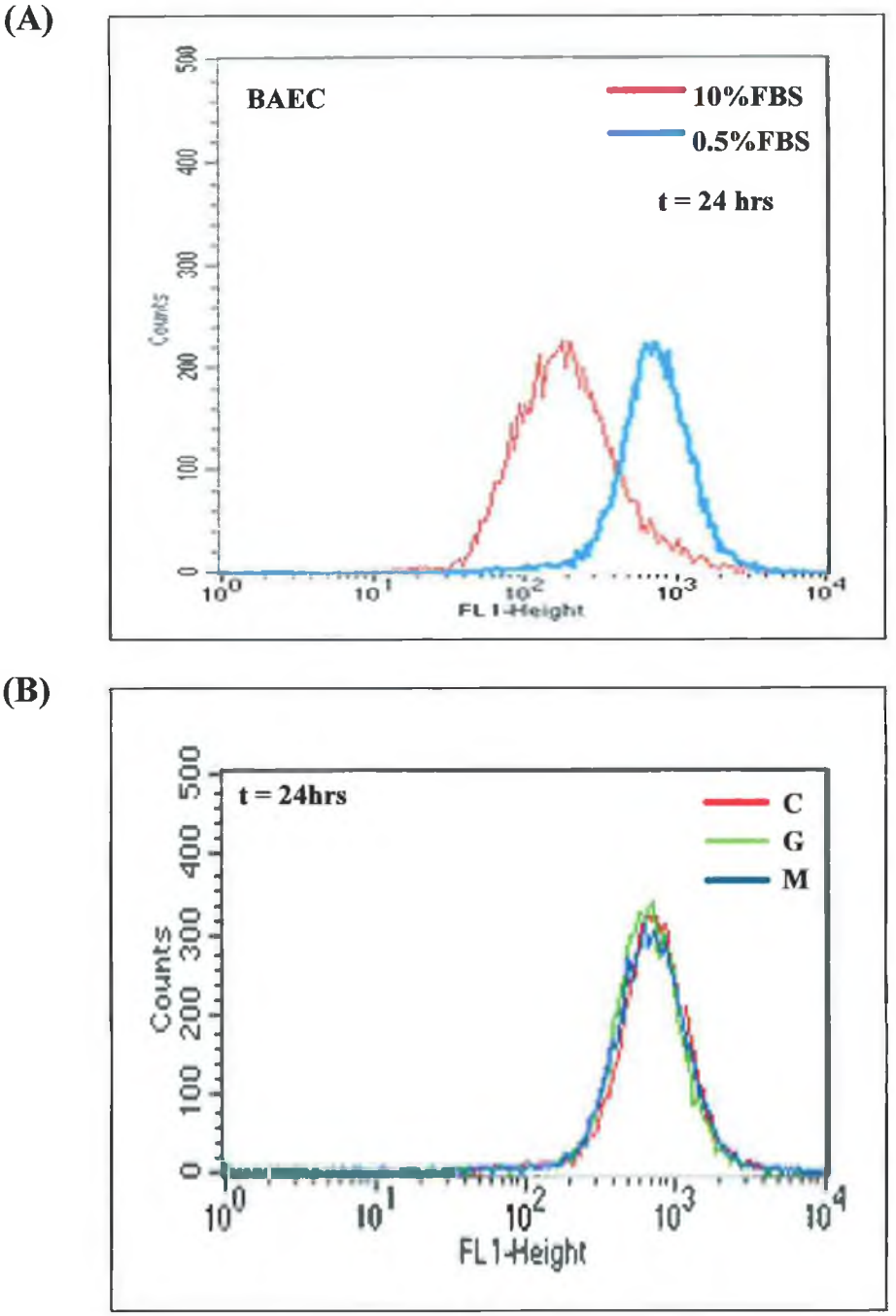


Fig. 3.11 Effects of serum starvation on BAEC proliferation

BAECs were cultured in 10% or 0.5% FBS for 24hrs in the absence or presence of high glucose (25mM). After these time points, cells were collected and analysed by FACS as described in the Methods section 2.4.8. **(A)** Comparison of cell proliferation over the 24hr period in 10% or 0.5% FBS **(B)** comparison of proliferation of normal glucose (5.5mM: C), high glucose (25mM: G) and mannitol (25mM: M) treated cells at 24hrs in the presence of 0.5% FBS. Data are representative of at least 3 independent experiments.

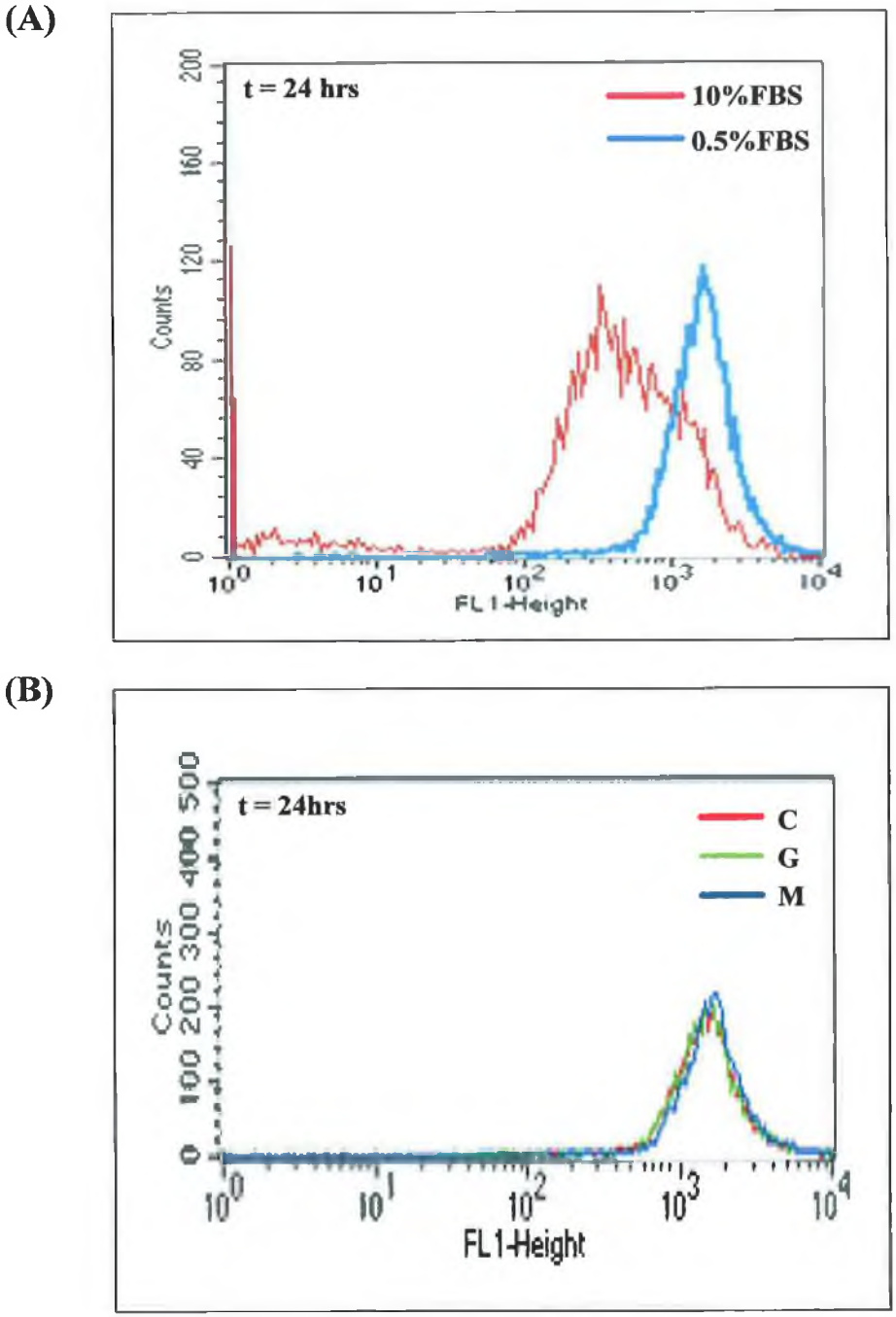


Fig. 3.12 Effects of serum starvation on BASMC proliferation

BASMCs were cultured in 10% or 0.5% FBS for 24hrs in the absence or presence of high glucose (25mM). After these time points, cells were collected and analysed by FACS as described in the Methods section 2.4.8. **(A)** Comparison of cell proliferation over the 24hr period in 10% or 0.5% FBS **(B)** comparison of proliferation of normal glucose (5.5mM: C), high glucose (25mM: G) and mannitol (25mM: M) treated cells at 24hrs in the presence of 0.5% FBS. Data are representative of at least 3 independent experiments.

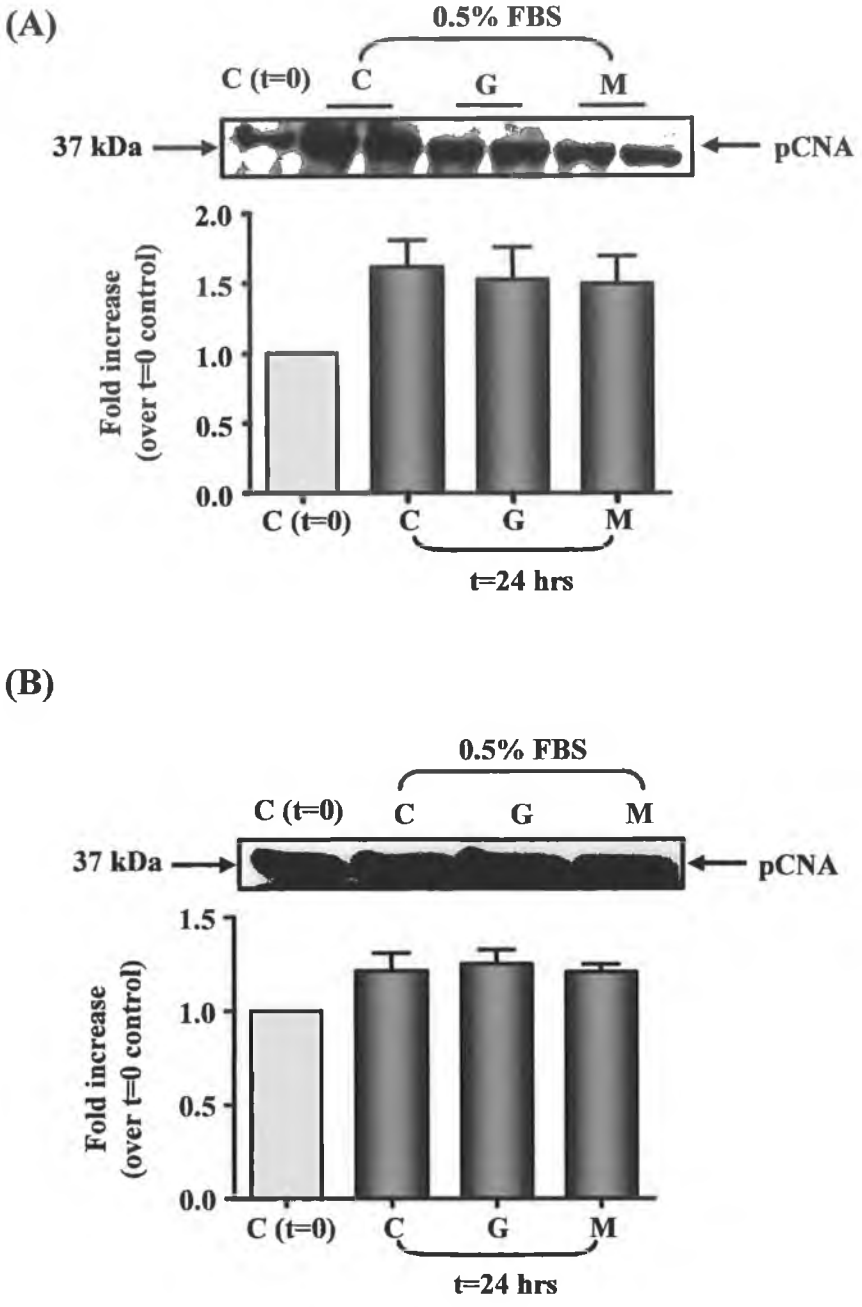


Fig. 3.13 **Effects of serum starvation and high glucose concentrations on pCNA expression**

(A) BAECs and **(B)** BASMCs cultured in 0.5% FBS were exposed to high glucose concentrations over a period of 5 days. Cells were harvested and cell lysates resolved by SDS-PAGE. Proteins were transferred to nitrocellulose and visualised by incubation with an anti-pCNA-specific antibody followed by HRP-conjugated anti-mouse secondary antibody. Blots were quantified by densitometric scanning and values represent mean ± SEM for three experiments.

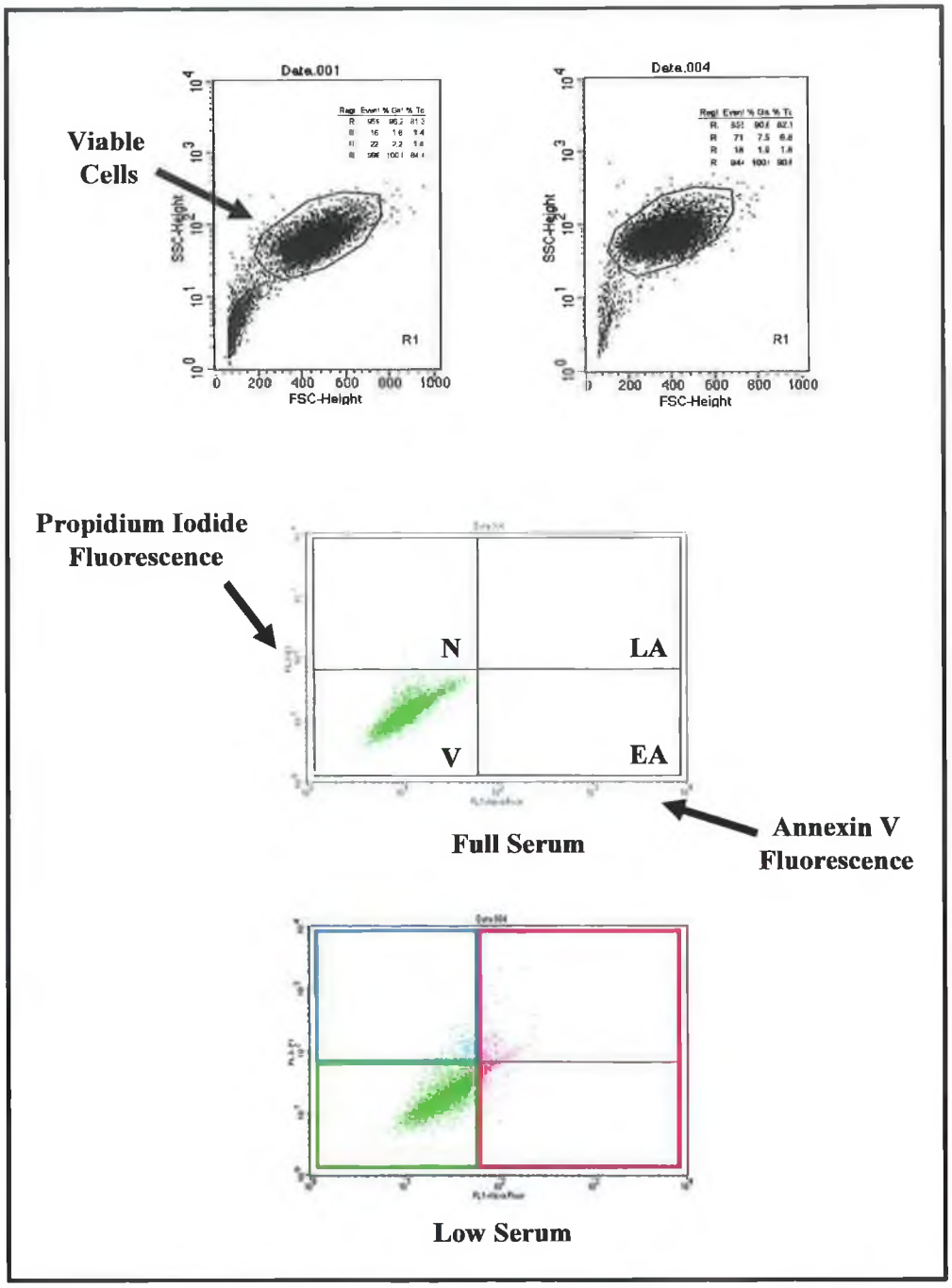


Fig. 3.14 Representative data obtained from FACS analysis for apoptosis

Cells were cultured in serum containing 10% or 0.5% FBS and subjected to FACS analysis and stained for apoptosis using the Vybrant® Apoptosis Assay Kit as described in the Methods section 2.4.7. Annexin V binds to phosphatidylserine located on the extracellular surface of apoptotic cells while PI is impermeable to live cells and apoptotic cells but stains necrotic cells with red fluorescence. Populations of cells are distinguished using flow cytometry and designated as viable (V), early apoptotic (EA), late apoptotic (LA) or necrotic (N) depending on the ratio of Annexin V:PI staining.

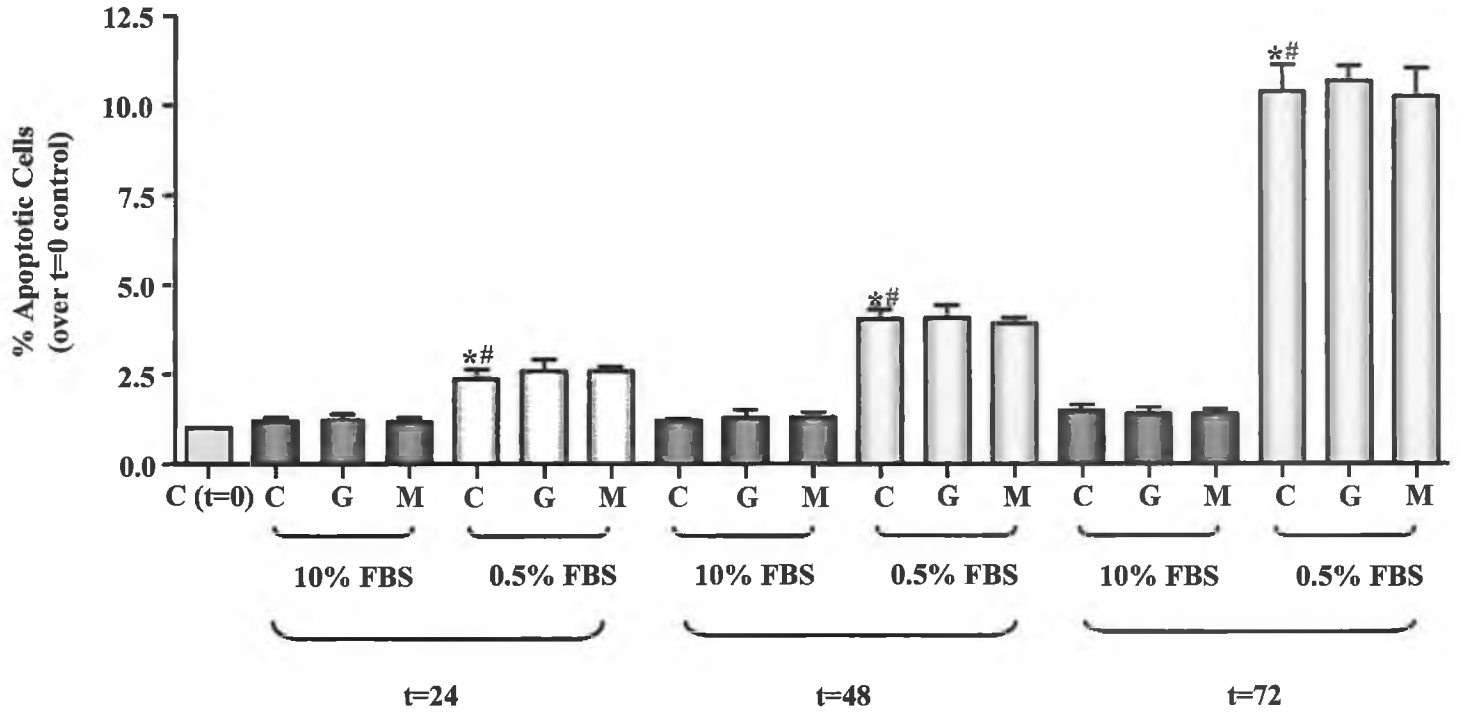


Fig. 3.15 Effects of serum starvation and high glucose concentrations on BAEC apoptosis as detected by FACS analysis

BEACs were cultured in media containing 10% or 0.5% FBS for 24hrs, 48hrs and 72hrs in the absence or presence of high glucose (25mM). After these time points, cells were collected and analysed for apoptosis by FACS as described in the Methods section 2.4.7. The values obtained for EA and LA cells were combined and plotted as a bar chart (n=3 $\pm$ SEM, *$p \leq 0.05$ compared to t=0 control, #$p \leq 0.05$ compared to 10% FBS control; normal glucose [5.5mM: C], high glucose [25mM: G] and mannitol [25mM:M]).

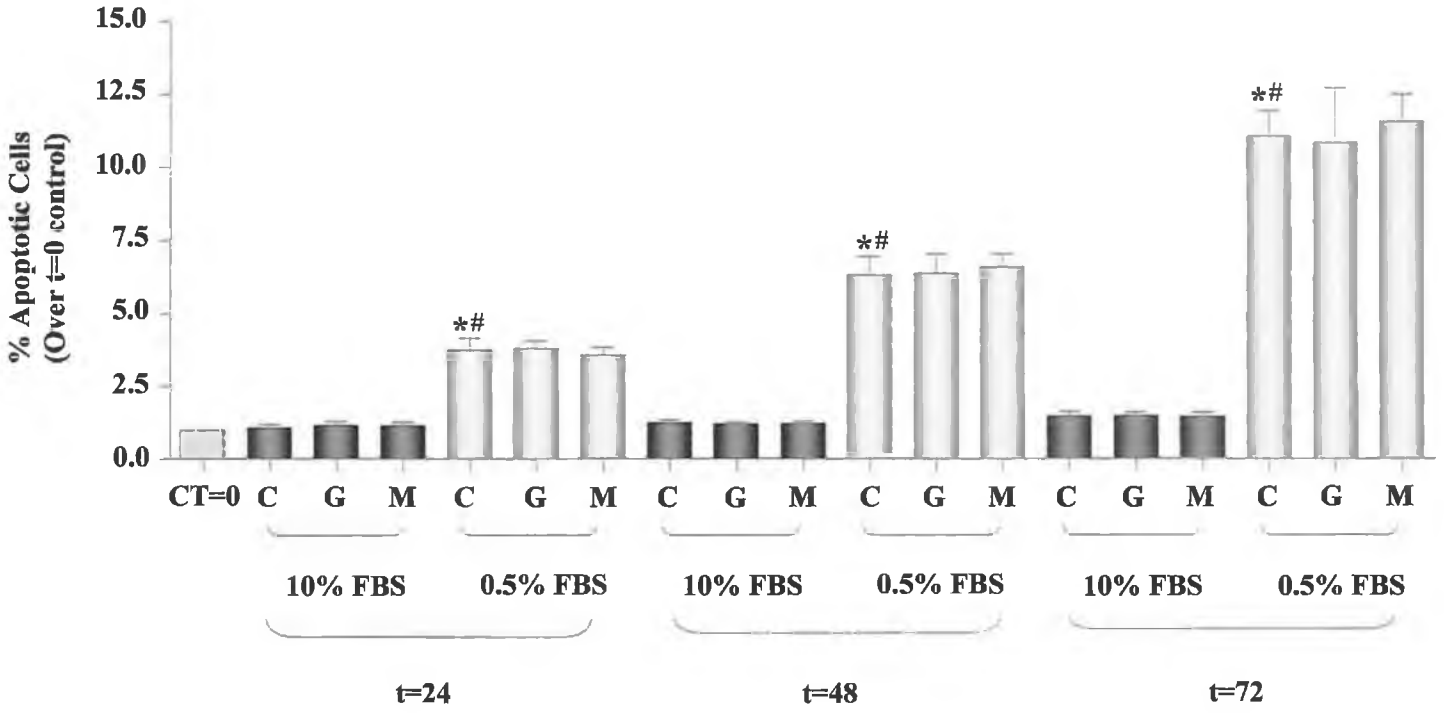


Fig. 3.16 Effects of serum starvation and high glucose concentrations on BASMC apoptosis as detected by FACS analysis

BESMCs were cultured in media containing 10% or 0.5% FBS for 24hrs, 48hrs and 72hrs in the absence or presence of high glucose (25mM). After these time points, cells were collected and analysed for apoptosis by FACS as described in the Methods section 2.4.7. The values obtained for EA and LA cells were combined and plotted as a bar chart (n=3 $\pm$ SEM, *$p \leq 0.05$ compared to t=0 control, #$p \leq 0.05$ compared to 10% FBS control; normal glucose [5.5mM: C], high glucose [25mM: G] and mannitol [25mM:M]).

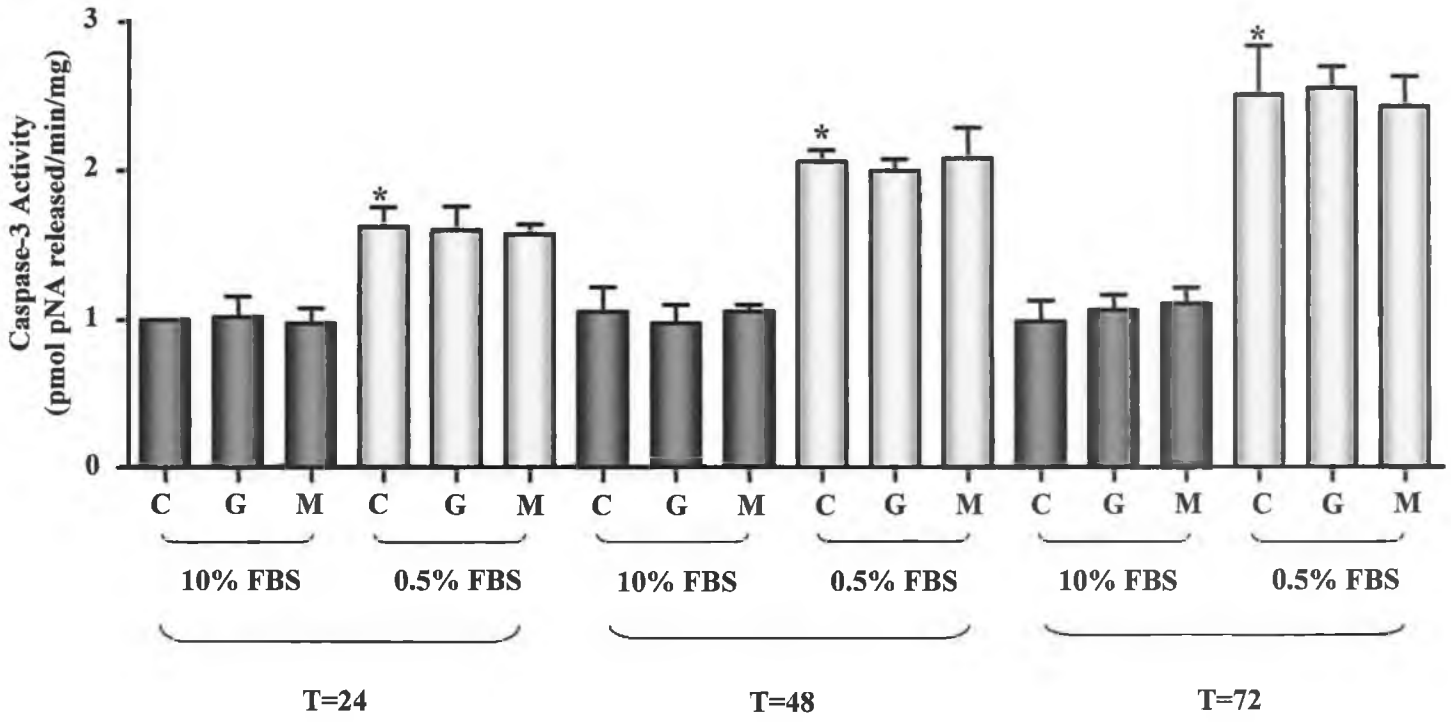


Fig. 3.17 Effects of serum starvation and high glucose concentrations on BAEC apoptosis as detected by Caspase-3 activity

BEACs were cultured in media containing 10% or 0.5% FBS for 24hrs, 48hrs and 72hrs in the absence or presence of high glucose (25mM). After these time points, cells were harvested and a colourimetric caspase 3 assay carried out on cell lysates as described in the Methods section 2.4.7. A pNitroanilide (pNA) standard curve (0-200μg/μl) allowed for the specific activity of caspase 3 to be calculated for each sample and expressed as pmol pNA released/min/mg. Data are representative of at least 3 independent experiments ± SEM *$p \leq 0.05$ compared to 10% FBS control; normal glucose [5.5mM: C], high glucose [25mM: G] and mannitol [25mM:M]).

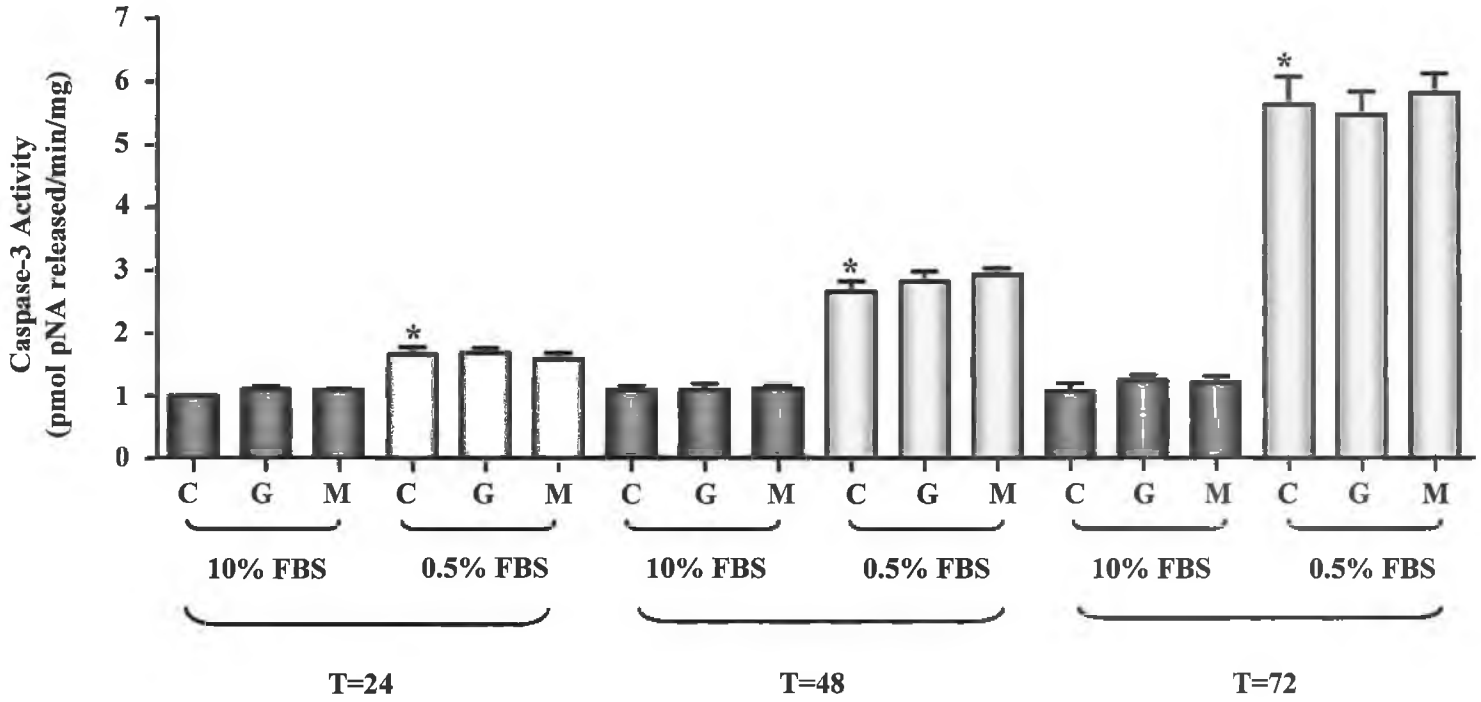


Fig. 3.18 Effects of serum starvation and high glucose concentrations on BASMC apoptosis as detected by Caspase-3 activity

BESMCs were cultured in media containing 10% or 0.5% FBS for 24hrs, 48hrs and 72hrs in the absence or presence of high glucose (25mM). After these time points, cells were harvested and a colourimetric caspase 3 assay carried out on cell lysates as described in the Methods section 2.4.7. A pNitroanilide (pNA) standard curve (0-200µg/µl) allowed for the specific activity of caspase 3 to be calculated for each sample and expressed as pmol pNA released/min/mg. Data are representative of at least 3 independent experiments ± SEM *$p \leq 0.05$ compared to 10% FBS control; normal glucose [5.5mM: C], high glucose [25mM: G] and mannitol [25mM:M]).

3.3 Discussion

It is well established that prolonged hyperglycaemia may be a key contributor in the development of vascular complications in diabetes leading to vascular disease. An important feature of vascular disease is abnormal growth, proliferation, migration and hypertrophy of macrovascular and microvascular cells, however, the precise molecular events linking hyperglycaemia with the abnormal macro- and microvascular cell functions remain poorly characterized.

Many studies have shown that hyperglycaemia can accelerate vascular endothelial and smooth muscle cell proliferation by activation of various pathways, such as reactive oxygen species (ROS) (Srivastava 2002), protein kinase C (PKC) (Patel et al. 1999), mitogen-activated protein kinases (MAPKs) (Amiri et al. 1999) and NF-κB activation (Fujita et al. 2002). The majority of studies focusing on the pathogenic mechanism of glucose in promoting vascular lesion formation have highlighted the proliferative effects of glucose on VSMC growth (Newby and Zaltsman 2000).

In this study, we focused on the effects of using pathophysiological levels of glucose (25mM) as detected in diabetic patients (Cai et al. 2002). We found that there was no notable change in BAEC and BASMC proliferation when compared to high glucose treated 10%FBS media in 24hrs or up to 7 days which is not consistent with previous studies. Various concentrations of glucose ranging from 22-60mmol/L has been using to stimulate proliferation in different cell types which causes either an increase or decrease in the proliferative effect during short-term (Sheu et al. 2005) or long-term (Esposito et al. 2001) exposure.

Short-term hyperglycaemia (45mmol/L glucose) produces oxidative damage and apoptosis which has been shown up to 6 hours in dorsal root ganglia neurons (Vincent et al. 2005). Studies on Bovine aortic and human microvascular endothelial cells also demonstrated a significant increase in cell number when exposed to high glucose (30mmol/L glucose) containing media compared with normal growth media (5.5mmol/L glucose) after 24 hours. However, this effect was not detectable at 48 hours. Long-term (8 weeks) exposure to high glucose did not modify the growth curve that was comparable to that of cells grown in low glucose containing media. High glucose induced a change in cell morphology after long-term compared with low glucose treated cells (Esposito et al. 2001).

As we described previously, FBS contains hormone and growth factors which can regulate cell growth. Since low serum conditions are used to quiesce the cells and deprive them of essential growth factors, one could expect that the addition of an energy substrate such as glucose could lead to an increase in proliferation of these cells. We have examined the effects of high glucose under low serum conditions (0.5% FBS) and shown no additive effect of HG treatment to BAEC and BASMC proliferation.

The reported effects of hyperglycaemia on endothelial and smooth muscle cells which are mixed and very dependent upon experimental conditions have been shown in several studies (Weimann et al. 1984; Umeda et al. 1991; Santilli et al. 1992; Kusuhara et al. 1997; Morishita et al. 1997). It has been shown that hyperglycaemia enhances proliferation of bovine carotid artery endothelial cells under low serum (0.5%FBS) stimulation but inhibited in the presence of 10% serum (Hayashi et al. 1991). In contrast, hyperglycaemia also inhibits the rate of proliferation in macrovascular endothelial cells under serum free media (0% FBS) and low serum media (2% FBS) over 2 days exposure (Santilli et al. 1992; Morishita et al. 1997)

Apoptosis is a finely regulated process that plays an important role in regulating cell number (Thompson 1995). More recently, apoptosis has been implicated in the development of arteries. Several studies have focused on apoptosis during limb vessel development (Cho et al. 1995) and apoptosis during the remodelling of human ductus arteriosis (Slomp et al. 1997). However, apoptosis is not limited to cell elimination during embryonic development. In recent years, apoptosis has been implicated in cardiovascular disease.

Recent studies have suggested that vascular remodelling and lesion formation are determined in part by the balance between cell proliferation and apoptotic cell death (Gibbons and Dzau 1994; Isner et al. 1995). It has been reported that reduction in blood flow in animal models resulted in reduction in VSMC number by apoptosis (Cho et al. 1997; Kumar and Lindner 1997). Acute arterial injury is followed by rapid induction of medial cell apoptosis from 30 minutes to 4 hours (Perlman et al. 1997; Pollman et al. 1999). In humans, restenosis after angioplasty has been reported to be associated with a decrease in VSMC apoptosis (Isner et al. 1995). A further example of apoptosis comes from aneurysm formation with advanced atherosclerosis (Henderson et al. 1999). Apoptosis of VSMC is increased in aortic aneurysms and is

associated with an increase in expression of a number of pro-apoptotic molecules. These studies support the concept that apoptosis is a pivotal regulator of cell number in the vessel wall.

The main intracellular effectors of apoptosis are a family of cysteine proteases known as caspases. Caspases are activated by cleavage of pro-caspases in a sequential manner during apoptosis. Caspase-3 is an effector caspase that plays a role in cell death induced by a variety of stimuli (Thornberry and Lazebnik 1998).

Rupture of atherosclerotic plaques is associated with a thinning of the VSMC-rich fibrous cap overlying the core. Rupture occurs particularly at the plaque shoulders, which exhibit lack of VSMCs and the presence of inflammatory cells. Apoptotic VSMCs are evident in advanced human plaques including the shoulder regions, prompting the suggestion that VSMC apoptosis may hasten plaque rupture. Indeed, increased VSMC apoptosis occurs in unstable versus stable angina lesions (Bennett 2002).

The disappearance of macrophages by apoptosis could have a positive effect on plaque stabilization. The death of macrophages would lead to decreased breakdown of collagen fibers. On the contrary, the disappearance of SMC from the fibrous cap or other vulnerable regions of the plaque could lead to destabilization of the plaque (Davies 1996; Libby et al. 1996).

A previous study showed that hyperglycaemia attenuated the rate of serum withdrawal-induced apoptosis on rat aortic A7r5 cells and attenuated capsase-3 activity, and also increased the abundance of transcript and protein levels of Bcl-2 and Bcl-$_{xl}$ (Li et al. 2005).

We investigated the apoptotic level of BAEC and BASMC in normal growth media (10%FBS) and low serum media (0.5%FBS) by FACS analysis. It is not surprising that low serum media induced apoptosis on both cell types. However there was no significant alteration observed when adding in high glucose. The percentage of apoptotic cells is varied in different cell types (0.2-10%) and is dependant on the environment. In our studies in BAEC and BASMC, the basal percentage of apoptotic cells is approximately 0.2-2%.

The significance of apoptosis in atherosclerosis appears to depend on the stage of the plaque , localization, and cell types involved (Kockx and Knaapen 2000). Glucose-induced suppression of apoptosis appears to be of particular importance in the initial events of vascular injury and thickening of the media of the vessels. It is

well documented that high glucose and advanced glycation end products increase NF-κB activity in rat VSMC (Lander et al. 1997; Yerneni et al. 1999) and Bcl-$_{xl}$ has been identified as an NF-κB-dependent transcriptional target (Barkett and Gilmore 1999). Therefore, high glucose concentrations are likely to have an effect on the activity of NF-κB activation and Bcl-$_{xl}$ expression, leading to apoptotic suppression in VSMC.

It is well established that the rate of apoptosis not only depends on the relative balance between pro- and anti-apoptotic signals but also on the target cells. Initial reports have shown that prolonged treatment of human umbilical vein endothelial cells with high glucose lead to increase DNA fragmentation and higher prevalence of apoptosis, while fibroblast were unaffected by sexposure to high glucose and no increase in apoptosis was observed (Baumgartner-Parzer et al. 1995). Moreover, the cellular mechanism by which the effect of high glucose modulates apoptotic events in the retina clearly appeared to be tissue-specific and cell-specific within tissues (Podesta et al. 2000).

Cellular responses to high glucose are numerous and varied ultimately resulting in functional changes and cell death. High glucose causes mitochondrial membrane depolarization and loss of uncoupling protein, resulting in increased oxidative stress as well as release of cytochrome *c* and activation of caspases (Leinninger et al. 2004; Vincent et al. 2004). Caspase-3 activity was also examined in this study and a similar effect was found when compared to FACS analysis. According to the studies by Leninger and Vincent which indicate caspase-3 activation pathway is causally involved in hyperglycaemia-induced myocardial apoptosis when exposed to 60 mmol/L glucose, whereas there was no significant alteration when treated with 33mmol/L glucose (Cai et al. 2002); we hypothesise that physiological high glucose concentrations (25mM) may not be sufficient to induce cell apoptosis.

Chapter 4

Effect of High Glucose on BAEC and BASMC Cell Fate (proliferation and apoptosis) Under Hypoxic Conditions

4.1 Introduction

Arterial wall hypoxia and associated vascular cell proliferation have been implicated in the development of atherosclerosis (Bjornheden et al. 1999; Lee et al. 2000) and has also been shown to be mitogenic to cultured pulmonary artery smooth muscle and endothelial cells (Dempsey et al. 1997; Lou et al. 1997; Cooper and Beasley 1999). Prevalence of tissues hypoxia and increased VSM cell proliferation is reported in experimental models of diabetes and hypertension (Miller and Wilson 1984; Bunkenburg et al. 1992; Alipui et al. 1993; Santilli et al. 1993; Sowers and Epstein 1995; Oikawa et al. 1996). Elevated glucose concentrations in media have been shown to produce both hypertrophic and hyperplastic effects in cultured porcine aortic smooth muscle cells (Natarajan et al. 1992) which hypoxia potentiates the effect of high glucose on the proliferation of VSM and mesangial cells (Sodhi et al. 2001; Sodhi et al. 2001). Therefore, these results strongly suggest an important role for hypoxia in accelerated cell proliferation in diabetes; however the mechanisms responsible for the accelerated cell growth and progression into cardiovascular disease in diabetes remain to be clearly defined.

Apoptosis is the dominant mechanism of cell death in rats undergoing coronary artery occlusion (Kajstura et al. 1996). Apoptosis may also make a major contribution to overall cell death in the infarcted human heart (Morrell et al. 1997) and is an important pathological feature of the hypoxic myocyte (Wardell 1977) and the reperfused myocardium (Brown and Sernia 1994; Cooper and Beasley 1999).

Apoptosis can be induced in response to hypoxia (Tanaka et al. 1994). The severity of hypoxia determines whether cells become apoptotic or adapt to hypoxia and survive. A hypoxic environment devoid of nutrients prevents the cell undergoing energy dependent apoptosis and cells become necrotic. Apoptosis regulatory proteins are delicately balanced (Yamamoto et al. 2001; Mayorga et al. 2004). Hypoxia is a common phenomenon and cells can adapt to this environmental stress so; that after repeated periods of hypoxia, selection for resistance to hypoxia induced apoptosis occurs.

Aim 2: To Investigate the Effects of Hypoxia (2% O_2) on BAEC and BASMC growth (proliferation *vs* apoptosis) in the absence or presence of HG (25mM).

4.2 Results

We have established that the addition of high glucose concentrations (25mM) to BAEC and BASMC cultured in either 10% or 0.5% FBS has neither positive or negative effect on the balance between cell proliferation and apoptosis. Consequently, we introduced a known stimulus of cell apoptosis, hypoxia, to determine the effect on BAEC and BASMC growth (apoptosis *vs* proliferation).

A Ruskinn Invivo$_2$ 400 Hypoxia Workstation was used to study the effects of lowering oxygen tensions *in vitro*. Using this apparatus, we exposed BAEC and BASMC to hypoxia at the level of 2% O_2 (Fig. 4.1).

BAEC and BASMC were cultured in 10% FBS for 5 days in either normoxic (5% CO_2, 95% air) or hypoxic (2% O_2, 5% CO_2, 93% N_2) conditions and the effects on proliferation and apoptosis were measured as described previously. FACS analysis for proliferation showed that BAEC and BASMC proliferation was significantly decreased in cells exposed to hypoxic conditions when compared to normoxic control (Fig. 4.2). Concurrent western blot analysis for pCNA expression confirmed the hypoxia-induced decrease in BAEC (4.63 ± 0.50-fold n=3 *vs* 8.97-fold ± 0.24-fold n=3; *p≤0.01) and BASMC (2.49-fold ± 0.12-fold n=3 *vs* 7.92-fold ± 0.61-fold n=3; *p≤0.01) proliferation when compared to normoxic control (Fig. 4.3).

We detected hypoxia induced apoptosis by using FACS analysis, representative data has been shown (Fig. 4.4(A)). When we compared hypoxia induced apoptosis to serum deprivation induced apoptosis (Fig. 3.14), significantly more early apoptotic and late apoptotic cells were detected suggesting that hypoxia is a greater stimulator of cellular apoptosis compared to serum deprivation.

Exposure of cells to hypoxic conditions increased the percentage of apoptotic cells in both BAEC and BASMC when compared to normoxic control (BAEC (21.83% ± 2.46% n=3 *vs* 2.71% ± 0.43% n=3 *p≤0.01; and BASMC 26.79% ± 1.14% n=3 vs 5.41% ± 1.12% n=3; *p≤0.01) (Fig. 4.4(B)).

Concurrent caspase-3 activity also showed the hypoxia induced apoptosis in BAEC (2.96-fold ± 0.20-fold n=4 *vs* 1.21-fold ± 0.08-fold n=4; *p≤0.01) and BASMC (4.12-fold ± 0.37-fold n=4 *vs* 1.21-fold ± 0.06-fold n=4; *p≤0.01). This result was also confirmed in caspase-3 protein expression by western blot in which hypoxia was seen to significantly enhance caspase-3 expression in BAEC (1.73-fold ± 0.11-fold n=3 *vs* 0.48-fold ± 0.07-fold n=3; *p≤0.01) and BASMC (2.79-fold ± 0.30-fold n=3 *vs* 0.87-fold ± 0.05-fold n=3; *p≤0.01) (Fig. 4.5).

Having established the effects of hypoxia on BAEC and BASMC proliferation and apoptosis, we then investigated the effects of hyperglycaemia under these same conditions.

BAEC and BASMC were exposed to normoxic or hypoxic conditions with high glucose (25mM) or mannitol (19.5mM) for 5 days prior to analysis of proliferation and apoptosis as previously described using FACS analysis, we determined that under normoxic conditions, high glucose did not alter proliferation of BAEC or BASMC as detailed in our earlier studies (Chapter 3). However, under hypoxic conditions, high glucose induced a marked increase in proliferation of BAEC and BASMC when compared to normal glucose and mannitol treated control cells (Figs. 4.6 & 4.7).

This increase in proliferation was confirmed by western blot for pCNA. BAEC and BASMC were harvested after 5 days treatment and cell lysates resolved by SDS-PAGE. Similar to FACS analysis, there was no change of pCNA expression on BAEC and BASMC under normoxic conditions with either normal glucose or high glucose. In contrast, pCNA expression of BAEC and BASMC were significantly increased in the presence of high glucose under hypoxic conditions when compared to hypoxic control (BAEC 7.33-fold ± 0.49-fold n=3 *vs* 4.63 ± 0.50 n=3 *$p \leq 0.01$; BASMC 5.07-fold ± 0.50-fold n=3 *vs* 2.49-fold ± 0.12-fold n=3; *$p \leq 0.01$) (Fig. 4.8).

Although high glucose increases BAEC and BASMC proliferation under hypoxic conditions, this increase did not return proliferation to the level of normoxic day 5 control (BAEC 7.33-fold ± 0.49-fold n=3 *vs* 8.97-fold ± 0.24-fold n=3 #$p \leq 0.05$; BASMC 5.07-fold ± 0.50-fold n=3 *vs* 7.92-fold ± 0.61-fold n=3; #$p \leq 0.05$) (Fig. 4.9).

This increase in the presence of high glucose on BAEC and BASMC proliferation under hypoxic condition was confirmed by FACS analysis. When compared to hypoxic control, BAEC and BASMC proliferation in the presence of high glucose significantly increased under hypoxic conditions, however, when compared to normoxic control, high glucose treated BAEC and BASMC (green line) under hypoxic condition did not reach the same level of normoxic control (Figure 4.9).

FACS analysis for BAEC and BASMC apoptosis showed that high glucose did not alter cellular apoptosis under normoxic conditions as previously described. When cells were exposed to hypoxic conditions, the percentage of apoptotic cells

was significantly increased on BAEC and BASMC compared to normoxic control (BAEC 21.83% ± 2.46% n=3 *vs* 2.71% ± 0.43% n=3 #p≤0.01; BASMC 26.79% ± 1.14% n=3 *vs* 5.41% ± 1.12% n=3; #p≤0.01). However, hypoxia induced apoptosis was inhibited on BAEC (10.06% ± 2.35% n=3 *vs* 21.83% ± 2.46% n=3; *p≤0.05) and BASMC (12.32% ± 1.38% n=3 *vs* 26.79% ± 1.14% n=3; *p≤0.01) in the presence of high glucose when compared to hypoxic control (Fig. 4.10).

Increased caspase-3 activity based on pmol pNA released/min/mg protein was detected in BAEC and BASMC exposed to hypoxic conditions compared to normoxic control (BAEC 2.96-fold ± 0.20-fold n=4 *vs* 1.21-fold ± 0.08-fold n=4 *p≤0.01; BASMC 4.12-fold ± 0.37-fold n=4 *vs* 1.21-fold ± 0.06-fold n=4; *p≤0.01). However, the presence of high glucose in hypoxia treated cells resulted in a significant decrease in caspase-3 activity when compared to hypoxic controls (BAEC 1.83-fold ± 0.19-fold n=4 *vs* 2.961 ± 0.1952 n=4 *p≤0.01; BASMC 2.72-fold ± 0.22-fold n=4 *vs* 4.12-fold ± 0.37-fold n=4;*p≤0.05) when compared to hypoxic control (Figure 4.11).

Expression of caspase-3 protein levels by western blot showed no change in caspase-3 protein expression under normoxic conditions in either BAEC or BASMC. High glucose did not alter caspase-3 protein expression under normoxic conditions. Similar to caspase-3 activity, protein levels were increased under hypoxic conditions when compared to normoxic control (BAEC 1.73-fold ± 0.11-fold n=3 *vs* 0.48-fold ± 0.07-fold n=3 #p≤0.01; BASMC 2.79-fold ± 0.30-fold n=3 *vs* 0.87-fold ± 0.05-fold n=3; #p≤0.01). The presence of high glucose under hypoxic conditions significantly decreased caspase-3 expression levels compared to hypoxic control (BAEC 1.09-fold ± 0.11-fold n=3 *vs* 1.73-fold ± 0.11-fold n=3 *p≤0.05; BASMC 1.35-fold ± 0.09-fold n=3 *vs* 2.79-fold ± 0.30-fold n=3; *p≤0.01) (Fig. 4.12).

Further analysis for apoptosis examined the regulation of pro- and anti-apoptotic proteins. Bcl-$_{xl}$ which belongs to the Bcl-2 family is an anti-apoptotic protein involved in regulating cell apoptosis. Bcl-$_{xl}$ protein expression was detected by western blot in BAEC and BASMC. Under normoxic conditions, high glucose showed no effect on Bcl-$_{xl}$ expression in either BAEC or BASMC after 5 days treatment. Bcl-$_{xl}$ expression was significantly decreased under hypoxic conditions when compared to normoxic control (BAEC 5.85-fold ± 0.87-fold n=4 *vs* 11.78-fold ± 0.50-fold n=4 #p≤0.01; BASMC 2.61-fold ± 0.36-fold n=3 *vs* 4.99-fold ± 0.27-fold n=3; #p≤0.01). However this effect was reversed in the presence of high glucose

when compared to hypoxic control (BAEC 9.55-fold ± 0.71-fold n=4 *vs* 5.85-fold ± 0.87-fold n=4 *p≤0.05; BASMC 5.64-fold ± 0.17-fold n=3 *vs* 2.61-fold ± 0.36-fold n=3; *p≤0.01) (Fig. 4.13).

In parallel, we examined the expression of the pro-apoptotic protein Bax; another member of the Bcl-2 family. Bax protein expression was measured by western blot as previously described. No change in Bax protein expression was found under normoxic conditions in either BAEC or BASMC when cells treated in high glucose media; under hypoxic condition, Bax expression was inhibited when compared to normoxic control (BAEC 1.12-fold ± 0.09-fold n=3 *vs* 1.58-fold ± 0.11-fold n=3 #p≤0.05; BASMC 1.11-fold ± 0.07-fold n=3 *vs* 1.52-fold ± 0.13-fold n=3; #p≤0.05). However, high glucose enhanced the expression of Bax under hypoxic condition on BAEC (1.48-fold ± 0.08-fold n=3 *vs* 1.12-fold ± 0.09-fold n=3; *p≤0.05) and BASMC (1.63-fold ± 0.07-fold n=3 *vs* 1.11-fold ± 0.07-fold n=3; *p≤0.01) when compared to hypoxic control (Fig. 4.14).

bcl-xl and *bax* gene expression in BAEC and BASMC was determined using quantitative RealTime RT-PCR (QRTPCR) following treatment. PCR product was resolved by 2% agarose gel. We found that mRNA levels of *bcl-xl* and *bax* did not change at 24 hours or after the 5 days treatment period (Figs. 4.15 & 4.16).

As both the pro- and anti-apoptotic proteins were seen to increase in the absence or presence of high glucose under normoxic conditions, we compared the increased ratio of Bcl-$_{xl}$/Bax expression. We found that Bcl-$_{xl}$ expression has a (11.78-fold ± 0.50-fold n=4,p≤0.01) increase over t=0 control when compared to a (1.58-fold ± 0.11-fold n=3,p≤0.01) increase of Bax expression over t=0 control on BAEC and has a (4.99-fold ± 0.27-fold n=3,p≤0.01) increase over t=0 control when compared to a (1.52-fold ± 0.13-fold n=3,p≤0.01) increase of Bax expression over t=0 control on BASMC.

Although high glucose increased both Bax and Bcl-$_{xl}$ expression under hypoxic conditions, we found that Bcl-$_{xl}$ expression has a 1.63-fold ± 0.13-fold increase over hypoxic control when compared to a 1.32-fold ± 0.08-fold increase of Bax expression over hypoxic control on BAEC and has a 2.16-fold ± 0.06-fold increase over hypoxic control when compared to a 1.61-fold ± 0.06-fold increase of Bax expression on BASMC.

Figure 4.1 Ruskinn Invivo$_2$ 400 Hypoxia Workstation

This workstation has two semidetached chambers, one of which enables the investigator to carry out cell culture under direct vision, and the other in a same controlled oxygen environment in which samples are allow to be removed without disturbing the oxygen level of the main chamber. The unit has built-in sensor for oxygen which is supplied with separate feeds of compressed air, nitrogen and carbon dioxide. Temperature is controlled by thermostatic monitoring and maximum humidity is limited by controlled condensation.

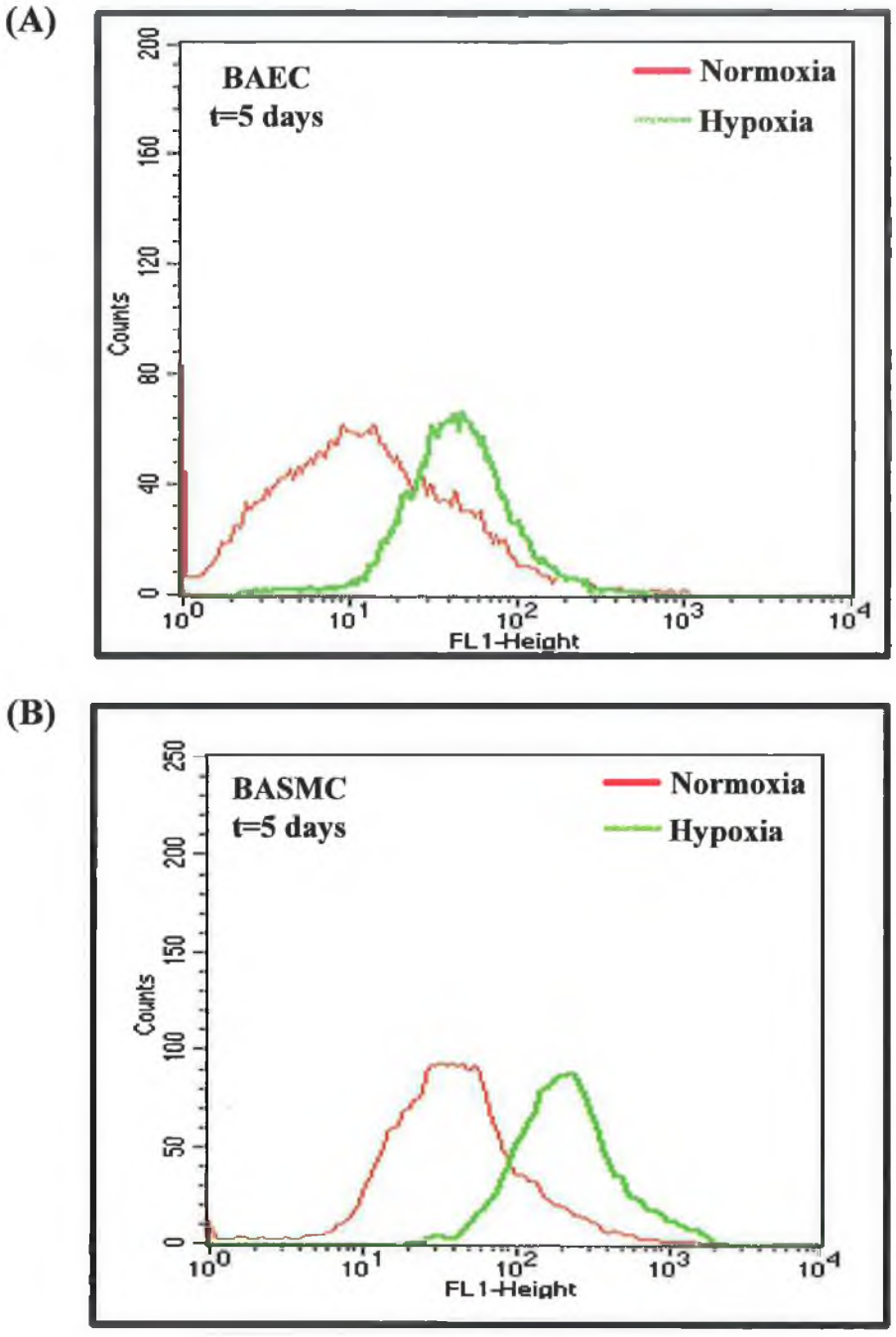


Figure 4.2 Hypoxia decreases BAEC and BASMC proliferation by FACS analysis

BAECs **(A)** and BASMCs **(B)** were cultured in 10% FBS for 5 days in either normoxic (5% CO_2, 95% air) or hypoxic (2% O_2, 5% CO_2, 93% N_2) conditions. After these time points, cells were collected and analysed by FACS as described in the Methods section 2.4.8. Data are representative of at least 3 independent experiments.

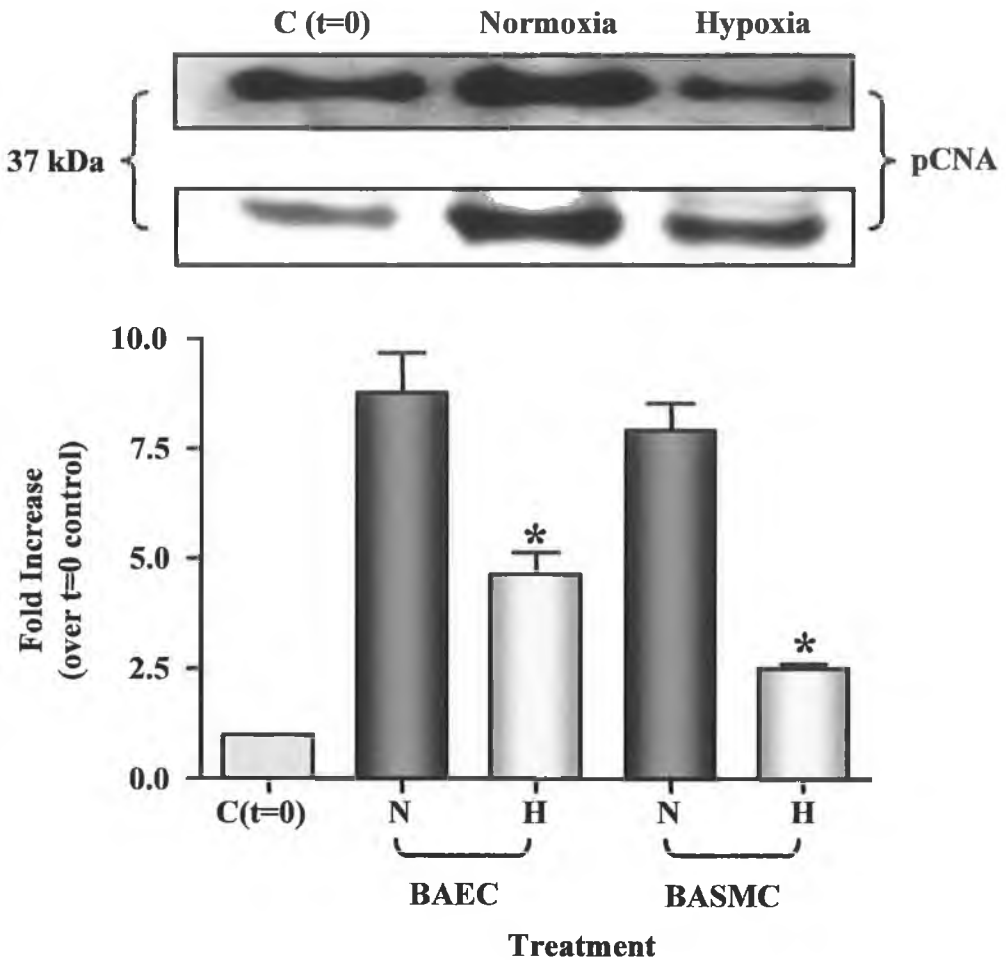


Figure 4.3 Hypoxia inhibits macrovascular pCNA expression

BAECs (top) and BASMCs (bottom) were exposed to normoxic (5% O_2, 95% air) or hypoxic (2% O_2, 5% CO_2, 93% N_2) conditions over a period of 5 days. Cells were harvested and cell lysates resolved by SDS-PAGE. Proteins were transferred to nitrocellulose and visualised by incubation with an anti-pCNA-specific antibody followed by HRP-conjugated anti-mouse secondary antibody. Blots were quantified by densitometric scanning and values represent mean ± SEM for three experiments; *$p \leq 0.01$ compared to normoxic controls.

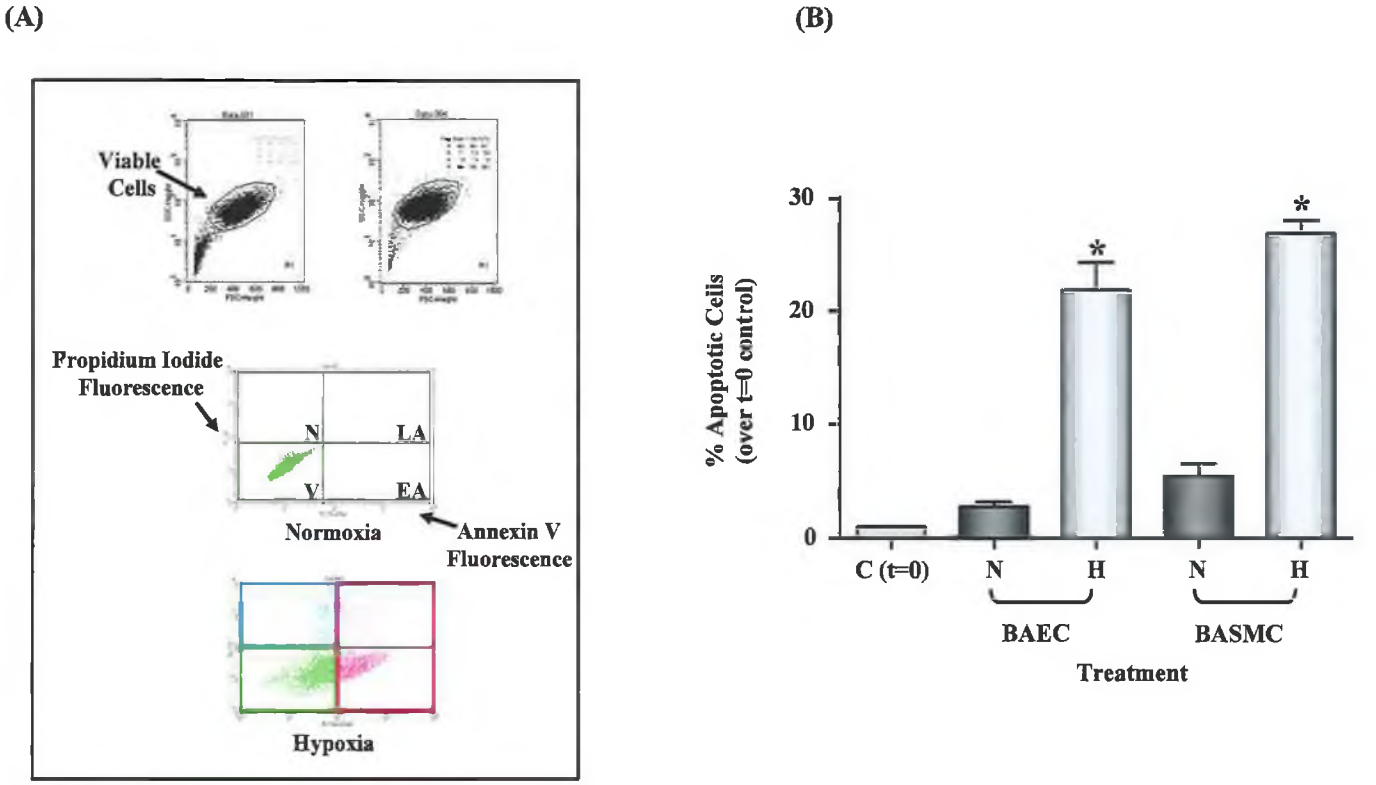


Figure 4.4 Hypoxia induces apoptosis in macrovascular cells

BAECs were cultured in media containing 10% FBS and exposed to either normoxic (5% CO_2, 95% air) or hypoxic (2% O_2, 5% CO_2, 93% N_2) conditions for 5 days. Cells were collected and analysed for apoptosis by FACS as described in the Methods. **(A)** representative example of FACS andalysis for hypoxia-induced apoptosis **(B)** The values obtained for EA and LA cells were combined and plotted as a bar chart (n=3, $\pm$ SEM, *$p \leq 0.01$ compared to normoxic controls).

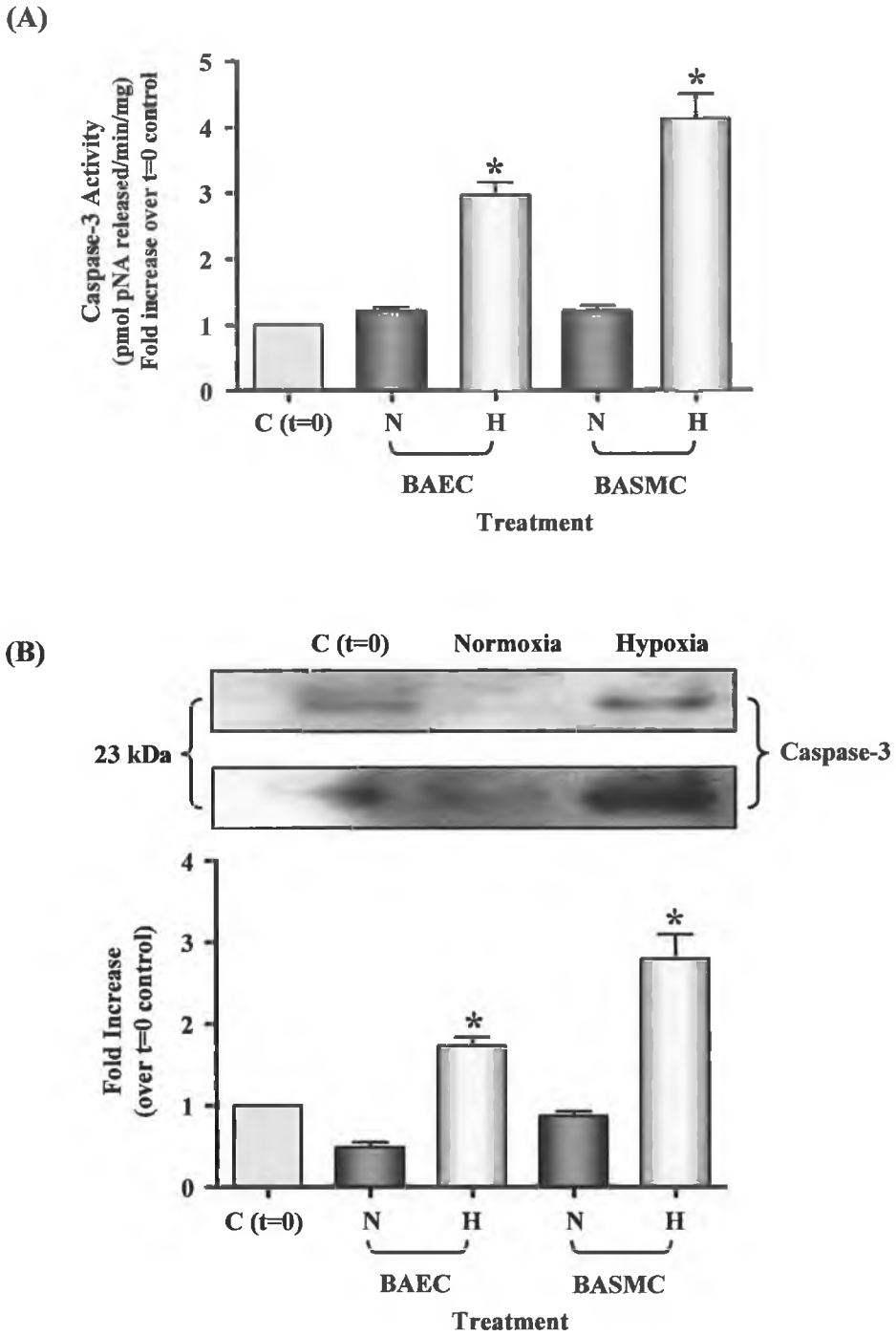


Figure 4.5 Hypoxia increases Caspase-3 activity and expression in macrovascular cells

BAECs and BASMCs were cultured in media containing 10% FBS for 5 days. Cells were then harvested for **(A)** Caspase-3 western blot (BAEC: top; BASMC: bottom) or **(B)** colourimetric caspase 3 assay as described in the Methods. Data are representative of at least 3 independent experiments ± SEM compared to normoxic controls; *$p \leq 0.01$

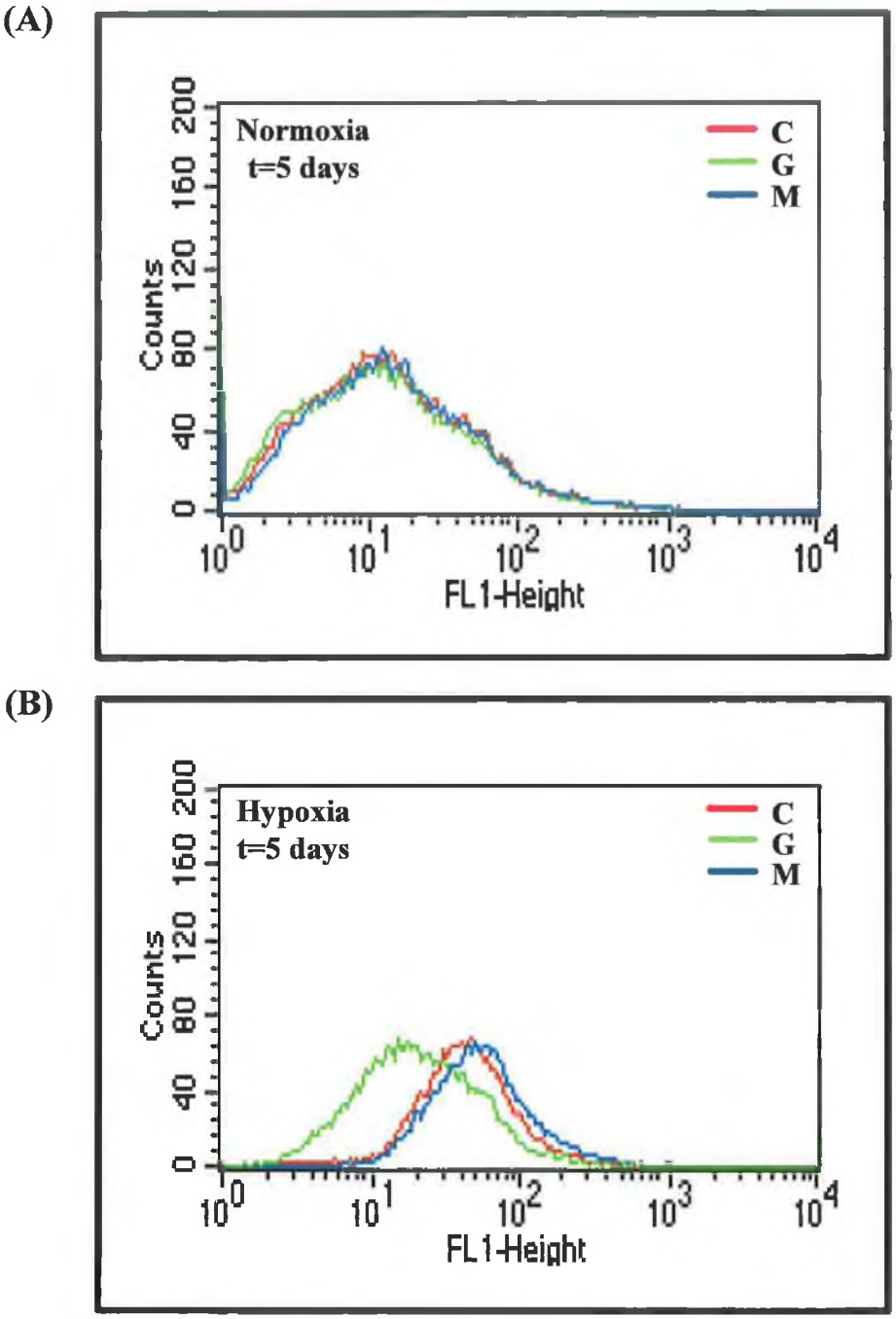


Figure 4.6 Effect of high glucose on hypoxia-induced proliferation in BAECs

BAECs were labelled with CFDA SE and cultured in 10% FBS for 5 days under **(A)** normoxic or **(B)** hypoxic conditions in the absence or presence of high glucose. Cells were then collected and analysed by FACS as described in the Methods. Panels show comparisons of cell proliferation over the 5 day period in 10% FBS with normal glucose (5.5mM: C), high glucose (25mM: G) and mannitol (25mM: M). Data are representative of at least 3 independent experiments.

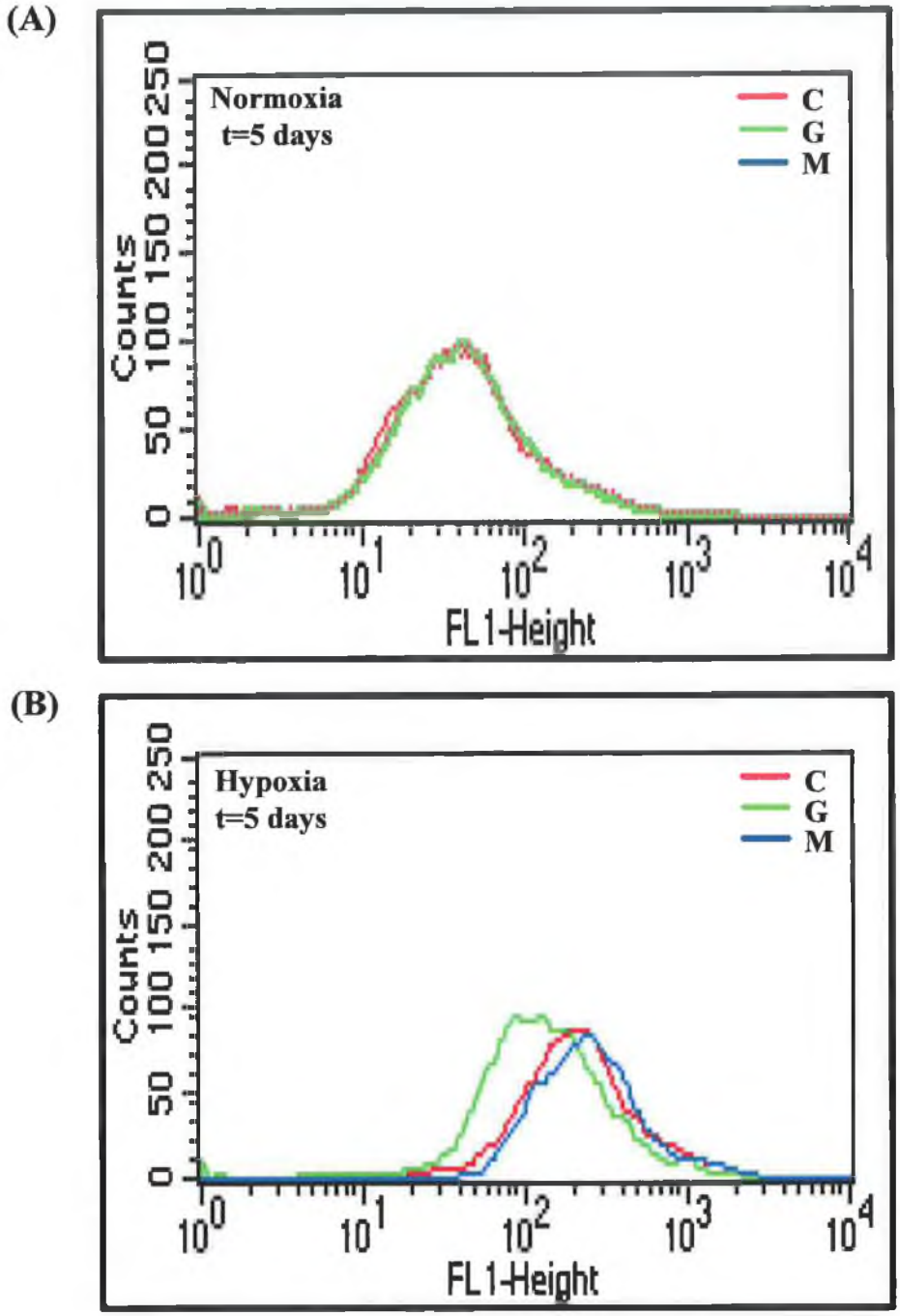


Figure 4.7 Effect of high glucose on hypoxia-induced proliferation in BASMCs

BASMCs were labelled with CFDA SE and cultured in 10% FBS for 5 days under **(A)** normoxic or **(B)** hypoxic conditions. Cells were then collected and analysed by FACS as described in the Methods. Panels show comparisons of cell proliferation over the 5 day period in 10% FBS with normal glucose (5.5mM: C), high glucose (25mM: G) and mannitol (25mM: M). Data are representative of at least 3 independent experiments.

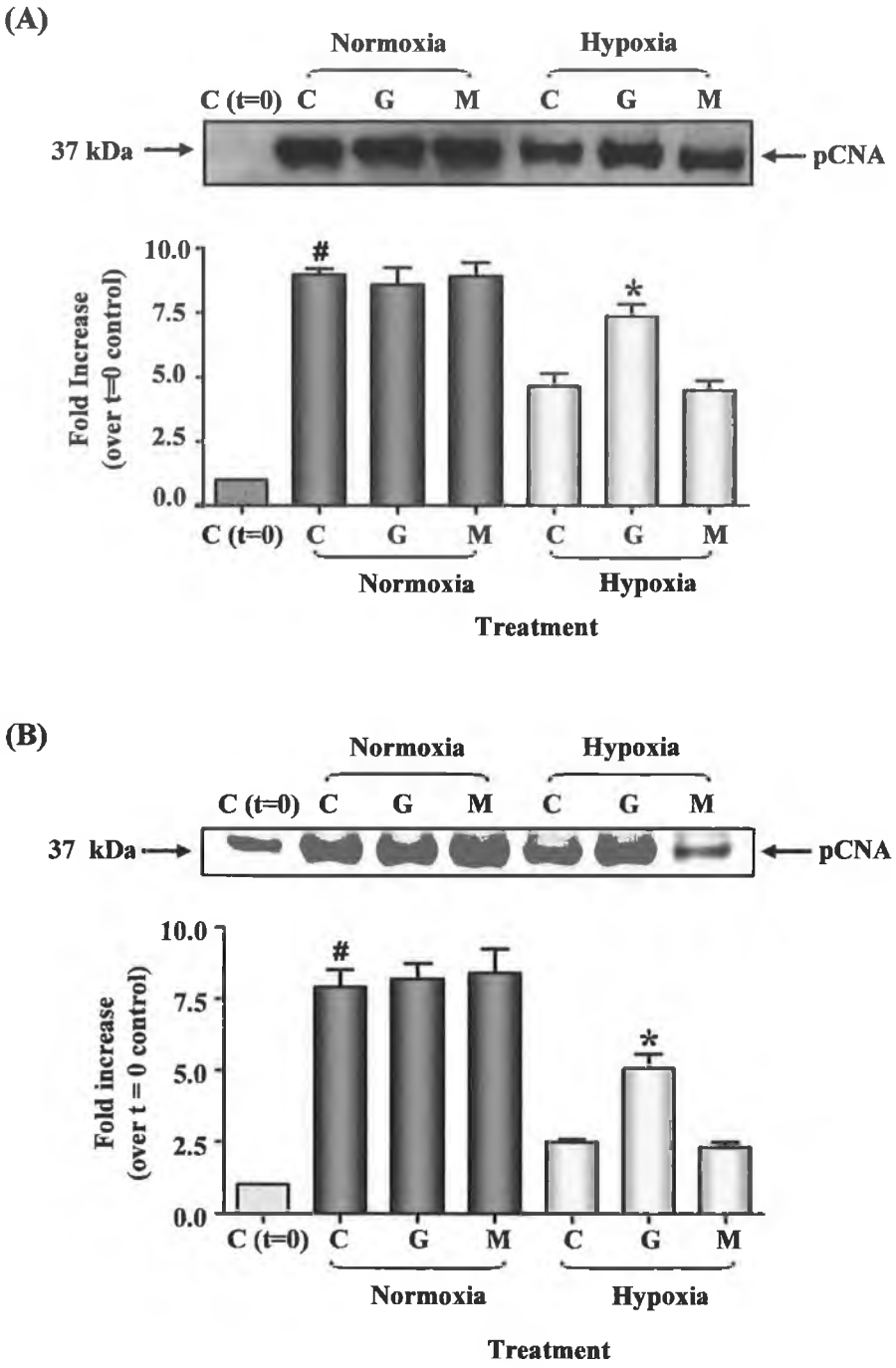


Figure 4.8 Effects of high glucose on hypoxia-induced decrease in pCNA expression

BAECs **(A)** and BASMCs **(B)** were cultured in 10% FBS under normoxic or hypoxic conditions for 5 days; in the absence or presence of high glucose (25mM). Cells were harvested and cell lysates resolved by SDS-PAGE. Proteins were transferred to nitrocellulose and visualised by incubation with an anti-pCNA-specific antibody followed by HRP-conjugated anti-mouse secondary antibody. Blots were quantified by densitometric scanning and values represent mean ± SEM for three experiments. #$p \leq 0.01$ compared to t=0 control, *$p \leq 0.05$ (BAEC), * $p \leq 0.01$ (BASMC) compared to hypoxic control.

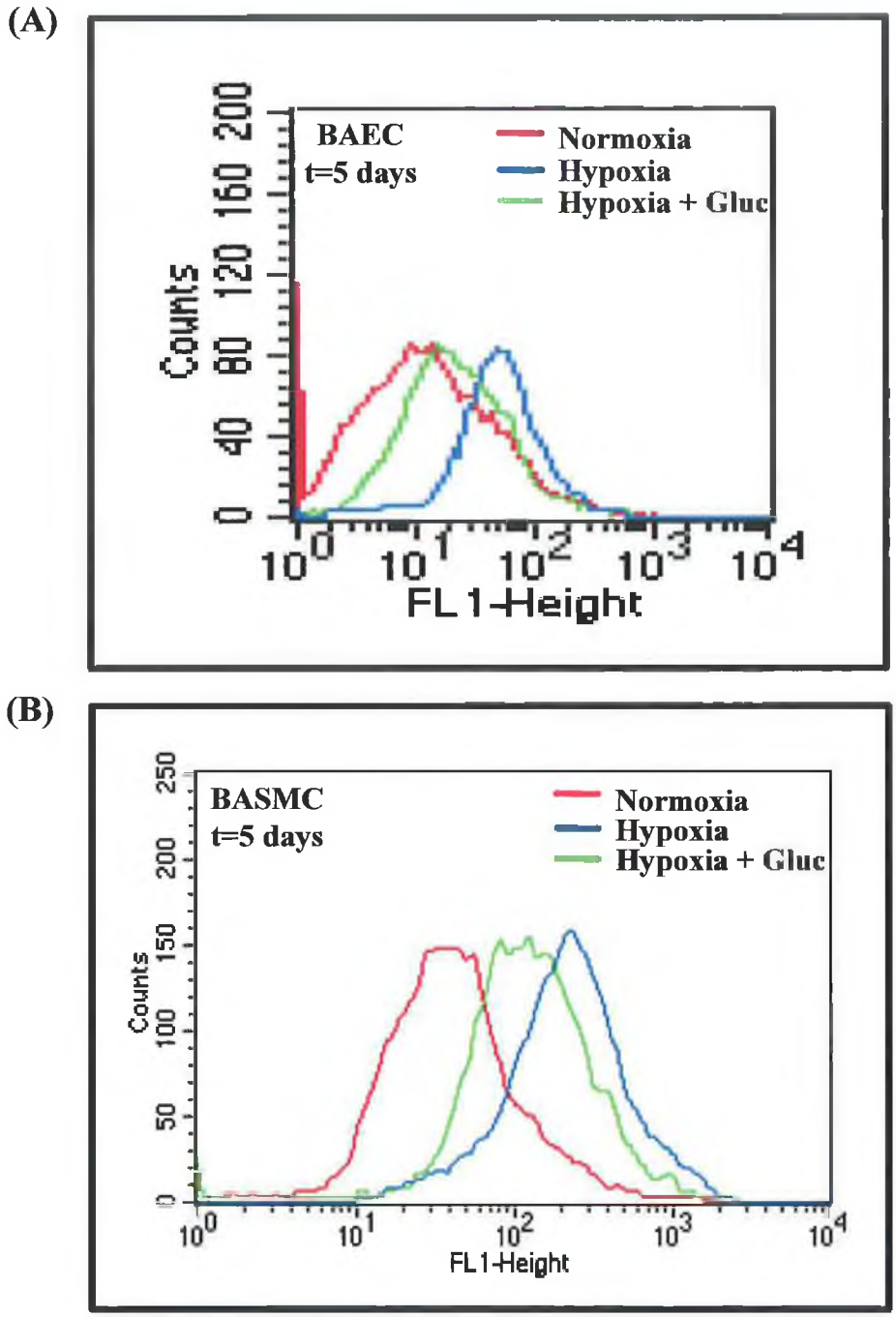


Fig. 4.9 **High Glucose in the Presence of Hypoxia Does Not Return Cell Proliferation to Normoxic Levels**

BAECs **(A)** and BASMCs **(B)** were labelled with CFDA SE and cultured in 10% FBS for 5 days under normoxic or hypoxic conditions. Cells were then collected and analysed by FACS as described in the Methods. Panels show comparisons of cell proliferation over the 5 day period in 10% FBS with normal glucose (5.5mM; Normoxia and Hypoxia), or high glucose (25mM: Hypoxia + Gluc). Data are representative of at least 3 independent experiments.

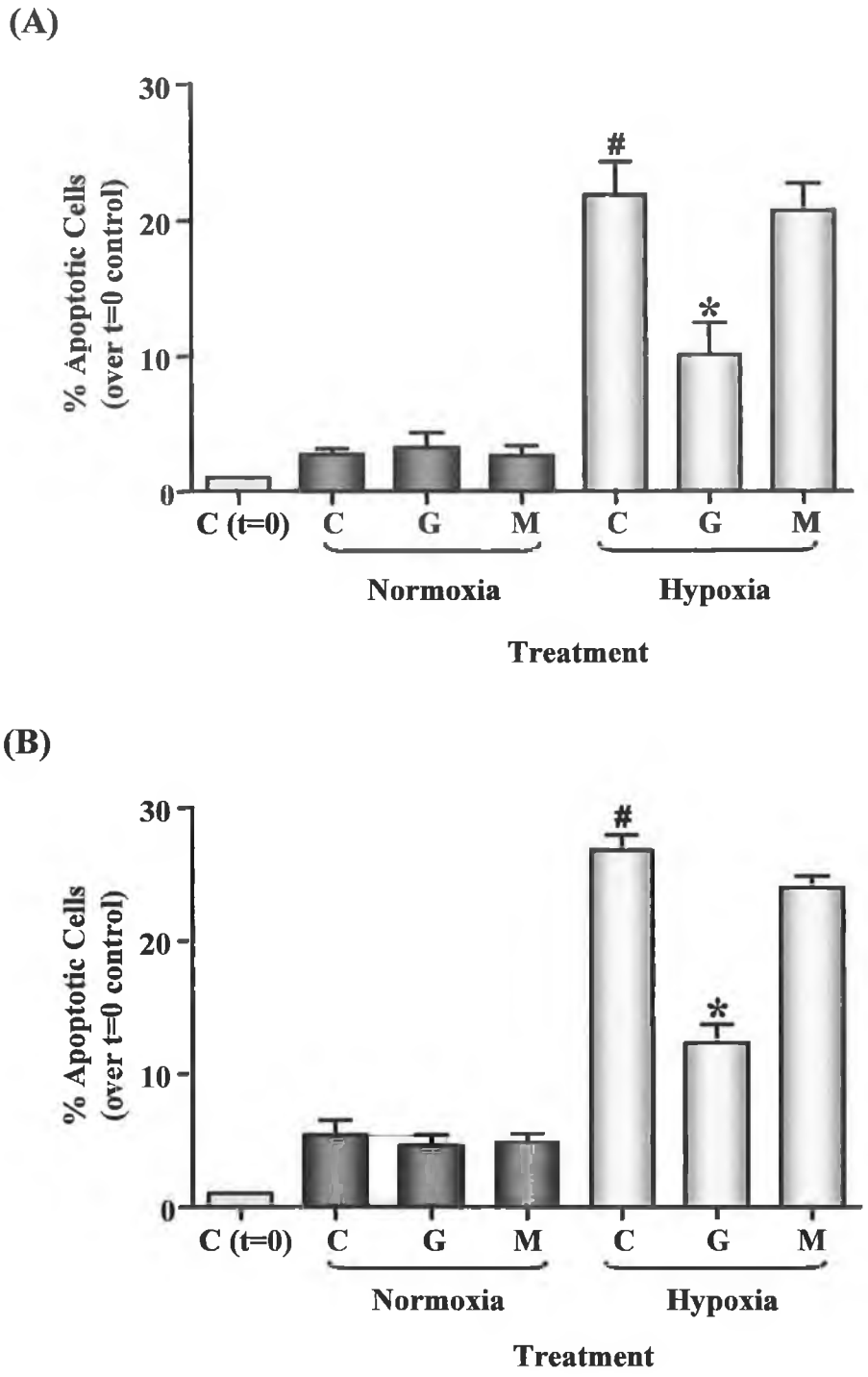


Figure 4.10 Effects of high glucose on hypoxia-induced increase in apoptosis

BAECs **(A)** and BASMCs **(B)** were cultured in media containing 10% FBS in the absence or presence of high glucose (25mM) and exposed to either normoxic (5% CO_2, 95% air) or hypoxic (2% O_2, 5% CO_2, 93% N_2) conditions for 5 days. Cells were collected and analysed for apoptosis by FACS as described in the Methods. The values obtained for EA and LA cells were combined and plotted as a bar chart (n=3, ± SEM, #$p \leq 0.01$ compared to normoxic control; *$p \leq 0.05$ (BAEC), *$p \leq 0.01$ (BASMC) compared to hypoxic control).

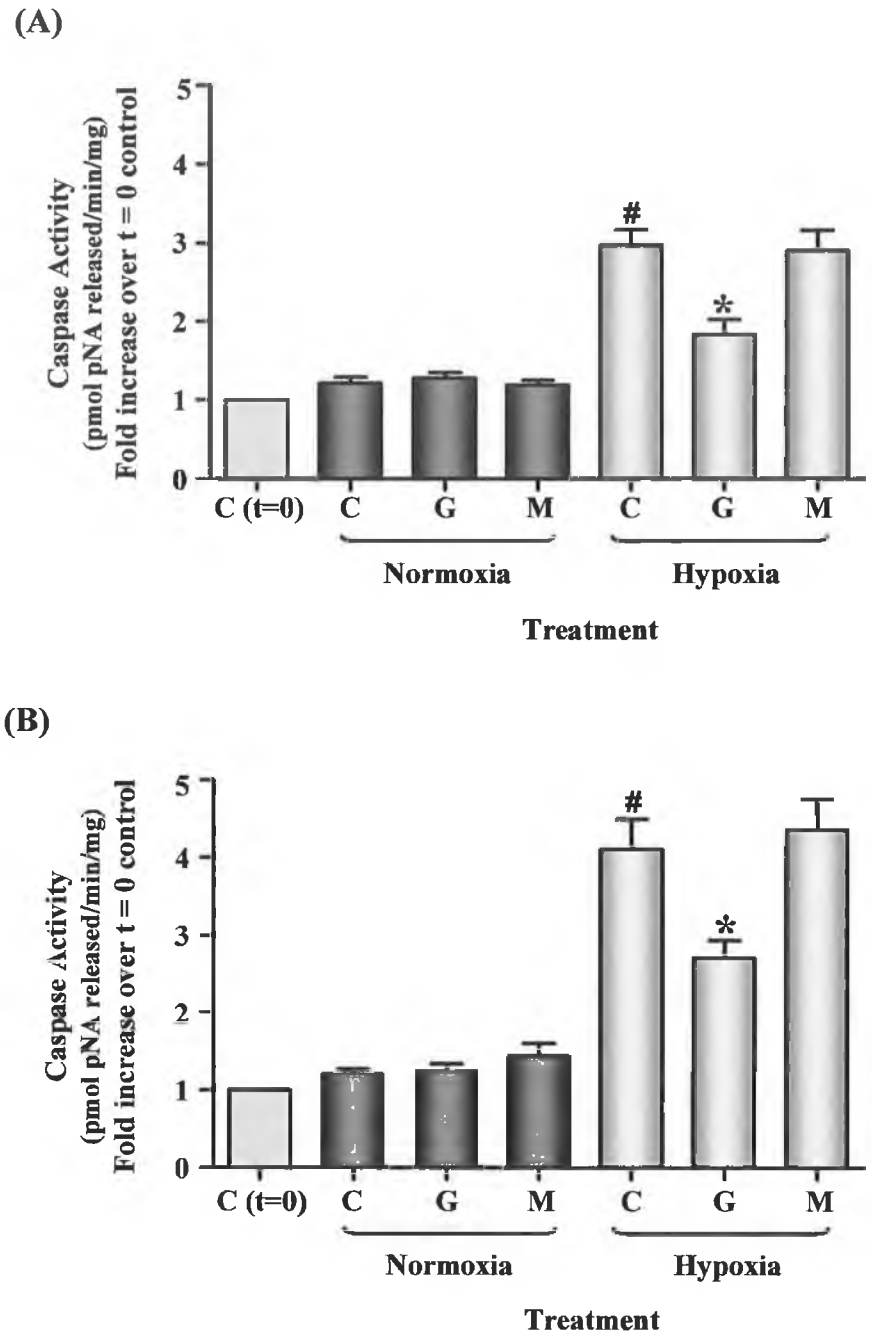


Figure 4.11 Effect of high glucose on hypoxia-induced Caspase-3 activity

(A) BAECs and **(B)** BASMCs were cultured in media containing 10% FBS for 5 days, in the absence or presence of high glucose (25mM) under normoxic or hypoxic conditions. Cells were harvested and a colourimetric caspase 3 assay carried out on cell lysates as described in the Methods. A pNitroanilide (pNA) standard curve (0-200µg/µl) allowed for the specific activity of caspase 3 to be calculated for each sample and expressed as pmol pNA released/min/mg. Data are representative of at least 3 independent experiments ± SEM. #$p \leq 0.01$ compared to normoxic control; *$p \leq 0.01$ (BAEC), *$p \leq 0.05$ (BASMC) compared to hypoxic control (normal glucose [5.5mM: C], high glucose [25mM: G] and mannitol [25mM:M]).

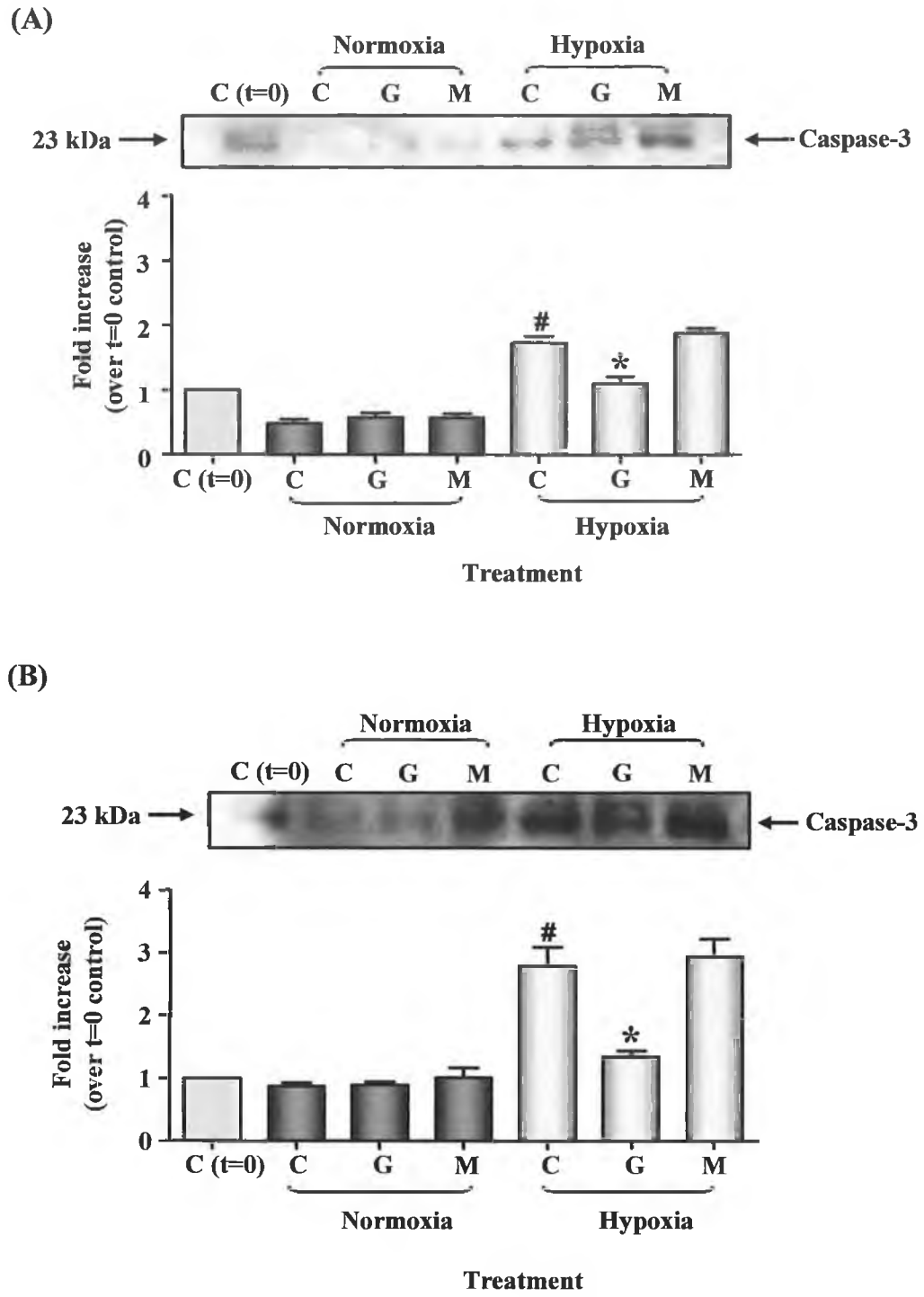


Figure 4.12 Effect of high glucose on hypoxia-induced Caspase-3 expression

(A) BAECs and **(B)** BASMCs were cultured in 10% FBS in the absence or presence of high glucose and exposed to normoxic or hypoxic conditions for 5 days. Cells were harvested and cell lysates resolved by SDS-PAGE. Proteins were transferred to nitrocellulose and visualised by incubation with an anti-Caspase-3-specific antibody followed by HRP-conjugated anti-rabbit secondary antibody. Blots were quantified by densitometric scanning and values represent mean ± SEM for three experiments, #$p \leq 0.01$ compared to normoxic control, * $p \leq 0.05$ (BAEC, * $p \leq 0.01$ (BASMC) compared to hypoxic control.

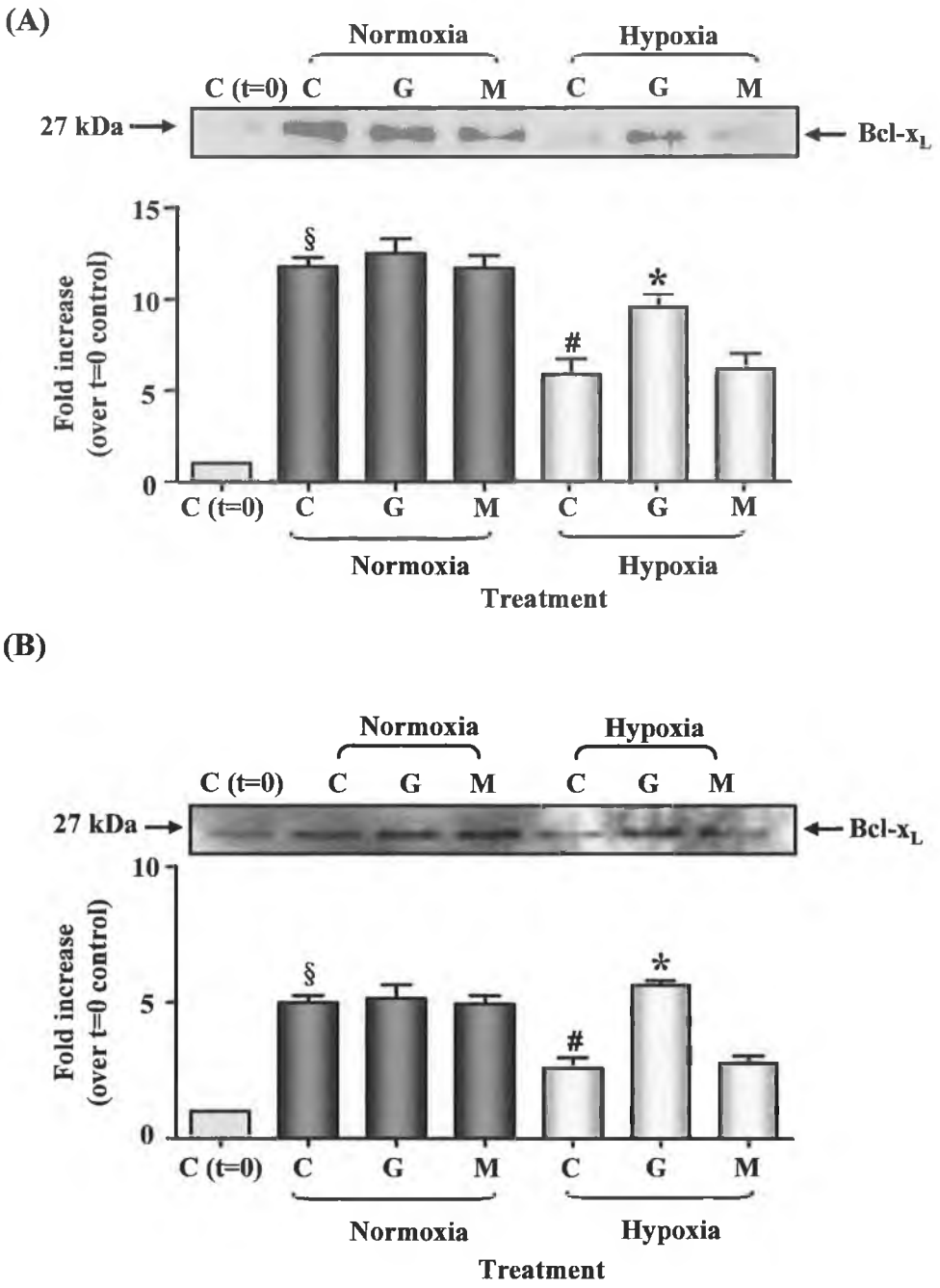


Figure 4.13 Effect of hypoxia and high glucose on expression of the anti-apoptotic protein Bcl-x_L

BAECs **(A)** and BASMCs **(B)** cultured in 10% FBS were exposed to high glucose concentrations over a period of 5 days under either normoxic or hypoxic conditions. Cells were harvested and cell lysates resolved by SDS-PAGE. Proteins were transferred to nitrocellulose and visualised by incubation with an anti-Bcl-x_L-specific antibody followed by HRP-conjugated anti-mouse secondary antibody. Blots were quantified by densitometric scanning and values represent mean ± SEM for three independent experiments, § $p \leq 0.01$ vs t=0 control; # $p \leq 0.01$ vs normoxic control; * $p \leq 0.05$ (BAEC), $p \leq 0.01$ (BASMC) vs hypoxic control.

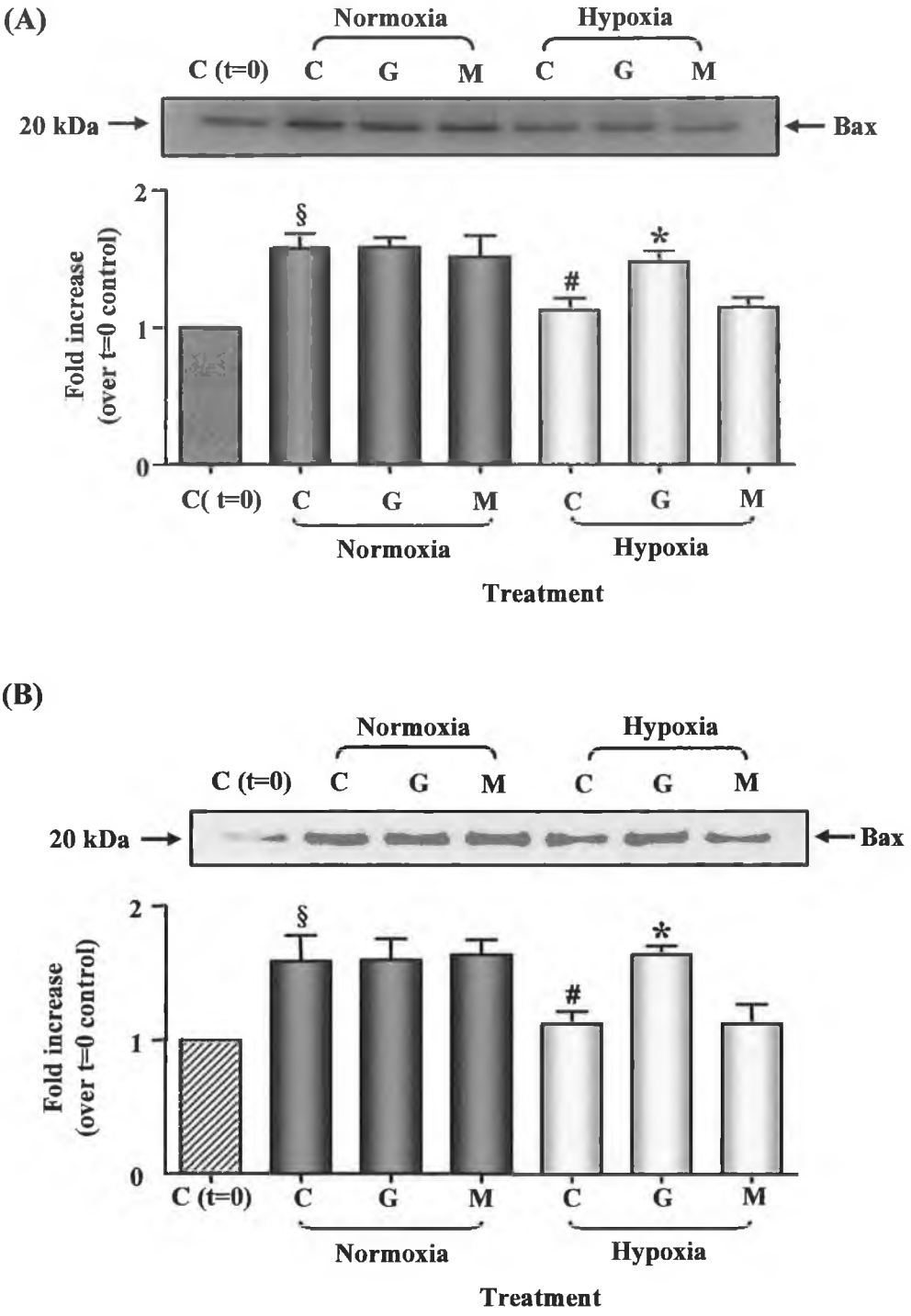


Figure 4.14 Effect of hypoxia and high glucose on expression of the pro-apoptotic protein Bax

BAECs **(A)** and BASMCs **(B)** cultured in 10% FBS were exposed to high glucose concentrations over a period of 5 days under either normoxic or hypoxic conditions. Cells were harvested and cell lysates resolved by SDS-PAGE. Proteins were transferred to nitrocellulose and visualised by incubation with an anti-Bax-specific antibody followed by HRP-conjugated anti-rabbit secondary antibody. Blots were quantified by densitometric scanning and values represent mean ± SEM for three independent experiments, § $p \leq 0.01$ vs t=0 control; # $p \leq 0.05$ vs normoxic control; * $p \leq 0.05$ (BAEC), $p \leq 0.01$ (BASMC) vs hypoxic control.

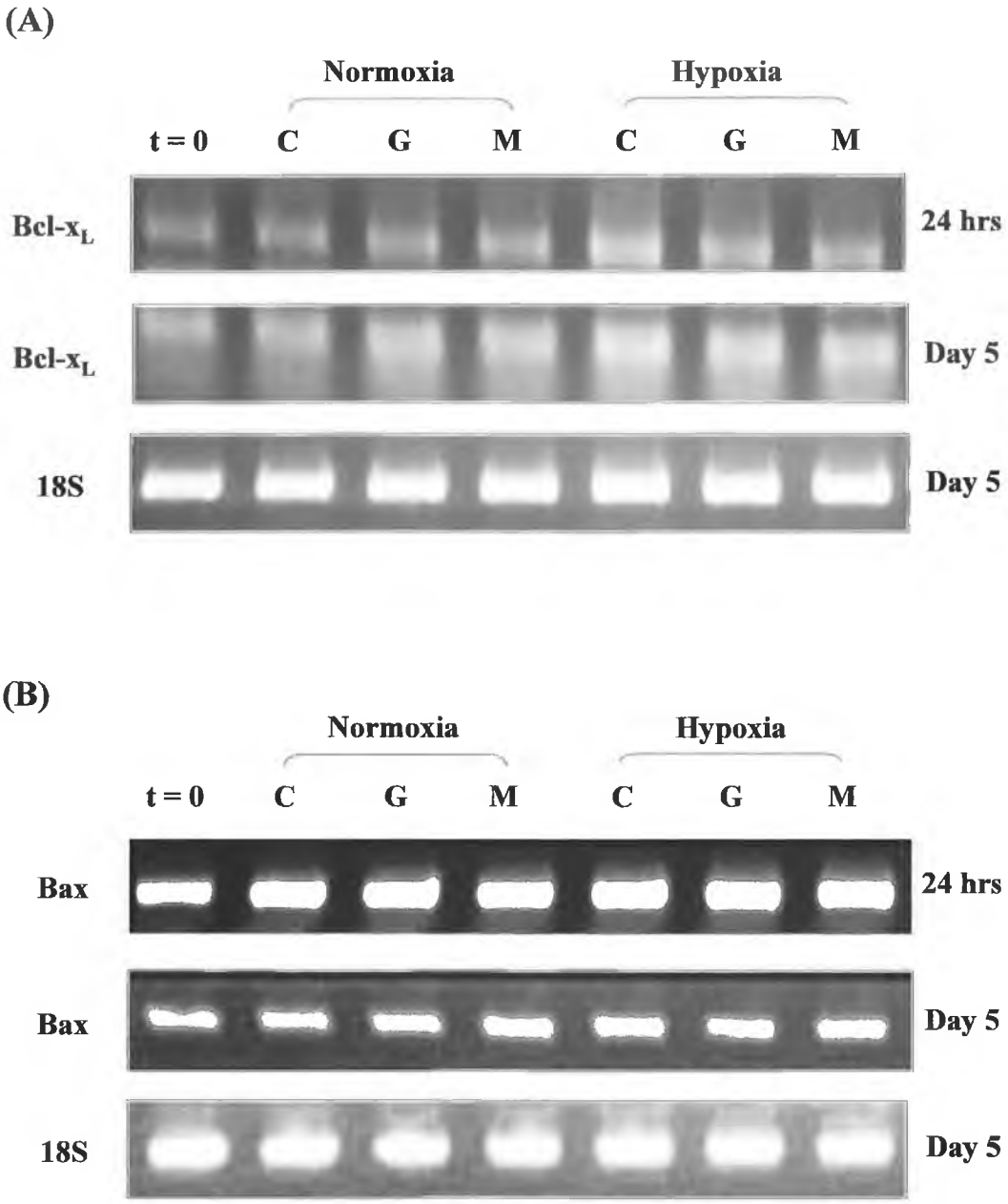


Figure 4.15 Effect of hypoxia and high glucose on Bcl-xL and Bax gene expression

BAECs cultured in 10% FBS were exposed to high glucose concentrations over a period of 5 days under either normoxic or hypoxic conditions. Cells were harvested, RNA extracted and QRTPCR carried out using specific primer sets for **(A)** Bcl-x_L and **(B)** Bax as described in the methods. The resultant PCR products were resolved by agarose gel electrophoresis (2%). Data are representative of at least three independent experiments showing similar results.

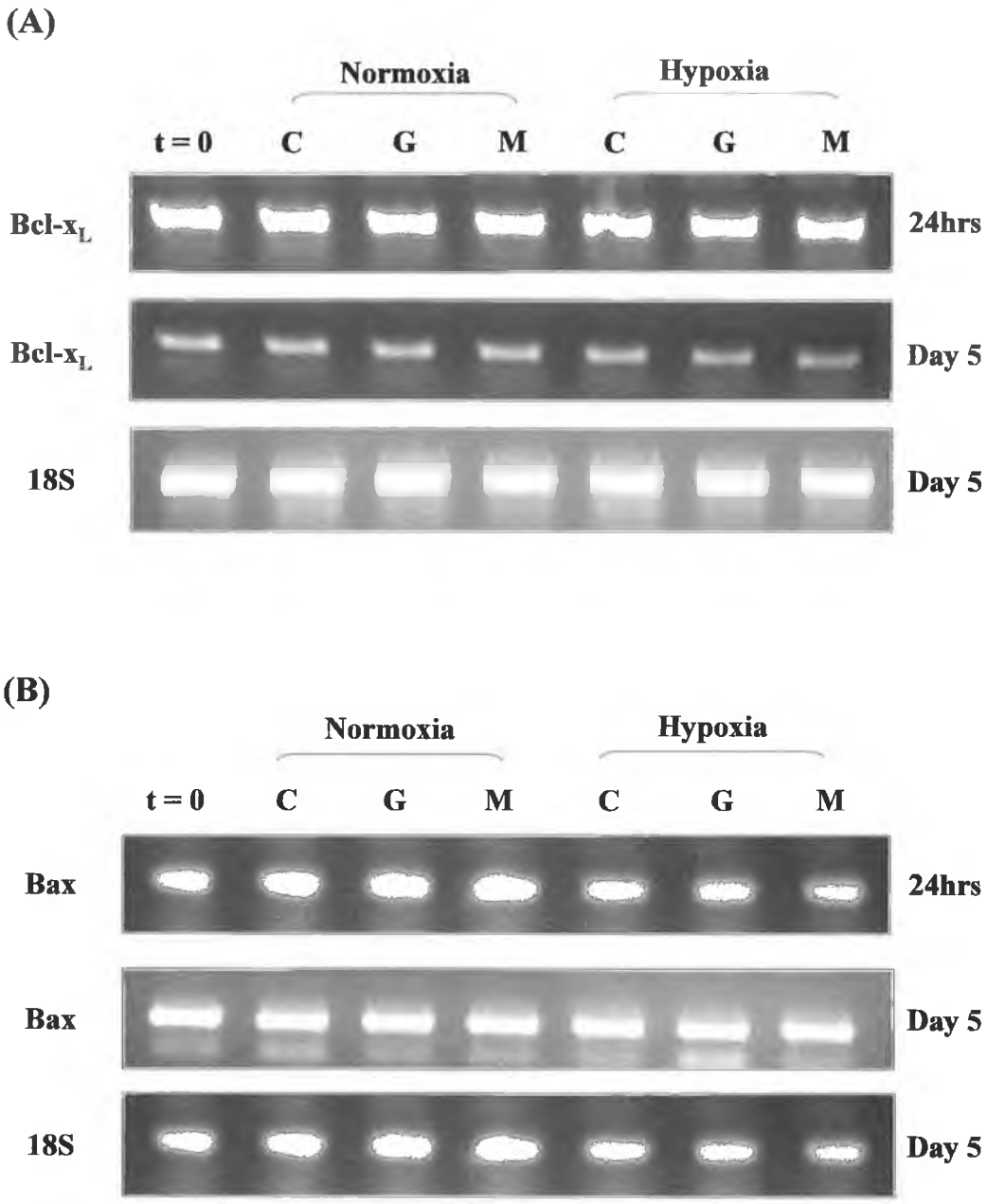


Figure 4.16 Effect of hypoxia and high glucose on Bcl-x_L and Bax gene expression

BASMCs cultured in 10% FBS were exposed to high glucose concentrations over a period of 5 days under either normoxic or hypoxic conditions. Cells were harvested, RNA extracted and QRTPCR carried out using specific primer sets for (A) Bcl-x_L and (B) Bax as described in the methods. The resultant PCR products were resolved by agarose gel electrophoresis (2%). Data are representative of at least three independent experiments.

4.3 Discussion

The present study demonstrates that hypoxia inhibits BAEC and BASMC proliferation and induces apoptosis compared with normoxia. However, high glucose significantly reverses this effect by increasing cell proliferation and decreasing cell apoptosis under hypoxic conditions. These findings suggest an important interaction between hypoxia and hyperglycaemia in vascular cells.

In our present study, hypoxia inhibits the proliferation of BAEC and BASMC which is consistent with previous observations (Benitz et al. 1986; Bai et al. 1997; Zhen et al. 2003). In contrast, hypoxia was found to induce cell proliferation in tumour cells such as follicular B-lymphoma (Shimizu et al. 1995) and others cell types such as rat mesangial cells and osteopontin (Sahai et al. 1997; Sahai et al. 1997; Sodhi et al. 2001). Increasing or decreasing cell proliferation under hypoxic conditions also depends on the oxygen level. 1% O_2 has been shown to inhibit proliferation and 3% O_2 increase proliferation in rat airway smooth muscle cells with lower oxygen level such as 0.5%-1% easily decreasing cell proliferation when compared with 2%-3% oxygen (Cogo et al. 2003).

Hypoxia inhibits cellular proliferation in a time-dependent manner (Galvin et al. 2004) which ranges from 5 mins (Liu et al. 2001) to 280 days (Stock and Vacanti 2001). Our initial hypoxic studies were carried out over a time period of 72 hours. However, we found no significant changes in cell proliferation until we extended the treatment period out to 5 days.

Hyperglycaemia and hypoxia are two major factors in diabetes. Some studies have shown an interaction between these two factors in which hyperglycaemia impairs hypoxia induced proliferation (Catrina et al. 2004; Nyengaard et al. 2004) or exaggerates cell growth (Sodhi et al. 2001).

In our present study, we found that high glucose increased cell proliferation in BAEC and BASMC under hypoxic conditions; however, this increase of proliferation did not reach the same levels as normoxic conditions; suggesting that although hyperglycaemia can upregulate cell growth under hypoxic conditions, this effect could not replace the normal cell growth in the vascular cells. This abnormal proliferation and response in the presence of high glucose to hypoxia may have a profound effect on the healing process in diabetes, particularly because hyperglycaemia is correlated to ischemia. Without an appropriate proliferative response, the subsequent phases of cell proliferation and matrix deposition are

delayed because the neovasculature is required for the deposition of matrix in healing process (Nissen et al. 1998; Singer and Clark 1999). On the other hand, acute hypoxia can induce cell apoptosis in minutes (Yu et al. 2004) with cell loss occurring at an early stage, decreasing cell proliferation in part.

Apoptosis clearly occurs in the myocardium during and after ischemia (Fliss and Gattinger 1996; Bialik et al. 1997; Haunstetter and Izumo 1998), although the relative contribution of apoptotic and non-apoptotic cell death in hypoxic cardiac injury is still unclear (Anversa and Kajstura 1998; Haunstetter and Izumo 1998). Many recent studies have indicated that either ischemia or hypoxia alone (Itoh et al. 1995; Saraste et al. 1997) or in combination with reoxygenation (Gottlieb et al. 1994; Fliss and Gattinger 1996; Aikawa et al. 1997) can trigger apoptosis. The mechanism by which hypoxia induces apoptosis remains unclear, although it is probably analogous to the pathways by which other cellular stresses initiate apoptosis. Cellular stresses, such as growth factor withdrawal, UV irradiation, or treatment with actinomycin D, glucocorticoids, or chemotherapeutic agents, cause apoptosis *via* a mitochondria-dependent release of cytochrome *c* and subsequent activation of the Apaf-1-caspase 9 complex or apoptosome (Green and Reed 1998).

Caspase 9 subsequently cleaves and activates downstream effector caspases such as caspase 3, which then mediate the biochemical features of apoptotic cell death (*e.g.* PARP and lamin cleavage). The triggers for cytochrome *c* release may include reactive oxidant species, increased cytoplasmic calcium concentration, decreased ATP levels, as well as activation or increased expression of the proapoptotic proteins p53 and Bax (Green and Reed 1998).

It has been shown that hypoxia induces cell apoptosis in many studies (Torres-Roca et al. 2000; Wu et al. 2004). In addition, hypoxia also inhibits the apoptosis of tumour cells (Kim et al. 2004). Some studies also show that hypoxia does not induce cell death (Galvin et al. 2004) and severe chronic hypoxia alone does not cause apoptosis of cardiac myocytes in culture (Webster et al. 1999).

In our study, we found that hypoxia significantly increased the percentage of apoptotic BAEC and BASMC when compared with normoxic control. Obvious protection against hypoxia-induced apoptosis was found in cells treated with high glucose. Therefore, it is logical to conclude that hyperglycaemia blocks one or more steps in the apoptotic pathway.

The regulation of apoptosis within the cell can be simplified into two major pathways (Roucou et al. 2001). One is initiated from death receptors at the cell surface and is mainly regulated by the tumour necrosis factor super family and includes, for example, the fas/CD95 receptor. Upon ligand binding to the death receptor, the receptor activates the apoptosis signalling pathway through activation of caspase-8 (Ashkenazi and Dixit 1998).

The other pathway is triggered by disruption of the mitochondria. Genotoxic agents, ischemia, oxidative stress, growth factor withdrawal, and many other stimuli require mitochondria to activate caspases. Unlike caspase-8, caspase-9 is involved in the mitochondrial pathway response to extracellular cues and internal insults. The pro-apoptotic signals regulate cytochrome *c* release from mitochondria and further activate caspase-9 through interaction with Apaf-1 (Green and Reed 1998; Zou et al. 1999). Both death receptor and mitochondrial pathways converge at the level of caspase-3 activation.

To further define the potential molecular mechanisms by which high glucose treatment modulates hypoxia induced apoptosis, we investigated the expression of pro- and anti-apoptotic proteins in BAEC and BASMCs.

Recently, Bcl-2 family proteins have been proved to be critical regulators of apoptosis. Bcl-2 was originally identified as a translocation breakpoint in human B-cell lymphoma (Reed 1995). It is now clear that Bcl-2 belongs to a large family of homologous proteins that can either promote or suppress apoptosis. The ratio between Bcl-2 to Bax and Bcl-$_{xl}$ to Bax determines cell survival or death after apoptotic stimuli. The expression pattern and role of different members of the family appear to be cell specific (Evan and Littlewood 1998).

The significance of apoptosis in atherosclerosis appears to depend on the stage of the plaque, localization, and cell types involved (Kockx and Knaapen 2000). Glucose-induced suppression of apoptosis under hypoxia appears to be of particular importance in the initial events of vascular injury and thickening of the media of the vessels. It is well documented that high glucose and advanced glycation end products increased NF-κB activity in rat VSMCs (Lander et al. 1997; Yerneni et al. 1999). Bcl-$_{xl}$ has been identified as an NF-κB–dependent transcriptional target (Barkett and Gilmore 1999). In our study, we found that high glucose increased the Bcl-$_{xl}$ protein expression under hypoxic conditions which suggests that high glucose concentrations are likely to have an effect on the activity of NF-κB activation leading to apoptosis

suppression in BAEC and BASMC. This notion remains to be tested directly and could provide a possible mechanism by which these cells become resistant to apoptosis in the presence of glucose in hypoxia.

Several studies have shown that anti-apoptotic protein Bcl-2 and Bcl-$_{xl}$ levels are dramatically either increased or decreased under hypoxic conditions, whereas the levels of the pro-apoptotic factors Bax and Bad remain unaltered (Schaffer et al. 2000; Yamamoto et al. 2004; Li et al. 2005). We focused on the expression of an anti-apoptotic molecule Bcl-$_{xl}$ which is a Bcl-2 related gene can function as a Bcl-2 independent regulator of apoptosis and a pro-apoptotic molecule Bax which heterodimerizes with Bcl-$_{xl}$ in mammalian cells.

Bcl-$_{xl}$ and Bax are homologous proteins that have opposing effects on cell growth and death, with Bcl-$_{xl}$ serving to prolong cell survival and Bax acting as an accelerator of apoptosis (Reed 1994). This present study revealed a significant decrease in Bcl-$_{xl}$ by hypoxic treatment which was reversed in the presence of high glucose. As our present data demonstrated marked down-regulation of Bcl-$_{xl}$ induced by hypoxia, the apoptosis induced by hypoxia may be due to an inappropriate decrease in anti-apoptotic factors.

Bax protein expression was also decreased under hypoxic conditions in our studies other than remaining unaltered; this finding is not consistent with previous reports (Kim et al. 2004; Yamamoto et al. 2004; Li et al. 2005). Recent studies demonstrated that the translocation of Bax from the cytoplasm to the mitochondrial membrane is critical to induce apoptosis (Murphy et al. 1999; Putcha et al. 1999). After delivery of death signals such as hypoxia to cells in culture, Bax moves to the mitochondria and other membrane sites and triggers a catastrophic transformation of mitochondrial function which suggests that Bax translocation from cytosol to the mitochondrial membrane occurred under a physiological condition, thereby leading to pathological tissue destruction (Yamamoto et al. 2004). Our data showed that high glucose upregulated protein expression of Bax under hypoxic conditions. It is important to note that while only total protein amounts of Bax were measured, our data cannot rule out the possibility that phosphorylated Bax is affected by hypoxia/hyperglycaemia. Moreover, Bax protein in cytosolic fractions was decreased and mitochondrial fractions was increased under hypoxic conditions in epithelial cells (Yamamoto et al. 2004). The time course of changes in Bcl-$_{xl}$ and Bax were different. Translocation of Bax to mitochondria occurred within 48 hours,

while a decrease in Bcl-$_{xl}$ occurred after 48 hours (Yamamoto et al. 2004). It is probable that the acute hypoxia-induced apoptotic process (within 48 hours) may depend on Bax, whereas the chronic hypoxia-regulated cell death process (over 5 days) may depend on Bcl-$_{xl}$

Therefore, we then compared the ratio of Bcl-$_{xl}$/Bax at the level of protein expression in the cells. We found a 9.5-fold increase of Bcl-$_{xl}$ levels when compared to a 1.48-fold increase of Bax levels under hypoxic conditions in BAEC and a 5.6-fold increase of Bcl-$_{xl}$ level when compared 1.6-fold increase of Bax level under hypoxic condition in BASMC (over t=0 control).

Considering the ratio of Bcl-$_{xl}$/Bax in the cells at different oxygen status, it appears that there is a phenomenon of levelling since the greater expression of Bcl-2 seems to neutralise and then block Bax function at the mitochondrial level.

In regard to cellular survival, the down-regulation of Bax under hypoxic conditions is important in chronically hypoxic cells which would render cells resistant to a variety of apoptotic stimuli. The evidence that Bax participates in hypoxia-induced cell death implies that its role in this pathway would be abolished in cells that adapt to survive under hypoxic conditions. The down-regulation of Bax would provide a survival advantage, as activation of Bax inactivates Bcl-2 and Bcl-$_{xl}$ (Letai et al. 2002). It has been shown that the down-regulation of Bax under hypoxic conditions was HIF-1-independent in human colon carcinoma cells which was not associated with decreased levels of *bax* mRNA and the observed reduction in Bax protein did not reflect increased proteosomal degradation (Erler et al. 2004) A global decrease in translation efficiency would be expected to contribute to the reduced expression of many proteins including Bax. Further studies are required to validate and expand this hypothesis.

It is well known that activation of Bax appears to involve subcellular translocation from the cytosol to mitochondria (Saikumar et al. 1998; Mikhailov et al. 2003). The signal directing the translocation remains to be elucidated. Regulatory proteins such as Bid have been reported to be involved in the modulation of cellular function, and localisation of Bax (Eskes et al. 2000; Nomura et al. 2003; Tsuruta et al. 2004). It is believed that cytosolic Bax fails to homodimerize in the presence of protective levels of Bcl-2 or Bcl-$_{xl}$, indicating a block at the death signal. Exploring various stimuli of Bax translocation could be important to understand the cell death system (Yamamoto et al. 2004). This should be further investigated to clarify the

exact role of Bax in this model, as it may serve as a point for various apoptotic signals.

Interestingly, a similar protective effect of chronic hyperglycaemia on cardiomyocytes was also reported in a model of hypoxia-induced apoptosis and necrosis (Schaffer et al. 2000).

Caspase-3 activity and caspase-3 protein expression were decreased following exposure to high glucose concentration under hypoxic conditions in our study. To date, over a dozen caspases have been identified, and about two-thirds of these have been suggested to function in apoptosis (Adams and Cory 1998). Caspase-3 is an effector enzyme for induction of DNA damage and for ensuring apoptotic cell death. Caspase function is positively or negatively modified by Bcl-2 family proteins, including Bcl-$_{xl}$, and Bax (Roucou et al. 2001). Our data also suggest that upregulation of Bax and Bcl-$_{xl}$ may also play a key role in the reduction of caspase-3 activity in BAEC and BASMC.

It has been recently demonstrated that the anti-apoptotic gene *bcl-xl* is unregulated within intimal VSMCs in animal models and human specimens of vascular disease (Pollman et al. 1998). Moreover, it has been shown that down regulation of Bcl-$_{xl}$ expression within intimal cells using antisense oligonucleotides induces VSMC apoptosis and regression of vascular lesions. A study also showed that acutely disrupting Bcl-2 expression in VSMCs altered cell cycle activity through the execution of the apoptosis pathway (Perlman et al. 2000). These findings indicate that Bcl-xl and Bcl-2 are important determinants of cell viability and lesion formation.

Our study demonstrates that there is no altered expression of *bcl-xl* and *Bax* mRNA by high glucose treatment in both cell types in hypoxia during 24hour and 5 days, while the expression of *bcl-xl* and *Bax* was not affected by both high glucose and mannitol treatments. Therefore, we propose that glucose-induced apoptosis suppression in BAEC and BASMC is partly due to upregulation of Bcl-$_{xl}$ and Bax protein expression as RNA levels did not change after the treatment period. The mechanisms for Bcl-2 and Bcl-$_{xl}$ anti-apoptotic function may include binding to Apaf-1 to prevent the activation of caspase cascade, altering mitochondrial membrane potential, and inhibiting cytochrome *c* release from mitochondria (Chinnaiyan et al. 1997; Decaudin et al. 1997; Kharbanda et al. 1997).

Although the present and previous studies clearly support the notion that chronic hyperglycaemia could provide protection against hypoxia-induced cell apoptosis and elevate cell proliferation, surviving cells should be considered as an important issue, since this elevated mortality rate of the diabetic patient after a myocardial infarction is greater than that of non-diabetic patients (Aronson et al. 1997). This elevated mortality rate has been largely attributed to the development of a cardiomyopathy. The present study suggests that the increased risk of developing hypoxia-induced morbidity, cell proliferation and apoptosis is linked to the preexisting diabetic cardiomyopathy (Aronson et al. 1997). This scenario would imply that chronic hyperglycaemia and hypoxia act as an alternative way in which on the one hand, they render the cell resistant to a hypoxic insult and one the other hand, the surviving cells exhibit abnormal contractile and transport properties which increase the risk of eventually developing severe heart failure.

Taken together, our findings suggest that BAEC and BASMC exposure to high glucose under hypoxic condition leads to increased anti-apoptotic and pro-proliferative events by altering the fine balance between the expression of anti-apoptotic and pro-apoptotic proteins and by increasing the expression of proliferating cell nuclear antigen. The relative concentration of these proteins is an important determinant of their final impact on cell fate, and promotion of anti-apoptotic and pro-proliferative events may play an important role in the development of macrovascular complications in diabetes.

Chapter 5

Effect of High Glucose on BAEC and BASMC Cell Fate (proliferation and apoptosis) Is HIF-1α-dependent

5.1 Introduction

All organisms possess mechanisms to maintain oxygen homeostasis, which are essential for survival. The hypoxia-inducible factor-1 (HIF-1), conserved during evolution from worms to flies to vertebrates, is central to adaptation to low oxygen availability.

HIF-1, a master regulator, in turn regulates transcription of many genes involved in cellular and systemic responses to hypoxia, including breathing, vasodilation, anaerobic metabolism, erythropoiesis and angiogenesis. Therefore, *hif* represents a master gene in oxygen homeostasis during embryonic development and postnatal life in both physiological and pathophysiological processes (Semenza 1998).

Although oxygen availability regulates multiple steps on HIF-1 transcriptional activation, the dominant control mechanism occurs through oxygen-dependent proteolysis of HIF-α (Huang et al. 1996). The most extensively studied isoform of the α-subunits is ubiquitous HIF-1α.

The regulation of HIF-1α expression and activity *in vitro* occurs at multiple levels, including mRNA expression (Wiener et al. 1996; Yu et al. 1998; Bergeron et al. 1999), protein expression (Wang et al. 1995; Jiang et al. 1996; Pugh et al. 1997; Salceda and Caro 1997; Huang et al. 1998; Kallio et al. 1999), nuclear localization (Kallio et al. 1998) and transactivation (Jiang et al. 1997; Pugh et al. 1997; Kallio et al. 1998; Ema et al. 1999). The most intensively studied has been the regulation of steady-state HIF-1α protein level.

Studies have shown the interaction between hyperglycaemia and hypoxia-inducible factor-1α (Catrina et al. 2004; Nyengaard et al. 2004), even though hypoxia is the main regulator of HIF-1α, several other factors such as insulin and advanced glycosylation end products (AGE) (Zelzer et al. 1998; Feldser et al. 1999; Treins et al. 2001) influence HIF-1α expression and function also.

We therefore studied the effect of hyperglycaemia on the regulation of HIF-1α function under hypoxic conditions at the protein and mRNA level and investigated the interaction between high glucose and HIF-1α on BAEC and BASMC fate.

<u>Aim 3:</u> To Investigate the role HIF-1α as a target for hyperglycaemia (25mM HG) in hypoxia-induced apoptosis and proliferation

5.2 Results

Hypoxia activates a number of genes which are important in the cellular adaptation to low oxygen conditions and this elegant response is principally mediated through HIF-1. Only active as a heterodimer, HIF-1 is composed of HIF-1α and HIF-1β. While HIF-1β is readily found in cells under all oxygen conditions, HIF-1α is virtually undetectable in normal oxygen conditions. For the HIF-1 transcriptional complex to be functional, HIF-1α levels must be induced.

Our initial studies investigated the expression of HIF-1α in BAEC and BASMC under conditions of normoxia and hypoxia. BAEC and BASMC were exposed to either normoxia or hypoxia for 5 days. Whole cell lysates were immunoprecipitated and analysed by western blot. Under normoxic conditions, no HIF-1α protein could be detected by western blot analysis of whole cell extracts in either BAEC or BASMC. In contrast, strong expression of HIF-1α protein level was observed under hypoxic conditions in both cell types (Figure 5.1).

Concomitantly, we examined the mRNA expression of HIF-1α under the same conditions on BAEC and BASMC. RNA was extracted and subjected to QRTPCR using pre-optimized primers (Appendix 1). PCR product was resolved by 2% agarose gel and normalized to 18S RNA. No change in mRNA was observed in either normoxia or hypoxia in both cell types corresponding with previous studies. High glucose did not alter mRNA expression under either condition suggesting that HIF-1α expression is controlled at the protein level in response to hypoxia as has been previously described (Figure 5.2).

Having confirmed the expression of HIF-1α under our experimental conditions, we examined the activity of HIF-1α by luciferase assay. BAEC and BASMC were transfected with a luciferase reporter construct containing multiple copies of a hypoxia response element (HRE) upstream of the luciferase gene (HRE-Luc). Cells were exposed to normoxic or hypoxic conditions for 24 hours. Hypoxic treatment of cells over-expressing HRE-Luc showed a significant increase in luciferase activity when compared normoxic controls (BAEC 3.48-fold ± 0.21-fold n=3 *vs* 1.05-fold ± 0.06-fold n=3 *$p \leq 0.01$; BASMC 3.37-fold ± 0.29-fold n=3 *vs* 1.21-fold ± 0.12-fold n=3 *$p \leq 0.01$) which confirmed that hypoxic regulation of HIF-1α expression can result in activation of genes contain HRE sequences (Figure 5.3).

Having shown that hypoxia can regulate HIF-1α expression and activity and that hyperglycaemia can attenuate the proliferative and apoptotic response to hypoxia; we went on to investigate the role of HIF-1α in hypoxia/hyperglycaemic-induced cell fate changes. BAEC and BASMC were cultured in either normoxia or hypoxia for 5 days in the absence or presence of high glucose and whole cell lysates extracted and analyzed by immunoprecipitation using an HIF-1α-specific antibody. HIF-1α expression was not detectable under normoxic condition with normal media or high glucose treated media. However, hypoxia dramatically increased HIF-1α expression under hypoxic condition. This expression was significantly inhibited in the present of high glucose when compared to hypoxic controls (BAEC 0.51-fold ± 0.06 n=3 *vs* 1.00-fold n=3; *p≤0.01) and BASMC (0.59-fold ± 0.07-fold n=3 *vs* 1.00-fold n=3; *p≤0.01) (Fig. 5.4).

To detect HRE-Luc activity of BAEC and BASMC under either normoxic or hypoxic conditions in the presence of high glucose, cells were transfected with a luciferase reporter and exposed for 24 hours. A significant increase activity was found under hypoxic conditions when compared to normoxic controls (BAEC 3.48-fold ± 0.21-fold n=3 *vs* 1.05-fold ± 0.06-fold n=3 #p≤0.01; BASMC 3.37-fold ± 0.29-fold n=3 *vs* 1.21-fold ± 0.12-fold n=3 #p≤0.01). High glucose did not alter this effect under normoxic conditions. However, this activity was significantly reversed following treatment with high glucose under hypoxic conditions when compared to hypoxic controls (BAEC 1.25-fold ± 0.10-fold n=3 *vs* 3.48-fold ± 0.21-fold n=3 *p≤0.01; BASMC 1.21-fold ± 0.12-fold n=3 *vs* 3.37-fold ± 0.29-fold n=3 *p≤0.01). This data suggests that glucose-dependent rescue of hypoxia-treated BAEC and BASMC is *via* inhibition of a HIF-1α-dependent mechanism (Fig. 5.5).

In our studies, we have shown that high glucose enhanced proliferation, inhibited apoptosis and decreased HRE-Luc activity in BAEC and BASMC under hypoxic conditions. We therefore question if this effect is HIF-1α-dependent. Firstly, we knocked down HIF-1α gene expression by HIF-1α siRNA (siHIF-1α) using pre-optimized primers (Appendix 1) and detected transfection efficiency using QRTPCR. BAEC and BASMC were transiently transfected with siHIF-1α and scrambled siRNA (siScram) for 3 hours and recovered in 10% FBS overnight. Total RNA from cell pellets was extracted and PCR product was quantitated by QRTPCR analysis. HIF-1α mRNA expression was significantly suppressed by siHIF-1α (BAEC 23% ± 3.61% n=3 *vs* 102% ± 11.72% n=3 *p≤0.01; BASMC 34% ± 2.03%

n=3 *vs* 101% ± 6.36% n=3; *p≤0.01) when compared to siScram. There was no significant change of none-transfected control on BAEC (100% n=3 *vs* 102% ± 11.72% n=3) and BASMC (100% n=3 *vs* 101.3% ± 6.36% n=3) when compared to siScram (Fig. 5.6).

Based on this successful siRNA transfection, BAEC and BASMC were transiently transfected with siHIF-1α or siScram for 3 hours and recovered overnight in 10% FBS. Cells were then exposed to high glucose in normoxia or hypoxia for 5 days. Proliferation was measured by FACS analysis. Under normoxic condition, siHIF-1α did not show any effect in the presence of high glucose, the effect of transfected siScram control was correlated to siHIF-1α transfected control. Under hypoxic condition, siHIF-1α transfected cells showed a significant increase proliferation when compared siScram transfected control on both cell type. However, due to ~77% knocked off HIF-1α on BAEC and ~67% on BASMC, hyperglycaemia-induced cell proliferation was impaired on both cell types when compared to siHIF-1α hypoxic control. This data suggests that hyperglycaemia upregulates cells proliferation under hypoxic conditions is HIF-1α-dependent (Fig. 5.7).

Although HIF-1α mRNA was not knocked down to approximately the same level in both BAEC (~77%) and BASMC (~67%), a slight increase in proliferation was observed in siHIF-1α BASMC under hypoxic conditions in the presence of high glucose; an effect that was not observed in BAEC (Fig. 5.8).

Concurrently, we examined the effect of apoptosis under the same conditions. BAEC and BASMC were transiently transfected with siHIF-1α or siScram for 3 hours and recovered in 10% FBS overnight, cells were then exposed to high glucose in either normoxia or hypoxia for 5 days. Cells were collected and the percentage of apoptotic cells was determined by FACS analysis. Under normoxic conditions, there was no change in apoptosis between siHIF-1α and siScram transfected cells, high glucose did not modify this effect of apoptosis on BAEC and BASMC. Under hypoxic condition, hypoxia-induced apoptosis was significant increased in siScram transfected cells when compared to normoxic siScram transfected control cells (BAEC 21.34% ± 1.37% n=3 *vs* 6.46% ± 0.78% n=3 § p≤0.01; BASMC 25.77% ± 1.2% n=3 *vs* 6.51% ± 0.33% n=3 § p≤0.01). However, this effect was significantly inhibited by siHIF-1α transfected cells (BAEC 15.34% ± 0.36% n=3 *vs* 21.34% ± 1.37% n=3 #p≤0.05; BASMC 16.20% ± 1.23% n=3 *vs* 25.77% ± 1.20% n=3 #p≤0.01)

when compared to siScram transfected hypoxic control. Since there was still 23% (BAEC) and 33% (BASMC) of HIF-1α mRNA in the siHIF-1α transfected cells, hyperglycaemia showed significant inhibition of apoptosis (BAEC 11.54% ± 0.94% n=3 *vs* 15.34% ± 0.36% n=3 *p≤0.05; (BASMC 11.88% ± 0.63% n=3 *vs* 16.20% ± 1.23% n=3 *p≤0.05) when compared to HIF-1α siRNA transfected hypoxic control (Figs. 5.9 & 5.10).

Similar results were observed by examining caspase-3 activity in BAEC and BASMC. Cells were treated under the same conditions for 5 days and caspase-3 activity assay was measured. Under normoxic conditions, high glucose had no effect on caspase-3 activity in either siHIF-1α or siScram transfected BAEC and BASMC. Under hypoxic conditions, caspase-3 activity significantly increased in siScram transfected cells (BAEC 4.45-fold ± 0.31-fold n=3 *vs* 1.45-fold ± 0.17-fold n=3 § p≤0.01; BASMC 3.34-fold ± 0.19-fold n=3 *vs* 1.44-fold ± 0.06-fold n=3; § p≤0.01) when compared to normoxic siScram transfected control. However, this hypoxia-induced caspase-3 activity was significantly decreased in siHIF-1α transfected cells (BAEC 3.01-fold ± 0.09-fold n=3 *vs* 4.45-fold ± 0.31-fold n=3 #p≤0.05; BASMC 2.23-fold ± 0.12-fold n=3 *vs* 3.34-fold ± 0.19-fold n=3; #p≤0.01) when compared to siScram transfected hypoxic control. High glucose showed a significant decreased in caspase-3 activity in siHIF-1α transfected BAEC (2.27-fold ± 0.19-fold n=3 *vs* 3.01-fold ± 0.09-fold n=3; *p≤0.05) and BASMC (1.65-fold ± 0.09-fold n=3 *vs* 2.23-fold ± 0.12-fold n=3; *p≤0.05) when compared to HIF-1α siRNA transfected hypoxic control (Figs. 5.11 & 5.12).

To further investigate the role of the apoptotic proteins, BNIP3L was introduced to this study. To examine BNIP3L protein expression in either normoxia or hypoxia in the presence of high glucose, BAEC and BASMC were exposed to either normoxic or hypoxic conditions with high glucose for 5 days and whole cell lysates were analyzed by immunoprecipitation. BNIP3L protein expression was not observed under normoxic conditions which correspond with previous studies. Under hypoxic conditions, the expression of BNIP3L protein was significantly increased in BAEC and BASMC. However, this expression was significantly inhibited by high glucose when compared to hypoxic control (BAEC 0.74-fold ± 0.05-fold n=3 *vs* 1.00-fold n=3 *p≤0.01; BASMC 0.59-fold ± 0.05-fold n=3 *vs* 1.00-fold n=3; *p≤0.01). This data suggests that hyperglycaemia may impair hypoxia-induced apoptosis *via* the inhibition of the pro-apoptotic protein BNIP3L (Fig. 5.13).

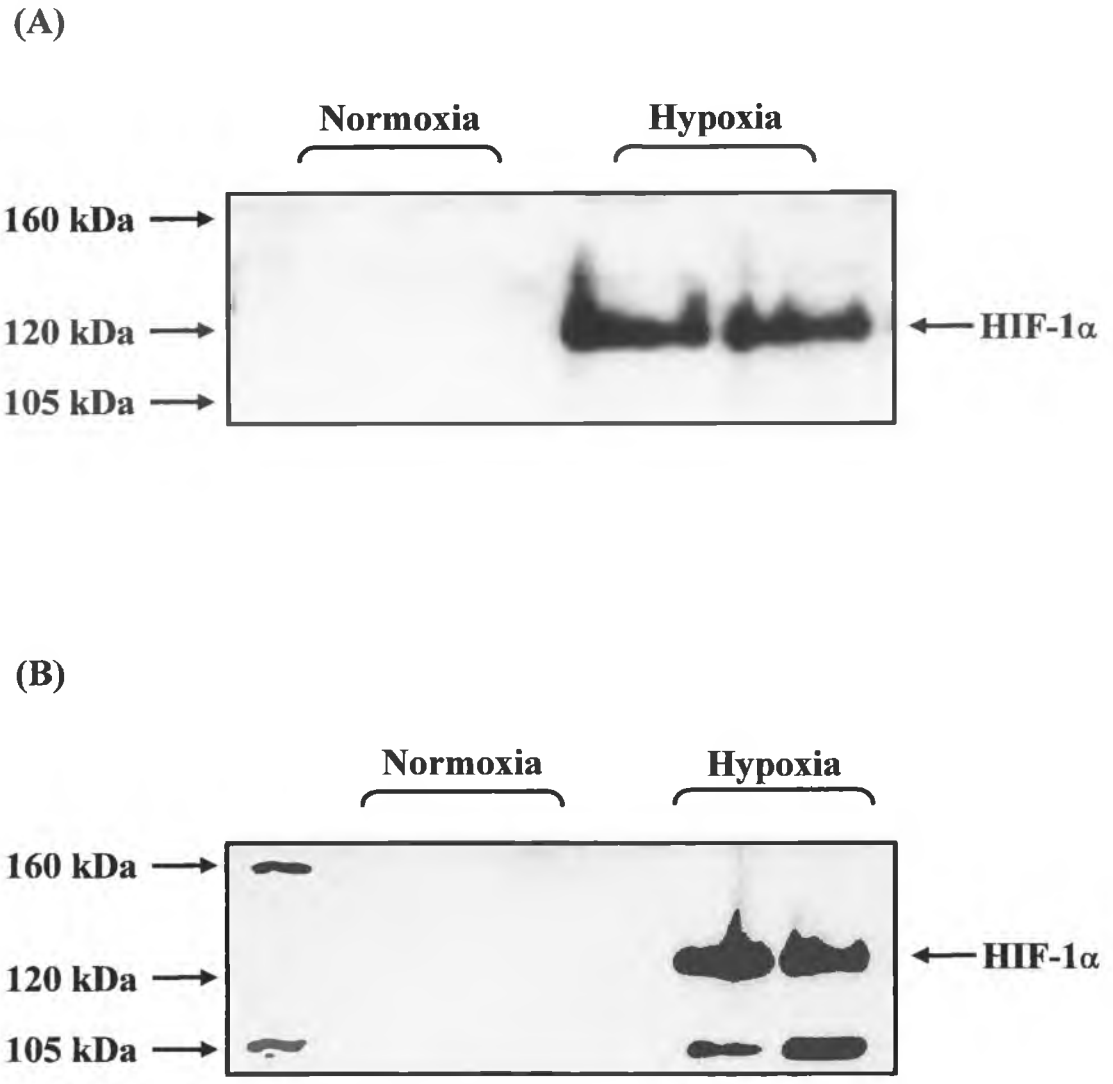


Fig. 5.1 Hypoxia Induces HIF-1α Protein Expression

BAECs **(A)** and BASMCs **(B)** were cultured in 10% FBS under normoxic or hypoxic conditions for 24 hours. Cells were harvested and cell lysates immunoprecipitated using an HIF-1α-specific antibody. Proteins were resolved by SDS-PAGE. and transferred to nitrocellulose. Visualisation of HIF-1α was by incubation with an anti-HIF-1α -specific antibody followed by HRP-conjugated anti-mouse secondary antibody.

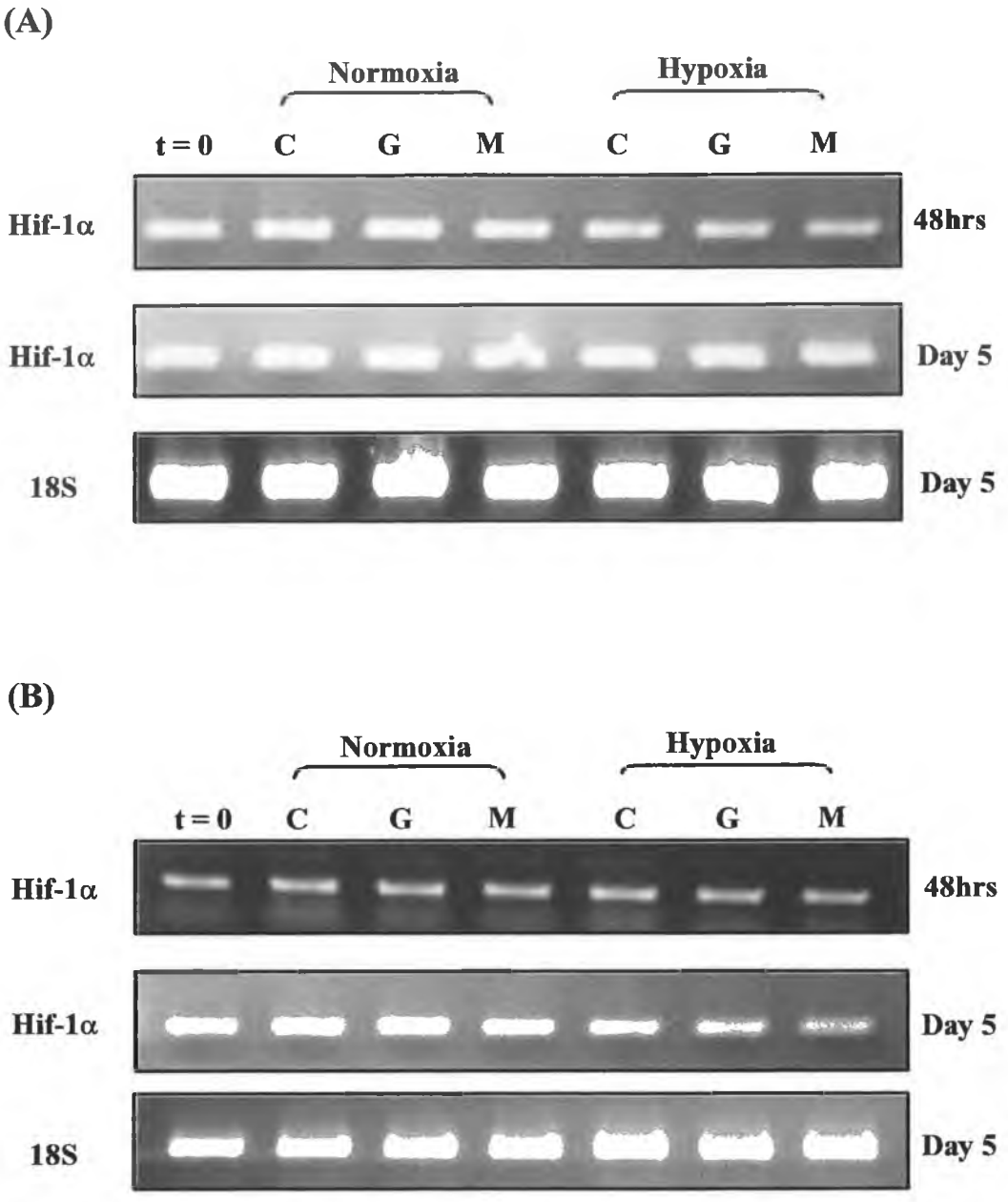


Fig. 5.2 **Hypoxia and High Glucose Concentrations Do Not Alter HIF-1α mRNA Expression**

(A) BAEC and **(B)** BASMC cultured in 10% FBS were exposed to high glucose concentrations for 48hrs or 5 days under either normoxic or hypoxic conditions. Cells were harvested, RNA extracted and QRTPCR carried out using specific primer sets for HIF-1α as described in the methods. The resultant PCR products were resolved by agarose gel electrophoresis (2%). Data are representative of at least three independent experiments showing similar results.

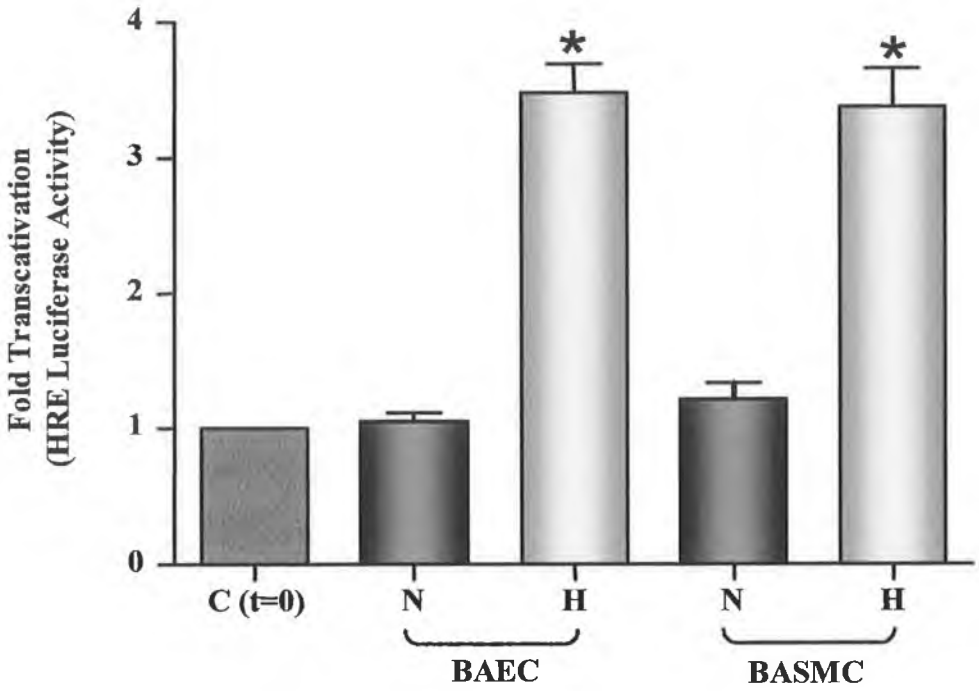


Fig. 5.3 Hypoxia Regulates Hypoxia Response Element Activation

BAECs and BASMCs were transiently transfected with an HRE-Luc reporter construct prior to treatment in the absence or presence of hypoxia for 24hrs. Cells were harvested and Luciferase assay carried out as described in the Methods. Values represent mean ± SEM for three experiments; *$p \leq 0.01$ compared to normoxic control.

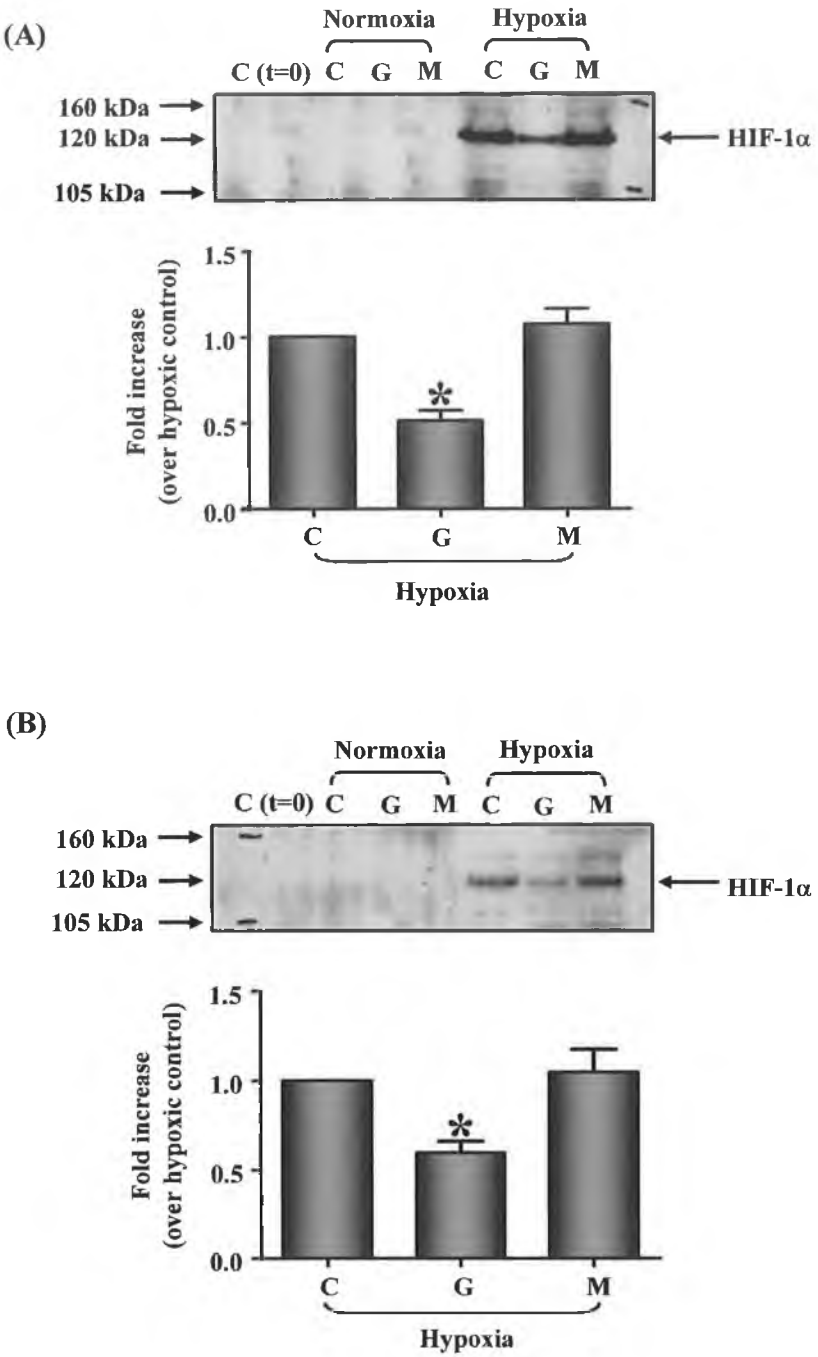


Fig. 5.4 Effect of High Glucose on Hypoxia-Induced HIF-1α Protein Expression

BAECs **(A)** and BASMCs **(B)** were cultured in 10% FBS under normoxic or hypoxic conditions for 24 hours. Cells were harvested and cell lysates immunoprecipitated using an HIF-1α-specific antibody. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Visualisation of HIF-1α was by incubation with an anti-HIF-1α -specific antibody followed by HRP-conjugated anti-mouse secondary antibody. Blots were quantified by densitometric scanning and values represent mean ± SEM for three experiments; *$p \leq 0.01$ compared to hypoxic control.

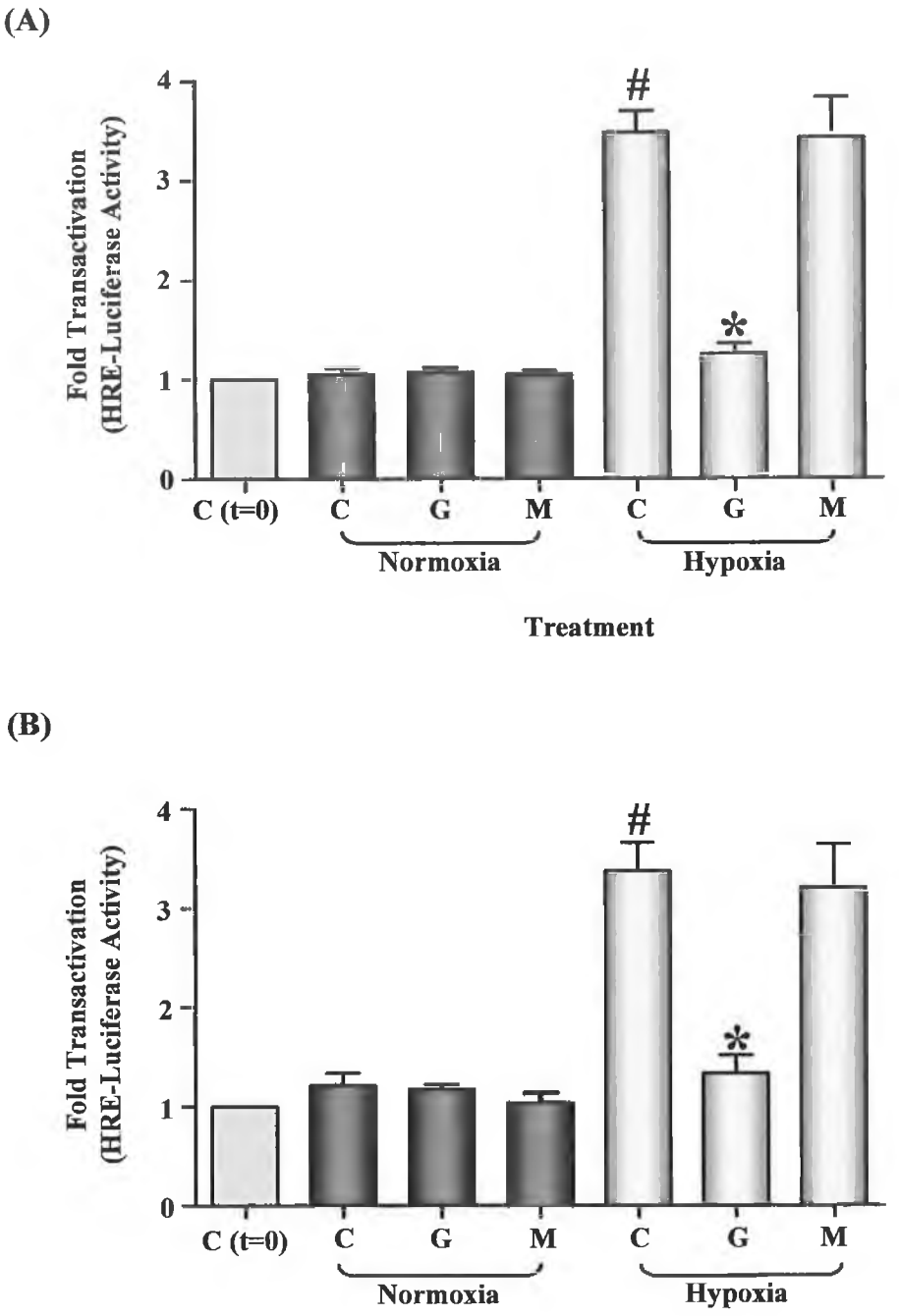


Fig. 5.5 High Glucose Inhibits Hypoxia-Induced Activation of HRE's

BAECs and BASMCs were transiently transfected with an HRE-Luc reporter construct prior to treatment in the absence or presence of hypoxia for 24hrs. Cells were harvested and Luciferase assay carried out as described in the Methods. Values represent mean ± SEM for three experiments; # $p \leq 0.01$ compared to normoxic control, *$p \leq 0.01$ compared to hypoxic control.

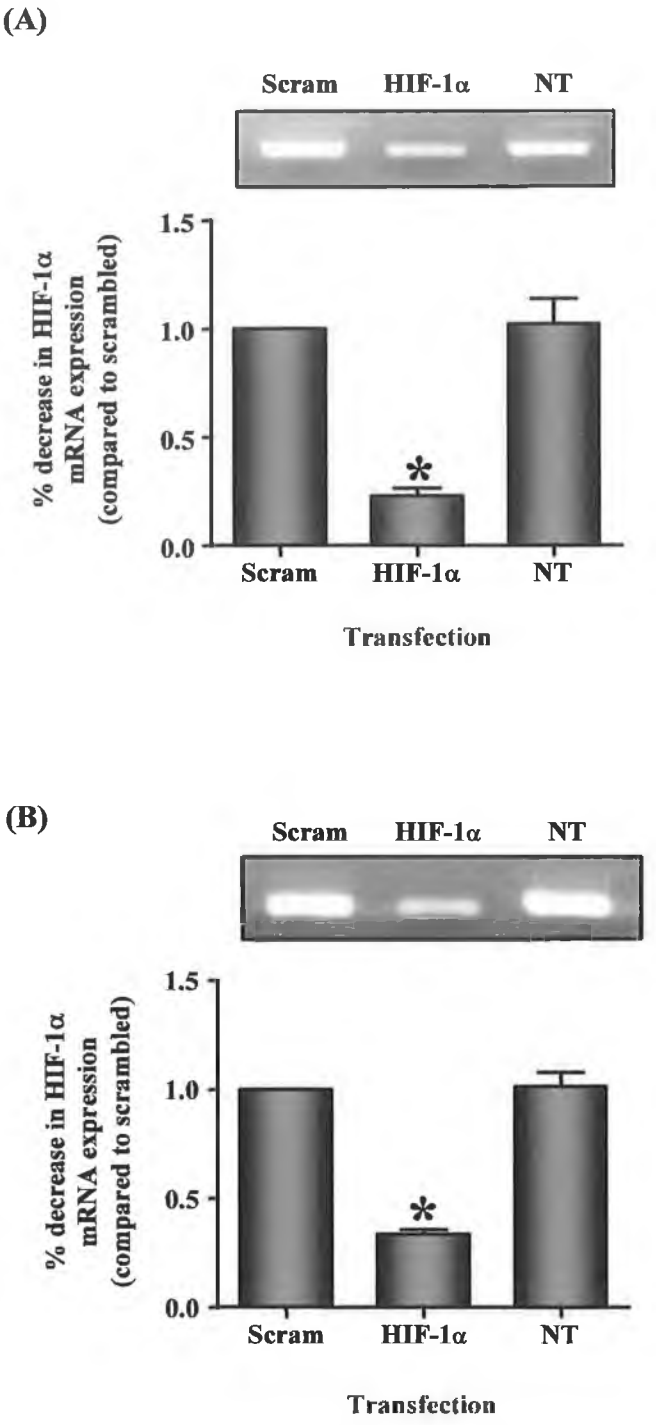


Fig. 5.6 Knockdown of HIF-1α mRNA using Small Interfering (si) RNA

BAEC's **(A)** and BASMC's **(B)** were transiently transfected with scrambled siRNA or siRNA specific to the bovine sequence of HIF-1α (Appendix 1). Cells were harvested, RNA extracted and QRTPCR carried out using specific primer sets for HIF-1a. The resultant PCR products were resolved by agarose gel electrophoresis. Data are representative of at least three independent experiments showing similar results. *$p \leq 0.01$ compared to scrambled control (NT: non-transfected)

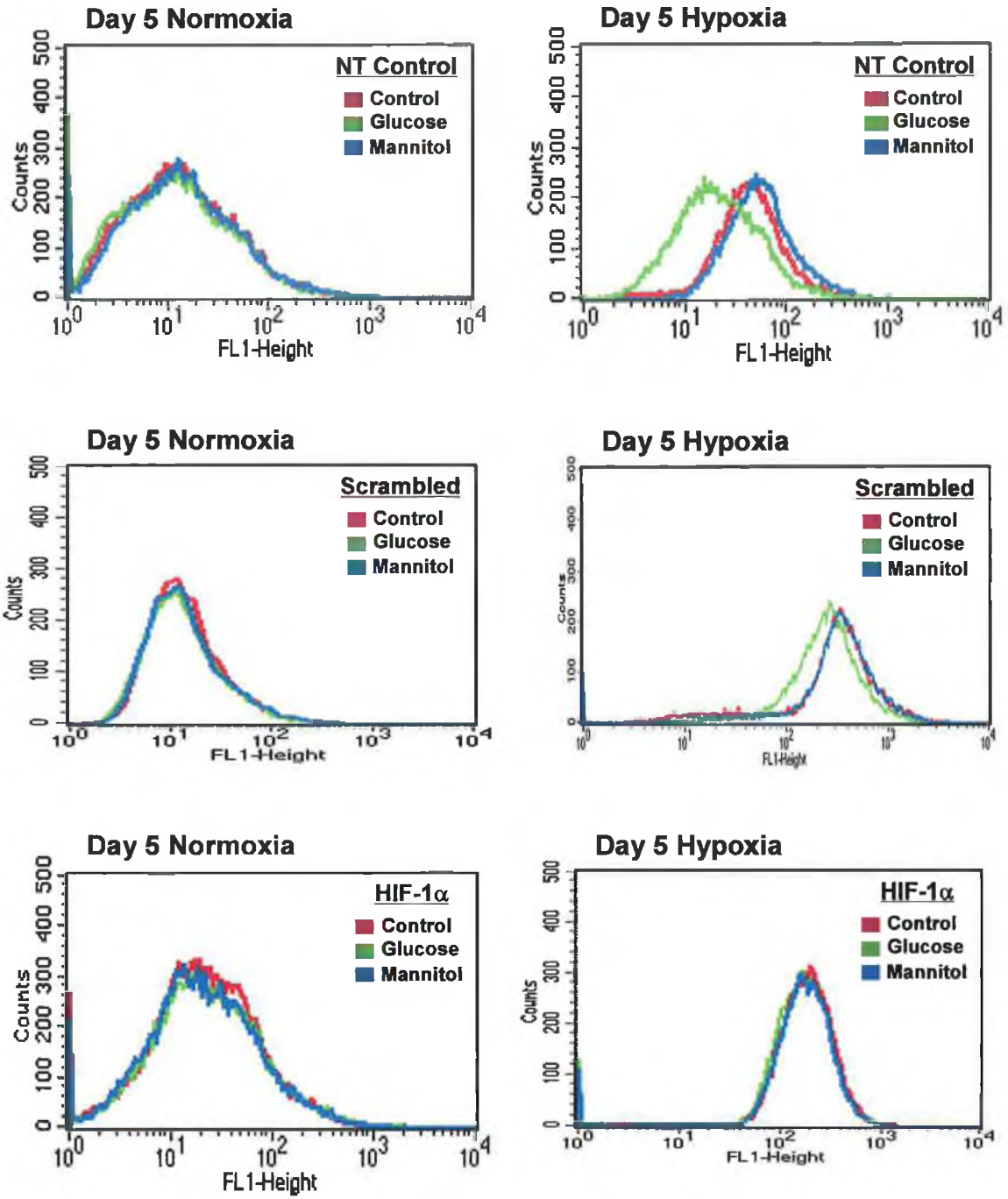


Figure 5.7 Effect of high glucose on hypoxia-induced proliferation in siRNA Transfected BAECs

BAECs were transiently transfected with siHIF-1α and labelled with CFDA SE prior to culture in 10% FBS for 5 days under **(A)** normoxic or **(B)** hypoxic conditions. Cells were then collected and analysed by FACS as described in the Methods. Panels show comparisons of cell proliferation over the 5 day period in 10% FBS with normal glucose (5.5mM: Control), high glucose (25mM: Glucose) and mannitol (25mM: Mannitol). Data are representative of at least 3 independent experiments.

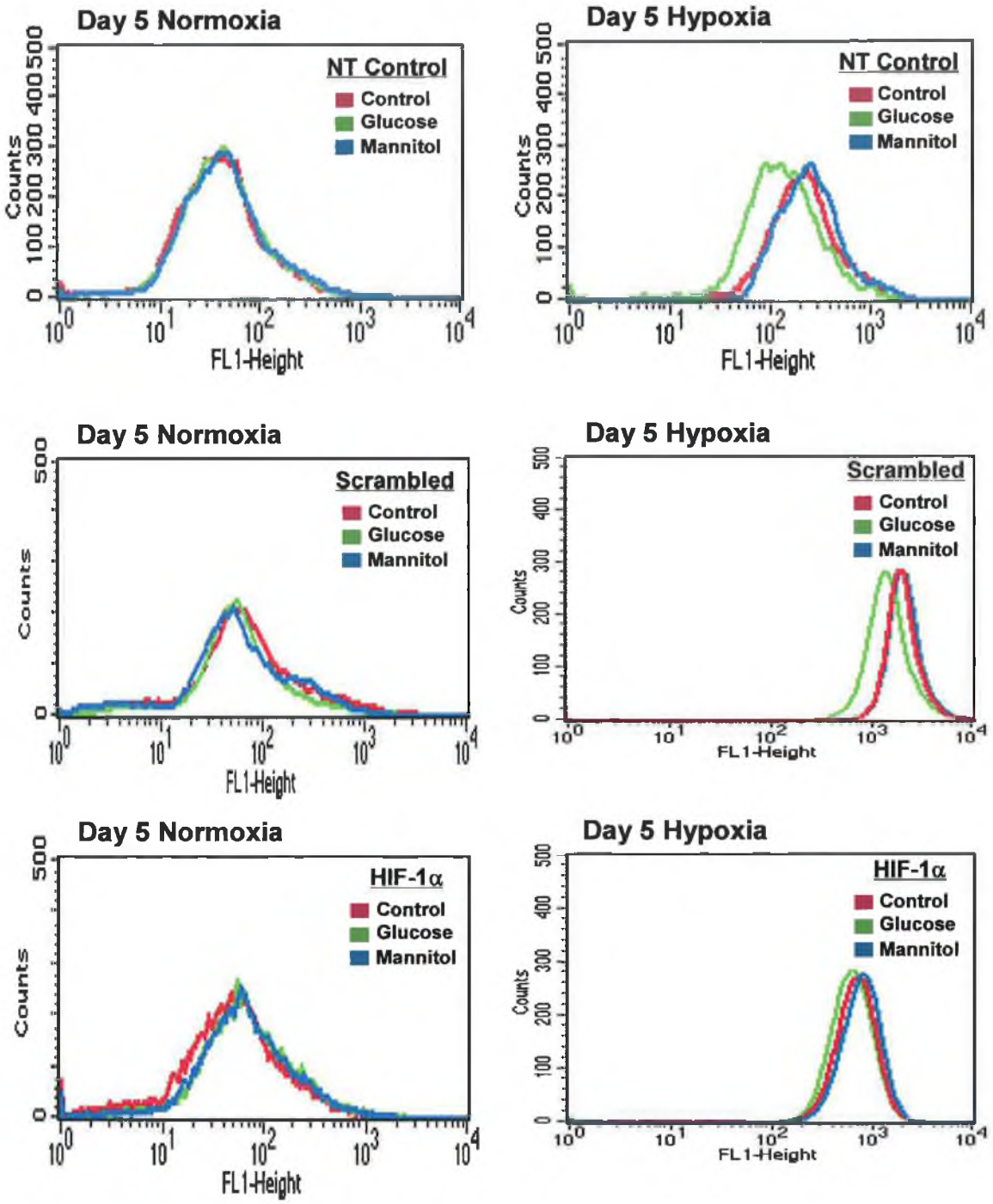


Fig. 5.8 **Effect of high glucose on hypoxia-induced proliferation in siRNA Transfected BASMCs**

BASMCs were transiently transfected with siHIF-1α and labelled with CFDA SE prior to culture in 10% FBS for 5 days under **(A)** normoxic or **(B)** hypoxic conditions. Cells were then collected and analysed by FACS as described in the Methods. Panels show comparisons of cell proliferation over the 5 day period in 10% FBS with normal glucose (5.5mM: Control), high glucose (25mM: Glucose) and mannitol (25mM: Mannitol). Data are representative of at least 3 independent experiments.

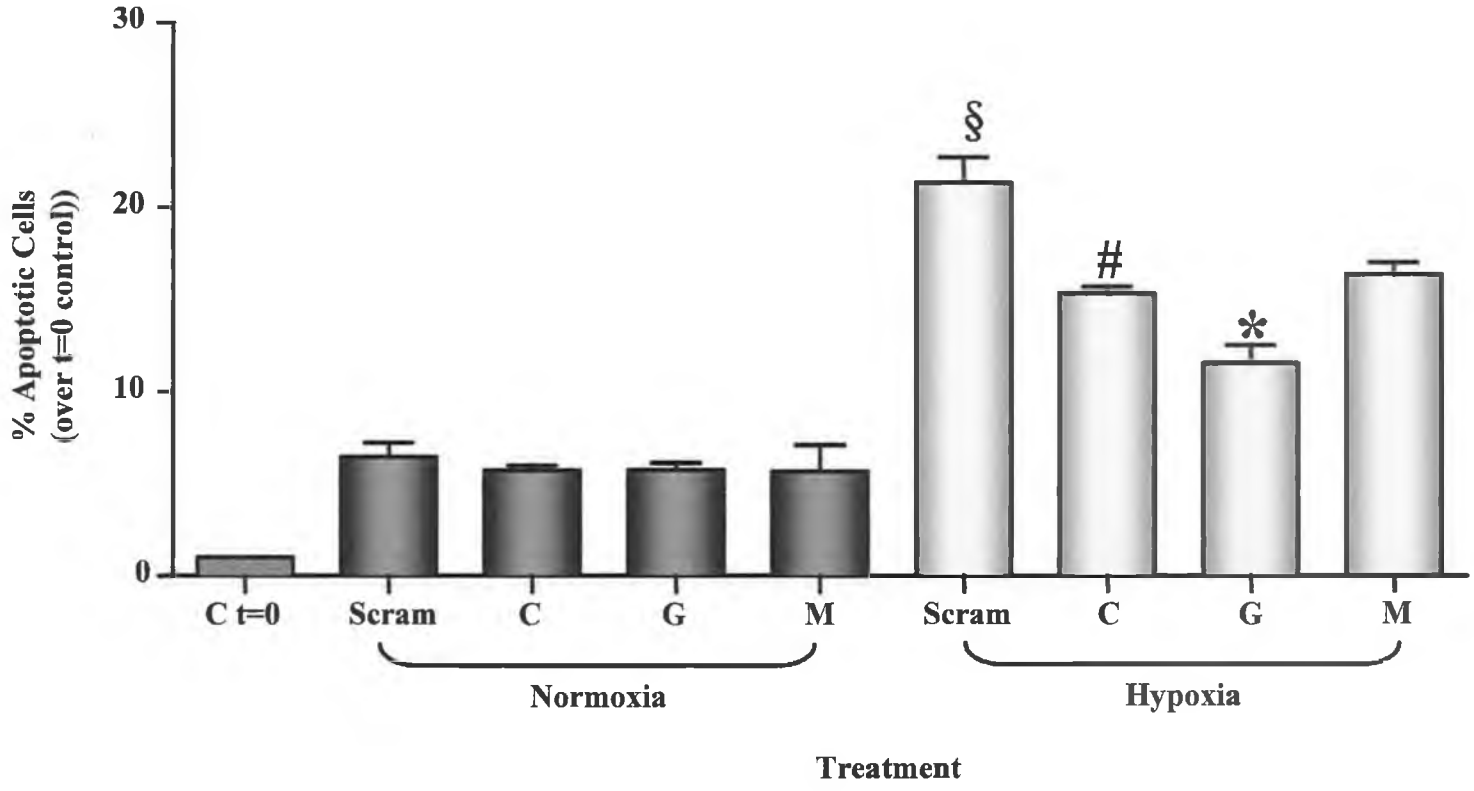


Fig. 5.9 Effect of High Glucose on Hypoxia-induced Apoptosis in siRNA-transfected BAECs

BAECs were transiently transfected with siHIF-1α prior to treatment in the absence or presence of high glucose under normoxic or hypoxic conditions for 5 days. Cells were collected and analysed for apoptosis by FACS as described in the Methods. The values obtained for EA and LA cells were combined and plotted as a bar chart (n=3, ± SEM, §$p \leq 0.01$ compared to normoxic scrambled control; #$p \leq 0.05$ compared to hypoxic scrambled control; *$p \leq 0.05$ compared to hypoxic siHIF-1α control).

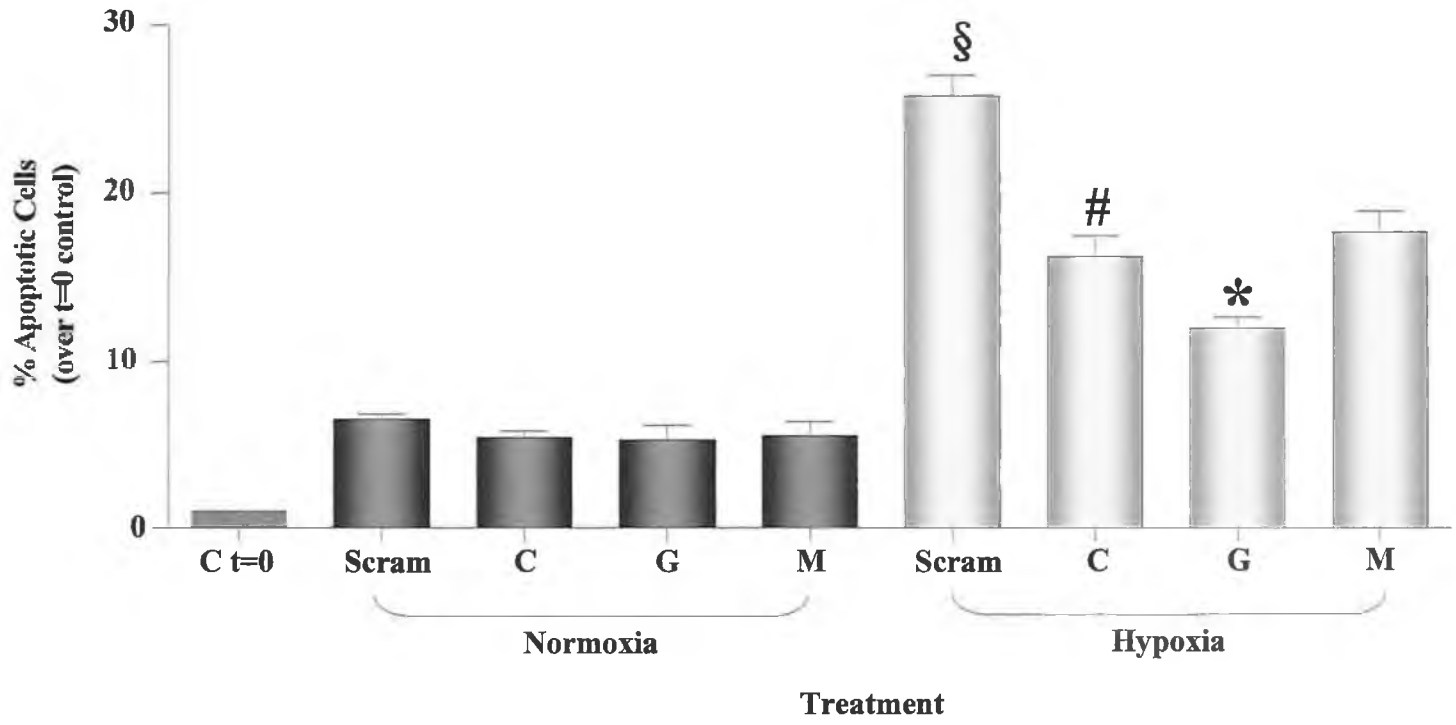


Fig. 5.10 Effect of High Glucose on Hypoxia-induced Apoptosis in siRNA-transfected BASMCs

BASMCs were transiently transfected with siHIF-1α prior to treatment in the absence or presence of glucose under normoxic or hypoxic conditions for 5 days. Cells were collected and analysed for apoptosis by FACS as described in the Methods. The values obtained for EA and LA cells were combined and plotted as a bar chart (n=3, ± SEM, §$p \leq 0.01$ compared to normoxic scrambled control; #$p \leq 0.01$ compared to hypoxic scrambled control; *$p \leq 0.05$ compared to hypoxic siHIF-1α control).

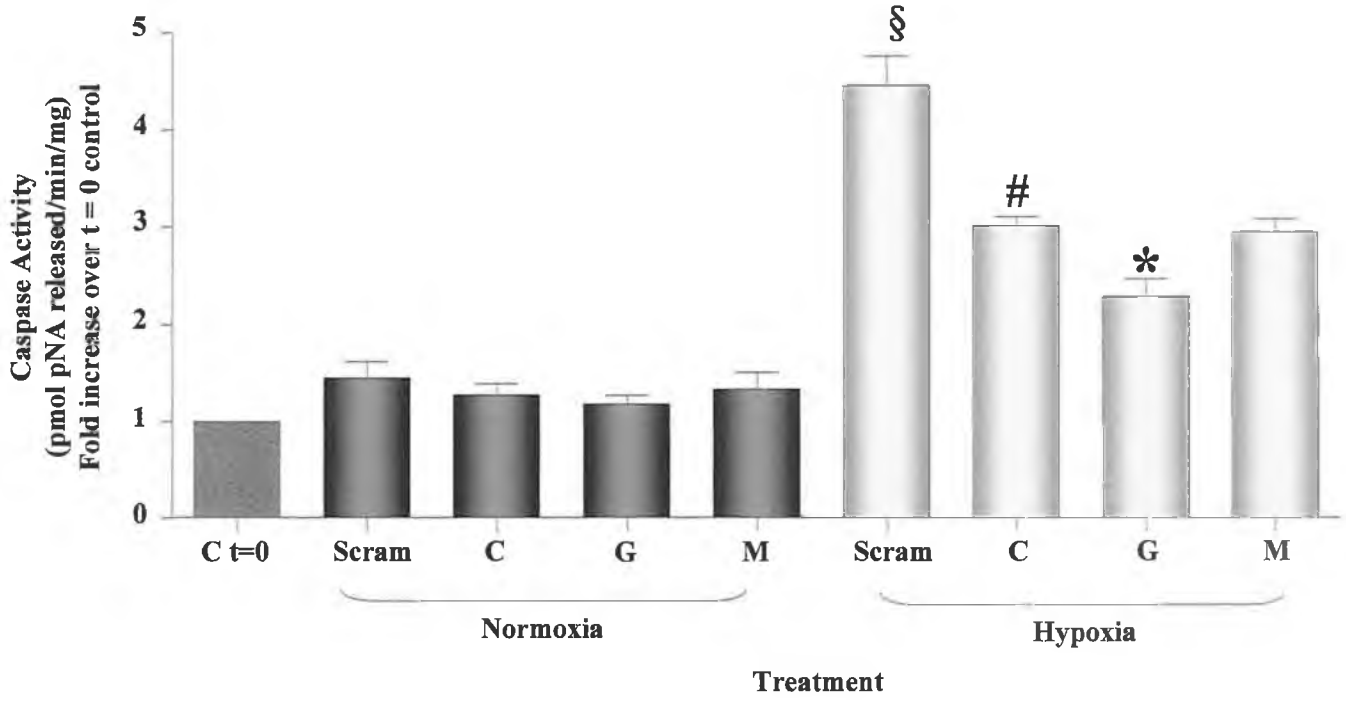


Figure 5.11 Effect of High Glucose on Hypoxia-induced Caspase-3 activity in siRNA Transfected BAECs

BEACs were transiently transfected with siHIF-1α and cultured in media containing 10% FBS for 5 days, in the absence or presence of high glucose (25mM) under normoxic or hypoxic conditions. Cells were harvested and a colourimetric caspase 3 assay carried out on cell lysates as described in the Methods. A pNitroanilide (pNA) standard curve (0-200µg/µl) allowed for the specific activity of caspase 3 to be calculated for each sample and expressed as pmol pNA released/min/mg. Data are representative of at least 3 independent experiments ± SEM; §$p \leq 0.01$ compared to normoxic scrambled control; #$p \leq 0.05$ compared to hypoxic scrambled control; *$p \leq 0.05$ compared to hypoxic siHIF-1α control

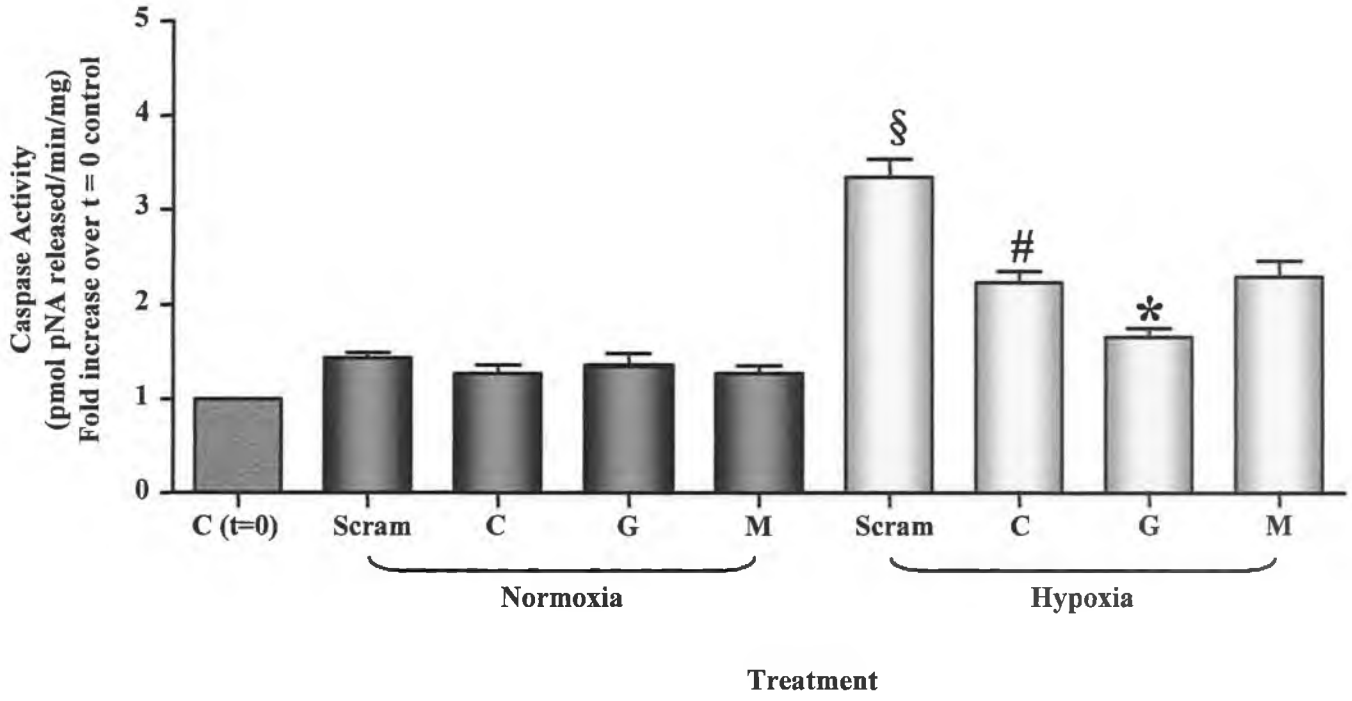


Figure 5.12 Effect of High Glucose on Hypoxia-induced Caspase-3 activity in siRNA Transfected BASMCs

BASMCss were transiently transfected with siHIF-1α and cultured in media containing 10% FBS for 5 days, in the absence or presence of high glucose (25mM) under normoxic or hypoxic conditions. Cells were harvested and a colourimetric caspase 3 assay carried out on cell lysates as described in the Methods. A pNitroanilide (pNA) standard curve (0-200μg/μl) allowed for the specific activity of caspase 3 to be calculated for each sample and expressed as pmol pNA released/min/mg. Data are representative of at least 3 independent experiments ± SEM; §$p \leq 0.01$ compared to normoxic scrambled control; #$p \leq 0.01$ compared to hypoxic scrambled control; *$p \leq 0.05$ compared to hypoxic siHIF-1α control

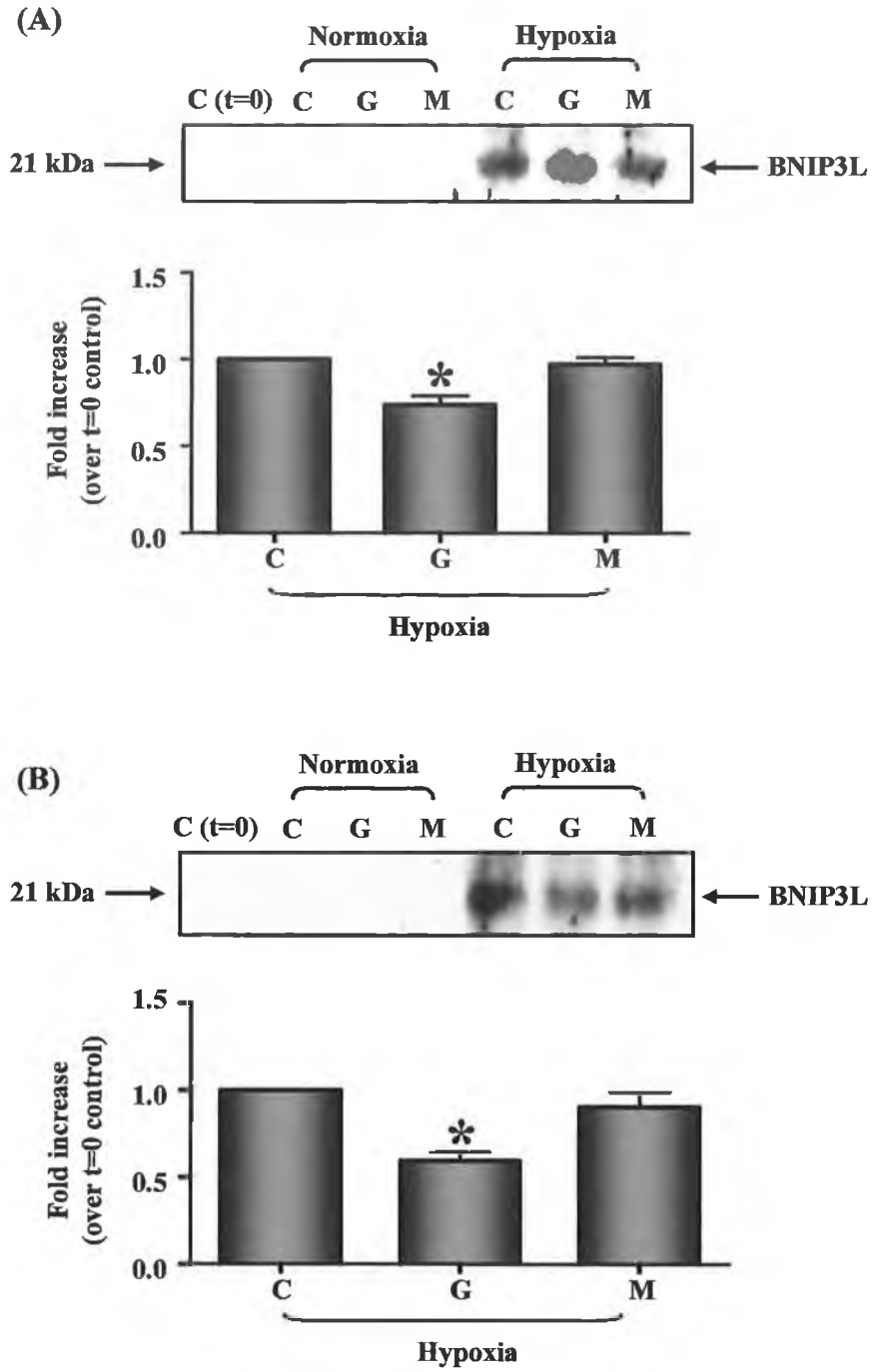


Fig. 5.13 Hypoxia and High Glucose Regulate Pro-Apoptotic BNIP3L Protein Levels

BAECs **(A)** and BASMCs **(B)** were cultured in 10% FBS under normoxic or hypoxic conditions in the absence or presence of HG (25mM) for 5 days. Cells were harvested and cell lysates immunoprecipitated using a BNIP3L-specific antibody. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Visualisation of HIF-1α was by incubation with an anti- HIF-1α -specific antibody followed by HRP-conjugated anti-rabbit secondary antibody. Blots were quantified by densitometric scanning and values represent mean ± SEM for three experiments; *$p \leq 0.01$ compared to hypoxic control.

5.3 Discussion

HIF-1α is a master gene controlling the hypoxic response in mammalian cells and thus plays an important role in cell apoptosis and proliferation. We have found that hyperglycaemia regulates cell apoptosis and proliferation under hypoxic conditions; we therefore studied the effect of hyperglycaemia on hypoxia induced HIF-1α expression and activity.

In this study, we demonstrated that inhibition of the expression of HIF-1α at the mRNA level resulted in an associated decreased protein levels which in turn increased hypoxia-induced proliferation and decreased hypoxia-induced apoptosis. We also showed that hyperglycaemia can inhibit hypoxia-induced HIF-1α expression at the protein level.

This observation suggests a mechanism of cross coupling between two of the most important determinates of the chronic complications in diabetes: hyperglycaemia and hypoxia. Both hyperglycaemia and hypoxia have been suggested to be important causative factors for diabetic retinopathy (Bursell et al. 1996), neuropathy (Newrick et al. 1986), arteriosclerosis (Santilli et al. 1993), kidney disease (Melin et al. 1997) and diabetic foot ulcers (Kalani et al. 1999). Moreover, blood glucose was shown to be in linear relation with fatal outcome after an acute hypoxic challenge such as acute myocardial infarction (Malmberg et al. 1999), suggesting a potential deleterious influence of hyperglycaemia on the capacity of tissue to adapt to low oxygen tensions.

We firstly investigated the HIF-1α protein and mRNA expression under normoxic and hypoxic conditions. HIF-1α protein levels were only detected under hypoxic conditions and high glucose did not have any effect on HIF-1α protein level under normoxic conditions. In contrast, high glucose significantly inhibits HIF-1α protein expression under hypoxic condition, however, high glucose does not regulate HIF-1α mRNA transcription which are correspond with previous studies (Catrina et al. 2004) and it is also in agreement with the observations made on normal retinal cells, where high glucose does not influence the normoxic levels of HIF-1α protein (Poulaki et al. 2002). However, the effect of glucose on HIF-1α protein levels seems to be cell dependent: in cancer cells, higher levels of HIF-1α are present at normal glucose concentrations than at low glucose concentrations (Lu et al. 2002).

In the present study, the impaired expression of HIF-1α protein with the glucose-induced decrease in functional activity of HIF-1α in hypoxic cells provides

an alternative mechanism of regulation of HIF-1α function by high glucose during hypoxia.

In the case of cellular responses to hypoglycaemia, a link between glucose concentrations and hypoxia signalling has been observed. Embryonic cells deficient in HIF-1α are unable to upregulate several HIF-1α target genes at low glucose concentration (Carmeliet et al. 1998; Iyer et al. 1998; Ryan et al. 1998). However, the modulation by hypoglycaemia of HIF-1α target genes seems to be dependent on the cell type varying from inhibition to stimulation (Williams et al. 2002).

Consistent with a negative effect of high glucose concentrations on hypoxia-induced protein stabilization, hyperglycaemia decreased the transcriptional activation function of HIF-1α as assessed by luciferase reporter gene assay. Glucose has been reported to interfere with hypoxia-dependent activation of the liver pyruvate kinase promoter (Krones et al. 2001). Due to the high homology between HRE and glucose responsive elements of this promoter, it has been proposed that the corresponding transcription factors HIF-1α and upstream stimulating factors such as VEGF, respectively, could compete for binding to either response elements, thereby inducing a decrease of the cognate activation pathway (Iyer et al. 1998).

We have shown that hyperglycaemia inhibits hypoxia-induced apoptosis, enhances hypoxia-induced anti-proliferation and interferes with HIF-1α protein level; therefore we investigated if these effects of hyperglycaemia were HIF-1α dependent. HIF-1α mRNA level was knocked down approximately 70% in both cell type using siRNA. We observed that hypoxia-induced apoptosis was significantly decreased in HIF-1α siRNA transfected cells when compared to hypoxic control, high glucose still inhibits hypoxia-induced apoptosis on HIF-1α siRNA transfected cells when compared transfected hypoxia control since there was still ~30% HIF-1α expression in the cells. However, the ratio of which high glucose inhibits HIF-1α siRNA transfected cells apoptosis was much smaller than high glucose inhibits none-transfected cells apoptosis under hypoxic condition.

The proliferation of HIF-1α siRNA transfected cells was increased when compared to none-transfected hypoxic control. Since ~70% HIF-1α mRNA has been knocked off, there was no significant increase proliferation by high glucose on HIF-1α siRNA transfected cell under hypoxia which suggests that high glucose inhibits cell apoptosis and upregulates cell proliferation under hypoxic condition may *via* a HIF-1α-dependant pathway.

Expression of the HIF-1α subunit is precisely regulated by the cellular O_2 concentration, such that the level of HIF-1α and the DNA binding activity of HIF1 increase exponentially as the O_2 concentration decrease. By binding to the hypoxia-response element (HRE) present in the promoter/enhancer region of target genes, HIF1 increases the expression of these target genes such as vascular endothelial growth factor A (VEGF-A), erythropoietin-1, heme oxygenase 1 (OX 1), glucose transporter 1 (GLUT 1) and other enzymes involved in glycolysis (Wenger and Gassmann 1997; Iyer et al. 1998) in response to hypoxia.

Here we showed that hyperglycaemia decreases HRE activation under hypoxic condition which suggests that hyperglycaemia interferes with HIF-1α by the inhibition of binding to HRE and interacting with target genes.

HIF-1α can induce apoptosis *via* two mechanisms. Firstly, it can increase the stability of the product of tumour suppressor gene p53. p53 induces apoptosis by regulating proteins such as Bax or can cause growth arrest which is mediated by p21 in environmental stress or DNA damage. Recently, it was shown that HIF-1α directly binds to the p53 ubiquitin ligase both *in vivo* and *in vitro* thereby stabilizing p53 (Chen et al. 2003). However, another report showed a direct binding of p53 to the ODD domain of HIF-1α (Hansson et al. 2002). HIF-1α interacts with wild-type p53 but not with tumour derived mutant p53 (An et al. 1998) which may reflect a difference in behaviour of HIF-1α in physiological circumstances compared with a tumour environment.

Secondly, HIF-1α can initiate apoptosis by inducing high concentrations of pro-apoptotic proteins Bcl-2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3). BNIP3 was initially related to mitochondrial functions (Chen et al. 1997; Ray et al. 2000) and has been shown to form heterodimers with anti-apoptotic Bcl-2 family members such as $Bcl\text{-}_{XL}$ and Bcl-2 and may promote apoptosis by sequestering these factors (Boyd et al. 1994; Chen et al. 1997; Yasuda et al. 1998; Ray et al. 2000) at the level of the mitochondria (Vande Velde et al. 2000).

We studied BNIP3 because it has been implicated in hypoxia-controlled, pro-apoptotic processes controlled by HIF-1 (Bruick 2000). BNIP3 protein level could not be observed under normoxic condition and BNIP3 protein expression was dramatically increased under hypoxic condition which is correspond with several studies (Bruick 2000; Guo et al. 2001; Schmidt-Kastner et al. 2004). However, hypoxia-induced BNIP3 protein level accumulation was significantly inhibits in the

presence of high glucose in our study which suggest that high glucose decreased hypoxia-induced apoptosis by diminishing the expression of BNIP3.

Mitochondrial damage and release of cytochrome *c* from the mitochondria to the cytosol accompany apoptotic cell death induced by various stimuli (Liu et al. 1996; Kluck et al. 1997; Nicholson and Thornberry 1997; Yang et al. 1997). Together with dATP, cytochrome *c* triggers the association of pro-caspase-9 with Apaf-1, the formation of a cytochrome *c*/dATP/caspase-9/Apaf-1 complex leads to the cleavage and activation of caspase 9 which in turn cleaves and activates caspase 3 and executes apoptosis in various models.

Therefore, we investigated caspase-3 activity and found that the inhibition of hypoxia-induced caspase-3 activity in the present of high glucose is dependent on HIF-1α mRNA expression which suggests that high glucose decreases caspase-3 activation under hypoxic condition is *via* HIF-1α-dependent pathway.

Taken together, in our study high glucose inhibits hypoxia-induced apoptosis and enhances hypoxia-induced anti-proliferation under hypoxic condition is HIF-1α-dependent.

Chapter 6

Discussion & Perspectives

Vascular complications are a major cause of morbidity and mortality in diabetes and are the leading cause of death in adults with diabetes mellitus (Getz 1993). Compared with other individuals with equivalent risk factors, people with diabetes have a four- to fivefold increase in mortality from vascular disease (Getz 1993). The factors responsible for this increased risk of cardiovascular disease are not known, but studies in patients with types I and II diabetes have demonstrated that good metabolic control is able to prevent early changes of atherosclerosis and decrease the risk of coronary heart disease events and deaths (Kuusisto et al. 1994; Jensen-Urstad et al. 1996).

Abnormalities in vascular cells such as increased permeability, enhanced expression of cell adhesion molecules and a reduced response to nitric oxide (NO) are thought to contribute to both micro- and macrovascular complications of diabetes (Hink et al. 2001; Wautier and Wautier 2001). Injury to endothelial and arterial smooth muscle cells triggers the formation of fibrous lesions within high glucose exposure further aggravating their formation (Ross 1993). Results from the Diabetes Control and Complications Trial (DCCT 1993) and United Kingdom Prospective Diabetes Study (UKPDS 1998) indicated that it is persistent hyperglycaemia of uncontrolled diabetes that causes vascular complications, however, whether there is a direct effect of glucose on vascular cells or an indirect effect to the modification of other risk factors is not known.

Previous studies have demonstrated that high concentrations of glucose cause death of vascular cells as a result of increased apoptosis (Wu et al. 1999; Nakagami et al. 2001; Ido et al. 2002; Liu et al. 2004) and cause the inhibition of vascular cell growth (Lorenzi et al. 1987; Curcio and Ceriello 1992; Graier et al. 1995; Morishita et al. 1997; Kamal et al. 1998).

In our initial studies, we firstly investigated the effect of high glucose (HG 25mM) on BAEC and BASMC apoptosis and proliferation and showed that hyperglycaemia alone in 10% FBS had no effect on proliferation and apoptosis. Experiments with mannitol excluded the possibility that the stimulus provided by HG is a function of hyperosmolarity which is consistent with previous findings (Lorenzi et al. 1985; Morishita et al. 1997; Morishita et al. 1997).

Previous studies using high glucose concentrations as stimuli for proliferation have examined these effects under low serum conditions ranging from 1-5% FCS (Oikawa et al. 1996; Sodhi et al. 2001). As low serum conditions are used to quiesce

the cells and deprive them of essential growth factors, we expect that the addition of an energy substrate such as glucose will lead to an increase in proliferation and decrease in apoptosis of these cells. To investigate this further, we then examined the effect of high glucose in cells cultured in 0.5% FBS. Although 0.5% FBS significantly increased cell apoptosis and decreased cell proliferation, high glucose did not alter these effects which suggests that concentrated high glucose (25 mM) is not sufficient to alter cell fate at physiological levels. Mannitol, as an osmotic control, failed to alter cell proliferation and apoptosis in our study which again correlates with previous studies (Graier et al. 1995; Morishita et al. 1997; Kamal et al. 1998).

Even though prolonged exposure of the tissues to hyperglycaemia seems to be the primary causative factor (DCCT 1993; UKPDS 1998), it has become increasingly evident that hypoxia plays an important role in all diabetes complications (Cameron et al. 2001). In addition to deficient blood supply as a consequence of micro- and macrovascular disease, it has been postulated that hyperglycaemia induces a pseudohypoxia state which is based on the finding that high glucose concentrations induce a high $NADH^+/NAD^+$ ratio in cells even when the oxygen tension is normal (Williamson et al. 1993).

Several studies have also observed that diseases characterized by chronic hypoxic episodes are associated with glucose intolerance (Austin et al. 1994; Brooks et al. 1994; Hardin et al. 1997). However, the effect of hyperglycaemia under hypoxic condition has not been fully studied.

Therefore, we introduced hypoxia into cells as a known stimulator of changes in cell fate. Hypoxia induces cell apoptosis and decreases cell proliferation which has been shown in many studies (Graeber et al. 1996; Shimizu et al. 1996; Webster et al. 1999). In contrast in tumour cell lines, hypoxia inhibits cell apoptosis and enhances cell growth (Cuisnier et al. 2003). We demonstrated that hypoxia induced cell apoptosis and decreased cell growth in both BAEC and BASMC by FACS analysis and western blot. However, these effects were altered in the presence of high glucose *in vitro*. A recent study showed that hypoxia-induced apoptosis in rat neonatal cardiac myocytes was prevented by the presence of as little as 1mM glucose in the medium (Malhotra and Brosius 1999). Glucose inhibited translocation of cytochrome *c* from mitochondria to the cytosol and cleavage of the death substrate poly ADP-ribose polymerase (PARP). Cleavage of PARP and DNA laddering were prevented by preincubation with caspase inhibitors indicating participation of

activated caspase in the apoptotic process (Lin et al. 2000). The alternate substrates lactate, pyruvate and propionate provided no protection from apoptosis and the effect of extracellular glucose on apoptotic biochemical and morphological changes was eliminated when glycolysis was blocked, indicating that glycolysis was necessary for glucose-induced protection (Malhotra and Brosius 1999).

The Bcl-2 family members are major regulators of the apoptotic process, whereas caspase are the major executioners. The cell death-regulating activity of the Bcl-2 family members appears to depend on their ability to modulate mitochondrial function. Bcl-2 family members are divided into pro- and anti-apoptotic molecules and it is suggested that protein-protein interactions between Bcl-2 family members are a major player in controlling the apoptotic process (Antonsson and Martinou 2000). Bax may form homodimers to accelerate cell death or heterodimers with either Bcl-2 or Bcl-$_{xl}$ to inhibit cell death. Therefore, the change in the ratio of Bcl-2 or Bcl-$_{xl}$ and Bax protein expression may attenuate the anti-apoptotic effect in apoptosis (Baghelai et al. 1999).

In our study, Bax and Bcl-$_{xl}$ proteins were expressed in a constitutive manner in BAEC and BASMC under normoxic conditions. Following hypoxia, Bax inserts into the mitochondrial membranes, while it is located in the cytosol and in peripheral association with intracellular membranes including mitochondria in normoxic conditions which suggests that Bax translocation from cytosol to the mitochondrial membrane occurred under a physiological condition, thereby leading to pathological tissue destruction (Yamamoto et al. 2004). Hyperglycaemia did significantly enhance the expression of Bax and Bcl-$_{xl}$ protein expression under hypoxic conditions when compared to hypoxic control, however; the ratio of increased Bcl-$_{xl}$ was found to be fivefold over Bax in BAEC and threefold over Bax in BASMC. This increased Bcl-$_{xl}$/Bax ratio correlates with previous studies; suggesting that the increase in Bcl-$_{xl}$ protein expression is involved in the inhibition of hypoxia-induced apoptosis(Clark et al. 1999; Riva et al. 2001). These results reveal a direct link between hypoxia and the regulation of proteins implicated in the control of cellular death. Conversely, these results are different from those where short ischemia and reperfusion did not alter Bcl-2 expression, but overexpression of Bax changed the ratio of Bax over Bcl-2 in the favour of apoptotic cell death (Moley et al. 1998; Nakamura et al. 2000; Podesta et al. 2000; Nakagami et al. 2001).

Since no change in *bcl-xl* and *Bax* gene expression was found in our studies

under either normoxic or hypoxic conditions in the absence or presence of high glucose, we propose that glucose inhibits cell apoptosis and enhances cell proliferation under hypoxic conditions in part by upregulation of Bcl-$_{xl}$ and Bax protein expression.

Bax promotes cytochrome *c* release (Appaix et al. 2000), which in turns activates caspase-9 and caspase-3 (Kluck et al. 1997; Yang et al. 1997; de Moissac et al. 2000). It was recently reported that caspases are important regulators of apoptosis and the release of cytochrome *c* from mitochondria activated caspase, especially caspase-3 (Green and Reed 1998). Our studies in which hyperglycaemia inhibits cell caspase-3 activation and caspase-3 protein expression in hypoxia to prevent cell apoptosis suggests that the protective action of high glucose appears to involved the caspase pathway *via* caspase-3, an effector molecule in the caspase-mediated cascade of apoptosis induction (Shi 2002).

Adaptive responses of cells to hypoxia are mediated by the hypoxia-induced factor-1 (HIF-1). Regulation of HIF-1 activity is critically dependent on the degradation of the HIF-1α subunit which, when stabilized against degradation, upregulates genes involved in angiogenesis, glycolytic energy metabolism, cell proliferation and survival (Huang et al. 1998; Kallio et al. 1999; Bruick and McKnight 2001; Epstein et al. 2001; Ivan et al. 2002).

The issue of whether HIF-1α is a pro- or anti-apoptotic protein is a matter of some debate (Piret et al. 2002) and there are published studies that are supportive of either viewpoint (Yu et al. 2004; Gwak et al. 2005; Luo et al. 2006). It has been shown that HIF-1α may indirectly produce a pro-apoptotic effect either by upregulating the expression of proteins in the Bcl-2 family that are known to mediate cell death or by associating with and stabilizing these proteins. The tumour suppressor protein p53 can activate target genes that initiate cell death. The accumulation of wild-type p53 in response to hypoxia was shown to be HIF-1α-dependent since p53 induction did not occur in a mutant hepatoma cell line that was incapable of synthesizing HIF-1α, whereas transfection with HIF-1α increased the amount of endogenous p53 in normoxic cells (An et al. 1998). Other investigator have demonstrated that hypoxic induction of the pro-apoptotic protein BNIP3 was mediated *via* HIF-1α in renal carcinoma and Chinese hamster ovary cell lines (Sowter et al. 2001).

In contrast to these observations, other evidence suggests that HIF-1α may have a protective role in limiting hypoxia-induced apoptosis. One study showed that pancreatic cancer cell lines that constitutively expressed HIF-1α were more resistant to apoptosis induced by hypoxia than were similar cell lines that lacked constitutive HIF-1α expression (Akakura et al. 2001). In addition, the induction of HIF-1α by hypoxia was shown to be protective against the apoptotic effect in a hepatoma cell line (Piret et al. 2002). Further evidence supporting an anti-apoptotic role for HIF-1α was demonstrated when neutralizing antibody against vascular endothelial growth factor (VEGF), the major target gene protein transactivated by HIF-1, was shown to block the anti-apoptotic effect of hypoxia (Baek et al. 2000).

Although in all cell lines tested, exposure of cells to hypoxia rapidly increased HIF-1α cellular protein levels; this expression is also induced by serum deprivation, hormones and growth factors in VSMC (Richard et al. 2000).

In our study, we observed that the HIF-1α protein level under hypoxic conditions was altered by high glucose and HIF-1α mRNA remained unchanged which supports previous reports that HIF-1α activation is regulated at the protein level (Maxwell et al. 1999; Lee et al. 2004). Under hypoxic conditions, HIF-1 has been shown to upregulate the expression of a number of genes (Bunn and Poyton 1996) among them those encoding glycolytic enzymes such as aldolase A and C (Ebert et al. 1996), lactate dehydrogenase A and phosphoglycerate kinase 1 (Firth et al. 1994). Moreover, during hypoxia, transportation of glucose into cells and glycolytic enzyme activity are enhanced and hypoxia upregulates expression of several key genes in the glycolysis pathway such as hexokinase 1 (HK1), phosphoglycerate kinase 1 (PGK1) and GLUT-1 which modulates glucose transportation and glycolysis under the control of HIF-1α during hypoxia. (Chen et al. 2001; Semenza 2003; Gao et al. 2004). It is important to note that in another model, hypoxic rat cardiomyocytes, glucose uptake and metabolism was found to be protective against hypoxia-induced apoptosis (Malhotra and Brosius 1999). The interaction of the signals of glucose and hypoxia was proposed also from experiments with mouse embryonic stem cells in which hypoglycaemia and hypoxia induced the expression of phosphoglycerate kinase 1, VEGF, lactate dehydrogenase and GLUT-1 (Krones et al. 2001).

When we exposed cells transfected with HIF-1α siRNA to hypoxia, it was found that cell apoptosis was decreased and proliferation was increased; suggesting

HIF-1α mediates cell fate by functioning as a pro-apoptotic protein. However, the effect of cell apoptosis and proliferation mediated by hyperglycaemia was impaired in HIF-1α siRNA transfected cells which suggest that alteration of hyperglycaemia on BAEC and BASMC cell fate (proliferation and apoptosis) is HIF-1α-dependent. Furthermore, concurrent results found in caspase-3 activity suggest that caspase-3 suppression was of particular mechanistic importance.

Moreover, HIF-1α overexpression significantly enhanced the growth inhibitory and pro-apoptotic response to hypoxia, whereas application of siHIF-1α markedly attenuated these features under hypoxic conditions in A549 cells (an alveolar epithelial cell line) (Krick et al. 2005) which correlates with our studies.

BNIP3 is a member of pro-apoptotic Bcl-2 family which has been shown to bind to Bcl-2 to inhibit its activity (Boyd et al. 1994), and BNIP3-Like (BNIP3L) and BNIP3 reveal a high degree of sequence conservation at the Bcl-2 homology (BH) 3 domain (Matsushima et al. 1998) and it has also been shown that BNIP3L directly targets the mitochondria to induce apoptosis-associated mitochondrial changes including membrane potential loss and cytochrome *c* release (Imazu et al. 1999). According to our studies, BNIP3L protein level was not detected under normoxia which correlates with previous studies (Kubasiak et al. 2002; Kothari et al. 2003; Schmidt-Kastner et al. 2004). As a pro-apoptotic protein, BNIP3L protein expression was significantly increased in hypoxia which supports previous studies that demonstrated that hypoxia-inducible expression of BNIP3L was HIF-dependent and caused apoptosis in neuroblastoma cells (Graeber et al. 1996). The response of BNIP3L to hypoxia in human cell lines was further characterized and BNIP3L was overexpressed in human tumours which implicated BNIP3L as an important mediator of HIF-1 signalling pathway leading to cell death (Mason et al. 2002). HIF-1α is essential for BNIP3 transcriptional activation under hypoxia, it has been previously shown that rodent BNIP3 promoter is activated by HIF-1α (Bruick 2000), however, direct interaction between BNIP3 promoter and HIF-1α was not demonstrated. Our studies demonstrated that BNIP3L expression was inhibited by high glucose under hypoxic conditions which suggests that hyperglycaemic protection of hypoxia-induced apoptosis may occur *via* the inhibition of BNIP3L protein expression or the interaction of with HIF-1α. Moreover, it has been shown that HIF-1α could directly bind to an HRE site on the human BNIP3 promoter and this binding is required for activation of the human BNIP3 promoter (Kothari et al. 2003)

(Fig. 6.1).

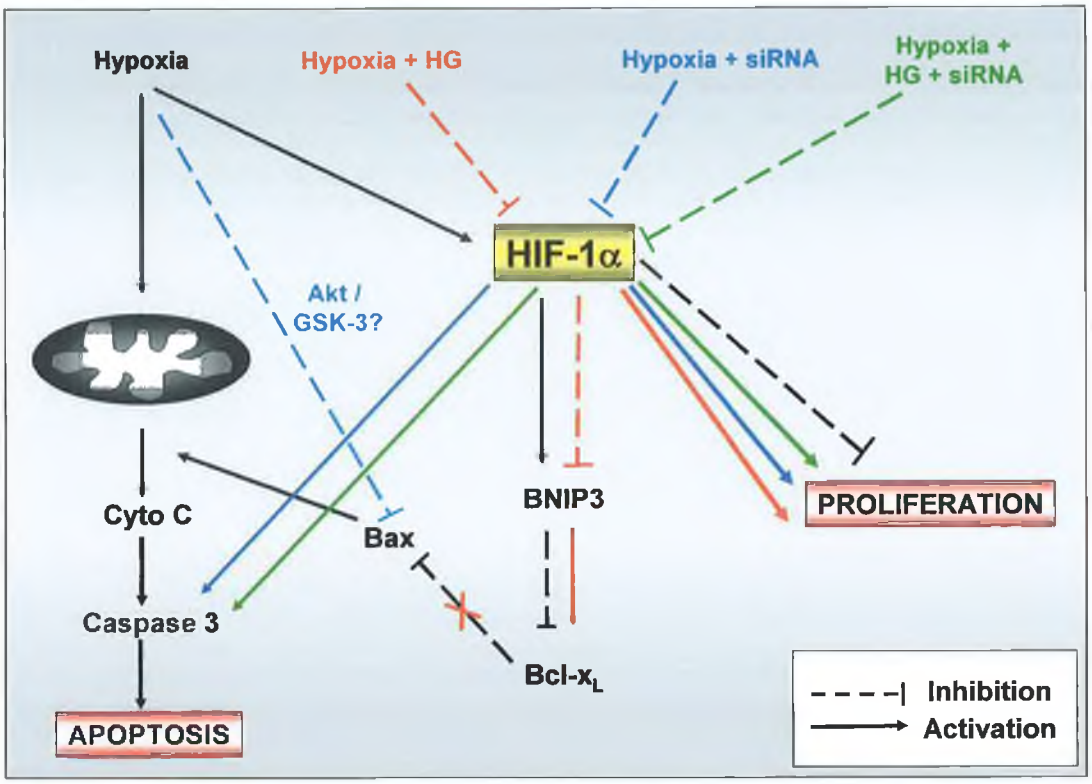


Fig. 6.1 **Diagrammatic representation of the effects of hypoxia and hyperglycaemia on BAEC and BASMC fate**

Many genes contain hypoxia response elements (HRE) which are required for transcriptional activation in response to reduced cellular O_2 concentration (Semenza and Wang 1992). Hypoxia response elements containing functionally essential HIF-1 binding sites with the consensus sequence 5'-RCGTG-3' (Semenza et al. 1996) has been identified in over 50 genes including those encoding transferrin, VEGF, inducible nitric oxide synthase (iNOS), heme oxygenase 1 (HO 1), glucose transporter 1 (GLUT-1) (Wenger and Gassmann 1997). Each of these proteins plays an important role in systemic, local, or intracellular O_2 homeostasis: transferrin delivers iron to the bone marrow for incorporation into haemoglobin; VEGF mediates vascularization; iNOS and HO 1 synthesize NO and CO respectively, which modulate vascular tone; and induction of GLUT-1 and glycolytic enzymes allows for increased anaerobic ATP synthesis.

Taken with our previous study that showed glucose inhibits HIF-1α protein expression; we hypothesis that this reduction in HIF-1α expression results in decreased binding of HIF-1α to HIF-1β (ARNT) thereby inhibiting the formation of

the functional HIF molecule that is required to bind to HRE's to regulate gene expression.

It has been shown that hyperglycaemia inhibits hypoxia-induced stabilization of HIF-1α protein levels against degradation and that mechanisms in addition to proline hydroxylation may be involved in primary human dermal fibroblasts and endothelial cells (Catrina et al. 2004). To further investigate how high glucose modulates HIF-1α protein levels under physiological conditions to modulate macrovascular cell growth, inhibition of HIF-1α proteosomal degradation could be carried out using proteosomal inhibitors to detect the hyperglycaemic effects on BAEC/BASMC cell fate in either normoxia or hypoxia.

Hyperglycaemia/hypoxia has shown parallel effects on BAEC and BASMC growth in mono-cultures in our present studies, however, as two different cell types, the dissimilarity of cell response to pathological conditions needs to be further investigated. Cyclic strain is a powerful stimulus and can also regulate cell fate decisions. Exposure of VSMC to cyclic strain leads to apoptosis *via* a p53 pathway, in contrast, cyclic strain can suppress EC apoptosis *via* AKT phosphorylation (Mayr et al. 2002; Persoon-Rothert et al. 2002). The relationship between circumferential stress and the structure of the vascular wall has been well established in macrovascular cell types. Increases in arterial pressure are associated with VSMC hypertrophy and increases in extracellular matrix (ECM) production. Conversely decreases in arterial pressure result in vessel atrophy (Bomberger et al. 1980). To examine the relations between BAEC and BASMC, the effects of exposing BAEC/BASMC co-cultures to pulsatile flow under normoxic/hypoxic conditions warrants further investigation.

In addition to controlling a switch to glycolytic metabolism and induction of erythropoiesis and angiogenesis, hypoxia promotes the undifferentiated cell state in various stem and precursor cell populations and the latter process requires Notch signalling (Gustafsson et al. 2005) (Fig. 6.2). Notch signalling is an attractive candidate for this process as it functions to maintain the stem/progenitor cell state such as myogenesis (Nofziger et al. 1999; Dahlqvist et al. 2003) and hematopoiesis (Varnum-Finney et al. 2000) and it is central to modulating fate decisions (proliferation, migration, differentiation and apoptosis) during embryonic development of the vasculature.

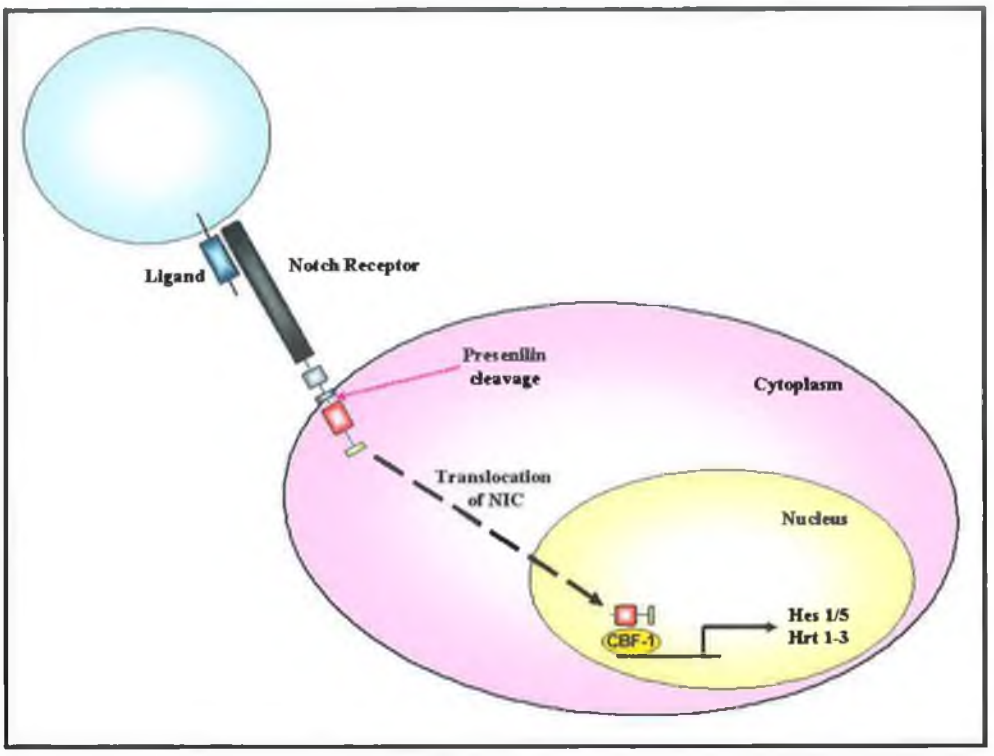
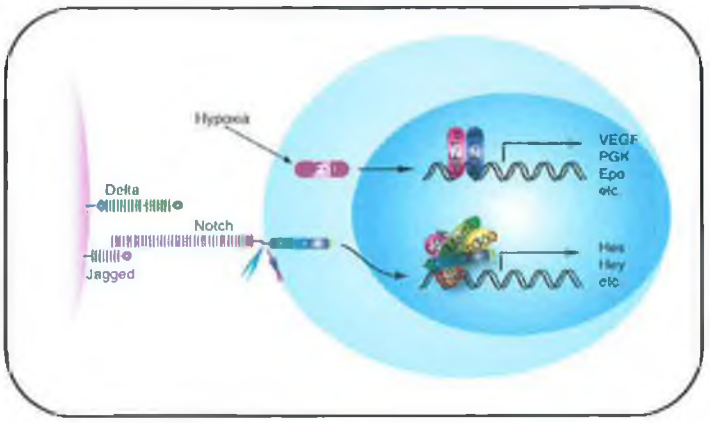


Fig. 6.2 Diagrammatic representation of the Notch Signalling Pathway

On binding of Notch ligand to receptor, cleavage by presenilin occurs and Notch IC is translocated to the nucleus. Notch IC then interacts with the CSL [CBF-1(RBP-jK)/Su (H)/Lag1] family of DNA binding proteins to form a transcriptional activator. This transcriptional activator turns on transcription of a set of target genes, including HRT-1, 2 and 3. Most Notch target genes encode transcriptional regulators. These are involved in modulating cell fate by affecting the function of tissue-specific basic helix-loop-helix transcriptional factors or through other molecular targets such as NF-kß.

Studies within our own laboratory have shown that the Notch signalling pathway is fundamental in controlling the balance between VSMC proliferation and apoptosis (Sweeney et al. 2004; Morrow et al. 2005; Morrow et al. 2005). The link between hypoxia and Notch may have ramifications for other aspects of hypoxia, such as tumour development, in which deregulation of both HIF-1α and Notch mediated signalling events have been implicated (Radtke and Raj 2003; Weng and Aster 2004). As many tumours show elevated expression of HIF-1α caused by hypoxia inherent to growing tumours and genetic loss of VHL (Kondo and Kaelin 2001), it will be interesting to investigate whether the elevated levels of HIF-1α are paralleled by increased Notch signalling and whether the ensuing Notch induction contributes to macrovascular cell growth (Fig. 6.3).

The canonical HIF-1α and Notch signalling pathways



Potential interactions of HIF-1α and NIC in the nucleus

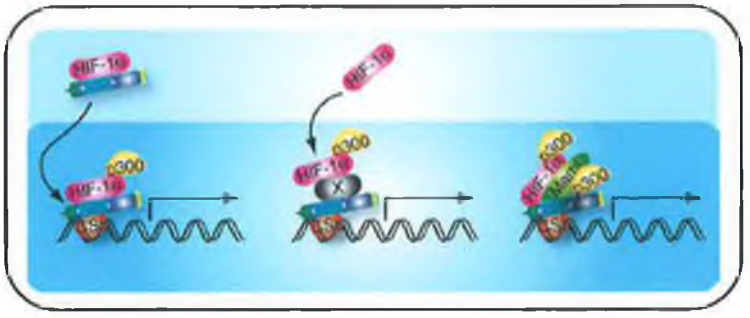


Fig. 6.3 Models depicting potential interactions of HIF-1α with cleaved Notch intracellular domain

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In conclusion, our results demonstrate that hyperglycaemia itself does not alter BAEC and BASMC cell fate (proliferation and apoptosis) in either full serum or low serum containing media under normoxic conditions. Hypoxia significantly induces cell apoptosis and decreases proliferation, which is impaired by hyperglycaemia *via* a HIF-1α-dependent pathway. Hypoxia and hyperglycaemia, as two very important factors, suggest playing an essential pathophysiologial role in the progression of diabetic vascular diseases.

Chapter 7

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Appendix

Appendix 1

Primer Name	Accession Number	Species	Primer Sequence
HIF-1α	NM_174339	Bovine	**For**: tca gct att tgc gtg tga gg **Rev**: tcg tgg tca cat gga tga gt
Bax	U92569	Bovine	**For**: tct gac ggc aac ttc aac tg **Rev**: tgg gtg tcc caa agt agg ag
Bcl-x_L	AF245487	Bovine	**For**: ggt att ggt gag tcg gat cg **Rev**: aag agt gag ccc agc aga ac
18S Ribosome	AF176811	Bovine	**For**: aaa cgg cta cca cat cca ag **Rev**: cgc tcc caa gat cca act ac
Hif-1α siRNA	NM_174339	Bovine	**5'-gag acu gau gac caa caa ctt-3'**

Appendix 2

Antibody	Dilution	Secondary Antibody Dilution & Species
pCNA	1:80	1:4000 anti-mouse
Caspase-3	1:1000	1:2000 anti-rabbit
Bcl-x_L	1:200	1:1000 anti-mouse
Bax	1:1000	1:2000 anti-rabbit
Hif-1α	1:250	1:250 anti-mouse
BNIP3L	1:500	1:1000 anti-rabbit

Appendix 3

Caspase-3 activity = $\frac{\mu mol\ pNA\ /\ 100\ \mu l\ x\ dilution\ factor}{Molar\ Extinction\ Coefficient\ (10.5)\ x\ Volume\ (ml)\ x\ Time\ (mins)}$

Specific Activity = $\frac{Caspase\text{-}3\ activity\ (\mu mol\ pNA/min/ml)}{\mu mol\ pNA/min/mg\ mg\ Protein\ x\ 0.1} = \mu mol$

Appendix 4

Introduction Figure References

Figure 1.2 Alberts, Johnson, Lewis, Raff, Roberts, Walter *Molecular Biology of the Cell* (1998)

Figure 1.5 Tedgui A, Mallat Z: Atherosclerotic plaque formation; *Rev Prat* 49(19):2081-6; (1999)

Figure 1.6 Zang, N., Hartig, H., Dzhagalov, I., Draper, D. and He, Y-W.: The Role of Apoptosis in the Development and Function of T Lymphocytes; *Cell Res* 15: 749-769; (2005)

Figure 1.7 Lavrik I, Golks A. and Krammer PH: Death Receptor Signalling; *J Cell Sci* 118: 265-267; (2005)

Figure 1.8 Bobé, P.: The Fas-Fas Ligand Apoptotic Pathway http://www.infobiogen.fr/services/chromcancer/Deep/Fas-FasLigandID220039.html

Figure 1.11 Kol A. Zarember and harry L.Malech; *J Clin Invest* 115: 1702-1704; (2005)

Figure 1.12 Miguel A Esteban and Patrick H Maxwell; *Expert Rev.Proteomics* 2: 307-314 (2005)

Table 1.1 Table adapted from McLellan and Schneider, 1997

Table 1.2 Table adapted from Eoin P. Cummins and Cormac T. Taylor, 2005

Discussion and Perspectives Figure References

Fig. 6.3 Pear, WS and Simon MC
Lasting longer without oxygen: The influence of hypoxia on Notch signaling; *Cancer Cell* 8(6):435-437, 2005