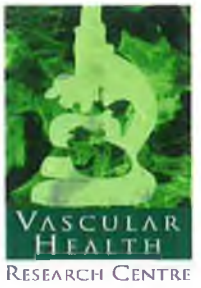
The Effect of the Dietary Constituent Conjugated Linoleic Acid and Homocysteine on Vascular Endothelial Cell Function



Thesis Submitted for the Degree of
Master of Science
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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 Master of Science is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Abbreviations

ADP	Adenosine Diphosphate
BAEC	Bovine Aortic Endothelial Cell
BCA	Bicinchoninic Acid
BK	Bradykinin
bp	Base Pairs
BSA	Bovine Serum Albumin
c	Cis
Ca	Calcium
CBS	Cystathionine b-synthase
CHD	Coronary Heart Disease
Cl	Chloride
CLA	Conjugated Linoleic Acid
Conc.	Concentration
Cox	Cyclooxygenase
Cu	Copper
DAN	2,3-diaminonapthalene
dATP	2-deoxyadenosine 5-triphosphate
dCTP	2-deoxycytidine 5-triphosphate
dGTP	2-deoxyguanosine 5-triphosphate
dTTP	2-deoxythymidine 5-triphosphate
EDTA	Ethylenediaminetetra acetic acid
EGTA	[Ethylenebis (oxyethyleneitrilo)] tetra acetic acid
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase

EtBr	Ethidium Bromide
ETOH	Ethanol
FCS	Foetal Calf Serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HBSS	Hanks Balanced Salt Solution
HCl	Hydrochloric Acid
HDL	High Density Lipoprotein
Hcy	Homocysteine
Hcy Thio	Homocysteine Thiolactone
ICAM	Intercellular Adhesion Molecule
LA	Linoleic Acid
LDL	Low Density Lipoprotein
Mg	Magnesium
MS	Methionine Synthase
MTHFR	Methylenetetrahydrofolate Reductase
Na	Sodium
NADPH	Nicotinimide Adenosine Diphosphate
NO	Nitric oxide
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PG	Prostaglandin's

PGHS	Prostaglandin H Synthase
pH	log of the reciprocal of the hydrogen ion concentration
RNase	Ribonuclease
rpm	Revolutions Per Minute
RT	Reverse Transcriptase
SDS	Sodium Dodecyl Sulphate
BASMC	Bovine Aortic Smooth Muscle Cell
SSB	Sample Solubilisation Buffer
t	Trans
TAE	Tris Acetone EDTA
Taq	Thermophilus Aquaticus
TE	Tris EDTA
TEMED	N, N, N, N' –tetramethyl ethylenediamine
THF	Tetrahydrofolate
Tx	Thromboxane
VLDL	Very Low Density Lipoprotein
xg	Centrifugal force

Units

A	Amp
Da	Dalton
^{o}C	Degrees Celsius
g	Gram
L	Litre
M	Molar
min	Minute
V	Volt
W	Watt

Prefixes

c	centi ($1x10^{-2}$)
m	milli ($1x10^{-3}$)
ì	micro ($1x10^{-6}$)
n	nano ($1x10^{-9}$)
p	pico ($1x10^{-12}$)

Abstract

The interaction between circulating dietary constituents and the vascular endothelial monolayer is vital to the proper maintenance of vascular homeostatic mechanisms, such as vasoregulation. Endothelial dysfunction with subsequent loss of vasoregulatory function are implicated in the early stages of atherosclerosis. Conjugated linoleic acid (CLA) and homocysteine are dietary constituents that have been implicated in the aetiology of atherosclerosis.

CLA is the collective term used for positional and geometrical isomers deriving from the essential fatty acid, linoleic acid. Previous studies have indicated an atheroprotective effect of CLA, manifested in slowing atherosclerotic plaque formation. CLA may be eliciting these effects via two potential mechanisms. Firstly, CLA may be auto oxidised to several furan derivatives that possess a redox buffering capacity, and may modulate endothelium exposure to oxidative stress. Secondly, the influence of CLA on the production of Cyclooxygenase (Cox) derived eicosanoids, such as prostacyclin, has also been suggested as a possible mechanism of action. Homocysteine is a sulphur-containing amino acid formed during the metabolism of methionine. Numerous studies have shown that hyperhomocysteinemia is an independent risk factor for atherosclerosis and atherothrombos. Prolonged exposure and high homocysteine concentrations result in impaired nitric oxide production and subsequent vascular endothelial dysfunction.

The objective of this study was to investigate whether both CLA and/or homocysteine modulate the expression and/or activity of two distinct vascular endothelial cell (EC) components; Nitric oxide synthase (eNOS) and Cox. Both eNOS and Cox, responsible for production of nitric oxide and prostacyclin respectively, have been strongly implicated in the endothelial maintenance of vascular hemodynamic mechanisms.

The results from this study suggest that CLA downregulates endothelial release of prostacyclin (PGI_2) and probably many other prostanoids (PGE_2 and PGD_2) CLA isomer mix does appear to putatively modulate prostanoid production and agonist-stimulated nitric oxide release from BAEC.

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Chapter 2 Materials and Methods

Chapter 1 Introduction

1.1 Atherosclerosis

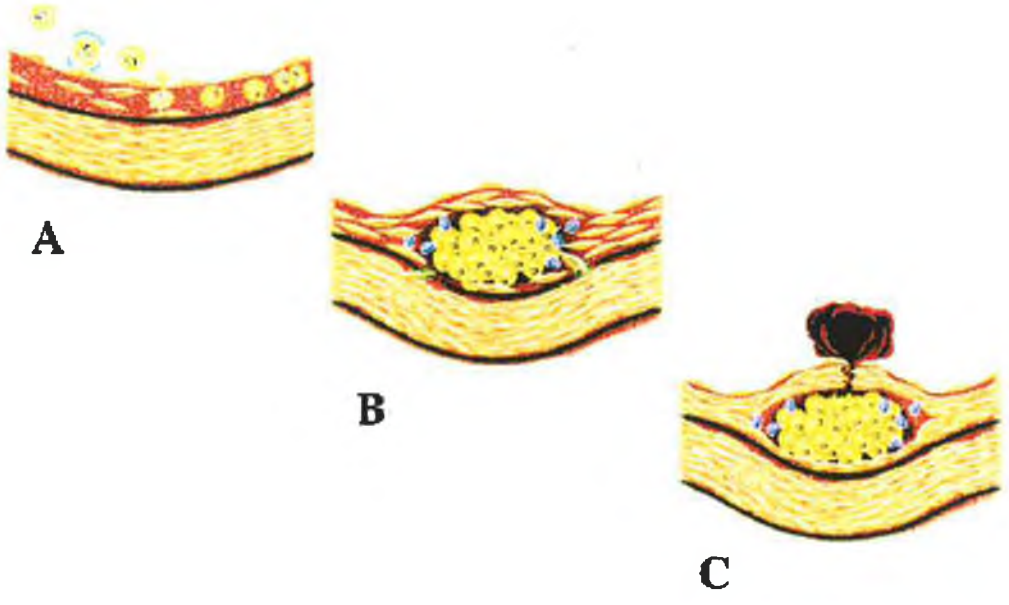
Atherosclerosis is a type of arteriosclerosis, and is the primary cause of all cardiovascular diseases. Atherosclerosis comes from the Greek words athero (meaning gruel or paste) and sclerosis (hardness). It is a type of pathogenic process in which deposits of fatty substances, cholesterol, cellular waste products, calcium and other substances build up beneath the endothelial layer of an artery. This build-up is called plaque. Plaques have various sizes and can be localized to areas of branching within the vasculature. Plaque build up is a natural process that occurs in everyone. The problem arises when people lead an "unhealthy lifestyle". Lack of exercise, a poor diet, smoking and drinking too much alcohol accelerates the process to a pathogenic level. 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. Plaques that rupture cause blood clots to form that can block blood flow or break off and travel to another part of the body. If either happens and blocks a blood vessel that supplies the heart, it causes a heart attack. If it blocks a blood vessel that supplies the brain, it causes a stroke. And if blood supply to the arms or legs is reduced, it can cause difficulty walking and eventually gangrene. Diagnosis of this disease is a problem. There are usually no symptoms until one or more arteries are so clogged with plaque that blood flow is severely reduced, this makes an early diagnosis very difficult.

Cardiovascular disease (CVD) is the major cause of death in the European Union. CVD causes 4 million deaths each year in Europe and over 1.5 million deaths each year in the European Union. Irish men and women have the highest rate of death before the age of 65 from coronary heart disease in the European Union. In 1993, 52 deaths per 100,000 population compared to the EU average of 27. CVD kills more Irish men and women of all ages than any other disease.

1.1.1 The molecular process of Atherosclerosis

Atherosclerosis is a slow, complex disease that typically starts in childhood and often progresses when people grow older. Exactly how atherosclerosis begins or what causes it isn't known, but some theories have been proposed. There is a complex concert of factors involved. The scientific consensus is that atherosclerosis begins when the vascular endothelium of the blood vessel becomes damaged. The three major causes of damage to the arterial wall are; Elevated levels of cholesterol and triglyceride in the blood, hypertension and cigarette smoke.

Figure 1.1.1 The formation and rupture of an atherogenic plaque



This damage causes activation of the vascular endothelium and initiation of an inflammatory response. The endothelium will start to express adhesion molecules which attract and recruit blood leucocytes, macrophage and oxidised LDL cholesterol (Fig. 1.1.1 A) (Li *et al*, 1993). The secretion of pro-inflammatory cytokines, which act as a chemotactic stimulus to the adherent leucocytes, direct their migration to the intima (Mach *et al*, 1999).

Inflammatory mediators act along with the expression of macrophage scavenger receptors, causing monocyte infiltration and accumulation in the arterial wall and subsequent transformation to foam cells after the uptake of modified lipoproteins, such as oxidatively modified LDL (OxLDL) (Quinn *et al*, 1987) (Rohrer *et al*, 1990). cells. In addition to MCP-1, macrophage colony-stimulating factor (M-CSF) contributes to the differentiation of the blood monocyte into the macrophage foam cell (Smith *et al*, 1995) (Qiao *et al*, 1997). Inflammatory mediators produced in plaques can promote the replication of macrophages within the intima as well (Ross, 1999) (Fig. 1.1.1, B). T lymphocytes join macrophages in the intima during lesion evolution. These leukocytes, as well as resident vascular wall cells, secrete cytokines and growth factors that can promote the migration of SMCs to the intimal layer and cause subsequent proliferation (Ross, 1999).

Inflammatory processes not only promote initiation and evolution of atheroma, but also contribute to precipitating acute thrombotic complications of atheroma (Libby, 2001) (Fig. 1.1.1C). Most coronary arterial thrombi that cause fatal acute myocardial infarction arise because of a physical disruption of the atherosclerotic plaque. The activated macrophage abundant in atheroma can produce proteolytic enzymes capable of degrading the collagen that lends strength to the plaque's protective fibrous cap, rendering that cap thin, weak, and susceptible to rupture (Libby *et al*, 1996). γ-Interferon arising from the activated T lymphocytes in the plaque can halt collagen synthesis by SMCs, limiting its capacity to renew the collagen that reinforces the plaque (Libby, 2001). Macrophages also produce tissue factor, the major procoagulant and trigger to thrombosis found in plaques. Inflammatory mediators regulate tissue factor expression by plaque macrophages, demonstrating an essential link between arterial inflammation and thrombosis (Libby and Simon, 2001).

1.1.2 The causes of Atherosclerosis

There are a number of well known risk factors that can accelerate this process towards a disease state. A person may have an inherited genetic predisposition to certain diseases, but the major cause of atherosclerosis are environmental factors. The following is a brief summary of the major environmental risk factors for atherosclerosis:

Cholesterol: Cholesterol is a soft, fat-like substance. It is found in the blood and in all cells. There are two sources of Cholesterol in the body. The liver synthesizes it and it is ingested as part of the diet. It is used in the body to form cell membranes, some hormones and other tissues. Cholesterol and triglycerides are insoluble in blood and so they have to be transported to and from the cells by special "carriers" called lipoproteins, which are synthesised by the liver and intestine. The three major forms of lipoproteins are High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL) and Very Low Density Lipoprotein (VLDL). It is the ratio of HDL to LDL that is important in determining the risk cholesterol presents. Elevated LDL and low HDL levels are key risk factors for the development of atherosclerotic vascular disease (Miller *et al*, 1977; Sharrett *et al*, 1994). Large-scale clinical trials have shown that reducing LDL levels confers a substantial relative risk reduction for major cardiac events (Brown *et al*, 1990).

Low-density lipoprotein (LDL) is retained in the intima, in part by binding to proteoglycan, and undergoes oxidative modification (Berliner *et al*, 1997; Williams and Tabas, 1998). Lipid hydro peroxides, lysophospholipids, carbonyl compounds, and other biologically active moieties localize in the lipid fraction of atheroma (Witztum and Berliner, 1998). These modified lipids can induce the expression of adhesion molecules, chemokines, proinflammatory cytokines, and other mediators of inflammation in macrophages and vascular wall cells (Witztum and Berliner, 1998).

Hypertension: High blood pressure can develop because of two main factors, a genetic predisposition to hypertension, or it may be environmentally induced through a poor diet and lack of exercise. Blood pressure is the measure of the force of the blood pushing against the walls of the arteries. When the heart contracts to pump out blood, pressure is highest. This measurement is called the systolic pressure. After pumping, the heart relaxes and pressure drops to its lowest point just before a new beat. This measurement is called the diastolic pressure. The measurement of an individual's blood pressure is always expressed as systolic pressure over diastolic pressure. For example, normal blood pressure for adults is considered to be in the range of 120/80 millimetres of mercury. Generally, blood pressure above 140/90 is considered to be high for adults, and blood pressure under 90/60 is considered to be too low.

Blood pressure levels are high in Ireland. Around 40% of men and women have raised blood pressure (more than 140/90mmHg) or are being treated for raised blood pressure. The prevalence of high blood pressure increases with age in both men and women. Around 85% of men and 80% of women with raised blood pressure are not receiving treatment. Of those that are treated, just under two-thirds remain hypertensive.

Smoking: Smoking increases the risk of atherosclerosis by 50% and advances the natural history by ten years. Smoking is the strongest risk factor for premature atherosclerosis Wilhelmsen, L., 1988). Smoking is an especially powerful risk factor for peripheral vascular disease (De Cesaris, R. *et al*, 1992). Atherosclerosis is promoted by smoking thorough several means. Smoking directly damages the endothelium, increases vascular tone, increases platelet activation and promotes LDL oxidation. (Carlson, 1979; Muscat, *et al*, 1991).

These are the main risk factors for cardiovascular disease, others include plasma levels of homocysteine, diabetes mellitus, obesity, physical inactivity and alcohol abuse. Diet has a major bearing on many of these risk factors. A healthy diet can greatly reduce the risk posed by these factors (Hu and Willett, 2002).

1.1.3 Atherosclerosis and the Diet

The relationship between diet and coronary heart disease has been studied intensively for nearly a century. In 1908, Ignatowski produced atherosclerosis in rabbits with a diet high in cholesterol and saturated fat (Antischkow, 1967): feeding the rabbits cholesterol alone produced identical lesions. In the early 1950s, controlled feeding studies demonstrated that saturated fatty acids and, to a lesser extent, cholesterol increased serum cholesterol concentration in humans (McGill, 1979). Meanwhile, epidemiological studies found that increased serum cholesterol predicted risk of coronary heart disease in human populations (Kato *et al*, 1973). These discoveries led to the classic diet-heart hypothesis, which postulated a primary role of dietary saturated fat and cholesterol in the cause of atherosclerosis and coronary heart disease in humans (Gordon, 1988). The diet heart hypothesis gained further support from ecological correlations relating saturated fat intake to rates of coronary heart disease in cohorts from different countries (Keys, 1980) and from studies of migrants from low- to high-risk countries (Kato *et al*, 1973).

Until recently, most epidemiological and clinical investigations of diet and coronary heart disease have been dominated by the diet-heart hypothesis. However, the original hypothesis was overly simplistic because the effects of diet on coronary heart disease can be mediated through multiple biological pathways other than serum total cholesterol or low-density lipoprotein cholesterol. Other factors implicated in heart disease include Lipid levels, Blood Pressure, Thrombotic Tendency, Cardiac Rhythm, Endothelial Function, Systemic Inflammation, Insulin Sensitivity, Oxidative Stress and Homocysteine Levels (Hu *et al*, 2002). The existence of these multiple pathways heightens the need to study clinical outcomes because the use of a single intermediate end point as a surrogate of coronary heart disease risk could be misleading. In the past 2 decades, understanding of the nutrients and foods likely to promote cardiac health has grown substantially owing to studies of the molecular mechanisms of atherosclerosis and the metabolic effects of various nutrients and foods, large and carefully conducted prospective cohort investigations, and dietary intervention trials. Although the search for the optimal diet for prevention of coronary heart disease is far from over, more specific and firmer evidence on diet and coronary heart disease is now available.

1.1.4 The Vascular Endothelium

Endothelial cell structure and functional integrity are important in the maintenance of the vessel wall and circulatory function (Lusher, 1990). As a barrier, the endothelium is semi-permeable and controls the transfer of small and large molecules (Charo *et al,* 1998). However, endothelial cells are dynamic and are capable of conducting a variety of metabolic and synthetic functions. These cells exert significant paracrine and endocrine actions through their influence on the underlying smooth muscle cells or on circulating blood elements, such as platelets and white blood cells (Diodati *et al*, 1998). Under basal conditions endothelial cells are intimately involved in maintaining the nonthrombogenic blood–tissue interface by regulating thrombosis, thrombolysis, platelet adherence, vascular tone and blood flow. They produce and release a variety of vasoactive substances, such as prostacyclin (Bunting *et al*, 1976) and nitric oxide (Ramsey *et al*, 1993) both of which inhibit platelet aggregation and cause vasodilation. These mediators are released in response to a range of chemical stimuli, such as thrombin, bradykinin, or ADP, as well as changes in haemodynamic forces, such as alterations in blood pressure or flow (Sumpio, 1993).

The myriad of functions of the endothelial cell makes the endothelium indispensable for body homeostasis, as is evident in its many finely controlled mechanisms. However, discordant stimulation of endothelial cells or an uncontrolled endothelial cell response are common events in many pathologic processes including atherosclerosis and diabetes (De Caterina, 2000). These diseases are related to endothelial injury, dysfunction and activation. Disturbed endothelial function plays a large role in cardiovascular disease (Drexler and Zeiher, 1991; Zeiher *et al*, 1991).

Nitric oxide synthase and cyclooxygenase are enzymatic pathways that are essential to the homeostatic function of the vascular endothelium (Drexler, 1999; Davidge, 1999). The production of nitric oxide and prostaglandin's are necessary for the regulation of platelet adherence, vascular tone and blood flow (Charo *et al*, 1998). The following sections review both enzymatic pathways and their importance to vascular function.

1.1.5 Endothelial Nitric Oxide Synthase (eNOS)

Nitric oxide is a potent vasodilatory gas in the cardiovascular system generated by the nitric oxide synthase (NOS) family of proteins (Mayer and Hemmens, 1997). NOS is NADPH dependent haem containing oxido-reductases that convert one of the guanidino nitrogen's of L-arginine to nitric oxide and the by product L-citrulline. There are three distinct NOS isoforms exist in mammalian cells: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) (Stuehr, 1997). eNOS and nNOS are generally referred to as constitutively expressed, Ca^{2+} -dependent enzymes, although eNOS can also be activated in a Ca^{2+} -independent manner (Ayajiki *et al*, 1997). Inducible NOS is expressed at high levels only after induction by cytokines or other inflammatory agents, and its activity is dependent on an increase in Ca^{2+}. All NOSs have approximately 60% the same amino acid identity and very similar primary structures.

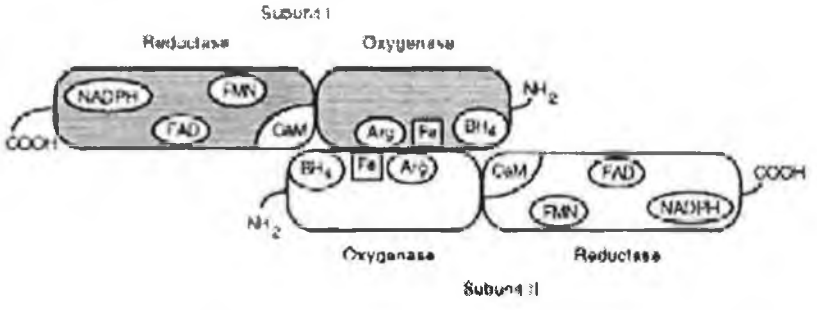


Figure 1.1.4.1 Scheme of the domain structure of the NOS dimer, showing cofactor and substrate binding sites

The enzyme functions as a dimer consisting of two identical monomers, which can be functionally (and structurally) divided into two major domains: a C-terminal reductase domain, and an N-terminal oxygenase domain (Fig 1.1.4.1). The former contains binding sites for one molecule each of NADPH, FAD, and FMN, in close homology with cytochrome P-450 reductase, whereas the latter binds haem and BH , as well as the substrate L-arginine. Between these two regions lies the calmodulin (CaM) binding domain, which plays a key role in both the structure and function of the enzyme.

The sub cellular localisation of eNOS and changes in its cellular compartmentalisation following cell stimulation are controversial. Reports have assigned eNOS to the golgi

apparatus (O'Brien *et al*, 1995), while others have localised eNOS in the plasma membranes at the plasmalemmal caveolae (Hecker *et al*, 1994). The truth lies somewhere in between as eNOS is associated with the plasma membrane and the perinuclear region, identified as the Golgi apparatus (Garcia-Cardena *et al*, 1996).

Biosynthesis of nitric oxide involves a two step oxidation of L-arginine to L-citrulline, with concomitant production of nitric oxide. The reaction consumes 1.5mol of NADPH, and 2 mol of oxygen per mol of L-citrulline formed. The proposed mechanisms involve an initial hydroxylation of L-arginine, which can also act as a substrate for NOS. This is followed by oxidation of the intermediate, using a single electron from NADPH, to form L-citrulline and NO (Griffith and Stuehr, 1995) (Fig. 1.1.4.2). The enzyme is also capable of catalysing the production of additional products, notably super oxide anion, depending on the conditions.

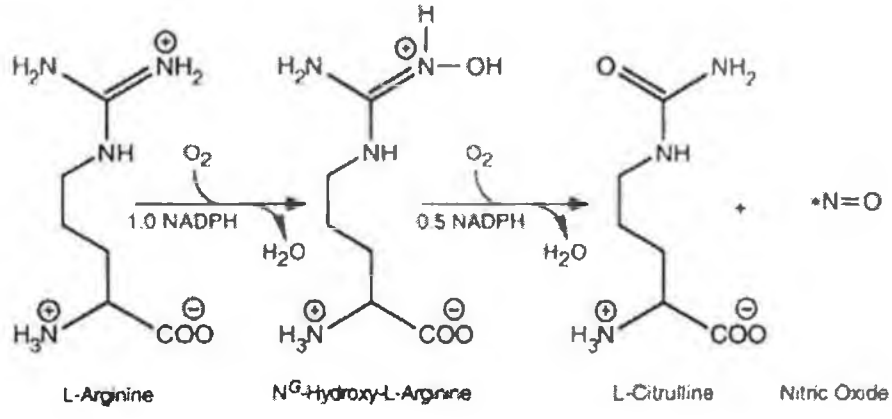


Figure 1.1.4.2 The NOS-catalysed reaction

Endothelium-derived nitric oxide is the most potent endogenous vasodilator known (Furchgott and Zawadzki, 1980). Nitric oxide induces vasodilation by stimulating soluble guanylate cyclase to produce cGMP (Ignarro *et al*, 1984). Nitric oxide has a short half-life and avidly interacts with sulfhydryl-containing proteins, haem proteins, and oxygen-derived free radicals to produce nitrite and nitrate (Kim *et al*, 2001). By virtue of its ability to nitrosylate proteins, it may change their activity or behaviour. The physiological importance of this endothelium-derived vasodilator is reflected by the significant increase in vascular resistance that is induced in animals and humans

exposed to pharmacological antagonists of nitric oxide synthase (Vallance *et al*, 1989; Rees *et al*, 1989). Endothelium-derived nitric oxide also inhibits platelet and leukocyte adherence to the vessel wall (Kubes *et al*, 1991; Tsao *et al*, 1994). This effect of nitric oxide is mediated in part by the activation of cGMP and phosphorylation of intracellular signalling proteins, such as vasodilator stimulated phosphoprotein (Smolenski *et al*, 1998). In addition, nitric oxide suppresses the expression of adhesion molecules and chemokines regulating endothelial interaction with circulating blood elements (Spiecker *et al*, 1998). Finally, endothelium-derived nitric oxide also inhibits vascular smooth muscle cell proliferation (Garg and Hassid, 1989). This is in part mediated by an effect of nitric oxide, an increase in vascular smooth muscle cell apoptosis (Weidinger *et al*, 1990). In contrast, nitric oxide is a survival factor for endothelial cells (Dimmeler *et al*, 1997). These observations are consistent with the view that nitric oxide is an endogenous antiatherogenic molecule. Impairment of endothelial NOS contributes to the pathological alterations in vascular reactivity and structure that are observed in atherosclerosis (Cooke and Dzau, 1997; Vanhoutte, 1997). Pharmacological inhibition or genetic deficiency of NOS inhibits endothelium-dependent vasodilation, impairs tissue blood flow, and raises the blood pressure (Cooke and Dzau, 1997) Furthermore, nitric oxide deficiency promotes the adherence and intimal accumulation of mononuclear cells and accelerates lesion formation in animal models of atherosclerosis (Cooke and Dzau, 1997; Gimbrone *et al*, 1995). By contrast, enhancing nitric oxide production in the vessel wall slows or even reverses atherogenesis or restenosis (Cooke *et al*, 1992; Von der Leyen *et al*, 1995; Wang, B.Y. et al, 1999).

eNOS is a calcium/calmodulin-dependent enzyme: in vascular endothelial cells, eNOS is activated in response to the transient increases in intracellular calcium initiated by the activation of diverse G protein-coupled receptors, including the bradykinin B2 receptor (Andrew and Mayer, 1999). eNOS is also activated by phosphorylation by protein kinase Akt, and eNOS is inhibited by the MAP kinases ERK1/2 (Bernier *et al*, 2000). eNOS phosphorylation by protein kinase Akt is promoted by vascular endothelial growth factor (Fulton *et al*, 1999; Michell, *et al*, 1999), by fluid shear stress (Dimmeler *et al*, 1999; Gallis *et al*, 1999) and stretch.

1.1.6 Cyclooxygenase I (Cox I)

Prostaglandin's (PGs) and thromboxane (Tx) are critical modulators of vascular tone in both physiological and pathophysiological conditions (Davidge, 1999). The production of these eicosanoids is regulated by the availability of arachidonic acid and the activity of prostaglandin H synthase (PGHS), also known as cyclooxygenase (Cox) (Smith *et al,* 2000). Liberation of arachidonate from membrane phospholipids is mediated through phospholipases. Once arachidonate is released it is converted to PGH_2 by Cox. Cox is a rate-limiting enzyme that exhibits a cyclooxygenase activity that incorporates two molecules of oxygen into arachidonic acid to form PGG_2 and a peroxidase activity catalysing a 2-electron reduction of PGG_2 to PGH_2. Cell-specific isomerisation or reduction of PGH_2 produces biologically active end products, such as prostacyclin (PGI_2) and thromboxane (Smith and Murphy, 2002). There are two known isoforms, Cox I and Cox II.

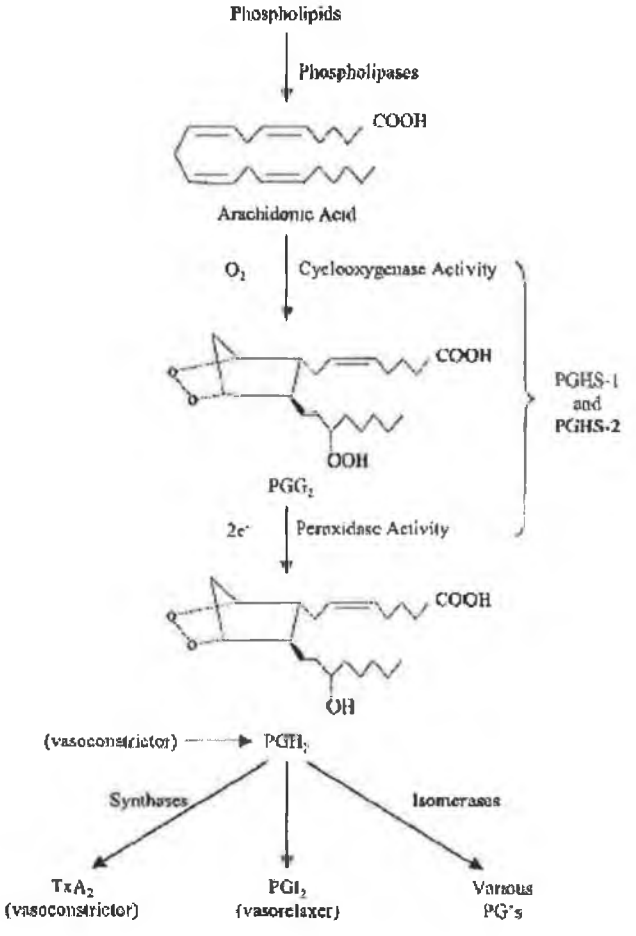


Figure 1.1.6: Cyclooxygenase pathway. Arachidonate is converted to PGH_2 by a cyclooxygenase activity and a peroxidase activity. Both the cyclooxygenase and peroxidase activities are associated with a single protein, Cyclooxygenase. Cell specific isomerisation or reduction of PGH_2 results in the major biological prostanoids.

Cyclooxygenase-I is a 69-kDa protein that is constitutively expressed but can also be induced. For example, shear stress induces expression of cyclooxygenase I protein in human umbilical vein endothelial cells (Doroudi *et al*, 2000). In addition, there are a number of putative promoter response elements for Cox I, including shear stress elements and transcription factor sites (Wang *et al*, 1993). Both the endothelium and smooth muscle cell contain Cox; however, endothelial cells contain up to 20 times more Cox than smooth muscle cells (DeWitt *et al*, 1983). In regard to sub cellular localization of Cox, immunogold-labeling microscopy has demonstrated that both Cox I and Cox II are present in equal proportions in the luminal surface of endoplasmic reticulum and in the inner and outer membranes of the nuclear envelope in human umbilical vein endothelial cells (Spencer *et al*, 1998). There does not appear to be a different sub cellular localization of Cox I versus Cox II.

Prostaglandin's (Prostacyclin, Thromboxane) are formed by most cells in our bodies and act as autocrine and paracrine lipid mediators (Smith and Murphy, 2002). They are not stored but are synthesized de novo from membrane-released arachidonic acid when cells are activated by mechanical trauma or by specific cytokine, growth factor, and other stimuli (e.g., collagen and ADP in platelets, bradykinin and thrombin in endothelium) (Davidge, 1999). Under normal physiological conditions, eicosanoids (primarily prostacyclin) produced by the cyclooxygenase pathway, primarily Cox I, generally induce vasorelaxation. However, in vascular pathologies, there may be an imbalance where PGHS-dependent vasoconstrictors become more predominant (Davidge, 1999).

Prostaglandin's are released from endothelial cells predominantly by facilitated transport through a known prostaglandin transporter (PGT) of the organic anion transporter polypeptide family, and potentially by other uncharacterised transporters (Schuster, 1998). Due to the evanescent nature of thromboxane and prostacyclin (which have half-lives on the order of seconds to a few minutes), these compounds must act near their sites of synthesis. There are at least 9 known prostaglandin receptor forms in mouse and man, as well as several additional splice variants with divergent carboxyl termini (Narumiya and Fitzgerald, 2001). Four of the receptor subtypes bind PGE_2

(EP_1-EP_4), two bind PGD_2 (DP_1 and DP_2) (Hirai *et al*, 2001) and the receptors that bind $PGF_{2\alpha}$, PGI_2, and TxA_2 (FP, IP, and TP, respectively) each derive from a single gene. The prostaglandin receptors belong to three clusters (on the basis of homology and signalling attributes rather than by ligand-binding properties) within a distinct subfamily of the G protein-coupled receptor (GPCR) super family of seven-transmembrane spanning proteins. The lone exception is DP_2, which is a member of the chemoattractant receptor sub grouping. The "relaxant" receptors IP, DP_1, EP_2, and EP_4 form one cluster, signalling through G_s-mediated increases in intracellular cyclic adenosine monophosphate (cAMP); the "contractile" receptors EP_1, FP, and TP form a second group that signals through G_q-mediated increases in intracellular calcium. The EP_3 receptor is regarded as an "inhibitory" receptor that couples to G_i and decreases cAMP formation. Although most of the prostaglandin GPCRs are localized at the plasma membrane, some are situated at the nuclear envelope (Bhattacharya *et al*, 1998).

1.2 Conjugated Linoleic Acid (CLA)

Conjugated linoleic acid (CLA) refers to a group of polyunsaturated fatty acids that exist as positional and stereo (cis, trans) isomers of conjugated dienoic octadecadienoate (18:2). The predominant geometric isomer in foods is the c9t11-CLA isomer (Ma *et al*, 1999), also called rumenic acid, followed by t7,c9-CLA, c11,t13-CLA, c8,t10-CLA and the t10,c12-CLA isomer. The three-dimensional stereo-isomeric configuration of CLA may be in combinations of cis and/or trans configurations.

Figure 1.2: Structures of trans-10,cis-12 CLA (upper panel),cis-9,trans-11 CLA (middle panel), and linoleic acid (lower panel)

A number of physiological properties have been attributed to CLA including action as an antiadipogenic, antidiabetogenic, anticarcinogenic as well as an antiatherosclerotic agent (Belury *et al*, 1997; Belury and Vanden Heuvel, 1997; Houseknecht *et al*, 1998). CLA also has effects on bone formation (Li and Watkins, 1998) and the immune system as well as fatty acid and lipid metabolism and gene expression in numerous tissues. Any

one of these effects would be considered advantageous, the possibility that CLA affects all of these is fascinating. On top of this, the fact that the richest sources of CLA, meat and dairy products, are consumed by people worldwide has massive implications and public health.

1.2.1 CLA in the Diet

CLA is found in foods such as beef and lamb, as well as dairy foods derived from these ruminant sources (Chin *et al*, 1992). The levels of CLA in various foods have been reported to vary from as low as 0.2mg per gram of fat in corn and peanut oil to as high as 17mg per gram of beef or 30mg per gram of milk fat. Trace quantities have been found in seafood's and vegetable oils.

The CLA in foods derived from ruminants relates to the bio hydration of unsaturated fatty acids by rumen bacteria (Bartlet and Chapman, 1961). Most of the studies investigating the sources of ruminant CLA have involved dairy cows and milk fat. The predominant isomer, representing 75-80% of total CLA, is cis-9, trans-11 CLA (Kelper *et al*, 1966). This isomer is formed as an intermediate in the microbial bio hydrogenation of linoleic acid. The second most prevalent CLA isomer in milk fat is trans-7, cis-9 and it originates almost exclusively from endogenous synthesis involving Δ^9-desatursae and trans-7 C18:1 produced in the rumen (Coral *et al*, 2002). Milk fat content is largely dependent on rumen outflow of trans-11 C18:1 and tissue activity of Δ^9-desaturase; both of these variables can be affected by diet and vary substantially among individuals. In a review paper by Parodi *et al* (1999) the early literature describing seasonal fluctuation of CLA in cows milk is summarised. The amounts in spring and summer, when cows pastured, were substantially higher than in fall and winter, when cows were stall fed.

Because of various food types throughout the world containing different levels of CLA human dietary intakes of CLA can differ substantially and because of the clear potential for various isomers of CLA to influence human health, documentation of dietary CLA is of interest. Various methodologies have been utilized to quantify intake of CLA,

including the use of disappearance data, dietary recalls, food frequency questionnaires and biochemical analysis of food duplicates. Researchers utilizing indirect methodologies have estimated CLA intakes in various locations including the United States, Australia, Germany and Finland to range from 50 to 1000mg/day (Lichtenstein, A. H., 2000). Using food duplicate technology, considered the gold standard, total CLA intakes are reported to be between 212 and 151mg/day for men and women.

1.2.2 CLA and atherosclerosis

1.2.2.1 *In vivo* research

There is a growing body of evidence that CLA reduces atherosclerotic plaque formation in experimental animals. When CLA (0.5g/rabbit/day) was added to a hypercholesterolemic diet and fed to rabbits for 12 weeks, serum triglycerides and low density lipoprotein cholesterol levels were significantly reduced compared with rabbits fed a diet without CLA (Lee *et al*, 1994). Importantly for heart disease risk, aortas of rabbits fed the CLA-containing diet exhibited less atherosclerotic plaque formation. In a subsequent study, hamsters were fed a diet with or without CLA designed to induce hypercholesterolemia (Nicolosi *et al*, 1997). The diet with CLA (1.0%) reduced plasma total cholesterol, non-high density lipoprotein-cholesterol, and early aortic atherosclerosis relative to a diet with linoleate (Wilson *et al*, 2000). In a similar model in hamsters fed a hypercholesterolemic diet, c9t11-CLA, the sole CLA isomer in the diet, had no effect on plasma lipids (Gavino, 2000). Because dietary CLA was associated with significantly reduced formation of dienes, it was concluded that the ability of CLA to reduce aortic plaque formation could be due to changes in low density lipoprotein oxidative susceptibility. In contrast to protective effects of CLA on atherosclerotic plaque formation in rabbits and hamsters, CLA inducted the formation of aortic fatty acid streaks in C57Black/6 mice (inbred atherosclerotic inducible mouse model) fed an atherogenic diet (Munday *et al*, 1999), suggesting a possible species specific effect.

The effects of CLA on thrombotic properties of blood cells have been studied in cultured platelets *in vitro* and in human subjects. In cultured platelets, CLA, c9t11-CLA, and t10c12-CLA inhibited arachidonic acid-, or collagen-, induced platelet

aggregation (Truitt *et al*, 1999). These findings were associated with reduced production of the proaggregatory cyclooxygenase products of ^{14}C-arachidonate, ^{14}C-thomboxane-A2, and ^{14}C-thromboxane-B2. In human subjects supplemented with CLA (3.9g/day) or placebo (sunflower oil) for 93 days, there were no differences in platelet aggregation or prothrombin time (Benito *et al*, 2001).

Because CLA appears to exert differential effects on lipid profiles as well as atherogenic markers in various animal models, further work is needed to demonstrate the mechanisms by which CLA may prevent atherosclerosis and to elucidate its role in modulating cardiovascular disease risk in humans.

1.2.2.2 *In vitro* research

There have been very few *in vitro* studies carried out to elucidate the cellular mechanisms by which CLA exerts it anti-atherosclerotic effects. The decrease in LDL cholesterol observed in the animal studies mentioned above, is consistent with both decreased apolipoprotein B secretion, observed in Hep G2 cells (Yotsumoto *et al*, 1999), and decreased intracellular triacylglycerol, observed in mouse adipocytes (Park *et al*, 1999). With regard to atherogenesis specifically and effects on vascular cells, little has been published. It has been shown that CLA affects arachidonic acid metabolism in human saphenous endothelial cells, inhibiting eicosanoid production (Urquhart *et al*, 2001) This suggests an anti-inflammatory action that may contribute to CLAs anti-atherogenic properties.

1.2.2.3 Proposed mechanisms behind CLAs anti-atherosclerotic properties

There are a number of hypotheses to explain the mechanisms of action of CLA. Originally, CLA was thought to protect LDL from oxidation because it was a more potent antioxidant than α-tocopherol (Ha *et al*, 1990). This theory was rejected though because it was found that CLA could not effectively protect membranes from oxidative modification under conditions of metal ion-dependant oxidative stress (Van den Berg *et*

al, 1995). Although CLA may not function as a true antioxidant, it has been found that auto-oxidation of CLA produced furan fatty acids, which may have the potential to protect against oxidant-mediated toxicity (Yurawecz *et al*, 1995).

CLA is incorporated into cell membrane phospholipids (Ha *et al*, 1990). This gives rise to the possibility that it may modify membrane fluidity and exert its effects by altering intracellular events like signal transduction pathways or eicosanoid synthesis. It has already been suggested that CLA alters eicosanoid synthesis, competing with linoleic acid and inhibiting eicosanoid production (Urquhart *et al*, 2001). Another study suggests that various isomers of CLA can be precursors for eicosanoid synthesis (Sebedio *et al*, 1997) as they are elongated and desaturated in a manner similar to linoleic acid. It thus seems possible that CLA derived eicosanoids may be produced. This could affect a number of pathways involved in lipid metabolism. There is also the potential for CLA to affect intracellular lipid metabolism. It has been shown that hepatic stearoyl coenzyme A desaturase mRNA expression is decreased by CLA (Lee *et al*, 1998), and that this down regulation is specific for the t10, c12 isomer (Choi, Y. *et al*, 2000). This enzyme is responsible for desaturating palmitic and stearic acids to palmitoleic and oleic acid, respectively.

Also, it has been established that CLA is both a ligand and an activator for the peroxisome proliferator-activated receptor α (Moya-Camarena *et al*, 1999). This nuclear transcription factor modulates gene expression for several enzymes and proteins involved in lipid metabolism, including lipoprotein lipase (Schoonjans *et al*, 1996), fatty acid binding protein (Sterschele *et al*, 1994) and acyl coenzyme A oxidase (Belury *et al*, 1997). Also different CLA isomers have different potencies for peroxisome proliferator-activated receptor α (Moya-Camarena *et al*, 1999).

1.3 Homocysteine

Homocysteine (Hcy) is a nonprotein-forming, thio-containing amino acid formed by demethylation of methionine. It is metabolised by remethylation to methionine or by transsulfuration to cysteine.

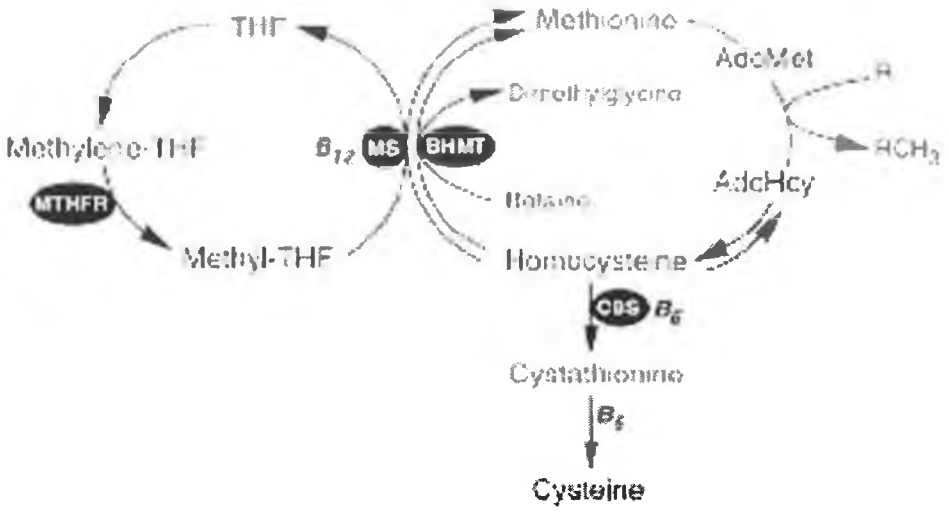


Figure 1.3 Homocysteine metabolism. AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; B6, vitamin B6 (pyridoxal phosphate); B12, vitamin B12 (methylcobalamin); CBS, cystathionine b-synthase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; THF, tetrahydrofolate.

The hypothesis that homocysteine is atherogenic was put forward more than 30 years ago by Kilmer McCully, who observed vascular lesions in children with inherited disorders of methionine metabolism (homocystinuria) (McCully, 1969). Homocysteine is now considered by many as a major and independent risk factor for atherosclerotic vascular disease (Boers, 2000).

1.3.1 Metabolism of Homocysteine

Homocystinuria is most commonly caused by a genetic deficiency for the enzyme cystathionine b-synthase (CbS). Individuals homozygous for this condition are unable to convert homocysteine to cystathionine, leading to high levels of circulating homocysteine, this in turn results in excretion of homocysteine in the urine. Homozygosity for the enzyme methylenetetrahydrofolate reductase (MTHFR) and, less commonly, methyltransferase deficiency also result in homocystinuria, causing skeletal and ocular defects and premature vascular events. Under normal conditions, homocysteine concentration is maintained at low levels by the enzymes CbS and MTHFR, and is removed from the body by remethylation to methionine, or trans-sulphuration to products that are cleared in the urine. Remethylation occurs by the action of the enzyme MTHFR with folate as a co-substrate, and by methionine synthase with vitamin B12 as a co-factor. Trans- sulphuration requires CbS, which uses vitamin B6 as a cofactor. Normal levels of fasting plasma homocysteine are considered to be between 5 and 15 μM. Moderate, intermediate and severe increases in the concentration of homocysteine refer to levels of 16-30, 31-100 and more than 100 μM respectively. Only a small fraction (<2%) of plasma total homocysteine circulates in the thiol form. The remainder is a mixture of disulfide derivatives, including homocystine, homocysteine-cysteine mixed disulfide, and protein bound disulfides (Mudd *et al*, 2000).

Moderate increases in homocysteine levels may result from a combination of genetic and dietary factors. High homocysteine levels can occur as a result of dietary vitamin deficiency without the genetic defects that cause homocystinuria (Miller *et al,* 1994). Dietary deficiencies in vitamin B12 and folic acid produce milder increases in plasma homocysteine concentration than found in homocystinuria, and dietary intake and plasma levels of these vitamins are inversely related to levels of plasma homocysteine (Rimm *et al*, 1998). Homocysteine levels begin to rise at plasma levels of folate, vitamin B12 and vitamin B6 that are regarded as within the low normal range (Selhub *et al*, 1993). Folic acid and vitamin B12 protect against the build-up of homocysteine in the blood by conversion to methionine. Vitamin B6 also prevents the accumulation of

homocysteine by converting it to cysteine and other compounds excreted in urine. Supplementation of diet with vitamins is now seen as an effective therapy for hyperhomocysteinaemia.

1.3.2 *In vitro* research

In vitro experimental research suggests that an increase in homocysteine concentration may cause atherosclerosis by a combination of endothelial injury (Woo *et al*, 1997) smooth muscle proliferation (Tsai *et al*, 1994; Tang *et al*, 1998), platelet activation and thrombogenesis (Den Heijer *et al*, 1996; Rodgers and Kane, 1986; Rodgers and Conn, 1990). Oxidative metabolism of homocysteine may play a role in endothelial dysfunction (Loscalzo, J., 1996). Oxidation of homocysteine stimulates the production of reactive oxygen species, such as super oxide anion, hydrogen peroxide and hydroxyl radical, all of which create a potent oxidative stress (Andersson, 1995; Misra, 1974).

Homocysteine interacts with nitric oxide to produce S-nitrosohomocysteine at physiological concentrations, which amplifies the vasoprotective effects of nitric oxide. Normal endothelial cells detoxify homocysteine by releasing nitric oxide, which in turn leads to the formation of S-nitrosohomocysteine (Stamler *et al*, 1993). This reaction decreases the production of hydrogen peroxide following oxidation of sulphydryl groups and represents a protective mechanism against the adverse effects of homocysteine. Although prolonged exposure and high homocysteine concentrations result in impaired nitric oxide production. Therefore chronically elevated homocysteine concentrations appear to cause a self-perpetuating cycle that eventually overwhelms the capacity of the endothelial cells to reduce the toxicity of homocysteine (Stamler *et al*, 1993).

These *in vitro* studies imply that homocysteine at high concentration may cause many of the pathological processes associated with atherosclerosis. The problem with many of these studies however is that the homocysteine concentrations used were in the pharmacological range (up to 1 mM) and not relevant to the levels present physiologically. The suggested effects proposed by the studies must, therefore, be interpreted with caution.

It is well know that endothelial damage has an adverse effect on flow mediated dilatation of large arteries, an endothelium dependent mechanism which is believed to be an early indicator of the atherosclerotic process. In a study by Woo *et al*, (1997) impairment of brachial artery endothelium-dependent dilatation in patients with raised homocysteine levels (mean(s.d.) 34.8(8.5) μM) was demonstrated. Although there were only 14 participants in the study, endothelial dysfunction was demonstrated in vivo at clinically relevant concentrations.

Vascular smooth muscle cell proliferation with subsequent formation of extra cellular matrix collagen is an important feature of atherosclerosis (Ross, 1999). Homocysteine has been shown to increase DNA synthesis with subsequent smooth muscle proliferation (Tsai *et al*, 1994; Tang *et al*, 1998) in a dose-dependent manner at plasma concentrations ranging from 25 to 500 μM.

The vascular endothelium maintains a non-thrombogenic surface through the antithrombin III and thrombomodulin-protein C anticoagulant pathways (Williams, 2001). Treatment of endothelial cells with homocysteine induces enhanced factor V activity (a procoagulant factor) (Rodgers and Kane, 1986) and reduced production of protein C (Rodgers and Conn, 1990). The concentrations used (up to 1 mM) were again very high compared to pathophysiological levels, although a study has shown that moderate increases in plasma homocysteine level is a risk factor for deep vein thrombosis (Den Heijer *et al*, 1996).

Leukocyte adhesion to the endothelium is an important part of the process of atherogenesis (Ross, 1999). The build up of leucocytes in inflamed tissues is caused by adhesive interactions with endothelial cells in the microcirculation (Granger and Kubes, 1994). A study by Pruefer *et al*, (1999) has shown increased adhesion and trans-endothelial migration of leucocytes when the mesentery of rats was perfused with plasma concentrations of homocysteine ranging from 1 to 5 μM. Immunohistochemical staining demonstrated significantly increased P-selectin and intercellular adhesion molecule I (ICAM) expression on the intestinal venules after homocysteine perfusion. These results were not supported by work from Dudman and Hale, (1997) who found

no increase in ICAM-1 expression on the endothelium following treatment with homocysteine. The possibility that homocysteine affects adhesion indirectly by activating leucocytes, which then interact with and cause pathological changes in the vascular endothelium, was then investigated. Endothelial-leukocyte interaction was seen to be mediated by the neutrophil-docking protein complex CD11b-CD18 (Dudman *et al,* 1999). Despite the impressive list of atherosclerotic effects of homocysteine, its biological relevance remains questionable. The effects described above occur only at concentrations of homocysteine in the pharmacological range. It is uncertain how lower concentrations might be associated with an increased risk of atherosclerotic disease.

1.3.3 *In vivo* research

Even though there is a lot of *in vitro* research to support a link between raised homocysteine concentration and atherosclerosis, clinical studies have produced mixed results. A number of prospective studies analysing the link between homocysteine and cardiovascular disease have been published (Nygard *et al*, 1997; Bostom *et al*, 1997; Wald *et al*, 1998). Verhoef *et al*, (1997) observed almost no association between plasma homocysteine level and risk of angina pectoris with subsequent coronary artery bypass surgery. In a study carried out by Folsom *et al*, (1998) it was found that fasting total homocysteine concentration was correlated positively and strongly at high levels with the age-, race- and centre-adjusted incidence of coronary heart disease in women, but not in men. Adjustment for other risk factors, however, abolished this association. This suggests that total homocysteine concentration was not independently associated with coronary heart disease. The Physicians' Health Study, after a 5-year follow-up, showed that plasma homocysteine levels above the 95^{th} percentile of the control distribution were associated with a 3.4-fold increased risk of myocardial infarction, however, after the 7.5 year follow up, the relative risk was only 1.7 and this was no longer statistically significant (Stampfer *et al*, 1992). This lack of association may be explained by a variation in homocysteine levels within subjects over time, which may have attenuated the effect. This result, however, questions the role of homocysteine in atherosclerotic disease.

Associations are described between plasma homocysteine concentration and carotid artery intimal-medial wall thickening (Malinow *et al*, 1993) and the prevalence of carotid artery stenosis (Selhub *et al*, 1995). Verhoef *et al*, (1994) in a sample of patients taken from the Physicians' Health Study, studied 109 patients who developed an ischaemic stroke over a 5-year period. They found no association between increased plasma homocysteine level and the risk of ischaemic stroke. A prospective study carried out in Finland examined the association between homocysteine and atherosclerotic disease in middle-aged men and women, the results showed no association with myocardial infarction or stroke (Alfthan *et al*, 1994).

Conflicting evidence was reported in a retrospective study (Perry *et al*, 1995), which showed a strong independent association between homocysteine level and stroke in a sample of middle-aged British men. This was consistent with the theory that raised homocysteine concentration caused stroke. In a study carried out by Taylor *et al*, (1999) it was reported that an increase of 1 μM in plasma homocysteine concentration was associated with a 5.6 per cent increased likelihood of death from vascular disease. After they carried out a retrospective study, although a similar age of onset of symptoms was noted in patients with raised and normal homocysteine levels, a significant progression in lower-limb occlusive disease and coronary artery disease was found in those with raised homocysteine levels. No such association was noted for cerebrovascular disease (Taylor *et al*, 1991). They followed this work with a prospective study of 351 patients with symptomatic peripheral arterial disease. No statistically significant relationship between decreasing ankle: brachial pressure index and increasing carotid stenosis was found in patients with increased homocysteine levels (Taylor *et al*, 1999).

Although some of the studies published do conflict, the consensus is that there is an undeniable link between raised homocysteine and cardiovascular disease (Eikelboom *et al*, 1999).

1.4 Objectives of this study

The objective of this study was to investigate whether both CLA and/or homocysteine modulate the expression and/or activity of two distinct vascular endothelial cell (EC) components; Nitric oxide synthase (eNOS) and Cox. Both eNOS and Cox, responsible for production of nitric oxide and prostacyclin respectively, have been strongly implicated in the endothelial maintenance of vascular hemodynamic mechanisms.

The experiments were designed to examine the effects of chronic treatments (up to 24) on the expression of eNOS and Cox I protein and steady state RNA. Nitrite and $PGF_{1\alpha}$ levels in the conditioned were assayed as an index of eNOS and Cox I activity, after acute (up to 180 min) and chronic (up to 72 hours) treatment.

Chapter 2 Materials and Methods

2.1 Materials

AGB Scientific (Dublin, Ireland)
Whatmann Chromatography paper

Alexis Biochemicals (Nottingham, UK)
2,3-diaminonapthalene

Amersham Pharmacia Biotech (Buckinghamshire, UK)
Anti-mouse 2° antibody, HRP conjugated
Anti-rabbit 2° antibody, HRP conjugated
ECL Hybond nitrocellulose membrane
ECL Hyperfilm
Rainbow molecular weight marker, broad range (6-175kDa)

Assay Designs (Michigan, USA)
EIA kit for 6-keto-Prostaglandin $F_{1\alpha}$

BioRad (Alpha Technologies, Dublin)
Bio-Dot SF Micro filtration apparatus

Bio Sciences Ltd (Dun Laoghaire, Ireland)
DMEM
dNTP's
DEPC-treated water
Trizol® reagent

Cayman Chemical Company (Michigan, USA)
Cox I monoclonal antibody
eNOS polyclonal antibody
Corriell Cell Repository (NJ, USA)

Bovine Aortic Endothelial Cells (BAECs)

MWG (Cork, Ireland)
Cox I primer set
Cox II primer set
eNOS primer set
β-actin primer set
GAPDH primer set

Nu-Chek-Prep Incorporated (Elysian, MN)
A preparation of mixed CLA was used in this study. The isomer composition was as follows; t9/c11-CLA + c9/t11-CLA (41%), c10/t12-CLA (44%), t10/t12-CLA (10%), t9/t11-CLA + c9/c11-CLA + c10/c12-CLA (5%).

Single isomers of CLA 29.5% c 9/ t 11 (18:2) and 29% t10/c 12 (18:2) were also obtained.

PALL Corporation (Dun Laoghaire, Ireland)
Biotrace nitrocellulose membrane

Pierce Chemicals (Cheshire, UK)
BCA protein assay kit
Supersignal West Pico chemilumescent substrate

Promega (UK)
Taq DNA Polymerase
MLV-RT
RNase H
Oligo dT

Sarstedt (Drinagh, Wexford, Ireland)

T25 tissue culture flasks

T75 tissue culture flasks

T175 tissue culture flasks

6-well tissue culture plates

5,10 and 25ml serological pipettes

15 and 50ml falcone tubes

Sigma Chemical Company (Poole, Dorset, England)

β-glycerophosphate

2-mercaptoethanol

Acetic Acid

Acetone

Agarose

Ammonium Persulphate

Bisacrylamide

Bovine Serum Albumin

Brightline Haemocytometer

Bromophenol blue

Chloroform

Calcium Ionophore A23187

DL-Homocysteine

EDTA

EGTA

Ethidium Bromide

Fetal Calf Serum

Glycerol

Glycine

Hanks Balanced Salt Solution

Hydrochloric acid

Isopropanol

Leupeptin

Methanol

Mineral oil (molecular grade)

Penicillin-Streptomycin (100x)

Ponceau S

Potassium Chloride

Potassium Iodide

Potassium Phosphate (Dibasic)

RPMI-1640

SDS

Sodium Chloride

Sodium Hydroxide

Sodium Nitrite

Sodium Orthovanadate

Sodium Phosphate

Sodium Pyrophosphate

TEMED

Tris Acetate

Tris Base

Tris Cl

Triton X-100

Trypsin-EDTA solution (10x)

Tween 20

2.2 Methods

2.2.1 Preparation and Storage of CLA stocks

A synthetic source of CLA isomers obtained from Nu-chek Inc. was used in this study. The mixture contained primarily 29.5% 9c,11t(18:2) and 29% 10t, 12c (18:2) CLA isomers but also contained trace amounts of other isomers. Individual purified solutions of 29.5% 9c,11t(18:2) and 29% 10t, 12c (18:2) CLA isomers were also used. Stock solutions of 100 mg/ml were made up in 95% ethanol (EtOH) and were stored at –20°C. Working stock dilutions were made up at concentrations of 10,000 and 2000 μg/ml and also stored at –20°C. Dilutions were made in RPMI 1640 tissue culture media to give treatment concentrations of 0-15 μg/ml (0-53 μM).

2.2.2 Cell culture Techniques

All cell culture was carried out in a sterile environment using a Bioair instruments aura 2000 MAC laminar airflow cabinet. Cells were visualized with an Olympus CK30 phase contrast microscope.

2.2.2.1 Culture of Bovine Aortic Endothelial Cells (BAEC)

The BAEC cell line (Corriell Cell Repository-NJ, USA) used in the study was maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin). Cells were cultured in 25cm^2 or 75cm^2 tissue culture flasks or 6 well plates. Only cells between passage 8 and 15 were used for experiments. Trypsination was carried out remove cells from the flask for passing. For trypsination, the media was removed and cells were washed in Hanks Balanced Salt Solution (HBSS) twice. For 25 cm^2 flask 1ml of 1X trypsin ethylenediamine tetracetic acid (EDTA) (10% v/v trypsin EDTA was made up with HBSS) was added to the flask. The flask was then incubated at 37°C for 5 minutes or until all the cells had detached from the surface. 10ml of growth medium was then added to the flask to inactivate the trypsin. The cell suspension was then removed from the flask and spun down in a centrifuge at 5000 xg for 5 minutes. The supernatant was then removed and cells resuspended in fresh growth medium. For routine passing of cells dilutions of 1:3 to 1:8 were made depending on when confluent cells were needed

again. Typically, 2 ml of the 10 ml cell suspension (1:5 dilution) was mixed with 13 ml of fresh growth medium and poured into a 75 cm^2 tissue culture flask. The cell line was incubated in a humid, 5% v/v CO_2 atmosphere at 37°C using a Hera water jacketed cell culture incubator.

2.2.2.2 Cell counts

Cell counts were carried out using a Sigma brightline haemocytometer. For experiments cells were seeded into 6 well plates and 25 cm^2 tissue culture flasks at densities of 10,000 to 50,000 cells per cm^2, depending on when confluent cells were needed. Briefly, a drop of the cell suspension was used to fill the counting chamber of the haemocytometer. The number of cells in each of the four quadrants was counted and averaged, the number of cells in the suspension was then calculated as follows,

The number of cells per millilitre = Number of cells counted per quadrant X dilution (if used) X 10,000

2.2.2.3 Storage of BAEC in liquid nitrogen

For long term storage the BAEC were maintained in liquid nitrogen in a cryofreezer unit. Cells to be stored were trypsinised and centrifuged as described in section 2.2.2.1. The pellet of cells was then resuspended in 1ml of freezing media (10% FCS, 1% DMSO) and then transferred to a sterile cryotube. The cell suspension was then graduately frozen in a Nalgene cryo freezing container, which was placed in a –80°C Freezer, at a rate of –1°C/min. After one night in the –80°C the cryovials were then transferred to the Thermoylen locator jr. cryostorage system. Cells were recovered from liquid nitrogen by thawing rapidly at 37C and then transferred to a sterile falcone tube containing 10 ml of fresh growth media. The cells were then spun down in a centrifuge at 5000 xg for 5 minutes. The supernatant was then removed and the cells resuspended in 15 ml of fresh growth media. The cell suspension was then transferred to a 75 cm^2 tissue culture flask and incubated in the Hera CO_2 incubator. These cells were then passed twice, to ensure complete recovery, before they were used in an experiment.

2.2.3 Preparation of whole cell lysates

Growth media was removed and cells were washed in HBSS twice. The cells were then harvested using a cell scraper into 5ml of HBSS. The cell suspension was then spun down in a centrifuge at 5000 xg for 5 minutes. The supernatant was removed and the cells were resuspended in 50-100 µl of 1X lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM B-glycerophosphate, 1 mM sodium orthovanadate, 1 µg/ml Leupeptin). The lysates were then frozen and thawed three times and then subjected to ultrasonication using a sonic dismembrator. Samples were then stored at –80°C prior to use for western blot analysis.

2.2.4 Bicinchoninic acid (BCA) protein microassay

This method combines the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the selective colorimetric detection of the cuprous cation using a reagent containing bicinchoninic acid. This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations over a broad working range of 20-2,000 µg/ml. The two separate reagents used were supplied in the commercially available assay kit (Pierce Chemicals): A, an alkaline bicarbonate solution and B, a copper sulphate solution. A working solution was prepared by mixing 1 part reagent B with 50 parts reagent A. On a microtitre plate 200 µl of the working solution was added to 2µl of the whole cell lysate or Bovine serum albumin (BSA) protein standard. The plate was then incubated at 37°C for 30 min. The absorbance of each well was then read at 560 nm using a micro plate reader. All samples and standards were tested in triplicate. Quantitation was carried out by interpolation from a BSA standard curve (0 – 10 µg/µl).

2.2.5 Preparation of total RNA from BAEC

Total RNA was isolated from BAEC using Trizol® reagent according to the method of Chomczynski and Sacchi (1987). The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is a modification on the isolation method developed by Chomcznski and Sacchi. Trizol reagent maintains the integrity of RNA while disrupting cells and dissolving cell components. Growth media was removed and cells were washed with HBSS twice. Cells were then lysed directly by adding trizol reagent to the flask, 1 ml per 10 cm^2. The lysate was transferred to a falcone tube and incubated for 5 minutes at 15°C to 30°C to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform per 1 ml of trizol was then added, the tube was then shaken vigorously for 15 seconds and then spun down at 12,000 xg for 15 min at 2°C to 8°C. The resulting aqueous phase was then transferred to a fresh tube. RNA was then precipitated by mixing with isopropyl alcohol, 0.5 ml per 1 ml of trizol. The samples were then incubated at 15°C to 30 °C for 10 min and spun down at 12,000 xg for 10 min at 2 to 8 °C. The RNA was then visible as a gel like pellet on the side and bottom of the tube. The supernatant was then removed and the pellet washed with at least 1ml of 75% ethanol per 1 ml of trizol used. The sample was then mixed by vortexing and spun down at 7,500 xg for 5 minutes at 2 to 8°C. The supernatant was removed and the pellet washed again in ethanol. After washing the pellet was air dried and the RNA resuspended in 30-50µl of RNase free water. All total RNA preparations were stored at -80 °C.

2.2.6 Quantitation of total RNA in samples

Quantitation of total RNA in samples was carried out by absorption spectroscopy. The absorption of the sample was measured at several different wavelengths to assess purity and concentration. Briefly, 2 µl of the sample was added to 1000 µl of Tris EDTA (TE) buffer (10 mM TrisCl, 1 mM EDTA, made up in RNase free water pH 7.4). The absorbance was then read using matched quartz quvettes, and using TE buffer as a blank. By measuring absorbance at 280 nm and 260 nm, purity was estimated using the A_{260}/A_{280} ratio. A ratio of 1.9 to 2.0 was indicative of a highly purified preparation of RNA. A ratio lower than this was indicative of protein contamination. Absorbance at 230 nm reflected contamination of the sample by phenol, while absorbance at 325 nm

suggests contamination by particulates or a dirty cuvette. The concentration of RNA in the sample was determined using the A_{260} reading as follows:

Concentration of single stranded RNA μg/ml = A_{260} x Dilution factor x 40

All samples were tested in triplicate and were kept on ice at all time during the experiment.

2.2.7 Design of PCR primer sets

A web based program called multialin was used to design the primer sets used in this study "Multiple sequence alignment with hierarchical clustering" (Corpet, 1988). This program allows alignment of sequences from a number of different species so primers can be designed from highly conserved areas. Primers were designed with ~50% GC content so the annealing temperature for all sets was ~55°C.

Cox I primer set, from bovine sequence fragment size: 650 base pairs
Forward primer 5' - AAT TCC AGT ACC GCA ACC - 3'
Reverse primer 5' - AGG TCT TGG TGT TGA GGC A - 3'

Cox II primer set, designed by consensus (rat, mouse and human sequences)
fragment size: 280 base pairs
Forward primer 5' - TTT GAA GAA CTT ACA GGA G - 3'
Reverse primer 5' - TAT TGC AGA TGA GAG ACT GA - 3'

eNOS primer set, designed from bovine sequence fragment size: 300 base pairs
Forward primer 5' - GCT TGA GAC CCT CAG TCA GG - 3'
Reverse primer 5' - GGT CTC CAG TCT TGA GCT GG - 3'

β-actin primer set, designed from mouse sequence fragment size: 550 base pairs
Forward primer 5' - ATC CTG CGT CTG GAC CTG GCT - 3'
Reverse primer 5' - CTT GCT GAT CCA CAT CTG GTG - 3'

GAPDH primer set, designed from bovine sequence fragment size: 341 base pairs

Forward primer 5' - TCC TGC ACC ACC AAC TGC TT - 3'

Reverse primer 5' - TGC TTC ACC ACC TTC TTG AT - 3'

2.2.8 Dot blot immunoassay

A dot blot apparatus was used to immobilize protein from whole cells lysates onto nitrocellulose membranes. The technique was used to optimise primary antibody concentrations for western blot analysis. A Bio-Dot SF Micro filtration apparatus (170-6542) from Bio-Rad was used for all dot blots. The dot blot procedure was carried out as follows: The Bio-Dot SF apparatus and gasket was cleaned and dried prior to assembly. The apparatus was assembled as described in the manufactures instruction manual. Three sheets of Whatmann chromatography paper were wetted in PBS. Nitrocellulose membrane was pre-wetted in distilled water and then wetted in PBS. The filter paper and membrane were then placed into the apparatus as described in the instruction manual. The four screws were then tightened in a diagonal crossing pattern and under vacuum to ensure there is no cross-well contamination. The membrane was rehydrated with 100 µl PBS per well. The flow valve was then adjusted so that the vacuum chamber was open to the atmosphere. Each well was filled with 200 µl of protein solution (BAEC whole cell lysate) to give a final amount of protein of 10 µg. The samples are then allowed to filter through the membrane by gentle vacuum. Each sample well was then washed with at least 200 µl of PBS. The wash liquid was then pulled through by applying gentle vacuum. After the wells were completely drained, the membrane was removed from the apparatus. The membrane was then blocked and incubated in primary and secondary as described in section 2.2.9.4. there was one variation, the dilutions of primary antibodies were numerous. The sample wells were cut into seven sections and incubated with seven different sections of primary antibody. Development and detection of the blot was carried out as described in section 2.2.9.5.

2.2.9 SDS PAGE and Western blot analysis

2.2.9.1 Sample preparation

An aliquot of the sample, giving a concentration of 25 µg protein, to be analysed was mixed 1:4 v/v with 4x Sample Solubilisation Buffer (SSB) (4 g Sodium dodecyl sulfate, 20 ml glycerol, 2 ml β mercaptoethanol, 0.04 g Bromophenol blue, 24 ml 0.25 M TrisCL, made up to 50 ml, pH to 6.8) and made up to 50 µl with lysis buffer in a sealed screw-cap micro centrifuge tube. The sample was then heated to 100^0C for 5 minutes. The sample was then placed on ice until ready to load onto gel. Prepared in this way there was enough volume to load two wells, 20 µl, or 10 µg, per well.

2.2.9.2 Preparation and running of gel

An Atto AE-6450 Dual Mini Slab Kit was used for all protein electrophoresis carried out during the study. Before running the gel, the apparatus needed to be set up and reagents made up. This was carried out as follows. All gel cast parts were cleaned with ethanol prior to use. The gel cast was prepared as described in the manufacturers operating instructions. Stock solutions of 4% (stacking), 7% (resolving) and 12% (resolving) unpolymerised Bis Acrylamide were made up and stored at 2-8^0C. As an example, 250 ml of the 4% stacking gel solution was made up as follows:

10% w/v Sodium dodecyl sulphate	-	2.5 ml
Stacking gel buffer (0.5M Tris/HCL, pH 6.8)	-	62.5 ml
Acrylamide/ Bis Acrylamide (40% Soln. 29:1 Ratio)	-	24.375 ml
Ultra pure water	-	160.625 ml

An aliquot of the resolving gel stock solution (10 ml per gel) was mixed with 50 µl of freshly prepared 10% Ammonium persulphate and 15 µl of TEMED, this was then poured into the gel cast. A few drops of water saturated butanol was then added to remove any bubbles, the gel was then allowed to polymerise for 10-20 minutes. When set the unpolymerised liquid was poured off. The stacking gel was prepared in the same manner. This was then poured onto the resolving gel, the 12-well comb was then put in place and again the gel was allowed to polymerise for 5-10 minutes. The chamber was then half filled with reservoir buffer (25 mM Tris, 192 mM Glycine, 0.1% Sodium

dodecyl sulphate). When the gel set, the comb, clips and casket was removed and the gel cast was clipped into the electrophoresis chamber. The chamber was then filled with reservoir buffer and the lanes flushed out to remove unpolymerised Bis Acrylamide. The lanes were then loaded with samples. The gel was then run at 200 V, 90 mA and 150 W until the dye front had migrated to the end of the gel.

2.2.9.3 Semi-dry electroblotting

An Atto AE-6675 semi-dry blotting apparatus was used for all electroblotting during this study. When the gel had finished running, it was removed from the cast and the stacking gel was removed from the resolving gel. The resolving gel was then soaked in semi-dry transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 5 minutes. Two stacks of 10 sheets of Whatmann chromatography paper was cut to exactly the same size of the resolving gel (9x 6 cm^2). The chromatography paper was also soaked in semi-dry transfer buffer. A sheet of PALL BioTrace nitrocellulose membrane was cut to the same size of the resolving gel (9x 6 cm^2) and pre-soaked in ultra pure water, then soaked in semi-dry transfer buffer. The transfer stack was set up as follows: 10 sheets of Whatmann chromatography paper, the nitrocellulose membrane, the resolving gel and finally 10 sheets of Whatmann chromatography paper. A pen was rolled over the stack to remove any air bubbles. The apparatus was then closed and run at 100 V, 600 mA, 150 W for 45 minutes.

2.2.9.4 Ponceau S staining, blocking and antibody incubations

When the transfer was finished the nitrocellulose membrane was removed and stained in Ponceau S for 5 minutes. The membrane was then rinsed briefly in ultra pure water to remove any background staining. The membrane was placed between 2 acetates and an image was taken with an Epson perfection 1200S scanner. The image was saved and used to asses equality of protein loading across the gel. Then membrane was then destained in Phosphate buffered saline-Tween 20 (PBS-T20) (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) until all Ponceau S stain was removed.

The membrane was then blocked in a 5% Bovine serum albumin solution (BSA) (5g of BSA in 100 ml of PBS-T20) for 2 hours at room temperature on an orbital shaker. The

membrane was then washed for 2x2 minutes in PBS-T20. The primary antibody was diluted in a 2.5% BSA solution. The dilution factor for each antibody was determined empirically. The membrane was incubated in primary antibody for 3-4 hours at room temperature on an orbital shaker.

The membrane was then washed 3 x 10 minutes in PBS-T20. The secondary antibody was diluted in a 2.5% BSA solution. The membrane was incubated in secondary antibody (conjugated to horse radish peroxidase) for 3 - 4 hours at room temperature on an orbital shaker. The membrane was then washed 3x10 times in PBS - T20. Excess wash buffer was drained from the membrane by holding gently in a forceps and touching the edge against a tissue.

2.2.9.5 Detection and development of blot

Supersignal west pico chemilumescent substrate from Pierce was the detection reagent used to develop western blots carried out in this study. This detection system employs a mix consisting of equal volumes of enhancer solution and stable peroxide solution. This solution is a highly sensitive enhanced chemiluminescent substrate for the development of utilising a horseradish peroxidase (HRP) label.

The detection reagents were removed from storage at 2-8^{0}C and allowed to equilibrate to room temperature before opening. An equal volume of reagent A was mixed with reagent B, usually a total of 1 ml for one membrane. The membrane was placed, protein side up, on an acetate and the detection reagent place on top ensuring the whole membrane was covered. A second acetate was then placed on top. The membrane was incubated for 10 minutes at room temperature. The excess detection reagent was then drained off by holding gently in a forceps and touching the edge against a tissue. The blot was placed down on to a fresh piece of cling film and wrapped up, any air bubbles were gently removed.

In the dark room, the wrapped blot was placed, protein side up in an x-ray film cassette. A sheet of Amersham Hyperfilm ECL auto radiography film was placed on top of the membrane, the cassette was closed and the film was exposed for between 10 seconds

and 5 minutes depending on the antibody. The film was then removed and developed in an Amersham hyperprocessor automatic developer.

2.2.10 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

2.2.10.1 Reverse transcriptase

All total RNA samples were prepared by the Trizol® method as previously described in section 2.2.5. RNA preparations were then quantified by absorbance spectroscopy as described in section 2.2.6. Reverse transcriptase was carried out using Promega Murine Leukemia Virus Reverse Transcripase (MLV RT) as follows. The initial amount of RNA used for each primer set was determined empirically to ensure a semi quantitative analysis section . The initial amount of RNA used for each primer set can be seen in Table 2.2.10.2. For the purpose of this protocol 2 μg of RNA was transferred to an RNase free microcentrifuge tube. To this 0.5 μg of Promega Oligo dT primer was added. This ratio of amount of RNA to Oligo dT remained constant for each primer set used. The volume was then made up 15 μl with RNase free water. The tube was heated to 70^0C for 5 minutes to melt secondary structure within the template. The tube was cooled immediately to prevent secondary structure from reforming. The following components were added to the annealed primer/template in the order shown:

MLV 5x Reaction buffer	5 μl
dATP, 10 mM	1.25 μl
dCTP, 10 mM	1.25 μl
dGTP, 10 mM	1.25 μl
dTTP, 10 mM	1.25 μl
MLV-RT	200 units

Make up to a total of 25 μl with RNase free water

The components were mixed by gently flicking the tube. The mix was then spun down in a microfuge and incubated for 60 minutes at 42^0C. 1 μl of RNase H (2 units/ μl). The mix was then incubated for 20 minutes at 37^0C. A negative control was also carried out with every RT reaction. The control contained no reverse transcriptase, any

amplification by RT PCR from the negative control was indicative of genomic DNA contamination. All RT samples were stored at -80^{0}C until needed for PCR.

2.2.10.2 Polymerase Chain Reaction (PCR)

All PCR was carried out using Promega Taq DNA Polymerase. A PCR mix was made up for each sample to be amplified. The mix was made up as follows:

RNase free water	36.5 µl
Buffer 10x	5 µl
dNTP 10mM	1 µl
$MgCl_2$ 25mM	3 µl
Forward primer 10µM	1 µl
Reverse primer 10µM	1 µl
Taq Polymerase 2-5u/µl	0.5 µl
RT sample	2 µl
Mineral oil	50 µl

When the reaction mixture was made up it was placed in the PCR thermocycler. All PCR was carried out in a Hybaid PCR thermocycler (SPRT 001). The program used was optimised for each primer set and is summarized in Table 2.2.10. For the purpose of this protocol the program was as follows:

94^{0}C – 3 minutes
94^{0}C – 1 minute
55^{0}C – 2 minutes
72^{0}C – 3 minutes
30 cycles
4^{0}C – Hold

When finished the samples were stored at –80^{0}C until needed for agarose gel electrophoresis.

Table 2.2.10.2: A summary of the empirically determined conditions employed for each primer set used in RT-PCR.

	Cox I	Cox II	eNos	GAPDH	β-actin
Initial RNA concentration	1 μg	1 μg*	0.2 μg	0.5 μg	0.2 μg
Volume of $MgCl_2$ (25 mM)	3 μl	3 μl	3 μl	3 μl	3 μl
Number of PCR cycles	35	40	25	25	25
Annealing temperature	55°C**	55°C**	55°C**	55°C**	55°C**

* The Cox II primer set was used qualitatively, not quantitatively and so the initial amount of RNA used did not need to be determined.

**Primers were designed with ~50% GC content so the annealing temperature for all sets was ~55°C.

2.2.10.3 Agarose gel electrophoresis

All DNA gel electrophoresis was carried out using a GibcoBRL Horizon 20.25 Gel electrophoresis Apparatus. Before use the gel box was cleaned with ethanol and the gel cast was set up as described in the manufacturers instruction manual. A 2.5% agarose gel stock was made up by dissolving 12.5 g of agarose in 500 ml of 1xTris Acetate EDTA (TAE) (40 mM Tris-Acetate, 1 mM EDTA). The agarose was dissolved by heating in a microwave (700 mHz) at full power for 5 minutes. 100 ml of the liquid agarose was then transferred to a fresh glass beaker. To this 250 μl of 200 μg/ml of Ethidium Bromide (EtBr) solution was added and mixed thoroughly to give a final concentration of 0.5 μg/ml EtBr. The agarose was then poured into the cast, the comb put into place and the gel allowed to set. Once set the comb was removed and the apparatus filled with 1x TAE buffer. The gel was now ready to load and run. The samples were prepared as follows: 13 μl of PCR product + 5 μl of 4x loading dye. 8 μl

was loaded each well in duplicate. The gel was run at 80 V, 110 mA and 150 W until the dye front had migrated ¾ length of the gel. 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. The gel was then disposed of in the appropriate ethidium bromide waste container.

2.2.11 Enzyme Immunoassay for 6-keto-Prostaglandin $F_{1\alpha}$

Assay designs correlate – Enzyme Immunoassay Kit (EIA) kits were used for quantitation of 6-keto-Prostaglandin $F_{1\alpha}$ in conditioned media during this study. The kit is a competitive immunoassay for determination of 6-keto-$PGF_{1\alpha}$. Briefly, a polyclonal antibody is employed to capture 6-keto-$PGF_{1\alpha}$ in the samples and standards. 6-keto-$PGF_{1\alpha}$ conjugated to alkaline phosphatase is used to compete for any antibody that might be left over. The mix is incubated for a short period, the excess reagents are then washed away and a substrate for alkaline phosphatase is added. After a short incubation time the enzyme reaction is stopped and the yellow colour generated is read on a microplate reader at 405nm. The intensity of the bound yellow colour is inversely proportional to the concentration of 6-keto-Prostaglandin $F_{1\alpha}$ in either standards or samples. For further explanation of the principles of immunoassays please see the books by Chard (1990) and Tijssen (1985).

Prostacyclin (PGI_2) is involved in platelet aggregation, vasorelaxation, and reproductive functions (Ylikorkala and Makila, 1985). However, PGI_2 has a half life of 60 minutes in plasma but only 2 to 3 minutes in buffer. The production of PGI_2 is typically monitored by measurement of 6-keto-$PGF_{1\alpha}$. 6-keto-$PGF_{1\alpha}$ is produced by the nonenzymatic hydration of PGI_2, and has been shown to be stable (Ylikorkala and Makila, 1985).

The assay was carried out as follows:
All reagents were brought to room temperature for at least 30 minutes prior to carrying out the assay. All samples and standards were run in triplicate.

1. 100 µl of standard diluent (growth media) was pipetted into the NSB and the Bo standard wells.
2. 100 µl of standards #1 to #7 were pipetted into the appropriate wells.

3. 50 µl of the samples were pipetted into the appropriate wells.
4. 50 µl of assay buffer was pipetted into the NSB wells.
5. 50 µl of blue conjugate was pipetted into each well, except the blank, TA and NSB wells.

   Note: Every well was green in colour except the NSB wells which were blue. The blank and TA wells were empty at this point and had no colour.
6. The plate was incubated at room temperature on a plate shaker for 2 hours at ~500 rpm. The plate was covered with the plate sealer provided.
7. The contents of the wells were emptied and each well was washed out with 200 µl of wash solution. This was repeated to give a total of 3 washes.
8. After the final wash the wells were emptied, and any remaining wash buffer was removed by firmly tapping on a lint free paper towel
9. 5 µl of the light blue conjugate (1:10 dilution) was added to the TA wells.
10. 200 µl of the p-Npp Substrate solution was added to every well. The plate was then incubated at room temperature for 45 minutes without shaking
11. 50 µl of stop solution was added to every well
12. The plate was then read immediately at an optical density of 405 nm.

The Results from the assay were calculated as follows:

1. The average Net Optical Density (OD) bound for each standard and sample was calculated by subtracting the average NSB OD from the average OB bound:

   Average Net OD = Average Bound OD – Average NSB OD
2. The binding of each pair of standard wells as a percentage of the maximum binding wells (Bo) was calculated using the following formula:

$$\text{Percent Bound} = \frac{\text{NetOD}}{\text{Net Bo OD}} \times 100$$

3 Using Microsoft Excel software a graph of Percentage Bound versus Log_{10} Concentration of 6-keto-$PGF_{1\alpha}$ for the standards was plotted . A fourth order polynomial trend line was fitted to the plot and concentrations of 6-keto-$PGF_{1\alpha}$ in the samples were determined by interpolation.

2.2.12 Fluorometric Nitrite assay (DAN assay)

This rapid and sensitive fluorometric assay for quantification of nitrite is based upon the reaction of nitrite with 2,3-diaminonaphthalene (DAN) to form the fluorescent product, 1-(H)-naphthotriazole. The assay can be used to detect 10 nM nitrite making it 50-100 times more sensitive than the well know Griess assay. The assay is a modification of the method of Damiani and Burini (Misko *et al*, 1986). The assay was carried out as follows: 10 µl of freshly prepared DAN (0.05 mg/ml in 0.62 M HCl) is added to 100 µl of the sample (conditioned media) or standard and mixed immediately. Standards are made up in the same media as the conditioned media. Sodium nitrite was used as the standard. After a 10 minute incubation at 20°C, the reaction is terminated with 5 µl of 2.8 N NaOH. The intensity of the fluorescent signal produced by the product is maximized by the addition of the base. Formation of the 2, 3-diaminonaphthotriazole was measured using a fluorescent plate reader with excitation at 365 nm and emission read at 450 nm.

2.2.13 Gel documentation system

Any results generated by Western blot and RT-PCR were photographed using a Kodak DC290 digital camera. The image generated was then analysed using Kodak 1D (version 3.5.4) densitometry imaging software. Briefly, a mean densitometric value was determined for each band (Western blot or RT-PCR). The values generated for treatment bands were then corrected against values generated for control bands (GAPDH, β-actin for PR-PCR) and lanes (ponceau S for Western blot). The corrected values were then expressed as fold change relative to negative control. These results were finally represented as a graph in an Excel spreadsheet.

2.2.14 Exposure of BAECs to cyclic stretch

The purpose of generating stretched samples was to use them as positive controls for the up regulation of eNOS and Cox I and II protein and RNA. BAECs were cultured in RPMI 1640 media supplemented with 10% fetal calf serum and antibiotics (50 U/ml penecillin, 50 mg/ml streptomycin). Cells were grown in a humidified atmosphere of 5% CO_2/95% air at 37°C. For all experiments, BAECs were seeded into 6-well Bioflex® plates (Dunn Labortechnik GmBH - Asbach, Germany) at a density of approximately $6x10^5$ cells/well. Bioflex® plates contain a pronectin-coated silicon membrane bottom which enables precise deformation of cultured cells by microprocessor controlled vacuum (Banes *et al*, 1985). When cells had reached approximately 80% confluency, a Flexercell® Tension Plus™ FX-4000T™ system (Flexcell International Corp. - Hillsborough, NC) was subsequently employed to apply a physiological level of cyclic strain to each plate (0-5% strain, 60 cycles/min, 0-24 h). Following all experiments, media was collected and cells were washed twice in PBS before being harvested using a cell scraper. Sample were then prepared as described in section 2.2.3 for western blot and section 2.2.5 for RT-PCR. All samples were stored at -80°C.

2.2.15 Exposure of BAECs to pulsatile shear stress

The CELLMAX^{TM} QUAD artificial capillary module (polypropylene cell-PPF70 from Spectrum Laboratories Inc.) was used to expose BAECs to pulsatile shear stress (Redmond, E.M. *et al*, 1995). The purpose of generating sheared samples was to use them as positive controls for the up regulation of eNOS and Cox I and II protein and RNA (Hendrickson *et al*, 1999).

The system consists of a bundle of 50 semi permeable fibronectin coated polyethylene tubes (outer diameter 630 μm, wall thickness 150 μm, luminal area 70 cm^2, outer surface area 100 cm^2, extra capillary volume 1.4 ml, 95% MWCO 0.5 μm) encased in a plastic holding. Cell culture medium is pumped at the chosen flow rate from a reservoir bottle through the lumen of the capillaries via silicone tubing. To maintain constant pH, pCO_2 and pO_2, the QUAD system was housed in a standard humidified cell culture incubator. Gaseous exchange occurs across the silicone tubing.

The module is equilibrated for 3 days, to remove any air bubbles, by circulating culture media throughout the tubing and capillaries. Following this, BAECs were harvested from conventional cell culture flasks using 0.125% trypsin-EDTA at 37°C and injected into the luminal area using a double syringe method. Briefly, cells are seeded (at densities of 10,000 to 50,000 cells per cm^2) using a syringe in one luminal port and the displaced media is withdrawn using another syringe through the other port. Cells were allowed to adhere for 3 hours, after which the pump is set to low flow (0.3 ml/min; 6 mm Hg; 0.5 dyn/cm^2) for at least 3 days. For the physiologically relative high flow rate (25 ml/min; 56 mmHg; 23 dyn/cm^2), the flow rate is increased steadily over ~5 hours until the desired flow rate is reached (t = 0). The cells were then harvested from their separate compartments after 24 hours. Briefly, the capillary bundle is first slowly flushed out with HBSS to remove any media containing FCS. BAECs are then incubated with ~5 ml of 1x trypsin EDTA for 5min at 37°C. The cell suspension is then washed out with 20 ml of fresh culture media. The cells are then pelleted at 5000 xg and samples are prepared as described in section 2.2.3 for western blot and section 2.2.5 for RT-PCR.

2.2.16 Statistics

Results are expressed as mean ± sem. Comparisons between control versus treated cells were made by Student's unpaired t-test, with statistical significance established at $P \leq 0.05$.

The module is equilibrated for 3 days, to remove any air bubbles, by circulating culture media throughout the tubing and capillaries. Following this, BAECs were harvested from conventional cell culture flasks using 0.125% trypsin-EDTA at 37°C and injected into the luminal area using a double syringe method. Briefly, cells are seeded (at densities of 10,000 to 50,000 cells per cm^2) using a syringe in one luminal port and the displaced media is withdrawn using another syringe through the other port. Cells were allowed to adhere for 3 hours, after which the pump is set to low flow (0.3 ml/min; 6 mm Hg; 0.5 dyn/cm^2) for at least 3 days. For the physiologically relative high flow rate (25 ml/min; 56 mmHg; 23 dyn/cm^2), the flow rate is increased steadily over ~5 hours until the desired flow rate is reached (t = 0). The cells were then harvested from their separate compartments after 24 hours. Briefly, the capillary bundle is first slowly flushed out with HBSS to remove any media containing FCS. BAECs are then incubated with ~5 ml of 1x trypsin EDTA for 5min at 37°C. The cell suspension is then washed out with 20 ml of fresh culture media. The cells are then pelleted at 5000 xg and samples are prepared as described in section 2.2.3 for western blot and section 2.2.5 for RT-PCR.

2.2.16 Statistics

Results are expressed as mean ± sem. Comparisons between control versus treated cells were made by Student's unpaired t-test, with statistical significance established at $P \leq 0.05$.

Chapter 3 Optimisation of RT-PCR and Western blot protocols

3.1 Introduction

The purpose of this chapter is to present the experiments that were carried out to optimise the western immunoblot and RT-PCR methods employed during this study. When employing RT-PCR for semi-quantitative analysis, a titre for initial total RNA concentration must be made for each primer set used. The reason for this is to ensure that any resulting bands are amplified within a linear range, and so semi-quantitative comparisons can be made. Initial RNA concentration is the only condition changed between each sample during the titre experiment. $MgCl_2$ concentration, annealing temperature and number of cycles remain constant. The optimised set of conditions for each primer set used in RT-PCR, as determined from these experiments, is summarised in Table 2.2.10.2.

For western immunoblot analysis, optimisation of the primary antibody concentration is required. The following experiments were carried out to empirically determine an optimal antibody concentration. A concentration of antibody needs to be used which would ensure maximum amount of binding to the target antigen, thus producing a maximal signal, with the lowest background binding. This is to ensure that any difference seen in target bands is not due to variation in antibody binding. The concentration of the secondary antibody and amount of protein loaded remain constant during the titre experiment.

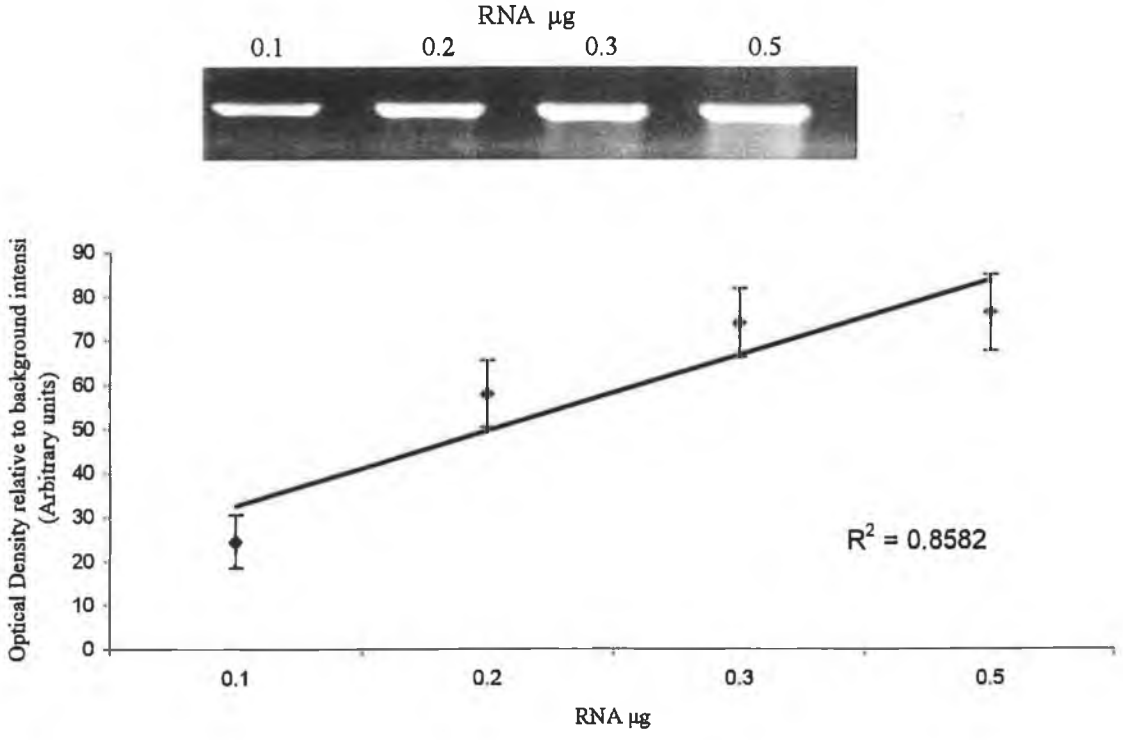
Once optimised the conditions employed for western immunobloting and RT-PCR remained constant throughout the study.

3.2 Optimisation of primer sets for RT-PCR

3.2.1 Optimisation of the β-actin primer set

In order to ensure that equal amounts of total RNA are used in each RT-PCR reaction mix a housekeeping gene, whose expression was not regulated by the treatment, was also amplified from each sample. B-actin is a standard housekeeping gene and was used during this study. The β-actin PCR product amplified between treatments must be equal before any observed change in eNOS or Cox I bands can be attributed to the treatment. If there was a difference it was compensated for by densitometric analysis.. The results from optimisation of the β-actin primer set are as follows:

Figure 3.2.1 Titre of initial BAEC total RNA concentration for the β-actin primer set used in RT-PCR.

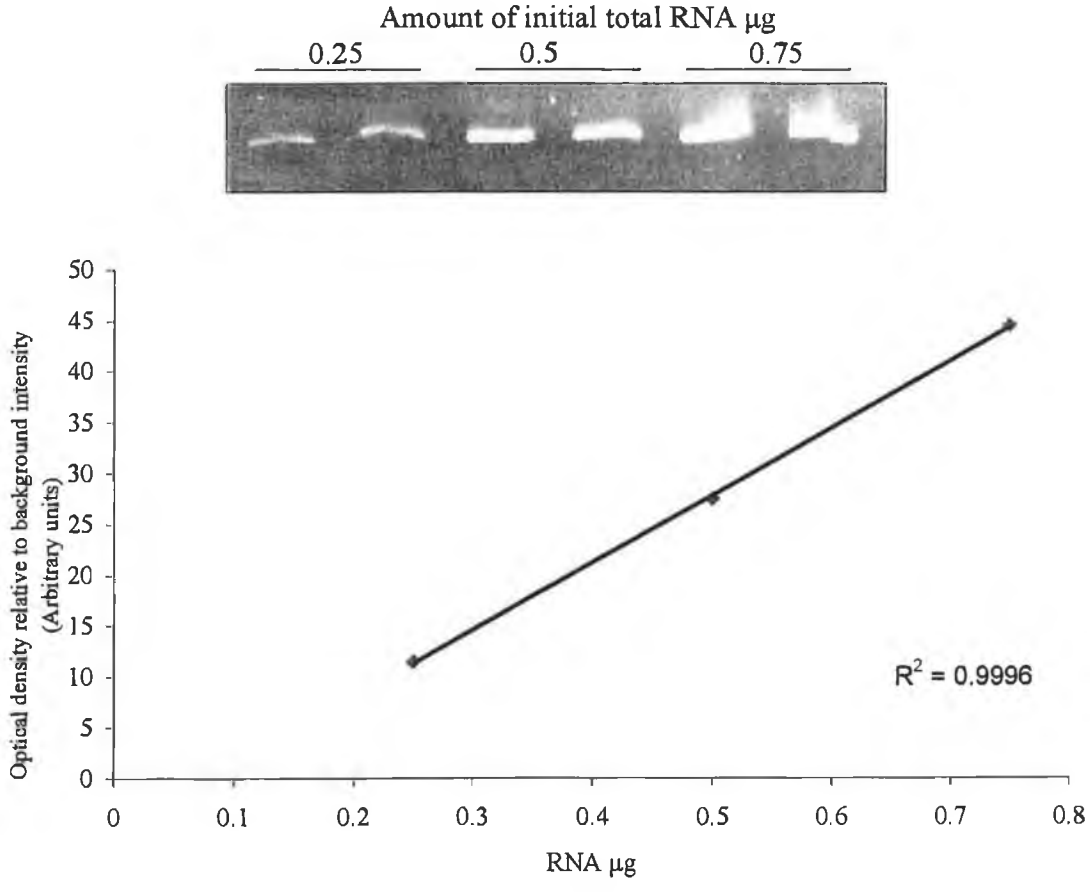


From this experiment it was determined that 0.2 µg of total RNA produced amplification within a linear range for the β-actin primer set used under the set conditions of RT-PCR.

3.2.2 Optimisation of the GAPDH primer set

A set of GAPDH primers were used as a housekeeping control for samples generated from shearing BAEC. These sheared samples were used as a positive control for the up regulation of Cox I. A titre for initial total RNA concentration was made for the GAPDH primer set used in RT-PCR. This is the only condition that changed between the samples. $MgCl_2$ conc., annealing temperature and number of cycles remained constant, Table 2.2.10.2. The results from optimisation of the GAPDH primer set are as follows:

Figure 3.2.2: Titre of initial BAEC RNA concentration for the GAPDH primer set used in RT-PCR.

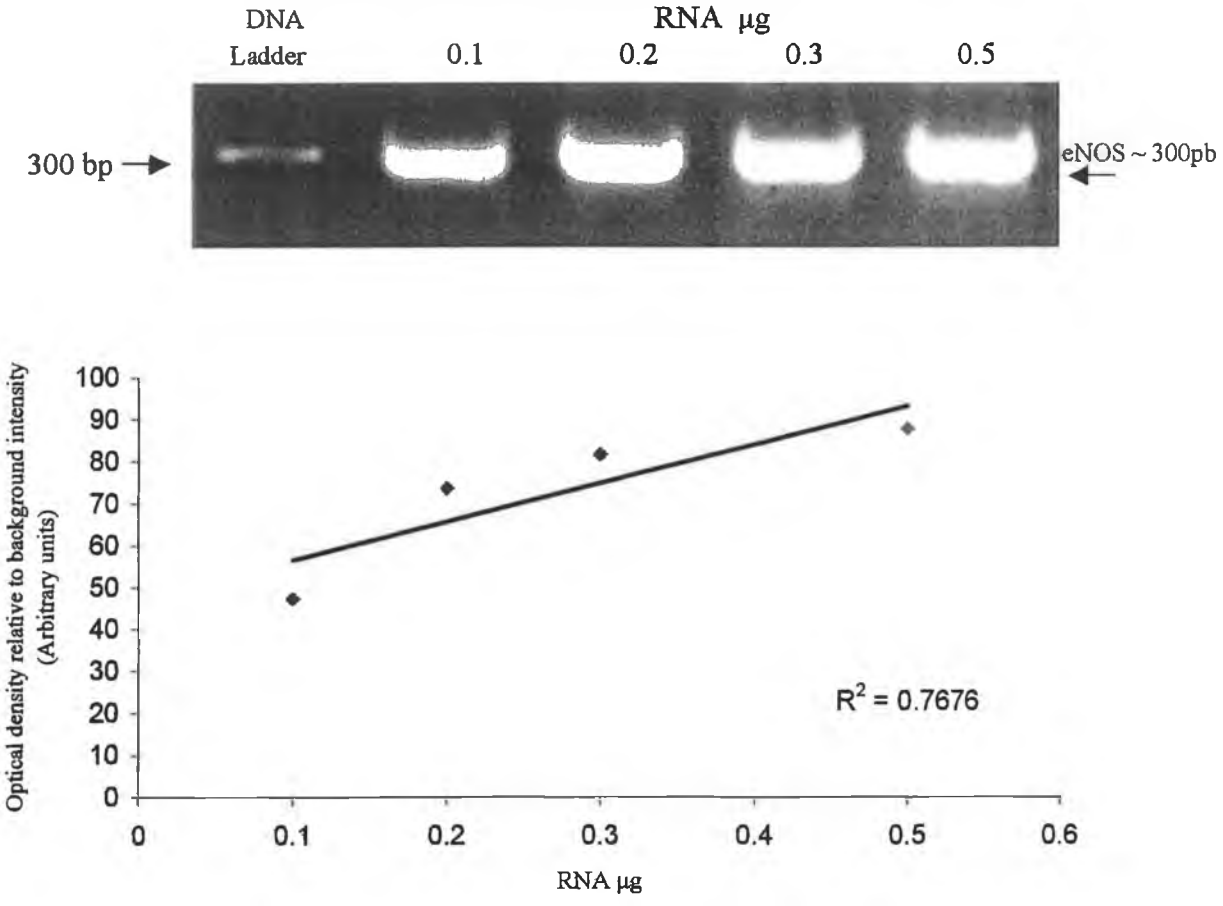


From this experiment it was determined that 0.5 µg of total RNA produced amplification within a linear range for the GAPDH primer set used under the set conditions of RT-PCR.

3.2.3 Optimisation of eNOS primer set

The results from optimisation of the eNOS primer set are as follows:

Figure 3.2.3 Titre of initial BAEC total RNA concentration for the eNOS primer set used in RT-PCR.

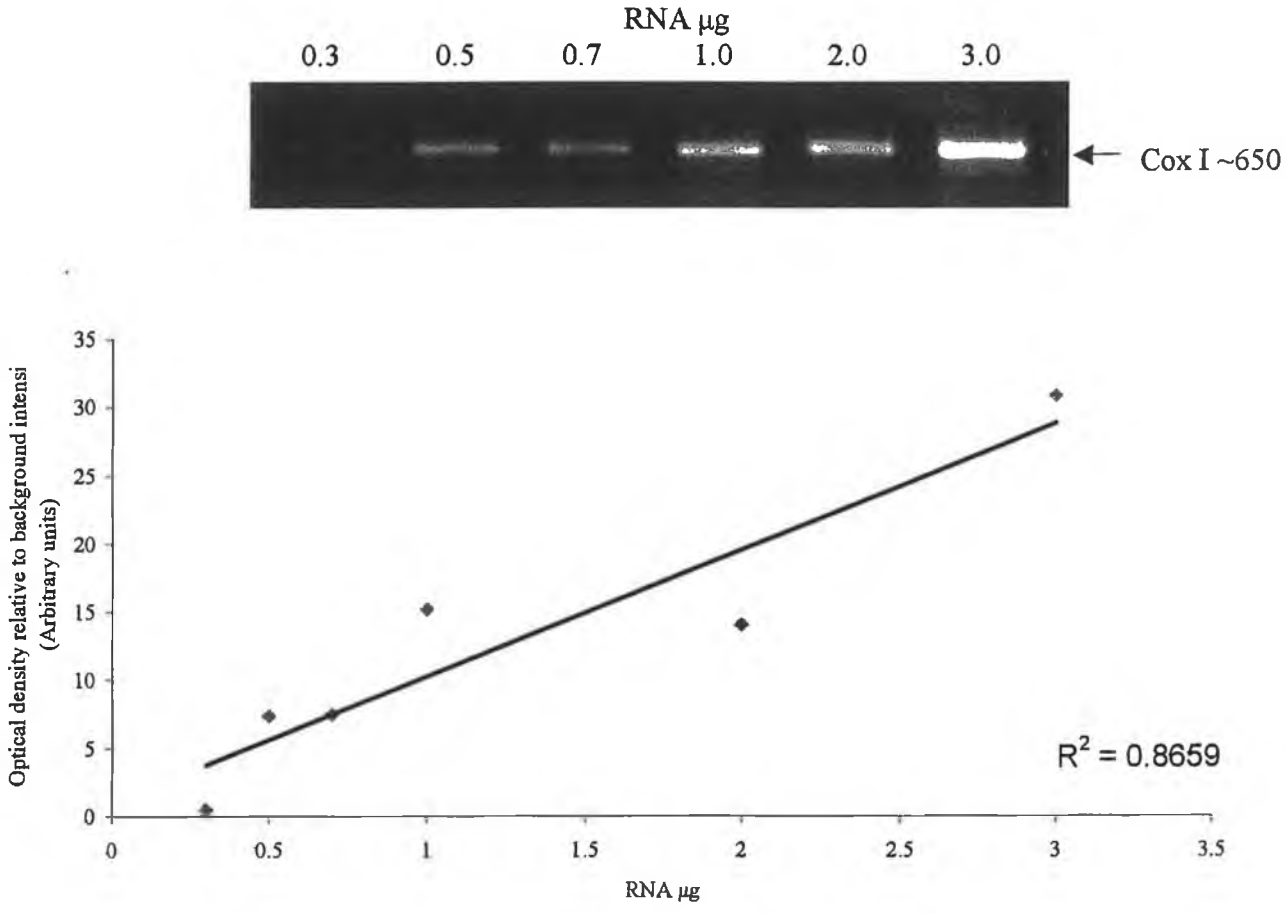


From this experiment it was determined that 0.2 µg of total RNA produced amplification within a linear range for the eNOS primer set used under the set conditions of RT-PCR, Table 2.2.10.2.

3.2.4 Optimisation of Cox I primer set

The results from optimisation of the Cox I primer set are as follows:

Figure 3.2.4: Titre of initial BAEC RNA concentration for the Cox I primer set used in RT-PCR.



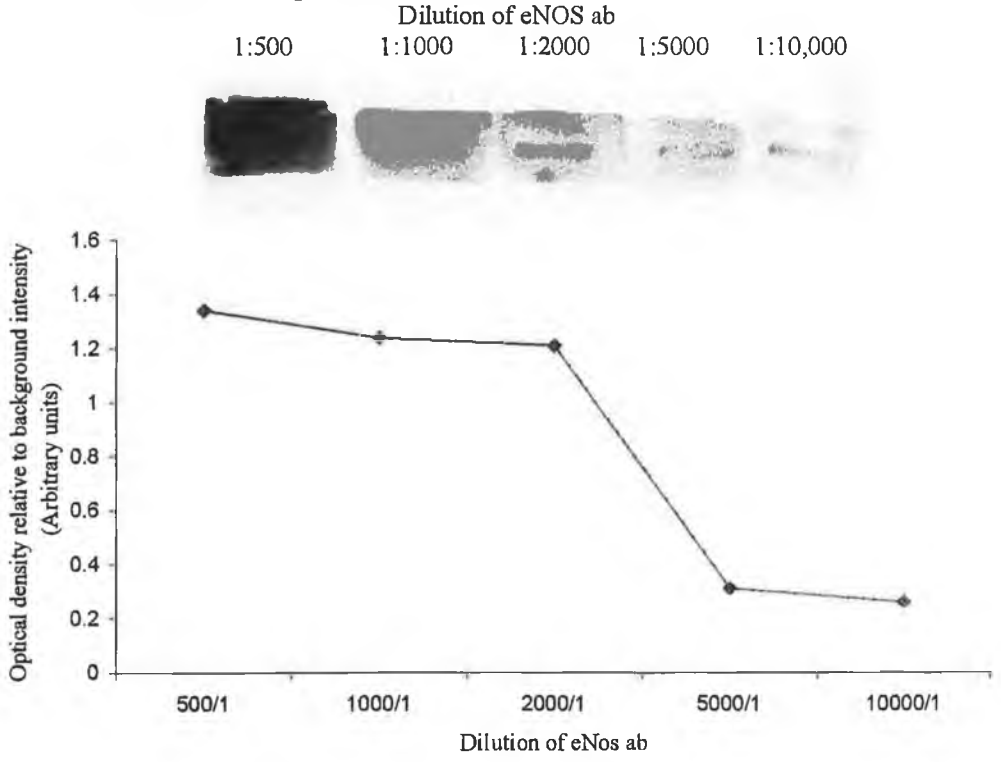
From this experiment it was determined that 1 µg of total RNA produced amplification within a linear range for the Cox I primer set used under the set conditions of RT-PCR, Table 2.2.10.2

3.3 Optimisation of primary antibody concentration for Western Blot

3.3.1 Optimisation of the eNOS primary antibody

The eNOS 1° antibody was optimised using the dot blot apparatus as described in section 2.2.8. Briefly, 10 μg of BAEC whole cell lysate was immobilized on to nitrocellulose membrane a number of times. The blot was incubated with various concentrations of eNOS 1° antibody and subsequently with a 1:15,000 dilution of anti rabbit 2° antibody. The blot was then developed as described in section 2.2.9.5. The purpose for this experiment was to empirically determine the optimum dilution of eNOS 1° antibody that will produce a maximal signal with 10 μg of BAEC whole cell lysate. The results were as follows:

Figure 3.3.1 Results from dot blot immunoassay with 10 μg of BAEC whole cell lysate. Various concentrations of eNOS 1° antibody were used to determine an optimum dilution.

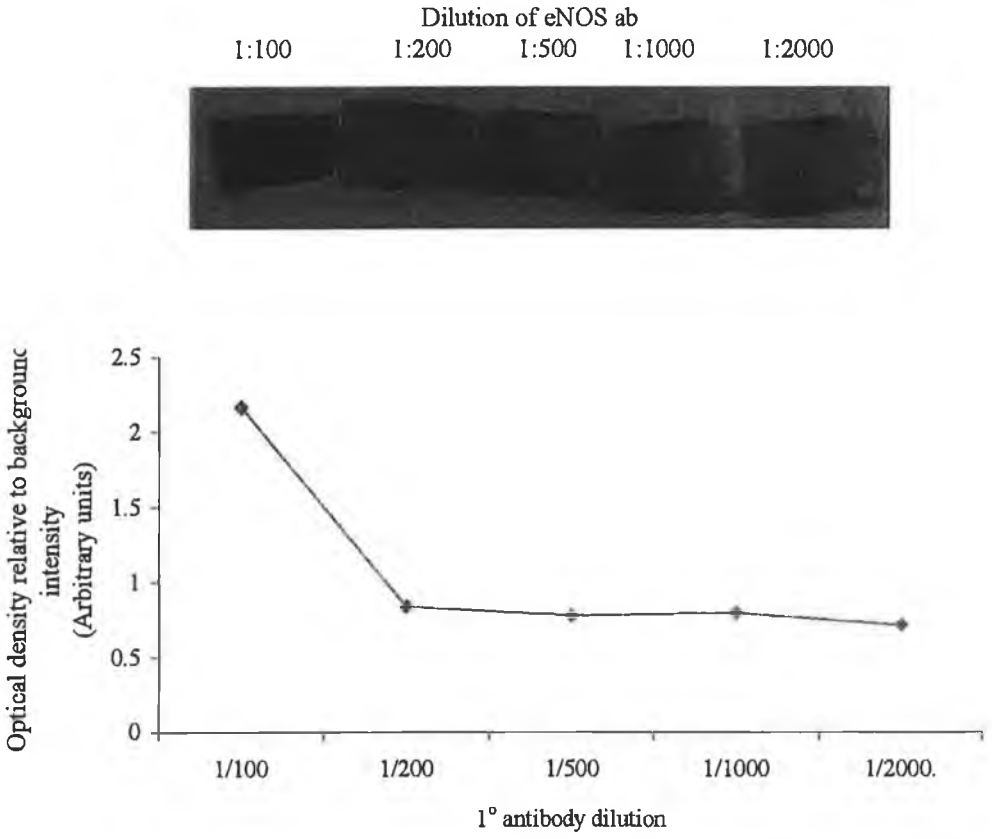


The results from this experiment suggest that a 1:2000 dilution of eNOS primary antibody and a 1:15,000 dilution of anti-rabbit 2° antibody will produce a maximum signal for eNOS from 10 μg of BAEC whole cell lysate.

3.3.2 Optimisation of the Cox I primary antibody

The Cox I 1° antibody was optimised using the dot blot apparatus as described in section 2.2.8. Briefly, the experiment was carried out in a similar fashion to that of the eNOS 1° antibody described in section 3.2.1.
The results were as follows:

Figure 3.3.2 Results from dot blot immunoassay with 10μg of BAEC whole cell lysate. Various concentrations of Cox I 1° antibody were used to determine an optimum dilution.



The results from this experiment suggest that a 1:100 dilution of Cox I primary antibody and a 1:8000 anti-mouse 2° antibody dilution will produce a strong, but not maximal, signal for Cox I from 10 μg of BAEC whole cell lysate. The signal intensity did not plateaux with this range of 1° antibody and so a dilution lower than 1:100 may have given a stronger signal.

3.4 Summary of Results

A summary of the optimised conditions determined for RT-PCR and Western Immunoblot is presented here. The conditions for each primer set were determined empirically in order that semi-quantitative comparisons could be made between samples. The following conditions were used throughout the rest of the study:

Table 3.4: A summary of the empirically determined conditions employed for each primer set used in RT-PCR.

	Cox I	Cox II	eNos	GAPDH	β-actin
Initial RNA concentration	1 μg	1 μg*	0.2 μg	0.5 μg	0.2 μg
Volume of $MgCl_2$ (25 mM)	3 μl	3 μl	3 μl	3 μl	3 μl
Number of PCR cycles	35	40	25	25	25
Annealing temperature	55°C**	55°C**	55°C**	55°C**	55°C**

* The Cox II primer set was used qualitatively, not quantitatively and so the initial amount of RNA used did not need to be determined.

**Primers were designed with ~50% GC content so the annealing temperature for all sets was ~55°C.

The optimal conditions employed for western immunoblot were also determined and are as follows:

- A 1:100 dilution of Cox I primary antibody and a 1:8000 dilution of anti-mouse 2° antibody will produce a strong, but not maximal, signal for Cox I from 10 μg of BAEC whole cell lysate.
- A 1:2000 dilution of eNOS primary antibody and a 1:15,000 dilution of anti-rabbit 2° antibody will produce a maximum signal for eNOS from 10 μg of BAEC whole cell lysate.

Chapter 4 The Effects of CLA on BAEC Function

4.1 Introduction

The effect of CLA isomer mix on steady state mRNA levels, protein expression and activity of Cox I, Cox II and eNOS in BAEC is reported in this chapter. The importance of correctly regulated expression and activity of these enzymes with regard to the vasoregulatory mechanisms of the vascular endothelium has already been discussed, section 1.1. The pretext for this study has been outlined in detail in section 1.2. Briefly, the results from a number of *in vivo* studies have shown that CLA may have an atheroprotective effect on the vasculature, through slowing the development of atherosclerotic plaque (Lee *et al*, 1994). It has been postulated that the effect is elicited via at least two potential mechanisms.

Firstly, by modulating endothelium exposure to oxidative stress. It has been shown that CLA may be oxidised to several furan derivatives that possess an oxidation/reduction buffering capacity (Yurawecz *et al*, 1995). It is postulated that this property of CLA may effect the expression and/or activity of eNOS through alteration of oxidative processes. Secondly, it has been shown that CLA is incorporated into fatty acid profile of the cell membrane and subsequently alters eicosanoid metabolism (Ha *et al*, 1990). This has previously been proven for a venous cell type although no data was presented for CLAs effect on Cox mRNA or protein expression (Urquhart *et al*, 2001). It is therefore hypothesised that CLA will putatively affect expression or activity of eNOS and/or Cox I in BAECs.

The experiments were designed to examine the effects of chronic treatments (up to 24 hours) of the CLA mix (0-10 μg/ml) on the expression of eNOS and Cox I protein and steady state RNA. The effect of CLA treatment on production of $PGF_{1\alpha}$ from un-stimulated BAEC was examined as was the effect of treatment on nitrite levels in conditioned media generated from un-stimulated and agonist stimulated BAEC. The rational behind examining the effect of CLA on agonist stimulated eNOS activity was that CLA treatment seemed to elicit no response in unstimulated BAEC. This is further explained in section 4.3.3.

4.2 The effect of CLA on eNOS expression

4.2.1 Dose dependent effect of CLA mix treatment on eNOS protein expression

To determine whether various concentrations of the CLA mix and LA had any effect eNOS protein expression, BAEC were incubated with 0-10 μg/ml of CLA mix and LA for 24 hours. It was found that concentrations over 10 μg of CLA resulted in cells that morphologically looked very unhealthy. A larger quantity of floating cells were also noted at concentrations above 10 μg.

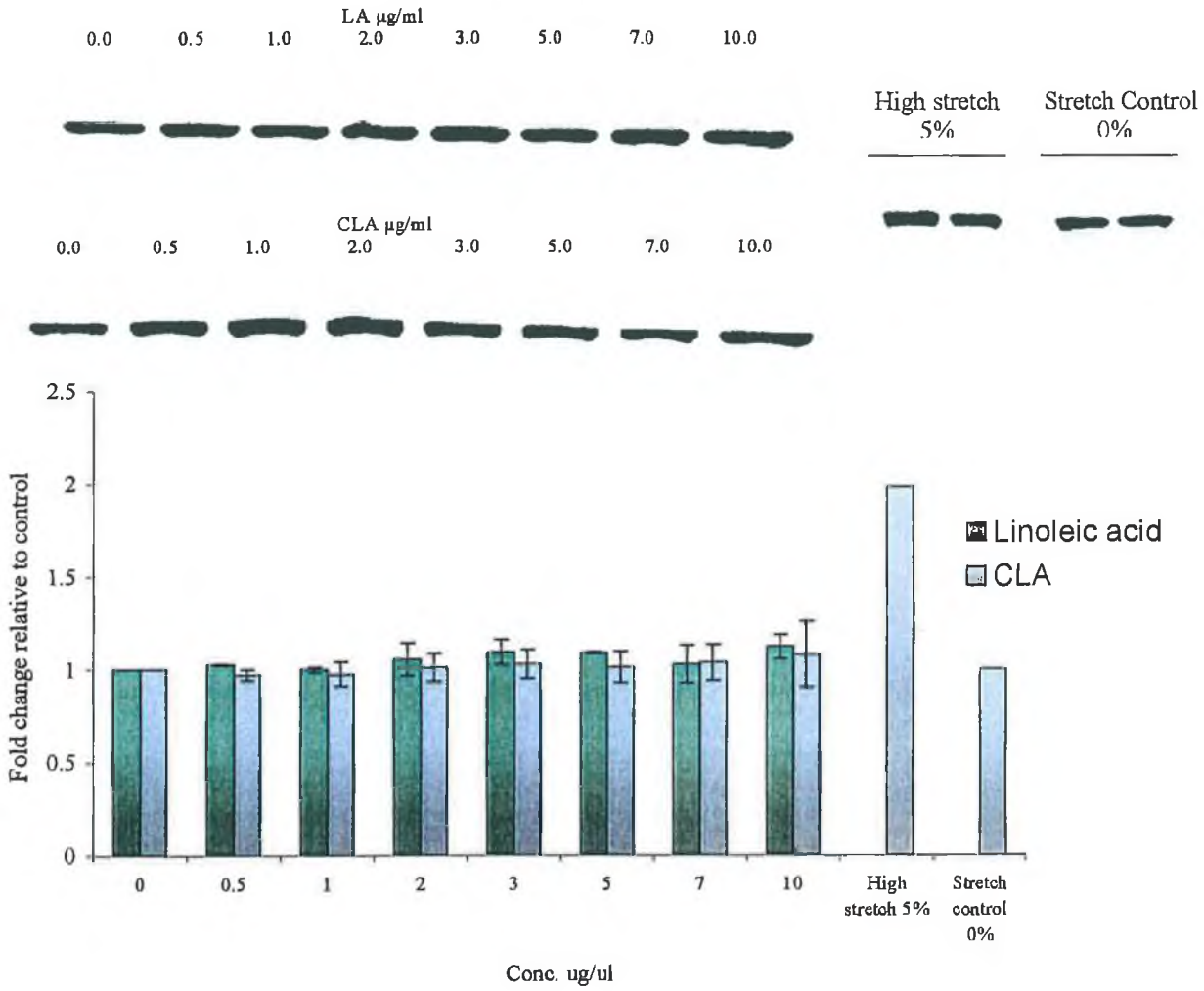
Stretched BAEC (5%) were used as positive control for the up regulation of eNOS. BAEC were stretched as described in section 2.2.14. Whole cell lysates were prepared as described in section 2.2.3. SDS PAGE and western blot analysis was carried out as described in section 2.2.9.

Linoleic acid was used as control to determine whether the conjugation of the CLA isomers was eliciting any effects on eNOS protein expression. Because all the treatments were dissolved in 95% ETOH, a vehicle control was included. The volumn of vechile control (95% ETOH) was the same as the treatment for all CLA treatments reported in this chapter.

The cells were quiesced for 24 hours before being treated. This was to ensure that the cells were all at G_o of the cell cycle before treatment.

The results were as follows:

Figure 4.2.1 Fold change in eNOS protein expression in BAEC after 24 hour treatment with 0-10 μg/ml CLA mix and LA as determined by western blot analysis. BAEC were quiesced by serum deprivation for 24 hours prior to treatment. Equality of protein loading was determined by Ponceau staining. The image presented is representative. The graph shown is derived from mean densitometric analysis of results from multiple analysis of samples generated from three separate experiments.

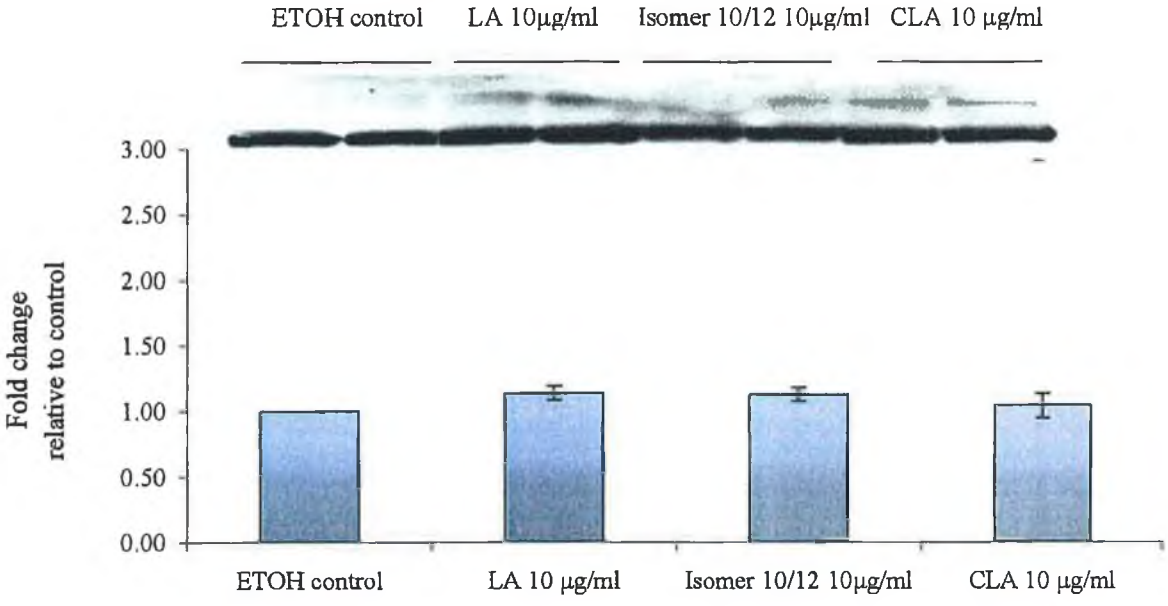


The results from this experiment suggest that CLA mix, at 0-10 μg/ml, had no effect on the expression of eNOS in BAEC at 24 hours. 10 μg/ml was also determined to be a maximum concentration for treatment due to the toxic effects of higher concentrations.

4.2.2 The effect of, CLA isomer t10/c12, CLA mix and Linoleic acid on eNOS protein expression

To determine whether the CLA isomer t10/c12 elicited any effect on eNOS compared to CLA mix and linoleic acid, BAEC were treated with 10 μg/ml for 24 hours. Because all the treatments were dissolved in 95% ETOH, a vehicle control was included. The volumn of vechile control (95% ETOH) was the same as the treatment for all CLA treatments reported in this chapter. Whole cell lysates were prepared as described in section 2.2.3. SDS PAGE and western blot analysis was carried out as described in section 2.2.9

Figure 4.2.2 Fold change in eNOS protein expression in BAEC after treatment for 24 hours with CLA mix, CLA isomer t10/c12, linoleic acid and ETOH control. BAEC were quiesced by serum deprivation for 24 hours prior to treatment. Equal protein loading was determined by Ponceau staining. The image presented is representative. The graph shown is derived from mean densitometric analysis of results from multiple analysis of samples generated from two separate experiments.

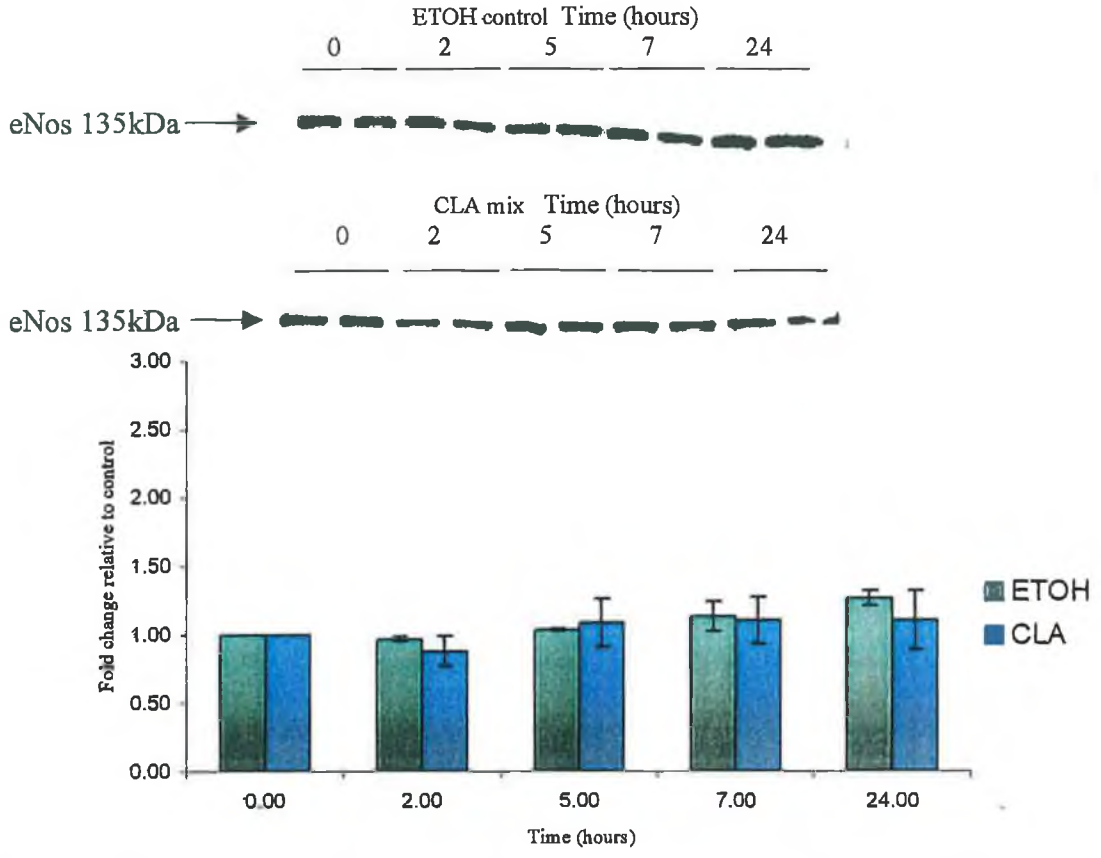


The results from this experiment suggest that CLA isomer t10/c12, at 10 μg/ml, had no effect on eNOS protein expression in BAEC at 24 hours compared to CLA mix and linoleic acid.

4.2.3 Temporal effect of CLA mix treatment on eNOS protein expression

To determine the effect of the CLA mix on eNOS protein expression over time, BAEC were incubated with 10 μg/ml of CLA mix over a time course up to 24 hours. Whole cell lysates were prepared as described in section 2.2.3. SDS PAGE and western blot analysis was carried out as described in section 2.2.9.

Figure 4.2.3: Fold change in eNOS protein expression in BAEC over a time course after treatment with CLA mix. BAEC were quiesced by serum deprivation for 24 hours prior to treatment. Equality of protein loading was determined by ponceau staining. The image presented is representative. The graph shown is derived from mean densitometric analysis of results from multiple analysis of samples generated from two separate experiments.

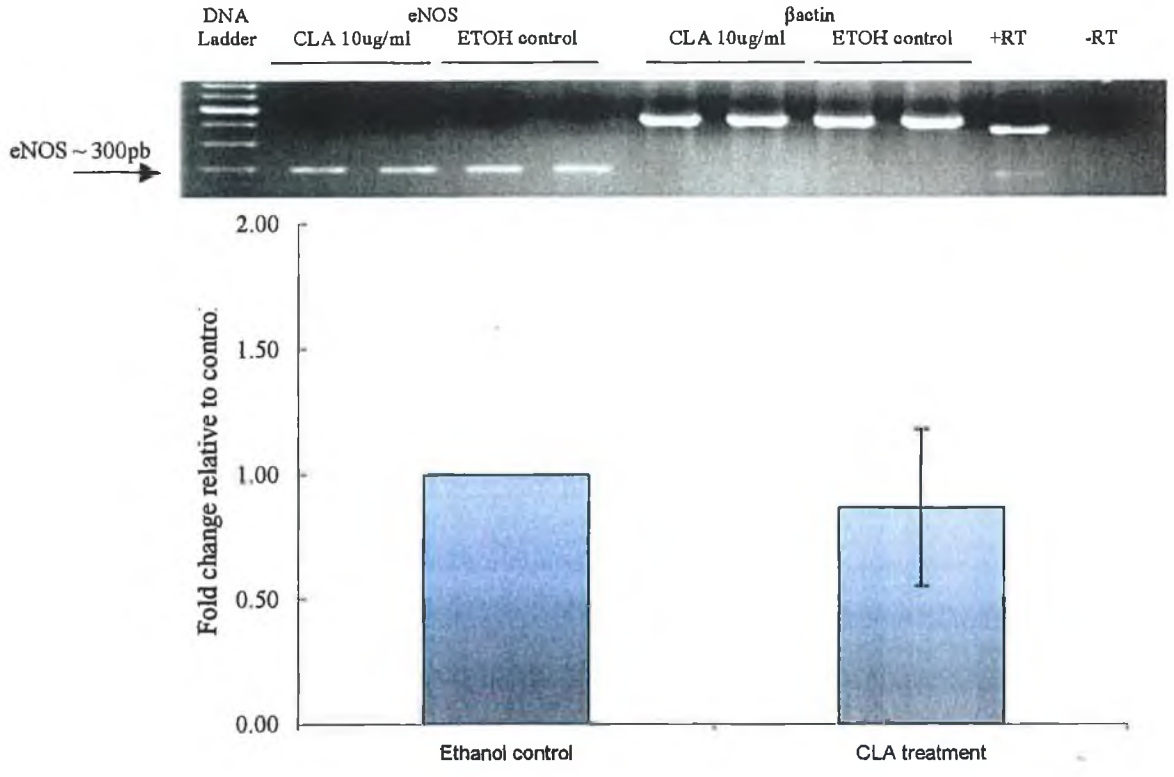


The results from this experiment suggest that 10 μg/ml of CLA mix had no effect on eNOS expression in BAEC over a 24 hour time course.

4.2.4 The effect of CLA mix on eNOS steady state mRNA levels in BAEC

To determine the effect of CLA mix on eNOS mRNA transcription, BAECs were treated with a single concentration of the mix for 24 hours. Total RNA was harvested by using the Trizol® method as described in section 2.2.5, RT-PCR was carried out as described in section 2.2.10. Control primers and control RNA was used for the +RT control, this was included as a positive control for the technique. A –RT control was included to ensure that amplification was not due to contaminating genomic DNA.

Figure 4.2.4 Fold change in eNOS mRNA in BAEC after 24 hours of treatment with 10 μg/ml CLA mix. BAEC were quiesced by serum deprivation for 24 hours prior to treatment. Results were normalised by amplification of β-actin. The image presented is representative. The graph shown is derived from mean densitometric analysis of results from multiple analysis of samples generated from four separate experiments.



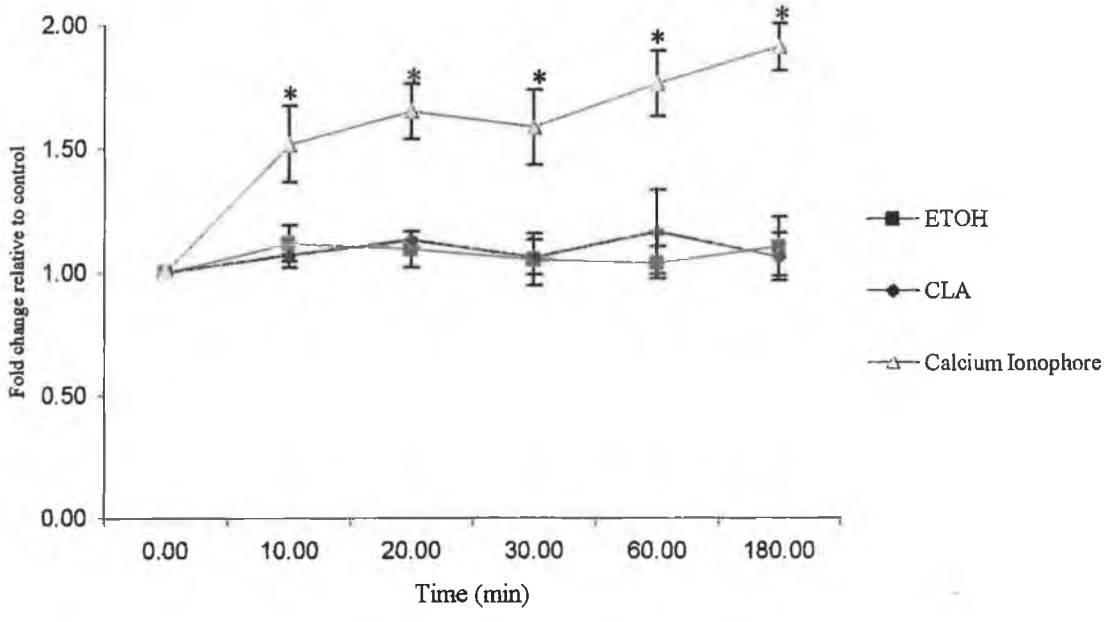
The results from this experiment suggest that 10 μg/ml of CLA mix had no effect on eNOS steady state mRNA levels in BAEC at 24 hours.

4.3 The effect of CLA mix treatment on eNOS activity

4.3.1 The effect of acute CLA mix treatment on nitrite levels in conditioned media from unstimulated BAEC

The effect of treatment on eNOS activity was determined by measuring the amounts of nitrite in conditioned media. eNOS produces nitric oxide which is rapidly converted to both nitrite and nitrate. Measurement of nitrite is an indirect index of eNOS activity. Nitrite was measured by the fluorometric DAN assay section 2.2.12. As a positive control for the assay conditioned media from Calcium Ionophore A23187 treated BAEC was assayed. Calcium ionophore treatment stimulates eNOS activity through a transient increase in cellular Ca^{2+}. The determined effects of CLA mix on eNOS activity over a 3 hour time course was follows:

Figure 4.3.1 Change in nitrite levels in conditioned media after treatment with CLA 10 μg/ml, ETOH control, and Calcium Ionophore A23187 10 μM. The graph shown is derived from mean statistical analysis of results from multiple analysis of samples generated from a least three separate experiments. * $P<0.05$ versus ETOH Control.

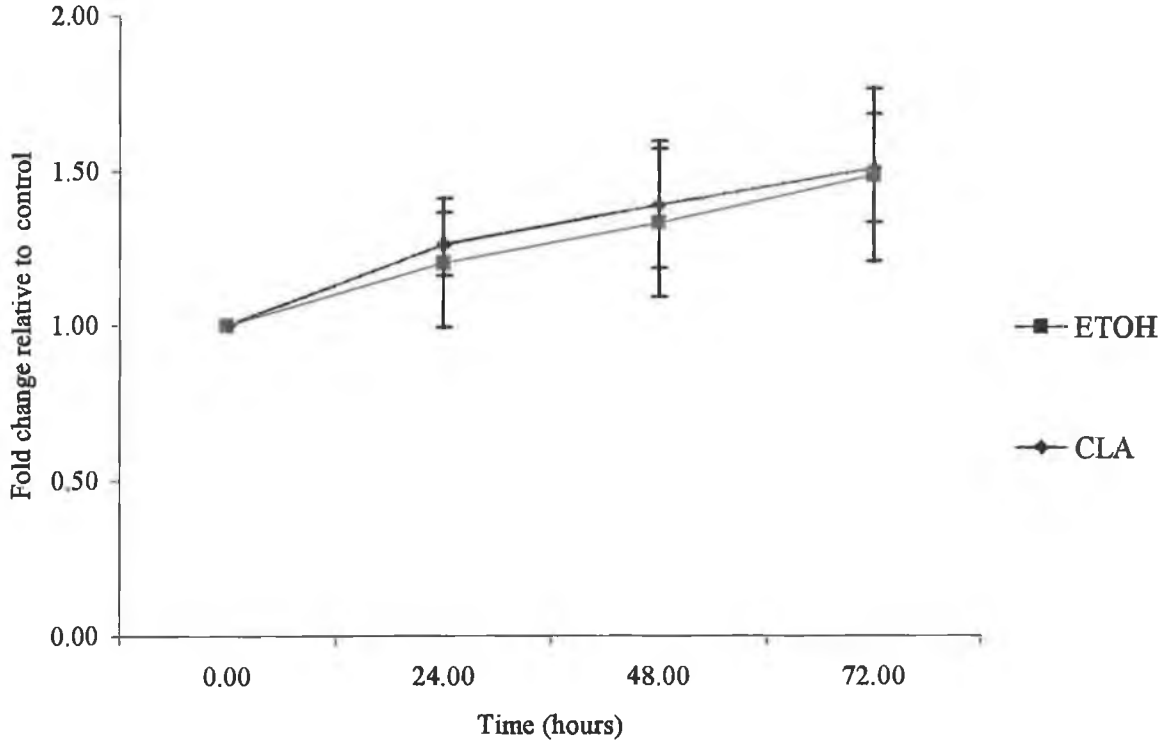


The results from this experiment suggest that CLA mix 10 μg/ml had no effect on the basal activity of eNOS over a 3 hour time course.

4.3.2 The effect of chronic CLA mix treatment on nitrite levels in conditioned media from unstimulated BAEC

Because there seemed to be no effect of CLA mix on eNOS activity over an acute time course, it was decided to examine the effects over a chronic time course. The determined effects of chronic exposure of CLA mix on basal eNOS activity was as follows:

Figure 4.3.2 Change in nitrite levels in conditioned media after treatment with CLA 10 μg/ml and ETOH control over a chronic time course. The graph shown is derived from mean statistical analysis of results from multiple analysis of samples generated from a least three separate experiments.

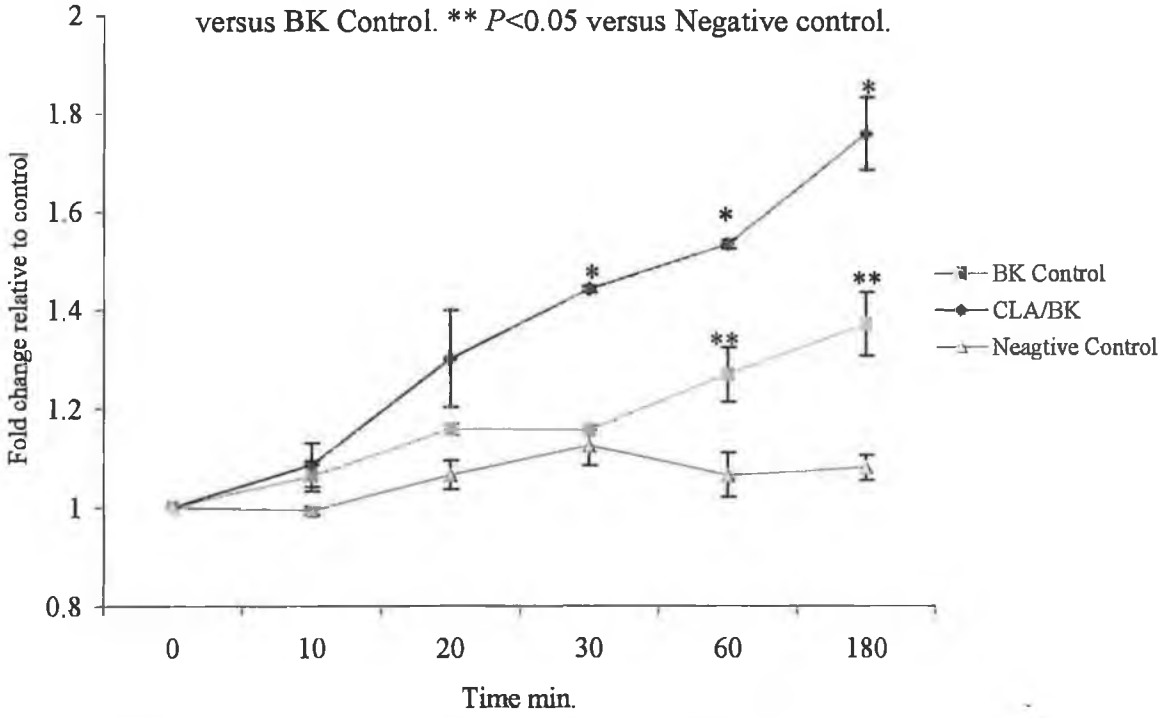


The results from this experiment suggest that CLA mix 10 μg/ml had no effect on the basal activity of eNOS over a 72 hour time course.

4.3.3 The effect of acute CLA mix pre-treatment on Bradykinin stimulation of eNOS by measurement of nitrite levels in conditioned media

Because CLA mix did not exert any effect on the activity of basal eNOS activity, it was decided to investigate the effect of CLA pre-treatment, both chronic and acute, on the ability of Bradykinin to stimulate eNOS activity. Bradykinin stimulates eNOS through a receptor mediated increase in cellular Ca^{2+}. After CLA mix treatment (10 μg/ml) for 3 hours, the CLA containing media was removed and BAEC were stimulated with 5 μM of Bradykinin in fresh media. Conditioned media was then harvested over a 3 hour time course and assayed for nitrite by the DAN fluorometric method as an index of eNOS activity. The negative control represents non stimulated nitrite build up over 3 hours. The results were as follows:

Figure 4.3.3 Change in nitrite levels in conditioned media over time. The graph shown is derived from statistical analysis of results from triplicate analysis of samples generated from two separate experiments. * $P<0.05$ versus BK Control. ** $P<0.05$ versus Negative control.

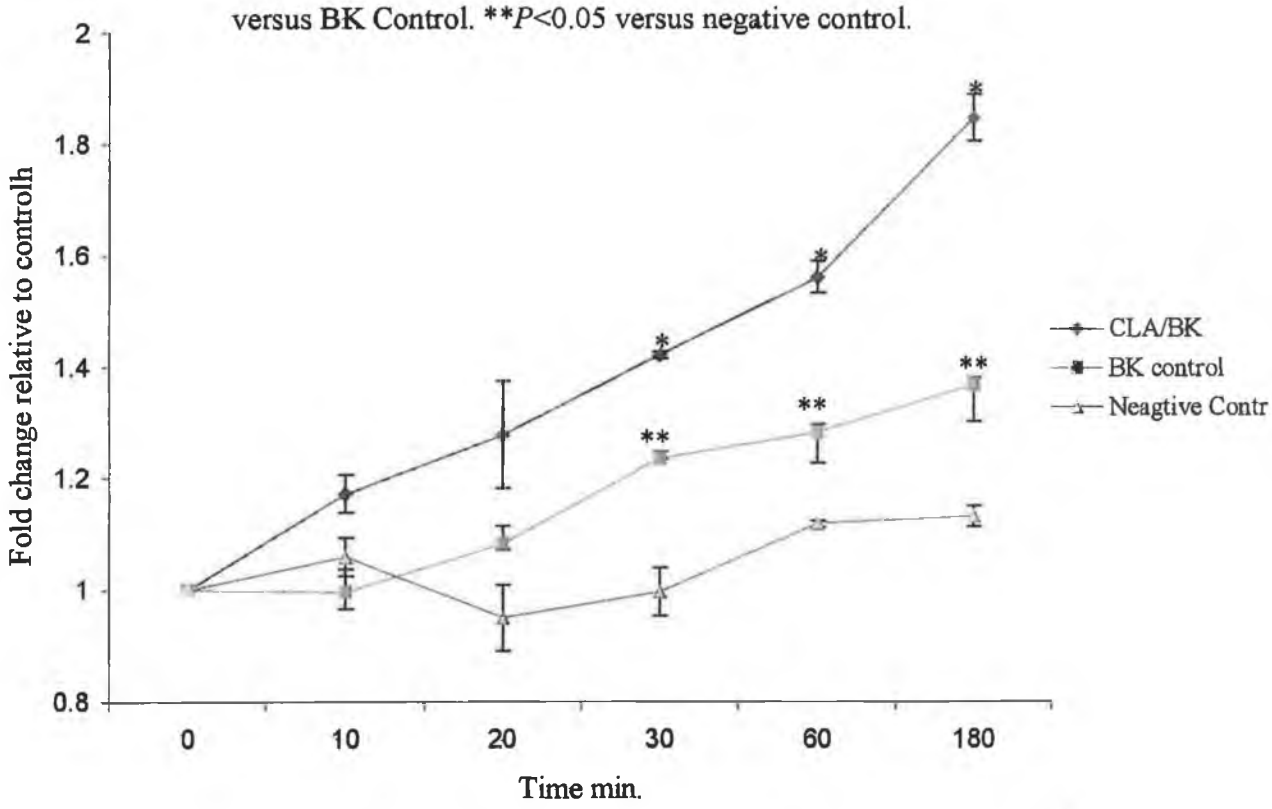


The results from this experiment suggest that a 3 hour pre-treatment with 10 μg/ml CLA mix potentates the stimulation of eNOS in BAEC by bradykinin over a 3 hour time course.

4.3.4 The effect of chronic CLA mix treatment on nitrite levels in conditioned media after stimulation of BAEC with Bradykinin

This experiment was carried out to determine whether CLAs effect on bradykinin stimulation of eNOS occurred with a chronic pre-treatment. After CLA mix treatment for 24 hours, BAEC were stimulated with 5μM of Bradykinin in fresh media. Conditioned media was then harvested over a 3 hour time course and assayed for nitrite by the DAN fluorometric method as an index of eNOS activity. The negative control represents nitrite build up without any treatment. The results were as follows:

Figure 4.3.4 Change in nitrite levels in conditioned media over time. The graph shown is derived from mean statistical analysis of results from triplicate analysis of samples generated from two separate experiments * $P<0.05$ versus BK Control. ** $P<0.05$ versus negative control.



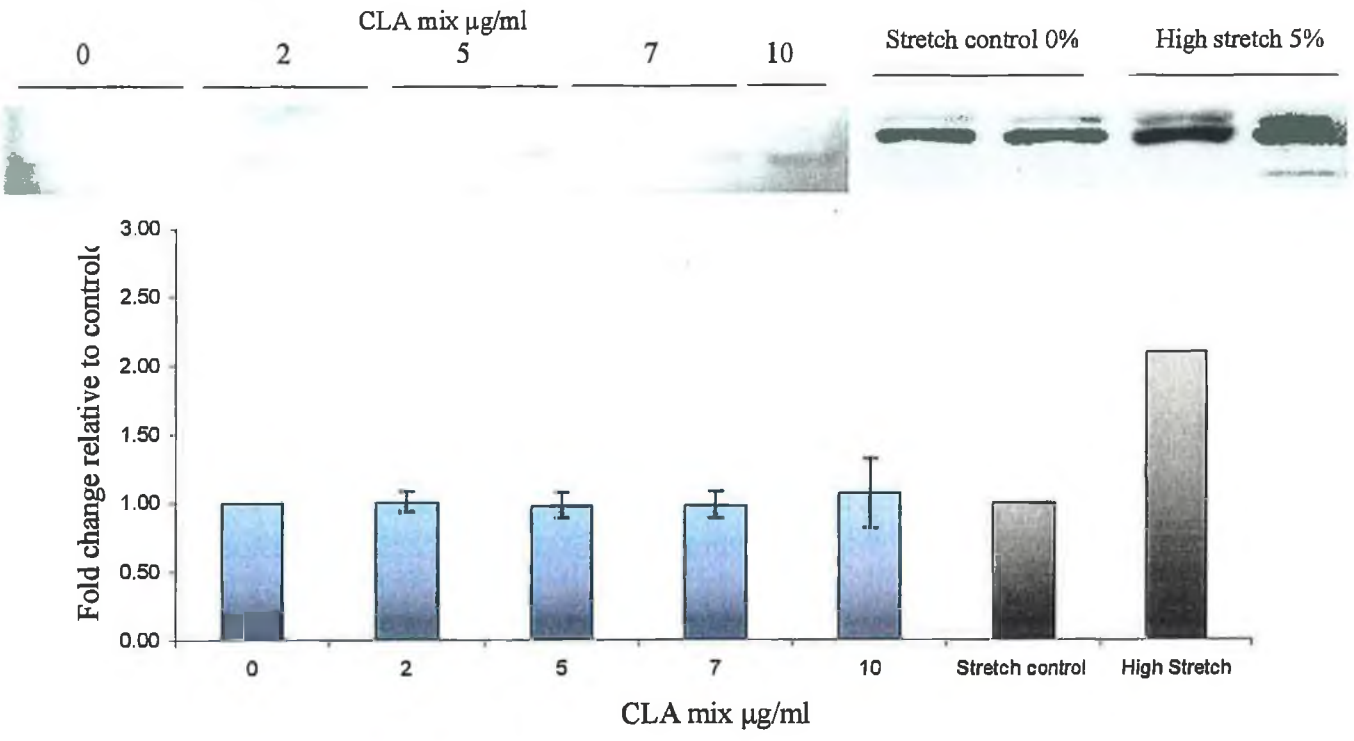
The results from this experiment suggest that a 24 hour pre-treatment with 10 μg/ml CLA mix potentates the stimulation of eNOS in BAEC by bradykinin over a 3 hour time course.

4.4 The effect of CLA mix on Cyclooxygenase expression

4.4.1 Dose dependant effect of CLA mix treatment on Cox I protein expression

In order to determine the effect of various concentrations of the CLA mix on Cox I protein, BAEC were incubated with between 0-10 µg/ml of the mix for 24 hours. BAEC that had been exposed to 5% stretch (as described in section 2.2.14) were also prepared as positive controls for the up regulation of Cox I protein. Whole cell lysates were prepared as described in section 2.2.3. SDS PAGE and western blot analysis was carried out as described in section 2.2.9.

Figure 4.4.1 Fold change in Cox I protein expression in BAEC after 24 hour treatment with 0-10 µg/ml of CLA mix. BAEC were quiesced by serum deprivation for 24 hours prior to treatment. Equality of protein loading was determined by ponceau staining. The image presented is representative. The graph shown is derived from mean densitometric analysis of results from multiple analysis of samples generated from three separate experiments.

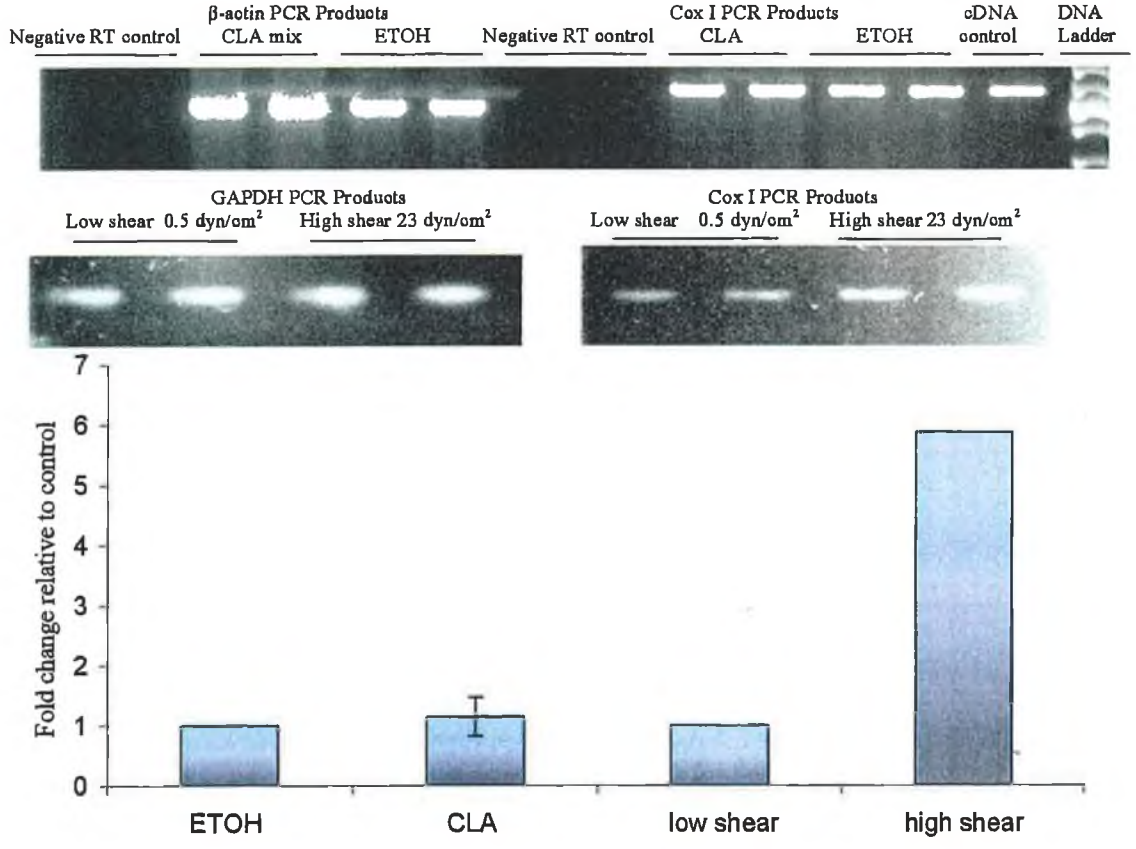


The results from this experiment suggest that 10 µg/ml of CLA mix had no effect on the expression of Cox I in BAEC at 24 hours.

4.4.2 The effect of CLA mix on Cox I steady state mRNA levels

To determine the effect of the CLA mix on Cox I steady state mRNA levels, BAEC were incubated with a single concentration of the mix for 24 hours. Samples generated from BAEC exposed to shear stress (23 dyn/cm^2, as described in section 2.2.15) were used as positive controls for the up regulation of Cox I mRNA. Cox I cDNA was amplified as a positive control for the Cox I primer set. Total RNA was harvested by using the Trizol® method, section 2.2.5. RT PCR was carried out as described in section 2.2.10.

Figure 4.4.2 Fold change in Cox I mRNA in BAEC after 24 hour treatment with 10 μg/ml CLA. BAEC were quiesced by serum deprivation for 24 hours prior to treatment. Results were normalised by amplification of β-actin or GAPDH. The image presented is representative. The graph shown is derived from mean densitometric analysis of results from multiple analysis of samples generated from four separate experiments.

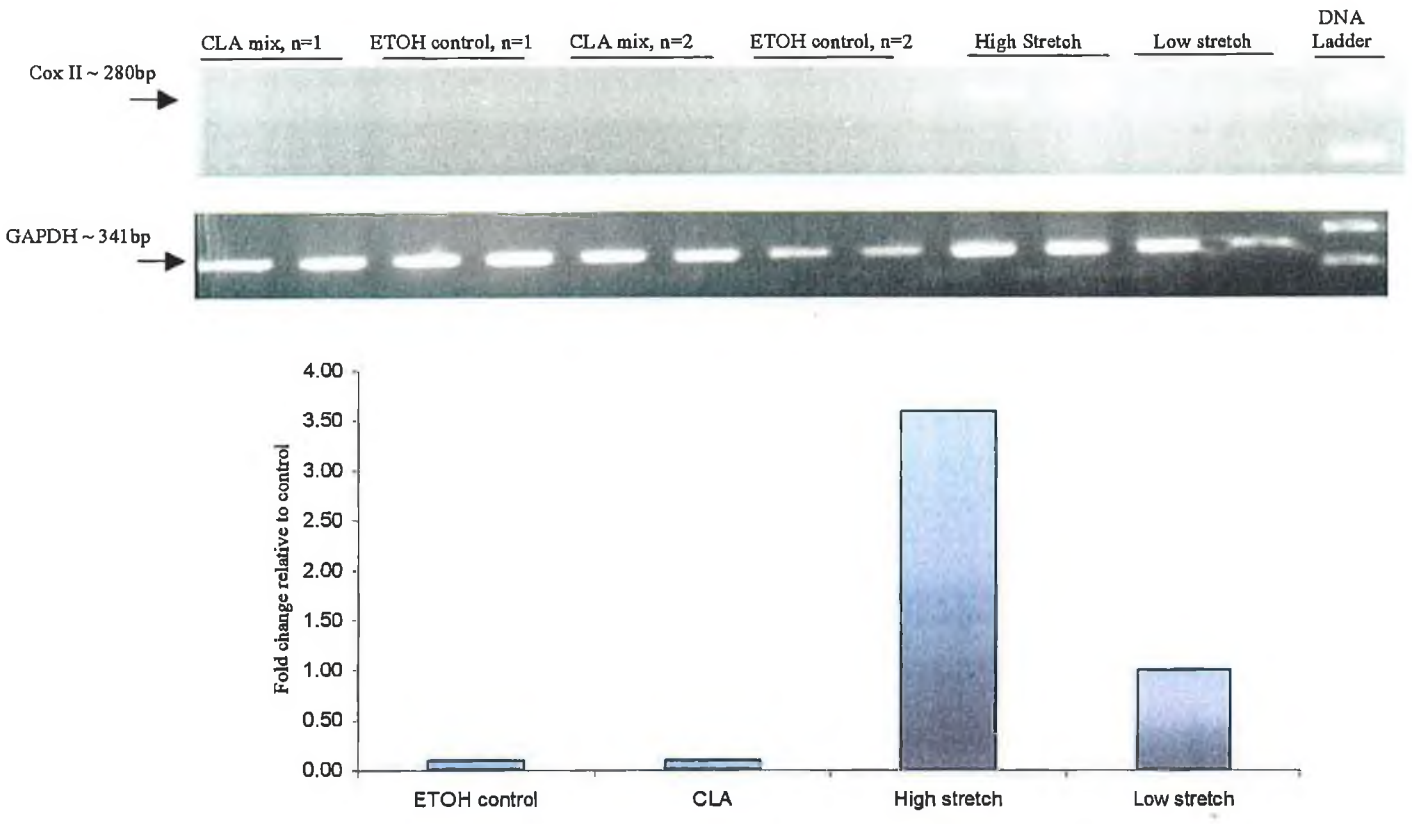


The results from this experiment suggest that 10 μg/ml of CLA mix had no effect on the expression of Cox I mRNA in BAECs at 24 hours.

4.4.3 The effect of CLA mix on Cox II steady state mRNA levels

To determine whether CLA mix had any effect on Cox II steady state mRNA levels, BAEC were incubated with a 10 μg/ml of the mix for 24 hours. Samples generated from BAEC exposed to cyclic stretch (5%) were used as positive controls for the up regulation of Cox II mRNA. Total RNA was harvested by using the Trizol® method as described in section 2.2.5. RT PCR was carried out as described in section 2.2.10.

Figure 4.4.3 Fold change in Cox II mRNA in BAEC after 24 hour treatment with 10 μg/ml CLA. BAEC were quiesced by serum deprivation for 24 hours prior to treatment. Results were normalised by amplification of GAPDH. The image presented is representative. The graph shown is derived from mean densitometric analysis of results from multiple analysis of samples generated from two separate experiments.



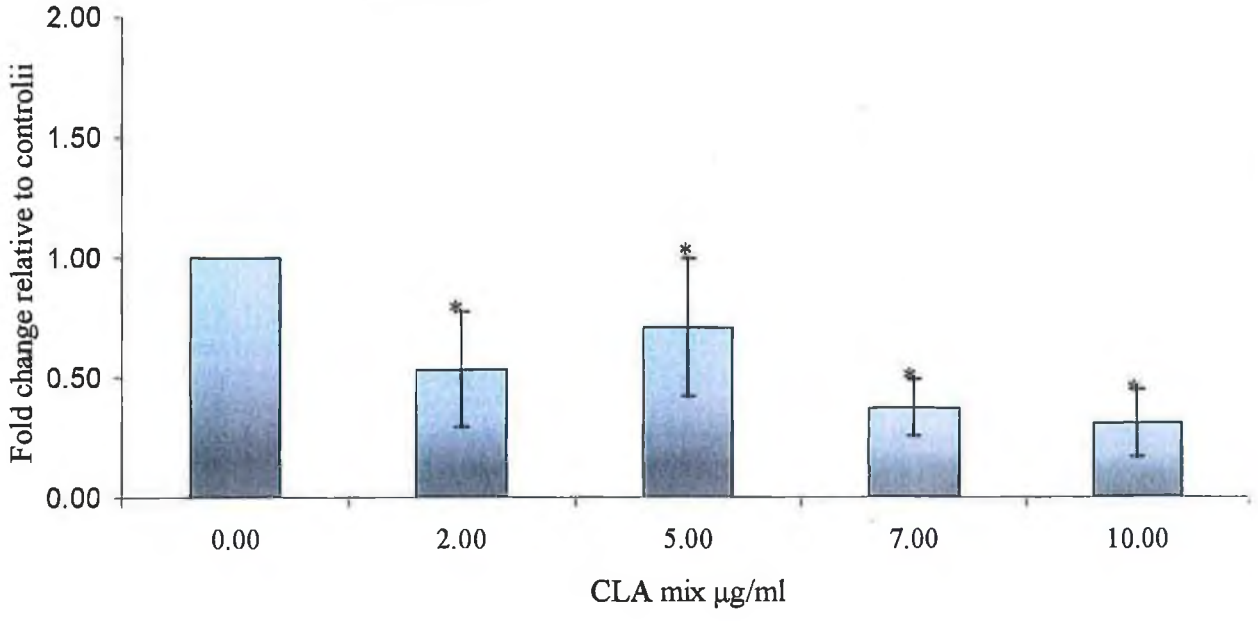
The results from this experiment suggest that 10 μg/ml CLA mix had no effect on the induction of Cox II transcription in BAEC after 24 hours.

4.5 The effect of CLA on Cyclooxygenase activity

4.5.1 Dose dependant effect of CLA mix on 6-keto-Prostaglandin $F_{1\alpha}$ levels in conditioned media after 24 hours

6-keto-Prostaglandin $F_{I\alpha}$ a stable downstream product of the cyclooxygenase pathway. The rate limiting enzyme of this pathway is Cox I. Cox I enzyme activity can be measured indirectly by monitoring the production of 6-keto-Prostaglandin $F_{1\alpha}$. The effects of the CLA mix on Cox I activity was indirectly measured by ELISA. The results were as follows.

Figure 4.5.1 Change in 6-Keto PGI $F_{I\alpha}$ concentration in conditioned media after 24 hour treatment with 0-10 μg/ml CLA mix as determined by ELISA. Cells were quiesced by serum starvation prior to treatment. The graph shown is derived from mean statistical analysis of results from triplicate analysis of samples generated from three separate experiments. * $P<0.05$ versus ETOH control

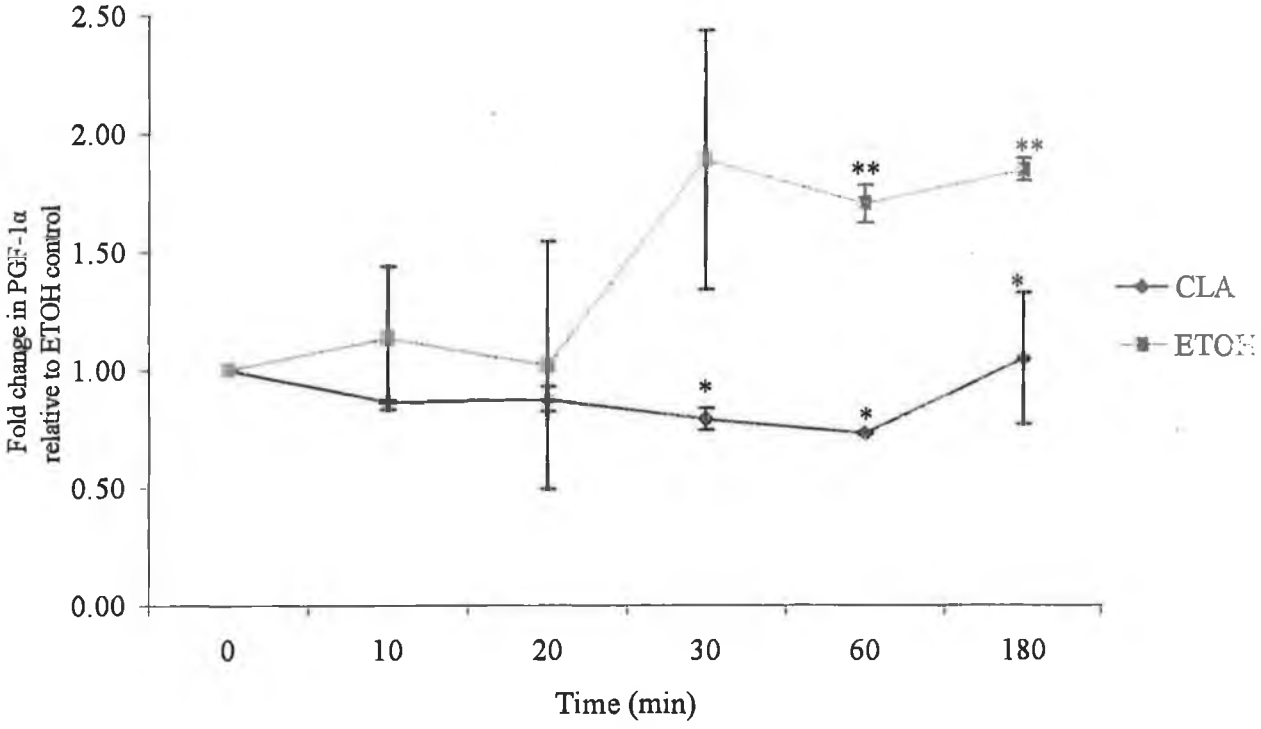


The results from this experiment suggest that CLA mix decreases the production of 6-Keto PGI $F_{I\alpha}$ with increasing dose at 24 hours. This is suggestive of a decrease in the production of PGI_2 and possibly a decrease in cyclooxygenase activity.

4.5.2 The effect of acute CLA mix treatment on 6-keto-Prostaglandin $F_{1\alpha}$ levels in conditioned media

To determine whether the decrease in the production of 6-keto-Prostaglandin $F_{1\alpha}$ caused by CLA occurred over an acute time course the following experiments were carried out. The dose of 10 μg/ml was chosen because it elicited the strongest response. The levels of 6-keto-Prostaglandin $F_{1\alpha}$ in conditioned media was measured by ELISA as described in section 2.2.11. The results were as follows:

Figure 4.5.2 Change in 6-Keto $PGF_{1\alpha}$ concentration in conditioned media after acute CLA mix treatment. Cells were quiesced by serum starvation prior to treatment. The graph shown is derived from mean statistical analysis of results from triplicate analysis of samples generated from two separate experiments * *P<0.05* versus ETOH control. ** *P<0.05* versus 0 min.

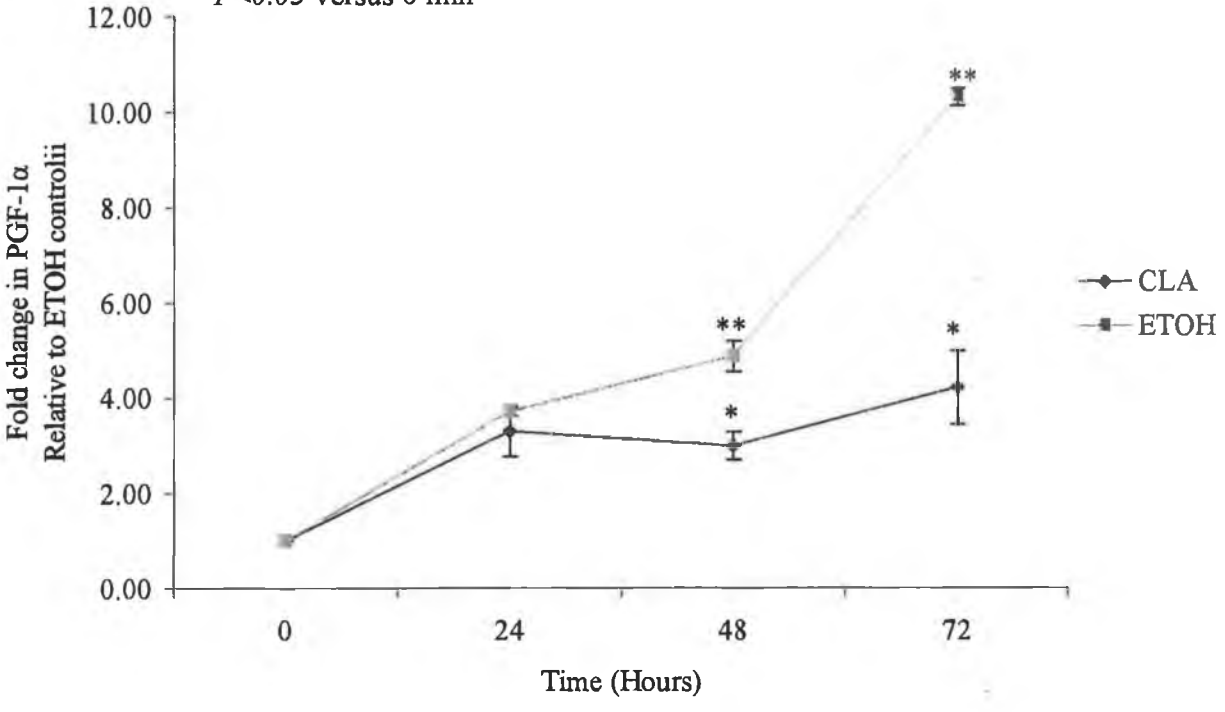


The results from this experiment suggest that CLA mix decreases the production of 6-Keto $PGF_{I\alpha}$ over a 3 hour time course. Again, this is suggestive of a decrease in the production of PGI_2 and possibly a decrease in cyclooxygenase activity. The increase in $PGF_{I\alpha}$ after ETOH treatment is a result of natural build in the media.

4.5.3 The effect of chronic CLA mix treatment on 6-keto-Prostaglandin $F_{1\alpha}$ levels in conditioned media

To determine whether the decrease in the production of 6-keto-Prostaglandin $F_{1\alpha}$ caused by CLA occurred over a chronic time course the following experiment was carried out. The dose of 10 μg/ml was chosen because it elicited the strongest response. The results were as follows:

Figure 4.5.3 Change in 6-Keto $PGF_{1\alpha}$ concentration in conditioned media after chronic treatment with CLA mix . Cells were quiesced by serum starvation prior to treatment. The graph shown is derived from mean statistical analysis of results from triplicate analysis of samples generated from two separate experiments * *P<0.05* versus ETOH control. ** *P<0.05* versus 0 min



The results from this experiment suggest that CLA mix decreases the production of 6-Keto PGI $F_{I\alpha}$ over a 72 hour time course. The increase in $PGF_{I\alpha}$ after ETOH treatment is a result of natural build in the media. Again, this is suggestive of a decrease in the production of PGI_2 and possibly a decrease in cyclooxygenase activity.

4.6 Discussion

The results from this study demonstrate that CLA has no dose dependant or temporal effect on eNOS protein expression, or steady state mRNA levels after 24 hours, under non-agonist stimulated conditions. The CLA isomer t10/c12 has been shown to posses distinct biological activity compared to other isomers of CLA (Khosla and Fungwe, 2001). The t10/c12 isomer also showed no regulation of eNOS protein expression. As a control for the conjugation of CLA, linoleic acid was also tested, and also had no effect on eNOS protein. Treatment also had no impact on basal levels of eNOS activity over an acute and chronic time course. Subsequently it was decided to examine whether CLA affected agonist-stimulated eNOS activity. Pre-treatment with CLA was shown to synergistically augment agonist activation of eNOS. Following pre-treatment of BAEC with CLA mix for 3 and 24 hours, Bradykinin stimulated release of nitric oxide was increased up to 28% relative to controls, for both treatments. These events are illustrated in the proposed model shown below.

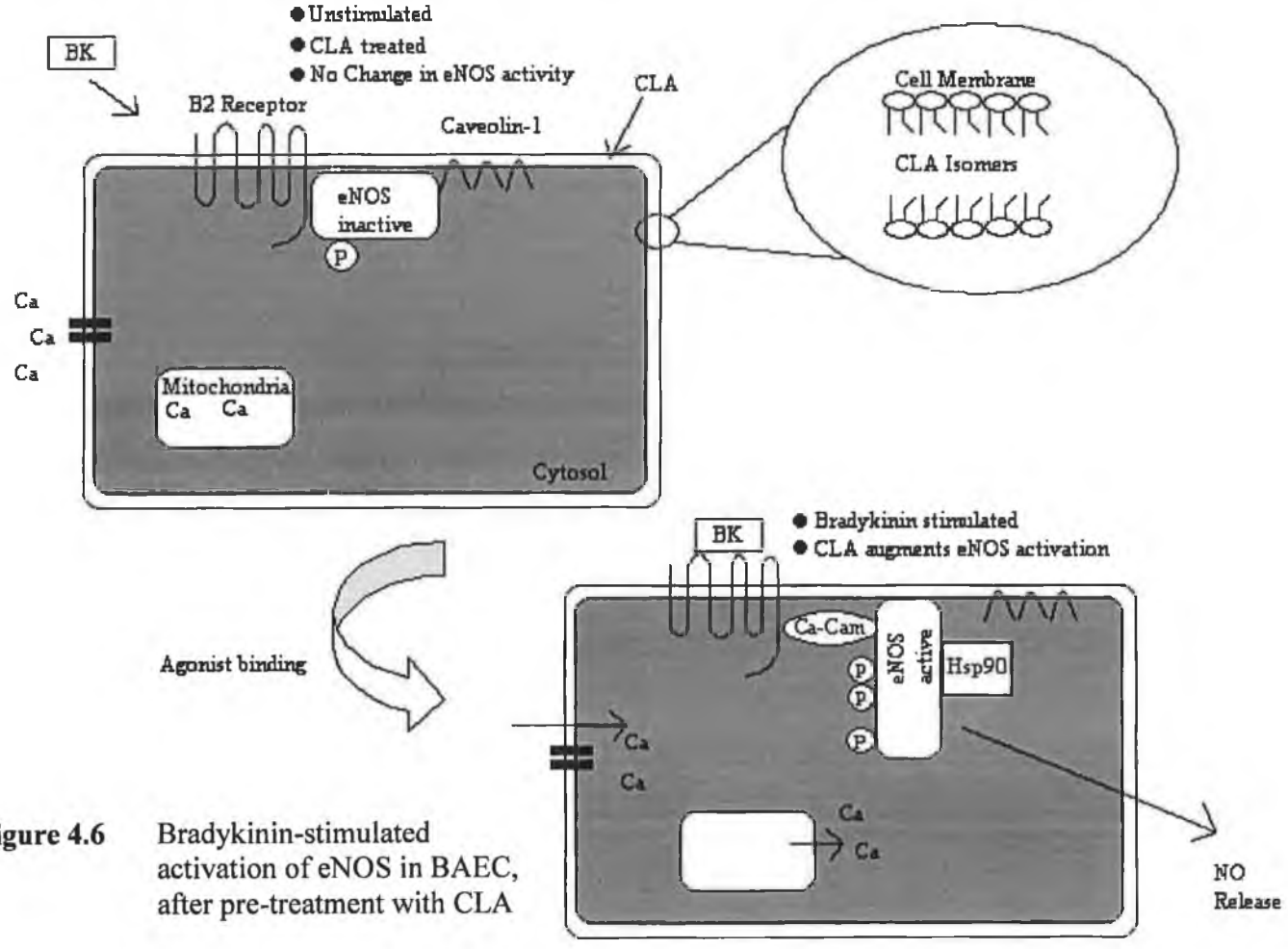


Figure 4.6 Bradykinin-stimulated activation of eNOS in BAEC, after pre-treatment with CLA

Bradykinin in plasma mainly acts on endothelial cells and stimulates them to release various agents associated with vascular tone and blood coagulation, such as nitric oxide, prostaglandin's, catecholamines, and cytokines, resulting in the maintenance of circulatory homeostasis (Dendorfer *et al*, 1998). Most of the cellular action of bradykinin are mediated through binding to the B_2 receptor (Figure 4.6). Bradykinin stimulation of BAECs causes a release of Ca^{2+} from intracellular mitochondria stores and a Ca^{2+} influx from outside the cell. Both, release and influx of Ca^{2+} lead to a transient increase in cytosolic Ca^{2+}, which promotes the binding of calmodulin (CaM) to eNOS. Subsequent phosphorylation, coupling with Heat Shock Protein 90 (Hsp 90) and release of eNOS from Caveolin-1 leads to enzyme activation and nitric oxide release. eNOS also forms complexes with the bradykinin B2 receptor, inhibiting activation, from which the enzyme is released in a Ca^{2+} and ligand-dependent manner upon B2 receptor activation (Venema, 2002). This is a common and significant form of eNOS regulation.

The role that CLA would have in augmenting this activation is unclear. CLA is incorporated into the phospholipid profile of the cell membrane (Belury and Kempa-Steczko, 1997). It could be possible that the altered lipid profile affects the 3D structure of the B_2 receptor resulting in a higher affinity for bradykinin and/or decreased affinity for inhibitory binding of eNOS, both of which would lead to increased nitric oxide production. This is speculative and needs further investigation. Such a study would initially need to examine whether CLA augmented receptor-independent activation in a similar manner. CLA may also alter the calcium influx elicited by bradykinin stimulation. Arachidonic acid, another cell membrane lipid, has previously been show to putatively modulate ion channel function (Devor and Frizzell, 1998). A previous study showed that in cultured keratinocytes, CLA reduced the incorporation of C^{14}-arachidonate (Liu and Belury, 1998).

eNos remains in an inactive state through binding to plasmalemmal caveolae (caveolin-1 and -3) (Andrew and Mayer, 1999). The distinctive lipid content of the caveolae is vital to the inhibitory function of Caveolin-1. A study carried out by Venema *et al*, (1995) demonstrated that eNOS activity could be negatively modified, through alteration of binding to caveolin-1, by anionic phospholipids present in the cell membrane. CLA present in the cell membrane may also modulate this inhibitory binding mechanism and therefore augment bradykinin-stimulated activation.

This synergistic augmentation of nitric oxide production may also be associated with other endothelial cell activators not examined in this study. CLA may have the same effect with Ca^{2+} independent activation by shear stress and cyclic strain, or by G-protein receptor independent activation by calcium ionophore A23187. Other eNOS activators that could be examined would include vascular endothelial growth factor (VEGF), acytlecholine and substance P.

CLA may also be modulating the availability of NO once it is released from the cell. The half life of NO is very short, on the order of seconds (Wink *et al*, 1998). NO reacts with O_2 to form various reactive NO species, as well as nitrite and nitrate (Kim *et al*, 2001). Because CLA may possess redox buffering capacity, it may be modulating the kinetics of these breakdown reactions resulting in higher available nitrite concentrations. Overall there are a number of different ways CLA could be eliciting the observed augmentation of bradykinin stimulated eNOS.

The second mechanism of action which may be eliciting the atheroprotective effects of CLA involves modulation of the cyclooxygenase pathway under non-stimulated conditions. The results demonstrate that CLA has no effect on Cox I steady state mRNA levels and protein expression. CLA did however down regulate the release of 6-Keto PGF Iα (a stable metabolite of PGI_2) in a time (60% decrease after 72 h) and dose (60% decrease with 10 μg/ml after 24 h) dependent manner. This down regulation of prostacyclin (PGI_2) suggests an inhibition of the cyclooxygenase pathway, consistent with this observation is a previous study which shows that CLA affects arachidonic acid

metabolism in human saphenous vein endothelial cells, inhibiting eicosanoid production (Urquhart, 2001).

There are a number of mechanisms by which CLA may be eliciting this effect. Like most other dietary polyunsaturated fatty acids, isomers and metabolites of CLA are readily incorporated into phospholipid and neutral lipid fraction of the cell membrane (Belury and Kempa-Steczko, 1997). This incorporation could be in competition with the uptake of linoleic acid, the precursor of arachadonic acid. Arachadonic acid is the natural substrate of the Cox pathway. This competition may have the effect of reducing the arachadonic acid complement of the cell membrane, which would subsequently cause a decrease in Cox activity and PGH_2 production (Belury, 2002). A previous study showed that in cultured keratinocytes, CLA reduced the incorporation of C^{14}-arachidonate (Liu and Belury, 1998). Another theory raises the possibility that CLA or elongated and desaturated products from CLA may act as substrates or antagonists for cyclooxygenase, resulting in reduced available enzyme for arachidonate. If CLA is metabolised by Cox it may give rise to a new profile of CLA derived eicosanoids (Belury, 2002).

Chapter 5 The Effect of DL Homocysteine on BAEC function

5.1 Introduction

The effects of the non protein amino acid homocysteine on steady state mRNA levels, protein expression and activity of eNOS enzyme is reported in this chapter. The importance of correct expression and activity of eNOS with regard to the vasoregulatory mechanisms of the vascular endothelium has already been discussed, section 1.1. The pretext for this study has previously been described in section 1.3.

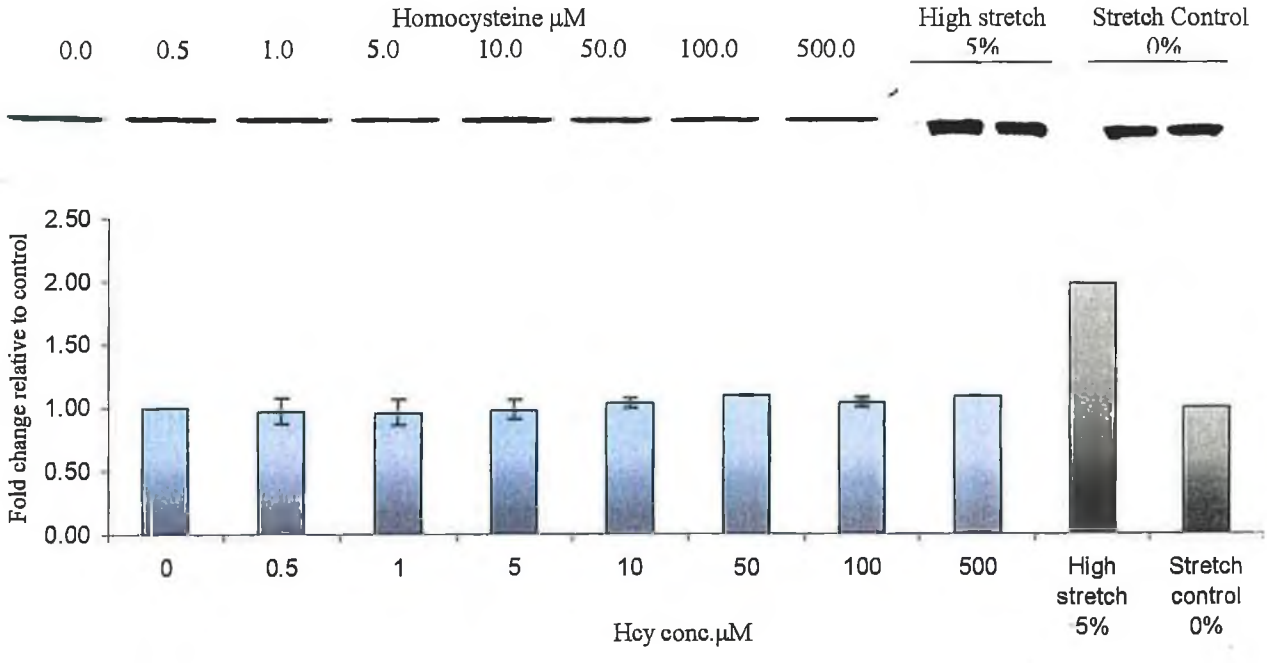
Briefly, homocysteine has previously been shown to interfere with the availability of nitric oxide produced by the vascular endothelium. Homocysteine interacts with nitric oxide to produce S-nitrosohomocysteine at physiological concentrations, which amplifies the vasoprotective effects of nitric oxide. Normal endothelial cells detoxify homocysteine by releasing nitric oxide (Stamler *et al*, 1993), which in turn leads to the formation of S-nitrosohomocysteine. This reaction decreases the production of hydrogen peroxide following oxidation of sulphydryl groups and represents a protective mechanism against the adverse effects of homocysteine. Although prolonged exposure and high homocysteine concentrations result in impaired nitric oxide production. Therefore chronically elevated homocysteine concentrations appear to cause a self-perpetuating cycle that eventually overwhelms the capacity of the endothelial cells to reduce the toxicity of homocysteine (Stamler *et al*, 1993).

From this pretext it is proposed that homocysteine may play a role in modulating the expression/ activity of eNOS protein and mRNA. The experiments were designed to examine the effects of chronic treatments (up to 24 hours) of the homocysteine (0-500 μM) on the expression of eNOS protein and steady state RNA. The effect of homocysteine treatment on nitrite levels over acute (0-3 hours) and chronic (0-72 hours) in conditioned media generated from un-stimulated BAEC was also investigated. Modulation of cyclooxygenase expression and activity was not investigated as there was not a strong pretext for such a study.

5.1.1 Dose dependent effect of DL - homocysteine treatment on eNOS protein expression

To determine the effect of various concentrations of homocysteine on eNOS protein expression, BAEC were incubated with 0-500 μM of Hcy for 24 hours. Stretched BAECs (5%) were used as positive control for the up regulation of eNOS. BAEC were stretched as described in section 2.2.14. Whole cell lysates were prepared as described in section 2.2.3. SDS PAGE and western blot analysis was carried out as described in section 2.2.9.

Figure 5.1.1 Fold change in eNos protein expression in BAEC after 24 hour treatment with various doses of Hcy. Cells were quiesced by serum deprivation for 24 hours prior to treatment. Equality of protein loading was determined by ponceau staining. The image presented is representative. The graph shown is derived from mean densitometric analysis of results from multiple analysis of samples generated from three separate experiments.

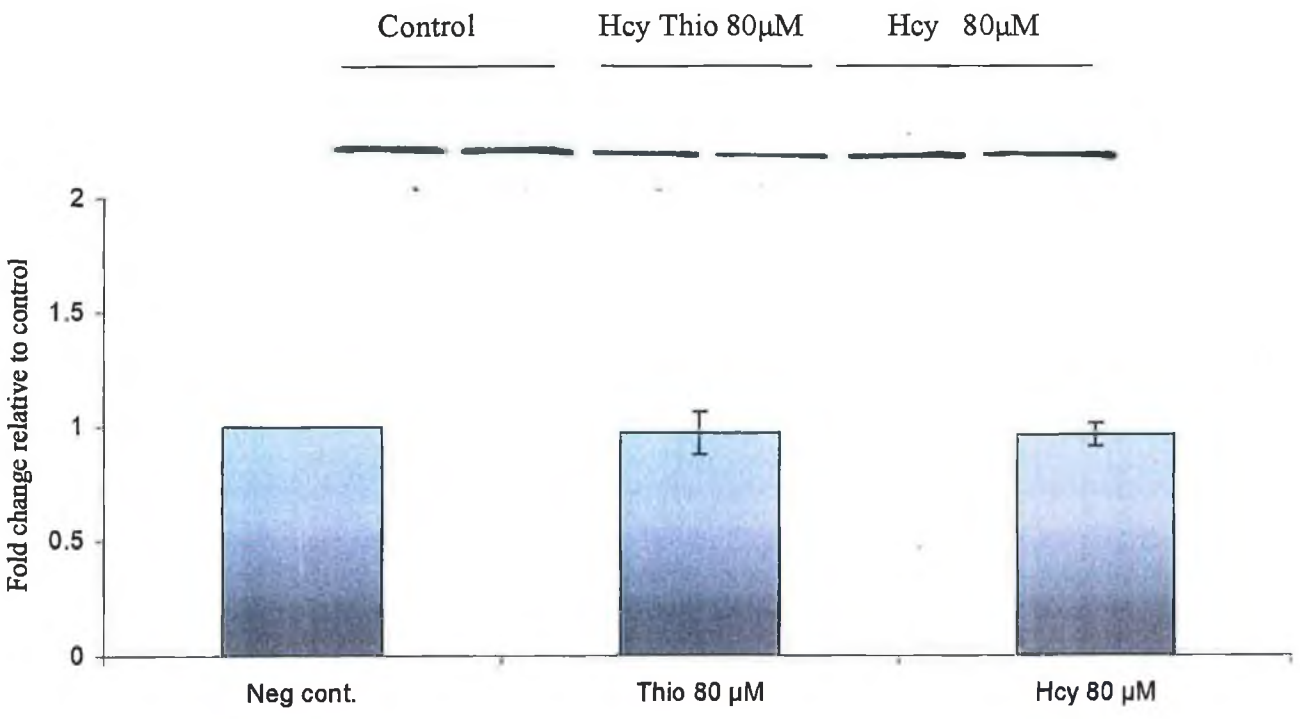


The results from this experiment suggest that 0-500 μM DL-homocysteine has no effect on the expression of eNOS protein in BAEC after 24 hours.

5.1.2 The effect of DL - homocysteine and homocysteine thiolactone treatment on eNOS protein expression

Because no effect was observed for DL - homocysteine, it was decided to examine the effects of homocysteine thiolactone on the expression of eNOS protein. Homocysteine thiolactone is a thiol form of circulating homocysteine that is also implicated in cardiovascular disease. A concentration of 80 μM was chosen because it is relevant to pathophysiological levels in a patient with hyperhomocysteine The results were as follows:

Figure 5.1.2 Fold change in eNOS in bovine aortic endothelial cells after 24 hour treatment with Hcy and Hcy Thio. Cells were quiesced by serum deprivation for 24 hours prior to treatment. Equality of protein loading was determined by ponceau staining. The image presented is representative The graph shown is derived from mean densitometric analysis of results from multiple analysis of samples generated from two separate experiments.

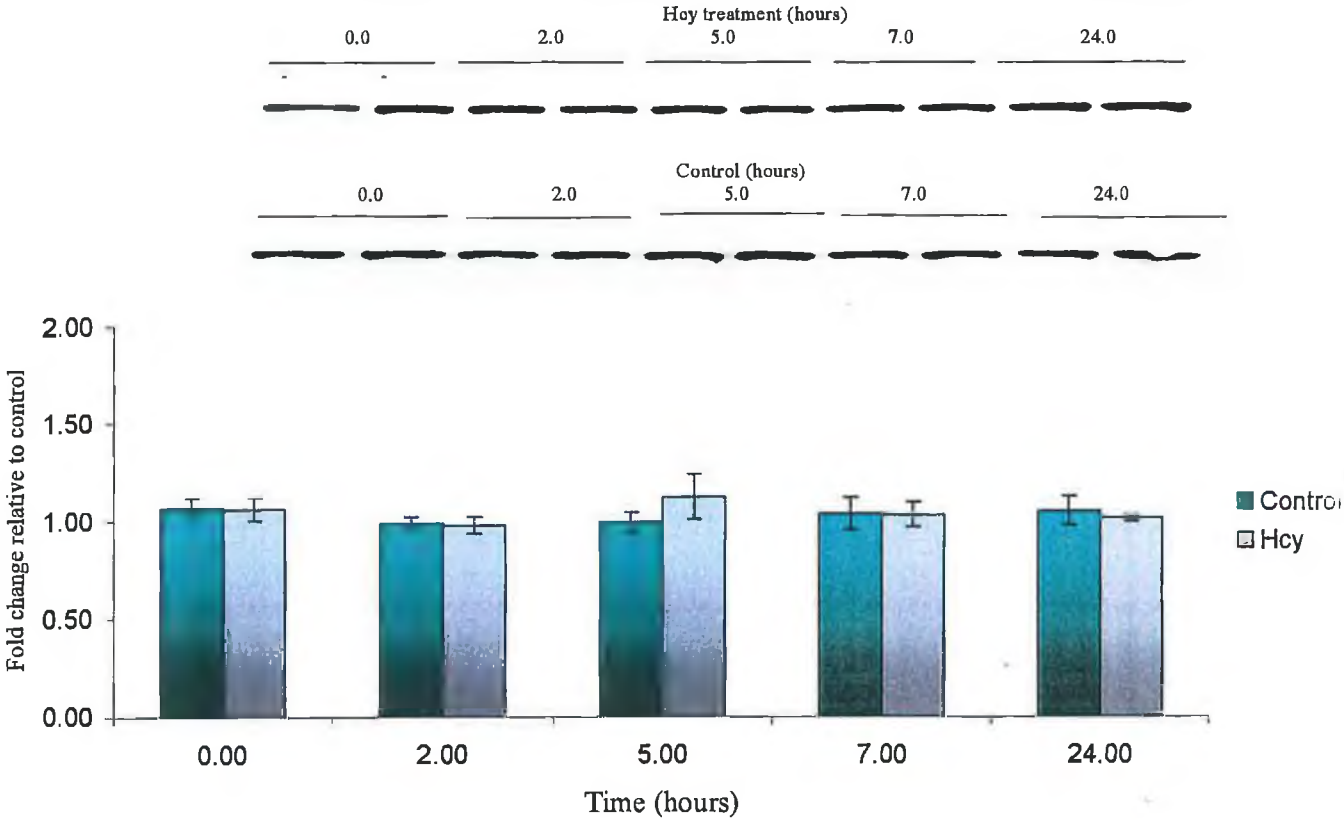


The results of this experiment suggest that 80 μM of DL - homocysteine and homocysteine thiolactone did not effect eNOS expression in BAEC after 24 hours.

5.1.3 Temporal effect of homocysteine treatment on eNOS protein expression

To determine the effect of homocysteine treatment on eNOS protein expression with respect to time, BAEC were incubated with a pathophysiological relevant concentration of Hcy, 80 μM, over a 72 hour period. Whole cell lysates were prepared as described in section 2.2.3. SDS PAGE and western blot analysis was carried out as described in section 2.2.9. The results were as follows:

Figure 5.1.3 Fold change in eNOS protein expression relevant to control in BAEC, over a 72 hour period. Cells were quiesced by serum deprivation for 24 hours prior to treatment. Equality of protein loading was determined by ponceau staining. The image presented is representative. The graph shown is derived from mean densitometric analysis of results from multiple analysis of samples generated from two separate experiments.

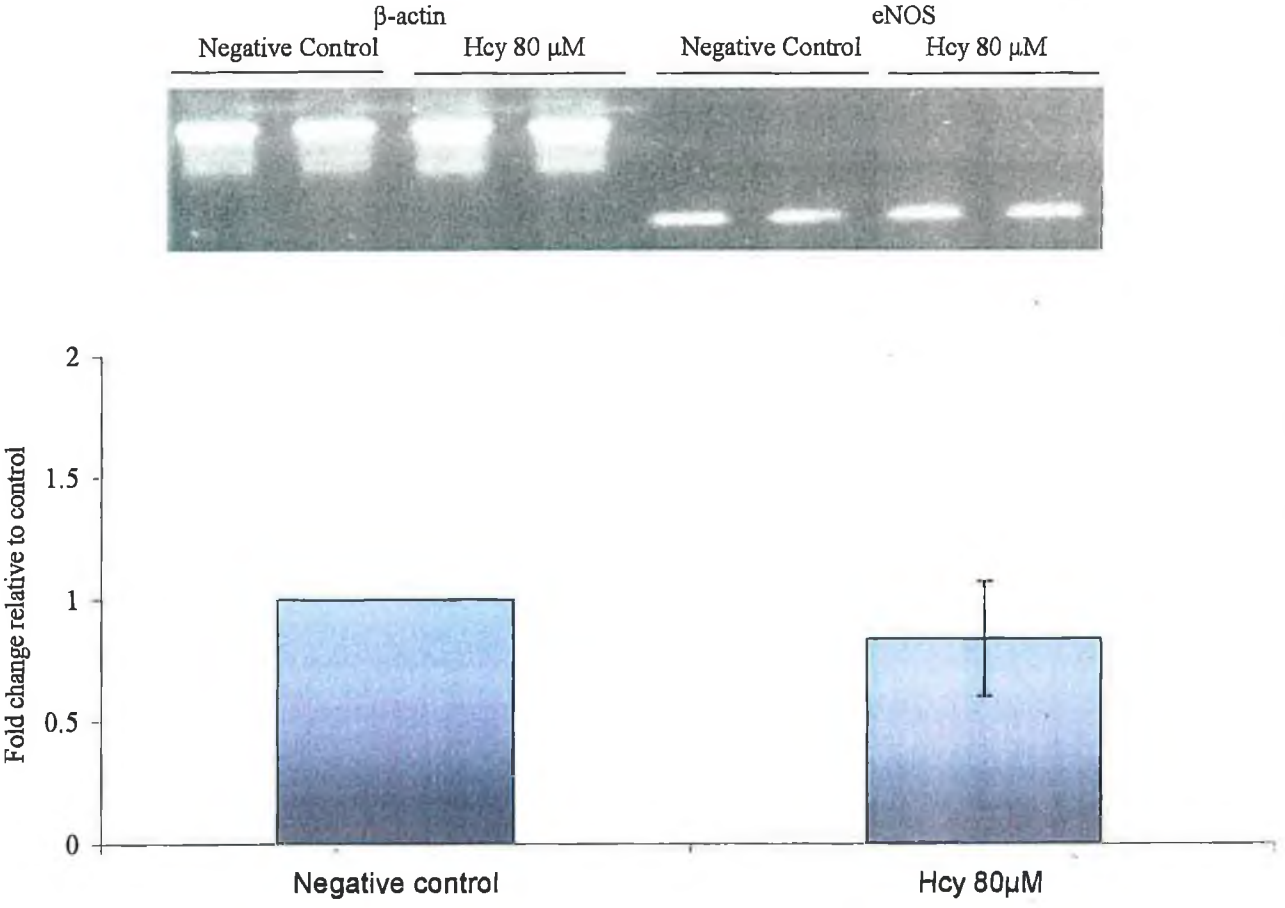


The results from this experiment suggest that 80 μM DL - homocysteine has no temporal effect on eNOS protein expression over a 24 hour period in BAEC.

5.1.4 The effect of DL - homocysteine on eNOS steady state mRNA levels

To determine whether Hcy had any effect on eNOS mRNA transcription BAEC were treated with 80 μM of Hcy for 24 hours. Total RNA then harvested by the Trizol® method as described in section 2.2.5. RT PCR was carried out as described in section 2.2.10. The results were as follows:

Figure 5.1.4 Fold change in eNOS mRNA in BAECs after 24 hours of treatment with Hcy 80μM as determined by RT-PCR. Cells were quiesced by serum deprivation for 24 hours prior to treatment. Results were normalised by amplification of β-actin. The image presented is representative. The graph shown is derived from mean densitometric analysis of results from multiple analysis of samples generated from two separate experiments.



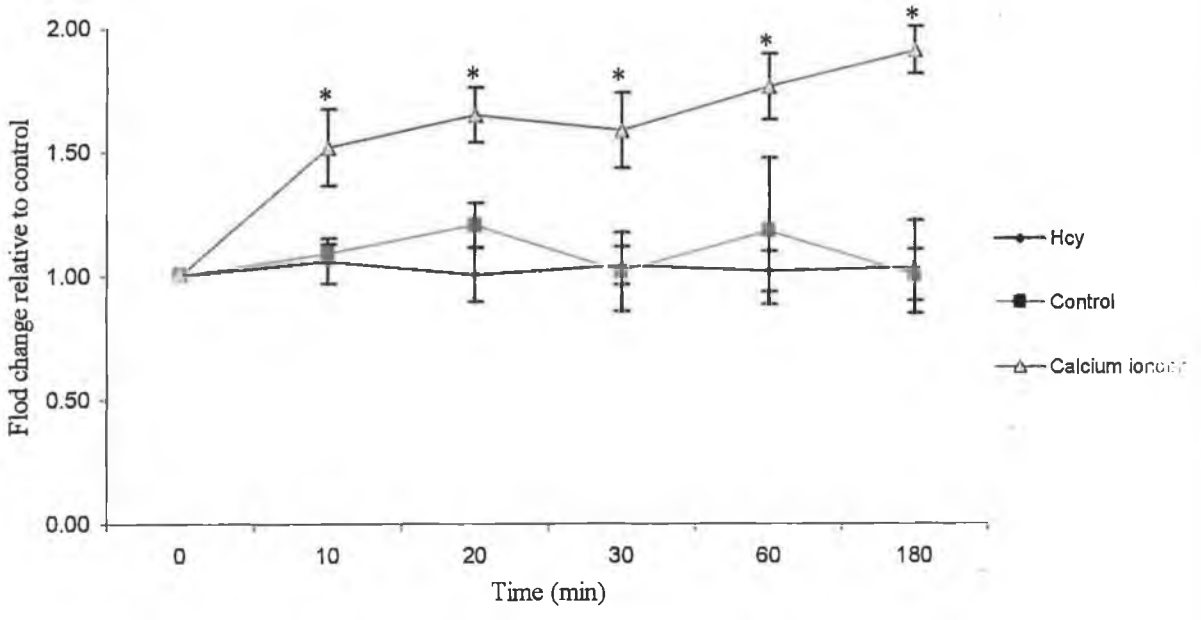
The results from this experiment suggest that 80 μM of DL - homocysteine have no effect on eNOS mRNA expression in BAEC after 24 hours.

5.2 The effect of DL - homocysteine on eNOS enzyme activity

5.2.1 The effect of acute DL – homocysteine treatment on nitrite levels in conditioned media of unstimulated BAEC

The effect of treatment on eNOS activity was determined by measuring the amounts of nitrite in conditioned media. eNOS produces nitric oxide which is rapidly converted to both nitrite and nitrate. Measurement of nitrite is an indirect index of eNOS activity. Nitrite was measured by the fluorometric DAN assay section 2.2.12. As a positive control for the assay conditioned media from Calcium Ionophore A23187 treated BAEC was assayed. Calcium ionophore treatment stimulates eNOS activity through a transient increase in cellular Ca^{2+}. The determined effects of DL - homocysteine on basal eNOS activity over a 3 hour time course was follows:

Figure 5.2.1 Change in nitrite levels in conditioned media after treatment with Hcy 80 μM, and Calcium Ionophore 10 μM over a three hour time course as measured by the DAN fluorometric assay. The graph shown is derived from mean statistical analysis of results from multiple analysis of samples generated from three separate experiments.

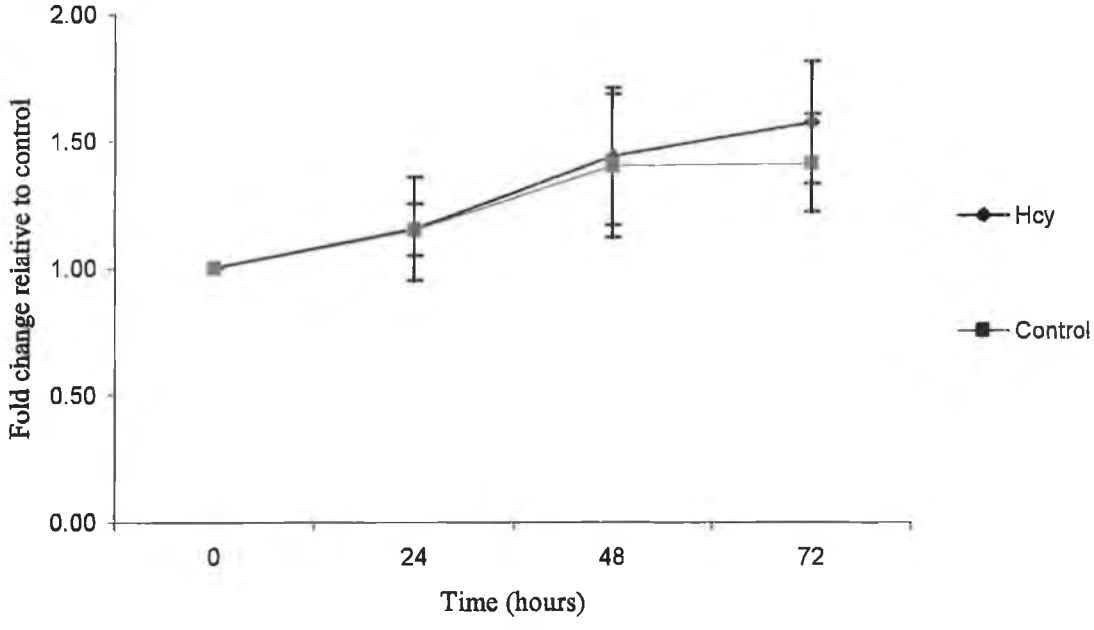


The results from this experiment suggest that DL - homocysteine has no effect on the basal activity of eNOS over a 3 hour time course.

5.2.2 The effect of chronic DL - homocysteine treatment on nitrite levels in conditioned media

Because there seemed to be no effect of homocysteine on eNOS activity over an acute time course, it was decided to examine the effects over a chronic time course. The determined effects of chronic exposure of Homocysteine on basal eNOS activity was as follows:

Figure 5.2.2 Change in nitrite levels in conditioned media after treatment with Hcy 80 μM over a seventy two hour time course as measured by the DAN fluorometric assay. The graph shown is derived from mean statistical analysis of results from multiple analysis of samples generated from three separate experiments.



The results from this experiment suggest that DL - homocysteine has no effect on the basal activity of eNOS over a 72 hour time course.

5.3 Discussion

The second aspect of this study focuses on homocysteine. The pretext for studying the effects of homocysteine was that it deleteriously modulates extra cellular oxidation/reduction processes.

Homocysteine has previously been shown to limit the availability of nitric oxide produced by the vascular endothelium. Normal endothelial cells detoxify homocysteine by releasing nitric oxide (Stamler *et al*, 1993), which in turn leads to the formation of S-nitrosohomocysteine. Acutely, and at low concentrations, this has the effect of improving the vasodilation function of the endothelium. However, at higher concentrations and with chronic exposure, bio availability of nitric oxide is drastically decreased, which is severely deleterious to endothelial vasodilatory function.

We subsequently hypothesized that homocysteine treatment may cause up regulation of expression or activity of eNOS to compensate for the lower levels of nitric oxide available to the vascular endothelium.

The results presented in this study suggest that DL - homocysteine treatment of BAEC has no effect on steady state mRNA levels and protein expression or activity of eNOS over acute and chronic time courses. Protein levels of eNOS were unaffected by DL - homocysteine and homocysteine thiolactone treatment at physiological and pharmacological concentrations. Nitrite levels in conditioned media remained unchanged after chronic and acute treatments.

One limitation of this study is the form that the homocysteine is presented to the cells. In vivo, homocysteine exists in a number of conjugated forms. A small fraction (<2%) of plasma total homocysteine circulates in the thiol form. The remainder is a mixture of disulfide derivatives, including homocystine, homocysteine-cysteine mixed disulfide, and protein bound disulfides (Mudd *et al*, 2000). It is possible that in vivo these forms may affect regulation of eNOS. In a human study by Chambers *et al*, (2001) it was demonstrated that reduced homocysteine is the deleterious form of homocysteine with

regards to vascular function and that other homocysteine species play a less important role.

Many of the studies to date have examined the effects of pharmacological concentrations of homocysteine, which is not accurately representative of the situation *in vivo*. For further *in vitro* studies it would be interesting to present a reduced homocysteine treatment, at physiological concentrations, to BAEC and then examine the effects on vasoregulatory function. This kind of treatment would need a validated manipulation of the oxidation status of the culture media to produce the desired reduced homocysteine.

A study carried out by Zhang *et al*, (2000) corroborates the non effect of homocysteine on eNOS expression presented here. The group did, however, report that pre-treatment with homocysteine resulted in a gradual decline in bradykinin, calcium ionophore and L-arginine stimulated nitric oxide production. An increase in reduced oxygen species was also reported with pre-treatment. A lack of consensus still exists, however, as to the effects of homocysteine on nitric oxide production. A study by Upchurch *et al*, (1997) report that homocysteine stimulates nitric oxide production, while De Groote *et al*, (1996) suggest that homocysteine downregulates nitric oxide production.

Whether or not homocysteine modulates eNOS activity, homocysteine does adversely affect endothelial function by modulating exposure to oxidative stress. A depletion in bioavailable nitric oxide, through alteration of oxidative stress has been reported (Stamler *et al*, 1993). Auto-oxidation of homocysteine results in the production reactive oxygen species such as of hydrogen peroxide and superoxide ion (Heinecke *et al*, 1987). Superoxide anion can react with nitric oxide to form peroxynitrite, which impairs its biological activity (Gryglewski, 1986). The role of superoxide formation in homocysteine-induced endothelial dysfunction is underscored by the demonstration of greater superoxide production in aortic tissue from mildly hyperhomocysteinemic mice than from wild-type mice (Eberhardt, 2000) and the finding that superoxide dismutase can reverse the decreased cerebrocortical blood flow during superfusion with homocysteine-containing buffer (Zhang, 1998). Earlier investigations have supported a

role for H_2O_2 in homocysteine-induced endothelial toxicity in vitro because catalase was found to inhibit the homocysteine-induced lysis of endothelial cells in the presence of transition metals or ceruloplasmin (Starkbaum, 1986).

Homocysteine-induced vascular oxidant stress may be additionally aggravated by a homocysteine-mediated decrease in the expression of the cellular isoform of glutathione peroxidase (GPx-1) (Weiss *et al*,2000). This key enzyme for the cellular defence against oxidant stress uses glutathione to reduce H_2O_2 and lipid peroxides to their respective alcohols (Flohe, 1989). H_2O_2 decomposes to the toxic oxygen species hydroxyl radical, which is highly reactive and causes lipid peroxidation, and hydroxide, which promotes tissue damage.

Chapter 5 Discussion

The interaction between circulating dietary constituents and the vascular endothelial monolayer is vital to the proper maintenance of vascular homeostatic mechanisms, such as vasoregulation. Endothelial dysfunction and subsequent loss of vasoregulatory function are implicated in the early stages of atherosclerosis. This study was carried out to determine whether the dietary constituents CLA and homocysteine can modulate normal endothelial function, with particular focus on eNOS and Cox I expression and activity.

Conjugated linoleic acid (CLA) is the collective term used for positional and geometrical isomers deriving from the essential fatty acid, linoleic acid. It has been postulated that CLA elicits atheroprotective effects in vitro by two potential mechanisms. Firstly, by modulating endothelium exposure to oxidative stress. CLA has been shown to possess antioxidant properties, in studies it was a more potent antioxidant than α-tocopherol (Ha *et al*, 1990). It has also been shown that CLA may be auto oxidised to several furan derivatives that possess a redox buffering capacity (Yurawecz *et al*, 1995). A factor in the aetiology of atherosclerosis (and diabetes) is that nitric oxide availability is impaired through increases in oxidative stress (Drexler *et al*, 1999). The hypothesis for this study was that the redox-buffering property of CLA may modulate the expression and/or activity of eNOS, through regulation of oxidative stress with subsequent impact on nitric oxide availability. Secondly, it has been shown that isomers and metabolites of CLA are readily incorporated into the phospholipid and neutral lipid fraction of the cell membrane (Ha *et al*, 1990) and may possibly exert its effects through altering eicosanoid synthesis. This has previously been proven for a venous cell type although no data was presented for the effect of CLA on Cox steady state mRNA levels or protein expression (Urquhart *et al*, 2001). It was therefore hypothesised that CLA would putatively affect expression and/or activity of eNOS and/or Cox I in BAEC.

The results from this study suggest that CLA downregulates endothelial release of prostacyclin (PGI_2) and probably many other prostanoids (PGE_2 and PGD_2). This effect on prostanoid production can be viewed in two ways. In one regard it would seem that

CLA is pro-atherogenic. Prostacyclin and other prostaglandin's are potent vasodilator, in balance with thromboxane, and also inhibits platelets aggregation. On the other hand a decrease in the production of prostaglandin's by the vascular endothelium may attenuate any pro-inflammatory pathogenicity and inhibit atherosclerotic plaque formation. In an animal study, carried out recently by Sinead Toomey *et al*, (2003) at the Royal College of Surgeons in Ireland, it was suggested that a CLA-supplemented diet resulted in a profound resolution of atherosclerotic lesion mediated by inhibition of prostaglandin formation and activation of PPARγ nuclear receptor. Based on the findings from other animal studies reviewed, it is most likely that CLAs effects are beneficial, and mediated, in part at least, by prostanoid downregulation.

There is also the possibility that CLA modulates the lipooxygenase and epoxygenase pathways. Arachidonic acid is also the natural substrate for these pathways. The possibility that CLA exerts its effects through all three eicosanoid pathways (Cyclooxygenase, Lipoxygenase and Epoxygenase) would be highly significant and could explain how CLA exerts many of its physiological roles in atherosclerosis, carcinogensis, diabetes, obesity and immunity.

CLA treatment also had no impact on basal levels of eNOS activity over an acute and chronic time course. Subsequently it was decided to examine whether CLA affected agonist-stimulated eNOS activity. Pre-treatment with CLA was shown to synergistically augment agonist activation of eNOS. The overall effect therefore suggests that CLA may be atheroprotective through a potentiation of agonist mediated production of nitric oxide. The endothelial vasodilator function, mediated in part by nitric oxide, is impaired in the early stages of atherosclerosis. CLA may protect against this vasodilatory loss. An increased level of nitric oxide may also protect against other early stage atherosclerotic events such as endothelium adhesion of leucocytes and platelets (Kubes *et al*, 1991) and vascular smooth muscle cell proliferation (Garg and Hassid, 1989).

It is also possible that the observed effects on nitric oxide and PGI_2 may be related. Endothelial cells release PGI_2 and nitric oxide in a coupled manner (Gryglewski *et al*, 1986). Nitric oxide has also been shown to highly stimulate prostaglandin production (Davidge *et al*, 1995). This suggests that CLAs inhibitory action against Cox in non-stimulated BAEC may be reversed in agonist stimulated BAEC, where nitric oxide may mediate Cox activation.

It was expected in this study that homocysteines induced oxidant stress may modulate nitric oxide breakdown to nitrite, however none was observed. Potential direction for further studies could include examining the specific role of reduced homocysteine, the most potent form of homocysteine, on vascular oxidant stress. Of the may forms of homocysteine present in blood plasma, reduced homocysteine has been shown to be the most deleterious to flow mediated vasodilatation (Chambers *et al*, 2001).

Treatment of non-stimulated BAEC resulted in a lack of modulation of nitrite levels. Because of this the effect of homocysteine on stimulated BAEC could also be examined. Bradykinin, calcium ionophore or acetylcholine would be suitable treatments for stimulation of eNOS (Venema, 2002; Taniguchi, 1999; Figueroa, 2002), although the physiologically predominant endothelial stimulators are haemodynamic forces such as shear stress and cyclic strain (Awolesi, 1994). The CELLMAX^{TM} QUAD perfused capillary co-culture system could be used to expose endothelial cells to physiologically relevant shear stress (Redmond *et al*, 1995).

This would also be a suitable model for examining subsequent alteration of vascular smooth muscle cell metabolism resulting from treatment of shear-stimulated endothelial cells. Previous reports have demonstrated that homocysteine induces DNA synthesis and proliferation (Taha, 1999; Chen, C., 2000) of vascular smooth muscle cells. It has been suggested this is mediated via MAP kinase activation (Woo, 2000) and a hydrogen peroxide–independent mechanism (Taha, 1999). Homocysteine also induces a release of intracellular Ca^{2+} from vascular smooth muscle cells and may induce vascular reactivity (Mujumdar, 2000). A problem with some of these *in vitro* studies may be that homocysteine is directly presented to vascular smooth muscle cells. The physiological

reality is that circulating homocysteine would primary influence endothelial cell function. It may prove interesting to investigate the subsequent effects of homocysteine induced endothelial dysfunction on vascular smooth muscle cell metabolism. The CELLMAX^{TM} QUAD perfused capillary co-culture system would again be a suitable *in vitro* model for such studies.

In conclusion while homocysteine does not appear to modulate eNOS expression or activity under basal conditions this study does however suggest two possible mechanisms of action for the anti-atherogenic effects of CLA previously observed in animal models. These mechanisms centre on the ability of CLA to modulate the activity of two enzymatic pathways, endothelial nitric oxide synthase and cycloxygenase. CLA isomer mix does appear to putatively modulate prostanoid production and agonist-stimulated nitric oxide release from BAEC.

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