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Endothelial physiology in young cigarette smokers: a multifunctional analysis of the effect of taurine supplementation

DECLARATION

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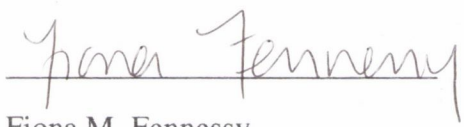

Fiona M. Fennessy

SUMMARY

The aim of this thesis is to determine the impact of cigarette smoking on endothelial dependent integration of the cardiovascular haemodynamic and barrier functions. Considering the antioxidant, anti-inflammatory and cytoprotective properties of taurine, and the relation of consumption of this amino acid to coronary heart disease in world-wide epidemiological studies, this thesis also investigates potential beneficial effects of taurine consumption in asymptomatic smokers on endothelial health.

Using duplex sonography, *in vivo* endothelial dependent and independent function was examined, and applanation tonometry was utilised to investigate ventricular-vascular interactions. As cigarette smoking is known to activate inflammatory cells, and monocyte-endothelial interactions are regarded as a critical early step in the atheromatous process, the circulating monocyte was used as a representative of the environment to which the endothelium of a smoker is exposed. In these *ex vivo* studies, the effect of monocyte conditioned medium on nitric oxide (NO) production from macro- and microvascular endothelial cells was examined to attempt to explain observed haemodynamic findings. As the maintenance of endothelial permeability is integral to normal haemodynamic functioning, the effect of monocyte conditioned medium on proliferation and apoptosis was also assessed. Circulating endothelial cells (CECs) were isolated to investigate the effect of smoking on endothelial cell desquamation, activation, phenotype and mode of death. All of the above studies were repeated post supplementation with taurine to assess the effect of this amino acid on endothelial cell function, viability and modificaction of the smoking induced inflammatory response.

These studies revealed that taurine supplementation for 5 days, in a dose that can be achieved by a daily fish diet of 100 gm/day, can restore the observed loss of flow mediated dilatation in young smokers. The response to a physiological stress, the cold pressor test, is abnormal in cigarette smokers in that the initial vasodilatatory

response is not seen. This response is partially restored by taurine supplementation, indicating that normal endothelial function can modify the response to a potent adrenergic stimulus. Applanation tonometry, i.e. measurement and analysis of central and peripheral waveforms, reveals that diastolic time is less and ventricular load greater in smokers, reflective of increased wave reflection suggesting altered compliance. Thus demand is increased and coronary perfusion decreased. The effects of taurine supplementation on central and peripheral haemodynamics reveals a beneficial effect on wave reflection from conduit vessels. However, taurine also induced a significant increase in blood pressure, suggesting a differential release of NO from the macro and microvasculature. This was subsequently examined by exposing endothelial cells of macro and microvasculature origin to the same cytotoxic stimulus of monocyte condioned medium, and a differential release in NO was found. These studies indicate that cigarette smoking has a differential effect on the vasculature of macro and microvessels. Taurine restores the smoking induced depressed expression of cNOS and release of NO from the macrovasculature. In contrast, smoking appears to be pro-inflammatory in the microvasculature, which is also attenuated by taurine supplementation. This finding was later supported by indentification of increased expression of activation markers on CECs in smokers. In addition to vasomotor control, barrier function was also assessed. A significant decrease in endothelial cell proliferation, with a corresponding increase in apoptosis, suggests the potential for a denuding injury in smokers. Taurine restored this function towards normal. This was supported by the finding of increased turmover, desquamation, activation and cell death in smokers.

Results obtained advocate the use of a multidimensional approach to physiological examination and investigation of therapy. The studies confirm the diverse nature of tobacco attack on the cardiovascular system and indicate that whilst micronutrient supplementation can ameliorate the abnormal physiology, it is unlikely to be sufficient for disease prevention.

"I was born not knowing

and have only had a little time to change that here and there"

Richard P. Feynman

This thesis is dedicated to my parents, Eamon and Mary, for their eternal love, support and friendship.

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Thank you to all friends, family, and Beaumont hospital employees (especially the portering and ancillary staff, and particular members of the doctor's residence and blood pressure unit!) who kindly donated blood samples and took part in the studies...over and over again....

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List of Abbreviations

AI	Augmentation index
CAI	Central augmentation index
CEC	Circulating endothelial cells
cNOS	constitutive nitric oxide synthase
CPT	Cold pressor test
DMEM	Dulbeccos Modified Eagle Medium
ECM	Extracellular matrix
EDRF	Endothelium derived growth factor
ET-1	Endothelin-1
FCS	Foetal calf serum
HBSS	Hanks balanced salt solution
HMVEC	Human microvascular endothelial cells
HUVEC	Human umbilical vein endothelial cells
ICAM	Intracellular adhesion molecule
IL-1α	Interleukin- 1 alpha
IL-1β	Interleukin- 1 beta
LDL	Low density lipoprotein
MCM	Monocyte conditioned medium
NO	Nitric oxide
PBS	Phosphate buffered saline
SMCs	Smooth muscle cells
TNFα	Tumour necrosis factor -alpha
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
VCAM	Vascular adhesion molecule

VEGF	Vascular endothelial growth factor

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Chapter 1

Introduction

1.1 Cardiovascular disease: an epidemic

Was there a cardiovascular disease epidemic during the twentieth century? A celebrated clinician, William Osler, wrote extensively about angina pectoris at the turn of the last century. In his 1897 lecture series he described angina pectoris as "a rare affliction in hospital practice....During the 7 years in which the Johns Hopkins Hospital has been opened.....there have only been four instances of angina pectoris" (Osler, 1897). By 1910 Osler reported having seen 208 additional cases and asked the question "...has angina pectoris increased in the community? Has the high-pressure life of modern days made the disease more common?" (Osler, 1910). The epidemiology of cardiovascular disease in the century now ending has moved this affliction to the forefront of public health; cardiovascular disease is now the leading cause of morbidity and mortality in the developed regions of the world (Newschaffer *et al.*, 1998) and in the developing countries (Murray & Lopez, 1997). To emphasise the point with figures, in the United States in 1995, 445 000 men and 505 000 women died of cardiovascular disease; that same year 281 000 men and 256 000 women died of cancer, the second leading cause of death (National Centre for Health Statistics, 1996).

Cardiovascular disease mortality appears to have increased in the first half of the twentieth century, plateaued out in the 1960s, and declined thereafter (Stallones, 1980; Higgins & Thom, 1989). If such sharp rises and falls in cardiovascular disease incidence actually occurs over a short time span, one would expect the main determinants to be environmental, rather than genetic or age-related, and thereby amenable to preventative strategies. As risk factors became identified and made aware of (Osler, 1908; Anitschoff & Chalatow, 1913; Kannal *et al.*, 1961), substantial preventative methods may have been the key determinants in cardiovascular disease mortality decline in the later half of this century. While more than 250 possible risk factors have been identified in the last 20 years (Hopkins & Williams, 1981; Hopkins

& Williams 1986), the principal environmental factor identified to date is dietary intake of animal fats.

1.1.1 Principal environmental factor: dietary lipid intake

The pivotal role of lipid metabolism, highlighted by the presence of lipid in the atheromatous plaque and the development of atheromatous lesions in animals fed high-fat diets (Hunt & Duncan, 1985; Berlin & Young 1983; Jacobsson & Lundholm, 1982; Mueller *et al.*, 1982) corresponds to the epidemiological evidence linking community and country dietary intake of animal fats (Walker, 1980; Oalmann *et al.*, 1981; Malcolm *et al.*, 1984; Zhdanov *et al.*, 1999; Fabsitz *et al.*, 1999); studies on the incidence of clinical atheromatous cardiovascular disease in populations migrating from low to high incidence countries demonstrating rapid equilibration. The central role of lipids has more recently been underscored by the clinical success of dietary and pharmaceutical lipid lowering strategies at an individual, community and national level (Corsini *et al.*, 1998; Bucher *et al.*, 1999; Hilleman *et al.*, 1999) The intriguing observation that improvement in morbidity and mortality in patients on HMG-coA reductase inhibitors, antedating decreases in lipid levels, has reignited interest in the role of the inflammatory process in atherosclerosis (Kano *et al.*, 1999; da Silva, 1999).

1.2 The atheromatous process

The understanding of the cellular and molecular mechanisms in the pathogenesis of atherosclerosis has largely been a product of the last 20 years, with subsequent substantial advances in interventional, surgical and medical treatment of cardiovascular disease. Much of the recognition of injurious agents and the mechanism of plaque formation has derived from the work of Russell Ross and his colleagues at the University of Washington in Seattle (Ross and Glomset, 1973; Ross,

1986; Ross, 1993). This "response to injury" hypothesis proposes that injury to the endothelium, through damage or activation, is the initiating event in atherogenesis. Endothelial injury leads to increased permeability so that lipoproteins and circulating monocytes adhere to the endothelium, migrate into the subendothelial space and form the initial lesion of atherosclerosis, the fatty streak. The molecular basis of this process has been clarified over the last decade and is now known to proceed through the orderly sequence of expression of adhesion molecules on both the inflammatory and endothelial cell. Increased adherence and infiltration of inflammatory cells further disrupts the endothelium and leads to exposure of thrombogenic surfaces to which platelets adhere. The damaged endothelium, platelets, monocytes and smooth muscle cells all have the potential to release growth modulatory factors which result in proliferation of smooth muscle cells and of fibroblasts, leading to the formation of the fibrous plaque. Such a sequence of events has already been established in the microvasculature during inflammation and is now believed to be active in the atheromatous process, an inflammatory fibroproliferative process which is a repair mechanism that then becomes harmful.

1.2.1 The role of the monocyte

The self-perpetuating process of chronic inflammation is dependent on elevated lipid levels in the arterial wall, evidence for which was first obtained in 1965 (Albrecht & Schuler, 1965). However, it could be argued that the rate-limiting process in atherogenesis is the monocyte-macrophage passage into the arterial intima. Circulating monocytes express adhesion markers and invade the intima of lesion prone areas in arteries. Monocytes scavenge and internalise lipoproteins (Goldstein *et al.*, 1979), in particular oxidised low density lipoprotein (LDL), and form foam cells. Once lipid-laden, foam cells migrate back into the bloodstream by crossing the arterial endothelium. The ratio of penetrating monocytes to emerging foam cells decreases as fatty cell lesions develop until a one-to-one ratio is achieved in late fatty lesions which

do not progress further. It would therefore appear that advancement of the lesion is at least partially a result of failure of the "monocyte clearance system", that is, failure of emergent monocytes to remove sufficient lipids (Gerrity, 1981).

Within plaque, huge numbers of macrophages can accumulate; some become very large and die and can release their lipid contents into pools of extracellular gruelly material, from which the name atheroma is derived (Greek for porridge). A specific role has been attributed to macrophages close to the lumen, which produce matrix metalloproteinases, render the plaque liable to rupture, and thereby initiating thrombosis (Galis *et al.*, 1995). The progression of the atheromatous plaque to ultimate rupture and thrombosis, which ultimately determine clinical outcome, is crucially dependent on a continual self-amplifying interaction between different cells, mediated by autocrine and paracrine release of cytokines and inflammatory mediators. An additional difficulty in establishing priority of processes and mechanisms is the fact that the presence of the plaque in turn alters systemic responses.

1.2.2 Endothelial cell activation

The adhesion and subsequent migration of inflammatory cells through the endothelium is induced by expression and activation of adhesion markers on the surfaces of the endothelium and of the circulating leukocytes. Expression of endothelial adhesion molecules Intracellular adhesion molecule-1 (ICAM-1) and Vascular cell adhesion molecule-1 (VCAM-1) are increased in the endothelium of rabbits in areas over atherosclerotic plaque (Cybulsky & Gimbrone, 1991), and ICAM-1 has also been detected in human atherosclerotic endothelium (Wood *et al.*, 1993). Chemoattractant and activating substances work synergistically with adhesion markers to induce leukocyte traffic. Plaque monocytes are known to produce the cytokines tumour necrosis factor (TNF) and interleukin-1 (IL-1), capable of inducing adhesion markers on endothelial cells, and the chemotactic cytokine, monocyte chemoattractant-1 protein. Oxidised LDL, present in plaques and often initially

oxidised by macrophages in the first place (Parthasarathy *et al.*, 1986), can also induce monocyte-endothelial adhesion (Kim *et al.*, 1994), which is thought to be through expression of the adhesion molecule P-selectin (Gebuhrer *et al.*, 1995). During enhanced monocyte and endothelial interactions (since monocytes remain in close proximity to endothelial cells for prolonged periods of time during the development of atherosclerotic lesions), monocytes are thought to modulate several endothelial functions through either cell-cell contact or through the release of humoral mediators such as tumour necrosis factor -alpha (TNF-α) (Hakkert *et al.*, 1992; Marczin *et al.*, 1996). It would therefore appear that the process of monocyte adhesion and migration involves an interplay between local cytokine activity and activation of and adhesion receptor expression on, both endothelium and monocytes.

1.3 The role of the endothelium in vascular homeostasis

1.3.1 The arachidonic pathway

The principal vasoactive eicosanoids are thromboxane A_2 and prostacyclin, both metabolites of arachidonic acid. Thromboxane A_2 is the principal arachidonic metabolite in platelets which, in addition to promoting platelet aggregation, is a powerful vasoconstrictor. Synthesised by the endothelial cells and smooth muscle cells, prostacyclin inhibits platelet aggregation and is a vasodilator (Oates *et al.*, 1988). Endogenous prostacyclin generators include bradykinin, choline esters, arachidonic acid, thrombin, trypsin, platelet-derived growth factor, epidermal growth factor, interleukin-1 and adenine nucleotides. Cyclo-oxygenase inhibitors (such as aspirin), glucocorticosteroids and vitamin K_1 inhibit biosynthesis of prostacyclin in the endothelial cell.

The platelet-suppressant and vasodilator actions of prostacyclin are mediated by adenylate cyclase in platelets and smooth muscle cells. While the vasodilatory effects of prostacyclin are somewhat overshadowed by EDRF (endothelium derived relaxation factor) in vascular homeostasis, prostacyclin is known to play significant clinical roles in promotion of skin healing of ischaemic ulcers (Nizankowski *et al.*, 1985), and a prostacyclin analogue "iloprost" has been shown to have some beneficial effects in Raynaud's disease patients (Rademaker *et al.*, 1989). It has been proposed that receptor-mediated release of prostacyclin and EDRF is coupled (De Nucci *et al.*, 1988), an increase in intracellular calcium being needed for both and protein kinase C being activated. These results suggest that these substances act as a common mechanism for endothelial defence.

1.3.2 Nitric oxide

The magnitude of response to oxidant/cytokine mediated endothelial activation, dysfunction and destruction is now known to be critically dependent on the bioavailability of the molecule nitric oxide (NO). NO is the most important regulator of endothelial function. The importance of this molecule in human health and disease has been recently acknowledged by the Nobel Assembly at the Karolinska Institute, when Robert F. Furchgott, Louis J Ignarro and Ferid Murad were awarded the Nobel Prize in Physiology or Medicine for 1998 for their discoveries concerning "Nitric oxide as a signalling molecule in the cardiovascular system" .

The key roles played by NO in atheromatous disease prevention include inhibition of leukocyte adhesion to the endothelium (Kubes *et al.*, 1991; Ma *et al.*, 1993; Lefer *et al.*, 1993) and also inhibition of platelet adhesion to the endothelium (Ramomski *et al.*, 1987). NO decreases vascular permeability and reduces lipoprotein flux into the vessel wall (Draijer *et al.*, 1995; Cardona-Sanclemente *et al.*, 1995). In addition, NO has been demonstrated to inhibit vascular smooth muscle proliferation and migration (Garg & Hassid, 1989; Cornwell *et al.*, 1994; Sarker *et al.*, 1995). In

fact, decreased availability of biologically active NO in the vascular wall may be one of the earliest detectable findings in atherogenesis.

1.3.3 cNOS (constitutive enzyme: nitric oxide synthase)

NO is produced within endothelial cells from the precursor L-arginine. A terminal guanidino nitrogen of L-arginine is transformed into NO by an oxidation pathway requiring the cofactors NADPH, tetrahydrobiopterin and calmodulin. L-arginine is converted into NO in endothelial cells through the constitutive action of the enzyme nitric oxide synthase (cNOS). Calcium appears to be the primary stimulus for activation of cNOS in the short term (Schini-Kerth and Vanhoutte, 1995). Stimuli that increase intracellular calcium will therefore also increase NO production, such as acetylcholine, bradykinin, substance P, adenosine diphosphate (ADP), thrombin, 5-hydroxytryptamine (serotoin) and calcium ionophore (Furchgott & Vanhoutte, 1989; Botting & Vane, 1990). Intracellular levels of the cofactor tetrahydrobiopterin may also determine cNOS activity.

1.3.4 iNOS (inducible enzyme: nitric oxide synthase)

In addition to cNOS, NO is also produced by distinctly different gene products of NOS. Neuronal constitutive NOS (ncNOS) and cytokine-inducible (iNOS) also exist. Both cNOS and ncNOS require the same cofactors, and generate NO continuously and at a lower concentration than *via* the iNOS pathway (Moncada *et al.*, 1991). The enzyme iNOS is calcium independent, but also requires NADPH and tetrahydrobiopterin as cofactors. It is found in macrophages, vascular smooth muscle cells, neutrophils, hepatocytes and endothelial cells (Billiar *et al.*, 1990; Feldman *et al.*, 1993; McCall *et al.*, 1989; Rosenkranz-Weiss *et al.*, 1994). Induction of iNOS occurs secondary to a number of stimuli such as lipopolyscaccharides, cytokines such as interferon-γ, tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1). As

induction of iNOS requires new protein synthesis, a longer time period (hours) is required to produce NO. Once produced, iNOS expression results in copious amounts of NO release, resulting in vasodilatation and hypotension (Petro *et al.*, 1991) and is thought to mediate the hypotension and myocardial depression associated with septic shock. It is considered that in the microvasculature, where blood flow is relatively slow and cellular interactions intimate, host defence and an inflammatory role for NO may pertain, with an aim to minimise the risk of injury to the host (Geiger *et al.*, 1997). The role of iNOS, however, in the chronic vasculopathies of dyslipidaemia, hypertension, diabetes mellitus and cigarette smoking is unclear.

1.4 The identification of EDRF

In 1980, Furchgott and Zawadzki described the actions of a labile substance they termed Endothelium Derived Relaxation Factor (EDRF). The role of EDRF in modulating vascular tone has provoked many studies to evaluate endothelial health by assessing vasoactivity. Clinical evaluation of endothelial function in forearm resistance vessels has been assessed by determining forearm blood flow by venous plethysmography in response to various pharmacological stimuli (Johnstone *et al.*, 1993; Chowienczyk *et al.*, 1993). The scope of this labile compound has become wider and more significant with time, as it is now known that not only does EDRF relay its effect on underlying smooth muscle cells (SMCs), but it also prevents platelet aggregation and adhesion (Jen et al., 1995)

1.4.1 Historical background of EDRF identification

The identification of NO as a critical molecule in the vascular system is one of the most interesting stories in vascular biology. The role which the endothelial cell was going to play in mediating dilatation was first brought to attention by experimental error. Fingertips of the investigator unintentionally rubbed off endothelial cells from

helical strips of rabbit aorta, and the experimental proposal of ACh inducing relaxation was not achieved. The importance of these cells in modulating the activity of vascular smooth muscle tone was published in 1980 by Furchgott and Zawadzki, demonstrating that ACh stimulated endothelial cells released a non-prostanoid, diffusible factor that acted on adjacent smooth muscle cells to produce relaxation (Furchgott & Zawadzki ,1980).

The possibility that flow-induced vasodilatation may be mediated by the endothelium was raised by Holtz *et al.* (1983). They noted that vasodilatation in response to increased flow was abolished by both mechanical and enzymatic removal of the endothelium in isolated perfused rings of canine coronary and femoral arteries. The question therefore arose as to how the endothelium induced relaxation of the smooth muscle cells in response to an increase in flow. The first *in vitro* work on EDRF was again carried out by the same group (Pohl *et al.*, 1986). It was found that dithiothreitol and haemoglobin, both inhibitors of EDRF, reversibly ablated the response of bioassay tissues to increased blood flow velocity and ACh. However, the mediator of flow-dependent vasodilatation in vascular segments was identified as EDRF in 1986, after comparing the characteristics of this compound to those of EDRF released by ACh (Rubanyi *et al.*, 1986).

The mechanism by which nitrovasodilators induced relaxation in vascular smooth muscle was proposed by Murad in 1977. These nitric oxide (NO) donors, through the stimulation of soluble guanylate cyclase and subsequent formation of cyclic guanosine 3',5' monophosphate (cGMP), induced relaxation of vascular smooth muscle cells (Katsuki *et al.*, 1977). It was almost a decade later that the similarities between the mode of action of EDRF and NO were recognised, and the idea that EDRF and NO were one and the same substance was proposed. EDRF was found to be chemically identical to NO (at least at the moment of its formation) by two separate investigators (Palmer *et al.*, 1987; Ignarro *et al.*, 1987).

1.4.2 Detection of flow stimulus by endothelial cells

The complex mechanotransduction pathways by which the frictional force and an increase in blood flow is detected by endothelial cells points towards an important role for transduction through the cytoskeleton to focal adhesion sites on the basal side of the cell. The focal adhesion sites are rich in integrins -transmembrane proteins that span the plasma membrane and bind to extracellular adhesion proteins-, connecting to the cytoskeleton *via* linker proteins on the inside of the cell (Davies, 1995). A second mechanotransduction pathway, implicating G proteins has also been proposed, but whether this involves basal focal adhesion sites is unknown (Gudi *et al.*, 1996). The response to an increase in blood flow therefore activates intracellular second messenger pathways either through focal adhesion sites or through stimulation of G proteins. These in turn lead to a characteristic immediate or delayed response to shear stress.

An acute response to flow induces a rise in intracellular calcium (Schwartz et al., 1992; Shen et al., 1992; Kuchan & Frangos, 1994). Other immediate responses to increased flow include the release of adenosine triphosphate (ATP) and substance P (Milner et al., 1990), which in turn stimulate NO release. A continued presence of shear stress is known to regulate endothelial gene expression (Nollert et al., 1992; Hsieh et al., 1992; Yoshizumi et al., 1989). A number of genes involved in cell growth include those known as "early growth response genes": c-*myc*, c-*fos* and c-*jun*, platelet-derived growth factors A and B (PDGF-A, PDGR-B) and transforming growth factor-β1(TGF-B1).

1.4.3 Flow and vascular remodelling

The vascular endothelium mediates the ability of blood vessels to alter their architecture in response to haemodynamic changes. If increased flow is maintained for

a considerable period of time, the arteries become permanently dilated, a process thought to be due to structural modification associated with an increase in lumen diameter and a greater medial cross-sectional area (Zarins *et al.*, 1987). Structural changes may result from hyperplasia and/or redistribution of vascular smooth muscle cells within the matrix, i.e. remodelling. The mechanisms involved in flow-induced hyperplasia are likely to involve increased expression of growth factor genes. A critical role for endogenous NO is as a negative regulator of vascular smooth muscle proliferation in response to a remodelling stimulus. It has been suggested that a primary defect in the cNOS pathway can promote abnormal remodelling and may facilitate pathological changes in the vessel wall morphology associated with atherosclerosis (Rudic *et al.*, 1998). In the absence of cNOS, luminal remodelling is impaired and also vessel wall thickness doubles due to proliferation of vascular smooth muscle cells. It is therefore fitting that after prolonged stimulation with increased shear stress, endothelial cNOS gene expression is increased *in vitro* and *in vivo* (Uematsu *et al.*, 1995; Awolesi *et al.*, 1995). NO derived from cNOS is therefore a major determinant of vessel architecture in response to a haemodynamic stimulus, and plays the role of an endothelium-derived mediator responsible for vessel remodelling.

1.4.4 Physiology Of Flow-Mediated Dilatation

The physiology of flow-mediated dilatation (FMD) involves 5 main steps:

1. An increase in blood flow is sensed by the endothelium.

2. Intracellular endothelial Ca^{2+} is increased (figure 1.4.1).

3. This increase in intracellular Ca^{2+} results in the production of NO from L-arginine (figure 1.4.2).

4. Within the smooth muscle cell NO activates soluble guanylate cyclase, which stimulates the production of cGMP from guanosine triphosphate (GTP).

5. cGMP dependent protein kinase stimulates phosphorylation of intracellular proteins.

This results in relaxation of the smooth muscle cell (figure 1.4.3).

Basal EDRF and agonist-induced EDRF synthesis both rely upon an increase in Ca^{2+} influx across the cell membrane, thereby requiring an initial increase in extracellular Ca^{2+} to commence this process (Singer and Peach, 1982).

A subsequent increase in intracellular Ca^{2+} may be mediated through three main mechanisms (figure 1.4.1):

1. Agonist-mediated phospholipase C/G protein interaction stimulates the release of Ca^{2+} from intracellular stores through the formation of inositol 1',4',5' triphosphate (IP_3) (Newby *et al.*, 1990).

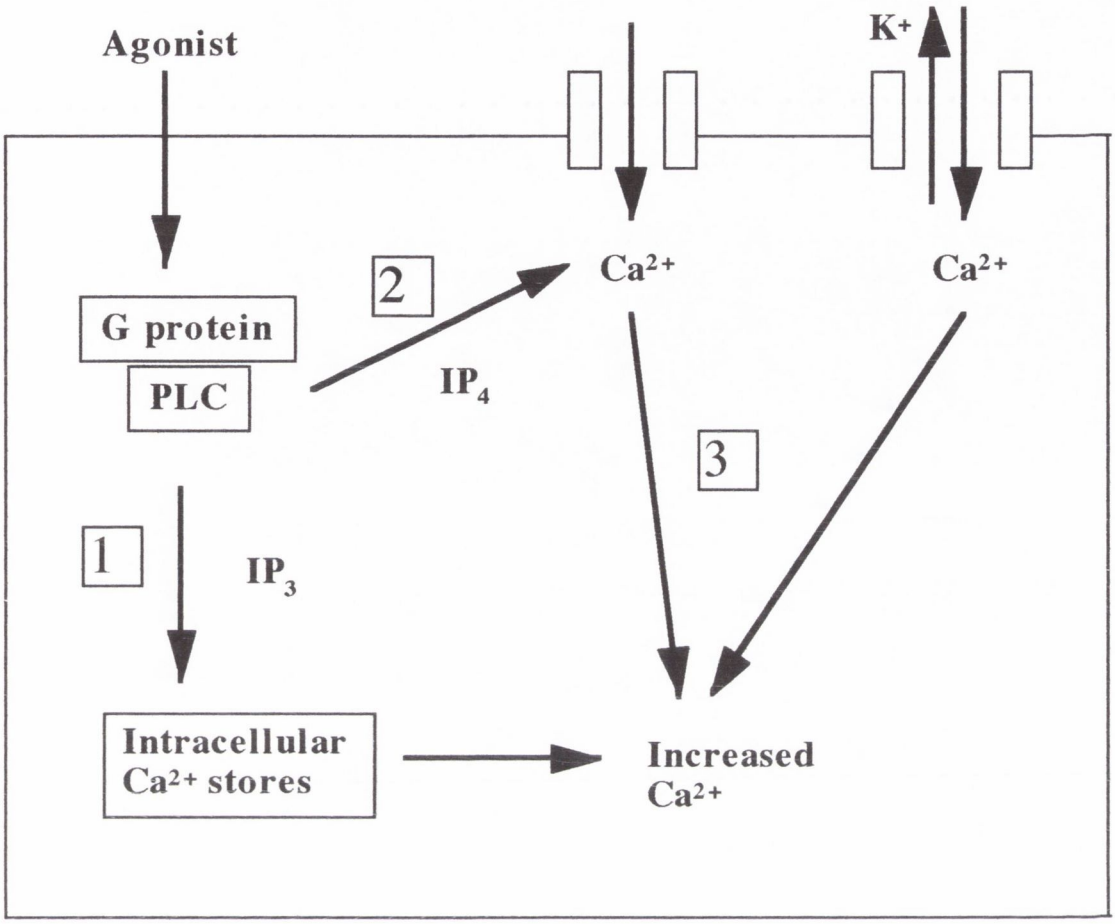
2. Agonist-induced activation of Ca^{2+} channels through stimulation of IP_4 (Luckhoff & Clapham, 1992).

3. A positive feedback mechanism exists whereby an increase in enhanced entry and an increase in intracellular Ca^{2+} stores stimulates Ca^{2+}-activated K^+ efflux, which results in membrane hyperpolarisation. Hyperpolarisation modifies the electrochemical gradient in favour of entry of extracellular Ca^{2+}. Studies on dependency of EDRF on Ca^{2+} are supported by bioassay experiments in which the removal of Ca^{2+} from endothelial cell perfusate resulted in a rapid cessation of production of EDRF, which, conversely, is restored by reperfusion of Ca^{2+} ions into the buffer (Griffith & Edwards, 1993).

1.4.5 cGMP

Ultimately, EDRF relaxes SMCs by cGMP-mediated effects on Ca^{2+}(Figure 1.4.3). The initial step in the SMC is the activation of soluble cytosolic guanylate cyclase, resulting in the formation of cGMP. cGMP-dependent protein kinase then

phosphorylates a number of intracellular proteins, which ultimately decreases intracellular Ca^{2+} in the SMC. A reduction in free cytosolic Ca^{2+} has been shown to be brought about through a decrease in Ca^{2+} influx (Collins, 1986). It has been demonstrated that an increase in cGMP can stimulate Ca^{2+} extrusion across the smooth muscle membrane (Popescu *et al.*, 1985).



ENDOTHELIAL CELL

Figure 1.4.1

Schematic representative of 3 possible mechanisms through which an increase in intracellular calcium may occur within the endothelial cell: 1) agonist-mediated phospholipase C (PLC)/G protein interaction through the formation of inositol 1',4',5' triphosphate (IP_3); 2) agonist-induced activation of Ca^{2+} channels through stimulation of IP_4; 3) a positive feedback mechanism exists whereby an increase in enhanced entry and an increased release of Ca^{2+} from intracellular Ca^{2+} stores stimulates Ca^{2+}-activated K^+ efflux, which results in membrane hyperpolarisation which modifies the electrochemical gradient in favour of entry of extracellular Ca^{2+}.

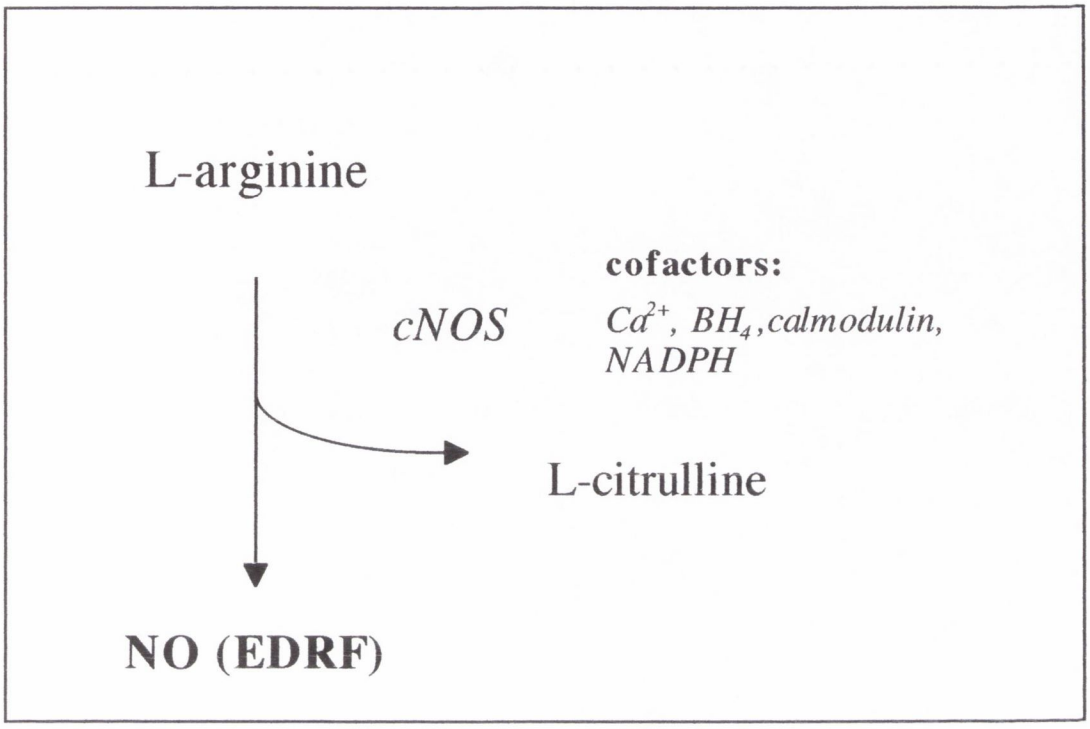


Figure 1.4.2

Schematic representative of NO production from L-arginine within the endothelial cell.

BH_4 = tetrahydrobiopterin; NADPH = nicotinamide adenine dinucleotide phosphate.

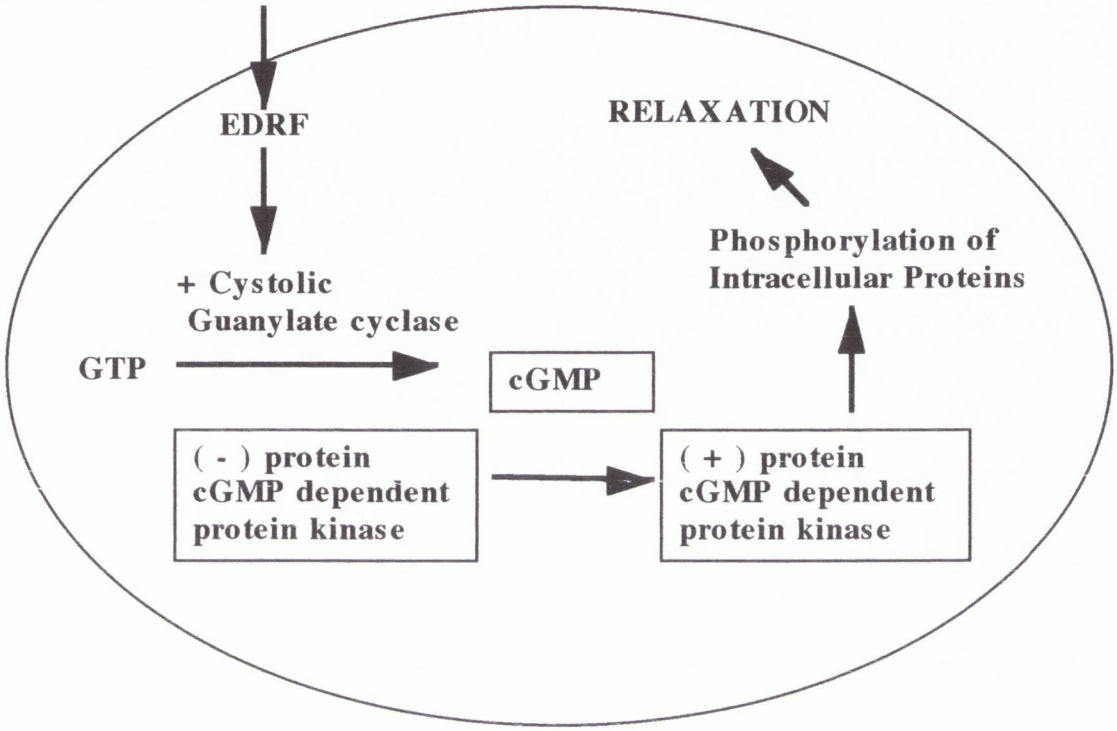


Figure 1.4.3

Schematic representation of the effect of EDRF/NO on smooth muscle cell relaxation. NO diffuses into smooth muscle cells and causes relaxation by increasing the formation of cGMP. cGMP-dependent protein kinase then phosphorylates a number of intracellular proteins, which ultimately decreases intracellular Ca^{2+} in the SMC, leading to relaxation.

1.4.6 Mitochondrial ATP

While stimulated pharmacological release of EDRF depends critically on mitochondrial ATP synthesis, basal release does not. This concept is supported by the finding that basal release is not affected by inhibition of glycolysis, oxidative phosphorylation or inhibition of mitochondrial ATPase (Griffith *et al.*, 1987). Flow-induced release of EDRF is thought to be the mediator of basal release. Evidence for this may be obtained from the fact that the normal moment to moment variations in flow are responded to by endothelial cells immediately, with slight variations in flow resulting in parallel changes in the diameter of coronary vessels (Holtz *et al.* 1984). It seems likely that normal variations in flow do not require extra mitochondrial ATP synthesis to mediate dilatation, cellular ATP stores being adequate, as endothelial cells have extremely high concentrations of ATP relative to other cell types.

However, it may be feasible to argue that severe sustained increments in shear stress (SS), such as in situations of loss of negative feedback in diseased vessels, may not be representative of the normal "basal" flow variations, and that such extremes of elevated pressures could require ATP levels in excess of those released from cellular stores, thereby necessitating stimulated mitochondrial ATP synthesis. This concept may be supported by the fact that endothelial cell (EC)-dependent relaxation is inhibited by severe anoxia (PO_2 < 10 mmHg), which is below the level required to support oxidative phosphorylation, and in turn, ATP synthesis. A scenario of persistently raised wall shear rate in a diseased vessel with loss of negative feedback, where the basal release of ATP is surpassed and stimulated ATP is necessary for EDRF release, could theoretically provide for a decreased O_2 supply to the EC, and a resultant decrease in production of EDRF.

1.4.7 EDRF and Free Radicals

It is worth noting the effects of oxygen derived free radicals on the production and functioning of EDRF. Rubanyi and Vanhoutte (Rubanyi & Vanhoutte, 1986 (a))

showed there to be a resultant decrease in ACh-induced relaxation of canine coronary arteries by superoxide anions. Infusion of superoxide dismutase into the perfused segment of an artery prolonged the half-life of EDRF from 6.3 sec to 15.7 sec (Rubanyi & Vanhoutte, 1986(b)), providing support for the hypothesis that these anions destroy EDRF produced by the endothelium.

However, the synthesis and biological activity of EDRF can be influenced in opposite directions by oxygen derived free radicals (hydrogen peroxide and hydroxyl radicals stimulate, while superoxide anions inactivate EDRF production), and trace elements (removal of superoxide anions and hence protection of EDRF by Cu(II) and stimulation of superoxide radicals by Fe(II)). The fact that EDRF is inactivated in conditions where superoxide anions are generated (Freeman & Crapo, 1982) or in hyperoxic conditions, suggests that the oxidised state of EDRF (for oxidative phosphorylation of guanylate cyclase) is necessary for its physiological role in regulation of blood flow, and maintaining homeostasis in vascular integrity. During situations such as ischaemia-reperfusion, hypertension or in the setting of cigarette smoking where free radical formation and changes in blood flow occur simultaneously, it is possible to believe that these O_2 derived free radicals play a modulatory role in EDRF formation.

1.4.8 NO abnormalities in cardiovascular disease

Under normal circumstances *in vivo*, NO activity is based upon a balance between its synthesis and breakdown. In the atheromatous process and in states of hypercholesterolaemia, the synthesis of NO is thought to be impaired, most likely due to uncoupling of the G-protein complex (Freeman *et al.*, 1996). The mechanism through which this occurs is considered to be secondary to reduced transcription and enhanced breakdown of NOS with increasing concentrations of oxidised LDL (Jessup, 1996). In addition, hypercholesterolaemia is associated with increased concentrations of endogenous cNOS inhibitors, a phenomenon that may be overcome

by L-arginine administration (Bode-Boger *et al.*, 1996; Cooke & Tsao, 1997). However, a reduction in NO synthesis due to reduced availability of the cNOS substrate L-arginine is unlikely the primary process involved in endothelial dysfunction in disease states, as it has been demonstrated that extracellular L-arginine concentration is an unlikely rate-limiting step due to the fact that intracellular L-arginine concentrations far exceed the K_m value of cNOS (Arnal *et al.*, 1995).

Alternatively, reduced NO activity in the presence of atheromatous disease may be secondary to enhanced catabolism of NO. The *in vivo* $t_{1/2}$ of NO is mainly determined by its reaction with superoxide and oxyhaemoglobin (Beckman & Koppenol, 1996). NO and superoxide act to form the powerful oxidant peroxynitrite ($ONOO^-$). As $ONOO^-$ is optimally formed from eqimolar concentrations of NO and superoxide, and under normal physiological conditions NO is formed in the picomolar to nanomolar range (Weyrich *et al.*, 1994), is not likely for high concentration of $ONOO^-$ to form. It is however possible that deleterious concentrations of $ONOO^-$ may from in atherosclerotic lesions, secondary to increased superoxide generation by endothelial oxidases, such as xanthine oxidase (Ohara *et al.*, 1993; White *et al.*, 1996). Particularly in the setting of hyperlipidaemia (Ma *et al.*, 1997), it is becoming more apparent that enhanced $ONOO^-$ formation contributes to lipid peroxidation in the atherosclerotic process.

1.5 Antioxidants

Subsequent to the realisation that enhanced oxidant formation is a predisposing factor for atheromatous disease, a lot of attention has been given to the role of antioxidant vitamins in cardiovascular disease prevention during the last 20 years. Exactly how cardiovascular disease risk may be minimised with antioxidant

supplementation is not clear. General antioxidant strategies may limit inflammation within plaques. A potential target for interrupting inflammatory activation in atheroma could involve inhibition of activation of a transcription factor nuclear factor κB(NFκB) (Collins, 1993). Interestingly, NO exerts anti-inflammatory effects on vascular walls by stabilising an endogenous inhibitor of NFκB, IκB-alpha (Peng *et al.*, 1995). Therefore, therapeutic strategies that augment local NO production or decrease its catabolism may limit vascular inflammation.

1.5.1 Antioxidants and cardiovascular disease

Much research in antioxidant protection during the 1970s and 1980s pointed towards a protective role for certain antioxidants against cardiovascular disease (in particular vitamin E and beta-carotene) (Hercberg *et al.*, 1998(a); Price *et al.*, 1997). Based on these observational studies, randomised clinical trials were developed at the end of the 1980s to assess the use of these simple, natural, very accessible nutrients in both cardiovascular disease and cancer prevention. The results of the Linxian study in China (Blot *et al.*, 1993), the Alpha-Tocopherol, Beta Carotene Cancer Prevention Study (ATBC) in Finland (The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group, 1994), the Carotene and Retinol Efficacy Trial (CARET) in the United States (Omenn *et al.*, 1996) and the effect of beta-carotene on cancer and cardiovascular disease in healthy United States physicians (Hennekens *et al.*, 1996), were dramatic and surprising.

While the Linxian study found beta-carotene, tocopherol and selenium at nutritional doses to have a non-significant 10% decrease in cerebrovascular mortality, the ATBC study revealed subjects receiving high dose vitamin E to have no significant decrease in cardiovascular disease morbidity, and those receiving beta-carotene to have an 11% increase in ischaemic heart disease deaths. In the treated group in the CARET study, a non-significant 26% increase cardiovascular disease mortality was

reported. It has been concluded from these studies that optimal protective effects may be expected from antioxidant intake, provided levels ingested are similar to those obtained in a healthy diet. This conception has provoked a large population-based intervention trial in France, the SU.VI.MAX Study (Etude Supplementation en Vitamins et Mineraux Antioxydant) (Hercberg *et al.*, 1998(b); Hercberg *et al.*, 1998(c)). The essential point obtained from these trials is that optimal blood concentrations of antioxidant vitamins associated with a decrease in cardiovascular disease risk are obtained through dietary intake levels, and not high levels of supplementation.

Apart from epidemiological trials, more recent studies have explored the effects of antioxidants on the pathophysiological processes involved in the atheromatous process. *In vitro* cell culture studies have demonstrated that vitamin E supplementation can reduce ICAM, VCAM and E-selectin endothelial expression, decrease monocyte adhesion to endothelial cells (Martin *et al.*, 1997; Wu *et al.*, 1997), and can also decrease chemokine MCP-1 and IL-8 production from stimulated endothelial cells. Similar *in vitro* findings have been observed with vitamin C, studies which have particularly focused on cigarette smoke-induced endothelial abnormalities (Lehr *et al.* 1994, Weber *et al.*, 1996). The effects of antioxidant supplementation on *in vivo* endothelial function have also been assessed. A local infusion of intra-arterial vitamin C was shown to markedly improve endothelial function (Heitzer *et al.*, 1996), while oral supplementation of vitamin E has provided conflicting results; supplementation of similar levels of vitamin E (300 IU/day for 8 weeks) showed improvement in endothelial function in some studies (Neunteufl *et al.*, 1998) and failed to do so in others (Elliot *et al.*, 1995; Gilligan *et al.*, 1995; Simmons *et al.*, 1999). It has been suggested however that these findings may be explained by variations in baseline antioxidant levels, higher pre-treatment levels contributing to lack of significant post-treatment effect.

1.6 Taurine

1.6.1 General properties

The failure to convincingly demonstrate a role for antioxidants in the management of cardiovascular disease may be related to the fact that in the supra-physiological doses employed, many of the agents used have potent pro-oxidant effects. As mentioned previously, NO bioavailability is critically dependent on cellular Ca^{2+} fluxes and scavenging of oxidants. Taurine is a conditionally essential amino acid; that is, it becomes essential when a cell must act in a stressed or hostile environment. It is an antioxidant whose effects on cellular unction are mediated through osmoregulation and control of Ca^{2+} fluxes. An inverse relationship between the consumption of taurine and the prevalence of cardiovascular disease has been suggested. However, its effect on the atheromatous process has not as yet been clarified. Taurine, a semi-essential amino acid, is the most abundant intracellular free amino acid present in mammalian cells. While it is not incorporated into proteins, it is found mainly in the free form (Hayes & Sturman, 1981). Taurine is taken in *via* the diet in carnivores and omnivores, but is also synthesised from cysteine or methionine present in the diet. Particularly high concentrations are found in the heart (25-30 mM) and lung (11-17 mM), but the highest concentrations of taurine are found in inflammatory cells, especially the neutrophil (around 50 mM), and also the retina (50-70 mM).

Synthesis is determined by the availability of cysteine and methionine, and cysteinesulfinic acid, the enzyme responsible for conversion of cysteinesulfinic acid to hypotaurine (the immediate taurine precursor that is oxidised spontaneously to taurine). As cysteinesulfinic acid is a pyridoxal phosphate-requiring enzyme, vitamin B_6 deficiency can lead to a reduction in endogenous taurine formation. The principal routes of taurine synthesis are demonstrated in figure 1.6.

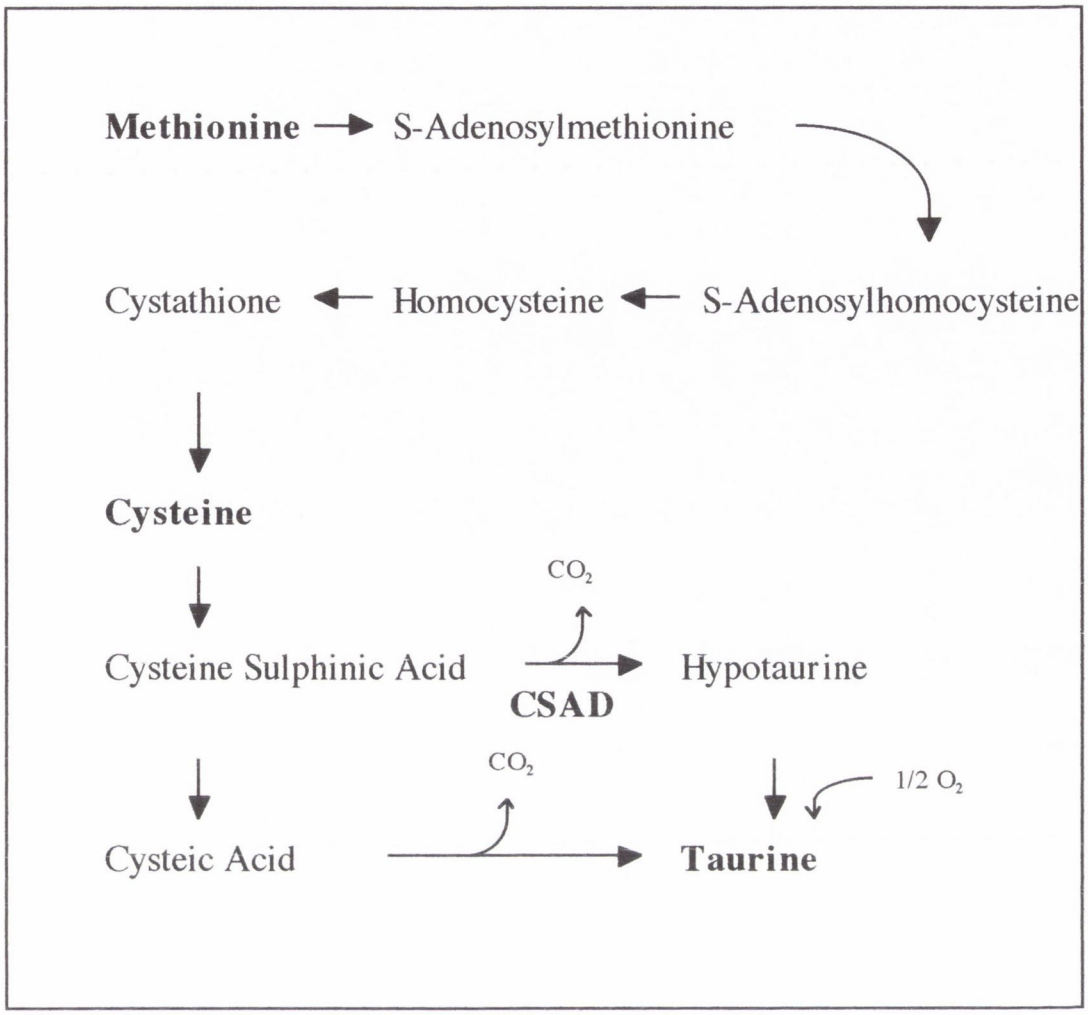


Figure 1.6 Taurine biosynthesis from methionine and cysteine

The principal routes of taurine synthesis include oxidation of cysteine to cysteine sulphinic acid and subsequently to cysteic acid, which is decarboxylated to taurine. Cysteine sulphinic acid may also be decarboxylated to hypotaurine, which is oxidised to taurine.

In man, taurine homeostasis is primarily controlled by the kidneys, which adapt to maintain plasma levels (Rozen and Schriver, 1982). If plasma concentration is depleted, renal reabsorption of taurine increases in an attempt to preserve stores. On the other hand, when the dietary supply increases, renal excretion is unregulated. For a long time taurine was viewed as a mere end product of sulphur amino acid metabolism with no physiological role except that of bile conjugation (Hastlewood, 1964). In recent years, however, it has been queried as to how a compound which is found in such high concentrations should have no cellular function. As a result, taurine has been established as playing a vital role in many physiological events including osmoregulation, detoxification, membrane stabilisation (Gaull and Rassin, 1979; Huxtable, 1992), neuromodulation (Barbeau *et al.*, 1975; Kuriyama, 1980) and neonatal brain development (Sturman *et al.*, 1986). Importantly, it has also been shown to play a role in cardiac function (Wright *et al.*, 1986), antioxidation (it reacts with and thereby removes hypochlorous acid a potent oxidising agent) (Kozumbo *et al.*, 1992) and modification of immune and inflammatory responses (Park *et al.*, 1993). Indeed, since the first description of taurine in 1827 (Tiedmann & Gmelin , 1827), the range of biological phenomena involving this substance has progressively expanded. Despite efforts to create a unifying theory for the actions of taurine, it is believed that "a limited number of basic mechanisms of action may underlie this confusing plethora of biological effects" (Huxtible, 1990). Understanding of the role of taurine in any organ or tissue is related to contributions of a combination of a number of mechanisms specific for that organ or tissue. For example, in the liver taurine conjugation with bile acids and lipids is its central role; in the heart taurine-Ca^{2+} interactions and osmoregulator functions seem primary.

1.6.2 Normal dietary intake of taurine

Early studies in the 1960s in the USA calculated dietary taurine intake to be in the range of 40-400 mg/day (Roe *et al.*, 1966). More recent studies measured taurine in the diets of a small group of British men and women and the average intake was found to be 76 mg and 43 mg /day for men and women respectively (Rana & Sanders, 1986). Dietary taurine comes almost exclusively from animal foodstuffs; particularly meat, offal and seafood. Seafood is the richest source of taurine, containing 827 mg taurine/100 gm of fish, whereas the majority of meats contain 40-60 mg taurine/100 gm of meat (Laidlaw *et al.*, 1990). In a world-wide epidemiological study on dietary prevention and cardiovascular disease, the CARDIAC study (WHO and WHO Collaborating Centres, 1986), an average of 100 gms of fish per day was sufficient to keep coronary artery disease mortality as low as that in the Japanese race.

1.6.3 The role of taurine in cell cycle homeostasis

Taurine has an established role in membrane stability and osmoregulation, thereby leading investigators to believe that this compound has a beneficial effect on cell viability. Indeed, *in vitro* studies have demonstrated taurine to have a beneficial effect on the prevention of human endothelial cell death (Wang *et al.*, 1996). In this study, taurine was reported to attenuate endothelial cell apoptosis induced by sodium arsenite alone and in combination with TNF-α. It was, however, also demonstrated to maintain endothelial function, as represented by increased endothelial intercellular adhesion molecule-1 (ICAM-1) expression and oxidative state, when added after the apoptosis-inducing insult. In addition, taurine was demonstrated to reduce polymorphonuclear neutrophil-mediated necrosis. A role for modulation of Ca^{2+} fluxes by taurine has been proposed as a mechanism by which taurine protects against endothelial cell death. Taurine has been found to protect against increased Ca^{2+}-

mediated cell damage in the heart, aorta, liver and cerebral cortex (Nakashima *et al.*, 1990; Satoh, 1994).

In addition, many studies have demonstrated a proliferative effect when cells were cultured with taurine. Taurine co-culture lead to a reversal of the inhibitory effect of high glucose and glycosylation end products on the growth of mesangial cells (Tractman *et al.*, 1994). In chick B lymphocytes, taurine increased ^{3}H-thymidine incorporation in cells that had been stressed with lipopolysaccharide, indicating increased proliferation (Porter *et al.*, 1993). A higher (12.5 mM vs. 0.1 mM) taurine dose failed to increase ^{3}H-thymidine incorporation in human lymphocytes, possibly indicating a dose-response effect (Pasantes-Morales & Leon-Cazares, 1981). In light of the essential role of retinal integrity in retinal function, there has been considerable interest in the proliferative effect of taurine in retinal cells. Large amounts of taurine are characteristic for the retina in all species tested, and taurine has been demonstrated to promote retinal pigment cells to enter S-phase thereby augmenting DNA synthesis, an observation that did not extend to scleral fibroblasts (Gabrelian *et al.*, 1992).

In contrast to these studies, taurine also has the ability to be anti-proliferative in conditions where proliferation is pathogenic. In radiation pulmonary fibrosis, the pulmonary fibroblasts proliferate and normal lung parenchyma is replaced with collagen which impairs respiratory function. This has been associated with excess reactive oxygen and cytokine function. *In vitro* studies have demonstrated that exogenous taurine can protect against radiation-induced pulmonary fibrosis by reducing the abnormal cell proliferation and production of collagen (Song *et al.*, 1998). Similarly, taurine also protects against ozone-induced lung inflammation and hyperplasia in rats (Scheller-Levis *et al.*, 1995).

1.6.4 Taurine and immune function

Taurine makes up about 50% of the free amino acid pool in the lymphocyte. It has been demonstrated to enhance recombinant IL-2 immunotherapy by increasing tumour cytotoxity of lymphocyte-activated killer cells (Finnegan *et al.*, 1998). Taurine also attenuates the microvascular endothelial cell-targeted injury associated with this form of immunotherapy.

Exceptionally high levels of taurine are found in polymorphonuclear neutrophils (13457±4743 nM per 10^9 cells), indicating a particular importance for the amino acid in this cell type. Taurine is known to preserve polymorphonuclear neutrophil phagocytic ability induced by hyperlipidaemia (Masuda *et al.*, 1986) and modulate myeloperoxidase, a key enzyme in the respiratory burst pathway (Stapleton *et al.*, 1988). Myeloperoxidase also catalyses the step that produces hypochlorous acid, a very potent and destructive reactive oxygen species. Even though it is necessary for bacterial killing, hypochlorous acid can also cause lethal tissue damage. Taurine can sequester hypochlorous acid production by using myeloperoxidase to form taurine-chloramine (McLoughlin *et al.*, 1991). It may be argued that by removing hypochlorous acid, taurine reduces the threat to host tissues; taurine may therefore have a negative effect on host defence as it binds a very efficient antibacterial agent. Taurine-chloramine is also bactericidal (Stapleton *et al.*, 1997), and has been shown to have immunomodulatory effects.

1.6.5 The antioxidant properties of taurine

Increased reactive oxygen species production is associated with decreased immune function and reactive oxygen scavengers are known to attenuate the deleterious effects of these toxic intermediates on immune function. Taurine has been proposed to act as both a direct and indirect antioxidant. Directly, taurine has been

proposed to act as a scavenger of hypochlorous acid (McLoughlin *et al.*, 1991; Stapelton & Bloomfield, 1993), whereas as an indirect antioxidant, taurine functions as a membrane stabiliser. It has been proposed that the protective activity of taurine may reside in its ability to become chlorinated in the presence of hypochlorous acid, thereby preventing the direct attack of this oxidant on cell membranes (Gordon *et al.*, 1986). It has also been suggested that taurine, *via* formation of its chlorinated derivative protects the lung from oxidant injury at least in part by inhibiting production of the proinflammatory mediators, TNFα and nitrite (Schuller-Levis & Sturman, 1994). When used as a prophylactant against reperfusion injury during myocardial revascularisation, the protective capacity of taurine was attributed to free radical scavenging (Milei at al., 1992). The use of supplemental taurine against lipid peroxidation was advocated in another study that demonstrated protection of hamster bronchioles from acute NO_2- induced alterations (Gordon *et al.*, 1986). In addition, it has been recently demonstrated that taurine protects against neutrophil-mediated pulmonary microvascular injury (Barry *et al.*, 1997), and a protective effect of taurine from neutrophil-induced reperfusion in an animal model, proposed to be mediated through it's antioxidant ability, has also been demonstrated (Raschke *et al.*, 1995).

1.6.6 The cardiovascular effects of taurine

Taurine has been reported to produce i) a positive inotropic effect in heart muscle (Sawamura *et al.*, 1983), ii) beneficial effects against treatment of congestive heart failure (Azuma *et al.*, 1984), and iii) protective effects against Ca^{2+} overload (Takihara *et al.*, 1980). These findings are thought to be due to a protective effect of taurine on both nuclear and cytosolic Ca^{2+}. Myocardial levels of taurine fall in ischaemia, hypoxia and cardiac failure, with depletion correlating well with the degree of mechanical dysfunction (Lombardini, 1980; Kramer *et al.*, 1981). The fact that

taurine is abundant in myocardium under normal healthy conditions suggests that taurine may be essential in maintaining cardiac function. As taurine has also been demonstrated to abolish arrhythmias in animal studies (Satoh, 1994; Satoh 1995), the cardioprotective activity is likely related to regulation of Ca^{2+} flux. Indeed, in clinical studies on diabetic subjects (Elizarova & Nedosugova, 1996), cellular release of taurine was shown to be impaired. It was proposed that taurine must leave the cell in response to an appropriate stimulus in order to influence Na^{2+} / Ca^{2+} exchange and hence cellular functions.

A world-wide epidemiological study on cardiovascular diseases and nutritional factors (CARDIAC study) demonstrated an inverse relationship between 24 hour urinary taurine excretion and blood pressures (Nara *et al.*, 1990). Moreover, in stroke-prone spontaneously hypertensive rats (a genetic hypertensive rat strain), characterised by high blood pressure, taurine has been shown to decrease blood pressure and attenuate blood pressure increases (Nara *et al.*, 1978; Yamamoto *et al.*, 1985). It was suggested that taurine decreased blood pressure through decreasing sympathetic nerve activity in the central nervous system. It has also been proposed that taurine may enhance catabolism of noradrenaline at vascular nerve terminals, or perhaps may desensitise adrenoceptors on vascular smooth muscle (Li *et al.*, 1996). The effect of taurine on the peripheral cardiovascular system remains unresolved.

However, among various biological markers of nutrition, taurine has been found to be inversely related to coronary heart disease mortality in a world-wide epidemiological survey (Yamori *et al.*, 1996). Taurine supplementation experimentally improves hypercholesterolaemia by accelerating cholesterol excretion into bile acids and also by attenuating intestinal cholesterol absorption, thereby playing a role in atheromatous disease prevention. These, and possibly other mechanisms, may contribute to the prevention of coronary heart disease through the direct or indirect improvement in risk factor states such as hypercholesterolaemia and hypertension.

1.7 Cigarette Smoking

An important risk factor for which taurine supplementation has not been investigated is cigarette smoking. Smoking contributes to approximately 30% of all ischeamic heart disease (IHD) deaths annually, a risk which is strongly dose-related (US Department of Health and Human Services, 1990(a); US Department of Health and Human Services, 1990(b)). The complex behaviour of smoking, which involves physiological and psychological dependence, works synergistically with other risk factors to further increase the risk of IHD and ischaemic stroke (Anderson *et al.*, 1989; Shinton & Beevers, 1989). Importantly, the benefits of smoking cessation become evident at an early stage. When patients with IHD quit smoking they have a very significant 50% decrease in the risk of re-infarction, sudden cardiac death and total mortality (Fielding, 1985; Salonen, 1980; Samet, 1991). Consistent with the realms of human nature, the likelihood of smoking cessation increases with the severity of signs and symptoms of disease (Hay & Turbott, 1970; Wilhelmsson *et al.*, 1975; Frid *et al.*, 1991). Considering the relatively rapid rate at which benefit is obtained post-cessation, one would expect the main determinants to encompass either a dampening of the inflammatory response to smoking or restoration of physiological function.

1.7.1 Mechanisms of smoking-induced arterial disease

The characterisation of the cellular mechanisms by which cigarette smoking initiates or contributes to the development of the atheromatous plaque is compounded by the fact that cigarette smoking is known to contain approximately 4,000 constituents (Hoffmann & Wynder, 1986). Mainstream smoke from a burning cigarette comprises 92% gaseous and 8% particulate compounds. 400-500 individual gaseous components comprise the gaseous phase, including carbon monoxide, nitrogen oxides, ammonia, hydrogen cyanide, formaldehyde, nitroso-compounds and

benzene as the major constituents. The particulate phase is comprised of compounds such as nicotine, phenol and nitrosamines. Efforts to identify specific individual atherosclerotic-inducing agents have not been successful.

However, multiple mechanisms by which cigarette smoking induces adverse vascular biological effects have been demonstrated. Smoking is an anoxic insult to the vascular wall, stimulating an influx of inflammatory cells (Gerlis, 1955). Monocytes are therefore primed to adhere to the endothelium (Lehr, 1993; Lehr *et al.*, 1993; Dovgan *et al.*, 1994), increased activation and adhesion markers being expressed on both the endothelium and the leukocytes. Cigarette smoking also modulates thrombotic mechanisms, increasing fibrinogen and hence increasing plasma viscosity; prothrombotic mechanisms being thought to account for a large part of the increased risk of cardiovascular disease in smokers (Meade *et al.*, 1987). Smoking also impairs platelet activity, promoting their tendency to aggregate and release vasoconstrictors (Nowak *et al.*, 1987).

1.7.2 Effect of cigarette smoking on vascular tone

Vascular tone is controlled both centrally by the release of catecholamines which induce vasoconstriction, and peripherally by the release of local mediators such as EDRF, prostacyclin, endothelin and thromboxane A_2. Cigarette smoking affects both the central and peripheral mechanisms of vasomotor control. The chemoreceptors of the carotid and aortic bodies are stimulated by minute doses of nicotine, with resultant effects such as cardiac acceleration, increase in blood pressure and peripheral vasoconstriction, secondary to an associated increase in plasma adrenaline and noradrenaline (Benowitz, 1991; Ward *et al.*, 1991). More in depth studies on cigarette smoking and sympathetic activity have indicated that smoking acts at peripheral sympathetic sites to enhance catecholamine release from chromaffin tissue and may also reduce the overall clearance of these substances at the neuroeffector junction. The

baroreflex is also impaired in smokers, indicating a decreased ability to reflexly counteract the effect of sympathetic activation centrally (Grassi *et al.*, 1994).

Cigarette smoking also interferes with the metabolism of the vasoactive eicosanoids thromboxane A_2 and prostacyclin, both metabolites of arachidonic acid. Metabolites of both thromboxane A_2 and prostacyclin are significantly higher in smokers, with a relatively greater increase in thromboxane A_2 metabolites, thereby favouring vasoconstriction in smokers (Lassila *et al.*, 1988).

There is also known to be a reduction in the release of a more important modulator of vasomotor tone, EDRF, in cigarette smokers. Impaired endothelial relaxation, dependent on NO, has been reported in the brachial artery of smokers without clinical evidence of atherosclerotic disease (Celermajer *et al.*, 1993). Endothelial dysfunction has also been demonstrated in the coronary circulation of young smokers with angiographically normal arteries (Nitenberg *et al.*, 1993). Other evidence for abnormalities in vascular release of NO has come from *in vitro* studies where NO production from saphenous veins excised from heavy smokers was found to be decreased (Higman *et al.*, 1993). While a direct effect of nicotine was not the culprit for this effect, activity of the enzyme cNOS was found to be reduced in venous extracts of smokers (Higman *et al.*, 1996). Importantly, this same group has shown there to be a slow recovery of endothelial function, over several months, in patients who stop smoking (Higman *et al.*, 1994).

1.8 Evaluation of endothelial function

The logistics of chemopreventative studies are such that dietary change or supplementation should be evaluated initially in pre-clinical (animal) studies, and short term clinical studies with measurable pathophysiological end-points. Doppler ultrasonic imaging of the brachial artery has greatly facilitated the *in vivo* evaluation

of conduit artery endothelial dependent vasomotor function. Applanation tonometry permits non-invasive evaluation of ventriculo-vascular interactions. An exciting new approach to the evaluation of endothelial function is the *ex vivo* examination of circulating endothelial cells.

The two most commonly employed methods for evaluating endothelial function are the responses of vessels to acetylcholine infusion and the response of the brachial artery to flow-mediated dilatation. Although the endothelium releases a number of products, no single blood test has proven useful in determining normal endothelial function or detecting early endothelial abnormalities. As previously discussed, Furchgott and Zawadzki identified the direct vasodilatory effect of acetylcholine (ACh) on functioning endothelium *ex-vivo*. This was subsequently followed by multitudinous studies of assessment of endothelial function (Ludmer *et al.*, 1986; Okumura *et al.*, 1998; Cooper A & Heagerty A.M., 1998; Quyyumi A.A, 1998; Hasadi D., 1998; Takase B, 1998).

1.8.1 Response to acetylcholine infusion

ACh produces endothelium-dependent relaxation in human renal, internal mammary, splanchnic, peripheral and pulmonary arteries. As cyclooxygenase inhibitors do not prevent the ACh response, and haemoglobin and methylene blue can inhibit it, EDRF is the most likely mediator of ACh effects (Bossaller *et al.*, 1987; Luscher *et al.*, 1988). Intracoronary infusion of ACh during cardiac catherisation dilates human epicardial coronary arteries (Ludmer *et al.*, 1986), thought likely to be due to activation of muscarinic receptors on the endothelium. In many studies the resultant increase in forearm blood flow was simultaneously measured using venous occlusion plethysmography, whereafter haemodynamic measurements were obtained.

1.8.2 Flow-dependent dilatation

While invasive drug infusions of the brachial artery and subsequent measurements of flow facilitated acquisition of important information, the availability of a non-invasive well-tolerated method for accessing endothelial function *in vivo* is far preferable, and has been under scrutiny since 1989 (Anderson & Mark, 1989).

A widely accepted measurement of arterial diameter changes in response to an increase in blood flow by direct visualisation of the vessel walls, suitable for superficial arteries only, was devised in 1992 by Deanfield (Celermajer *et al.*, 1992). The relationship between arterial diameter changes using a non-invasive method of assessing FMD and endothelial function is supported by a study by Uehata *et al.* which demonstrated a correlation within subjects between invasive monitoring of coronary artery responses to acetylcholine infusions and non-invasive brachial artery responses to reactive hyperaemia (Uehata *et al.*, 1993). It was therefore proposed that non-invasive measurement of endothelial responses to endothelium-dependent stimuli were an accurate and reliable method for assessing endothelial function (Sorensen *et al.*, 1995).

Using high-resolution ultrasound to determine peripheral arterial responses to changes in blood flow, normal subjects have been shown to demonstrate a significant increase in brachial artery diameter (around 10%) in response to hyperaemia. It is intriguing to note that the duration of brachial artery dilatation in response to an increase in blood flow is prolonged (up to 20 minutes), considering that the t1/2 of NO in biological tissues is of the order of 2 to 30 seconds (Cocks *et al.*, 1985; Lownstein *et al.*, 1994). As it has been demonstrated that NO activity is highest in large arterioles in which hydraulic resistance and shear stress are also highest (Griffith *et al.*, 1987), it is likely that mechanical factors such as pulsatility are involved. Abnormal endothelial responses have been demonstrated in various cardiovascular disease states, specifically, hypercholesterolaemia (Chowcenczyk *et al.*, 1992;

Creager *et al.*, 1990), hypertension (Panza *et al.*, 1990), diabetes mellitus (Johnstone *et al.*, 1993), proven atherosclerosis (Zeiher *et al.*, 1991; Drexler *et al.*, 1989) and in the preclinical phases of vascular disease in those with coronary risk factors (Celermajer *et al.*, 1995).

1.8.3 Cold pressor stimulation

Despite the pivotal role of *in vitro* studies in elucidating underlying mechanisms, the clinical implication of endothelial function and dysfunction can only be appreciated by *in vivo* human studies. Likewise, failure of vasodilatation in response to the cold pressor test (CPT), indicates abnormalities of endothelial functioning (Corretti *et al.*, 1995). The role of the endothelium in mediating vasomotion through adrenergic mechanisms has been assessed in normal and atherosclerotic coronary arteries (Nabel *et al.*, 1988; Zeiher *et al.*, 1989). Atheromatous disease likely impairs release of EDRF and perhaps other vasodilator substances as part of a generalised dysfunction of the endothelium, and therefore permit vasoconstrictor responses (Freiman *et al.*, 1986). The constrictor response of atherosclerotic arteries to cold exposure mimics the reactions of diseased epicardial arteries to the endothelium-dependent dilator and direct smooth muscle constrictor acetylcholine (Ludner *et al.*, 1986). Therefore, endothelial dysfunction present in atherosclerosis may result in a loss of dilator function and permit constrictor responses to a variety of stimuli.

These responses in diseased arteries may also be due to an initial decrease in sympathetic-induced NO production, either through failure of a sympathetic-induced increase in blood flow or failure of α_2 endothelial receptor stimulation (which stimulates NO release). Such failures of endothelial functioning prevent overpowering of the adrenergic-induced vasoconstrictor effects of the CPT on smooth muscle cells and immediate vasoconstriction ensues.

1.8.4 Applanation tonometry

Another such method for assessing endothelial function, and its interaction with the whole cardiovascular tree, is the use of applanation tonometry (sphygmocardiography). One of the earliest recorded medical skills - assessment of pulse character - has re-emerged with the advent of applanation tonometry, whereupon pulse wave analysis can be used to accurately record peripheral pulse waveforms and subsequently generate a corresponding central waveform. The arterial pulse is the most fundamental of clinical signs, and pressure pulse contour waveforms are accurately and non-invasively obtained using the applanation tonometer.

The instrument devised is a pencil-shaped probe held on the skin over the point of maximal arterial pulsation. The probe incorporates a sensitive micromanometer tip, the use of which is based upon the principle of applanation tonometry, as used in ocular tonometry for registration of intraocular pressure. Essentially, if one flattens or applanates the curved surface of a pressure containing structure, the circumferential stresses in the wall of the structure are balanced and the pressure registered by the sensor is the true intra-arterial pressure. The vessel must be compressed against underlying bone for a consistent signal to be achieved. The accuracy of this probe has been validated in animals and humans with indwelling radial artery lines (Kelly *et al.*, 1989). Subsequent to this, central aortic pressure waveforms were derived from radial tonometry pressure waveforms by mathematical transformation, a generalised transfer factor calculated by simultaneous invasive central aortic pressure and non-invasive radial pressure measurements (Chen *et al.*, 1997). This new methodology therefore provides information about vessel wall function and compliance (Wilkinson *et al.*, 1998; Cameron *et al.*, 1998), as well as central aortic pressures, which impact importantly on cardiovascular function.

1.9 Circulating endothelial cells

While abnormalities of function can be demonstrated *in vivo*, examination and investigation of the exact mechanisms by which risk factors adversely affect endothelial health is unclear. Isolation and examination of circulating endothelial cells (CECs) overcomes the problematical inaccessibility of the vascular endothelium *in vivo* and provides a useful tool for direct assessment of endothelial cells. Recent reports suggest that alterations in the mechanical, chemical or humoral environment of the endothelium may cause functional and structural endothelial disturbances and eventually lead to endothelial cell death (Meredith *et al.*, 1993; Chen *et al.*, 1997). The number of CECs isolated from the circulation have indeed been found to be increased in conditions associated with vascular injury such as myocardial infarction and angina (Hladovec *et al.*, 1978; Bouvier *et al.*, 1970), endotoxinaemia (Gerrity *et al.*, 1976), cytomegalovirus infection (Percivalle *et al.*, 1993; Grefte *et al.*, 1993), rickettsial infection (Drancourt *et al.*, 1992; George *et al.*, 1993), intravascular instrumentation (Sbarbati *et al.*, 1991; George *et al.*, 1991) and sickle cell anaemia (Solovey *et al*, 1997). While the exact stimuli for release of these cells into the circulation can only be speculated upon, the origin of these cells (expression of macro- or microvascular specific antigens), their activation status (detection of antigens expressed only or to a markedly greater extent on activation, such as P-selectin, E-selectin, VCAM or ICAM) and viability can be detected to provide for a more detailed explanation of endothelial pathobiology.

1.10 Research Objectives

Cigarette smoking is a major public health issue. Despite health promotion activities and public health policies, the incidence of smoking is increasing in young people. Smoking results in multiple adverse effects, most of which are mediated through the endothelium. Abnormalities in endothelial function have been previously identified in smokers. Knowledge to date of the antioxidant and immunoregulatory functions of taurine, and awareness of the relation of taurine consumption to coronary heart disease in world-wide epidemiological studies, would suggest that this micronutrient may have beneficial effects on endothelial function in young cigarette smokers. This thesis provides a multifaceted, comprehensive approach to assessing the components of endothelial dysfunction in this subject group. It also serves to investigate if taurine supplementation, at doses achievable at a dietary level, could offset the adverse physiological consequences of cigarette smoking, and to determine the mechanism of protection afforded by this amino acid.

Chapter 2

"Materials and Methods"

2.1 Materials

2.1.1 Equipment

Bench centrifuge (5403): Eppendorf-Netheler-Hinz-Gmbh, Hamburg, Germany

Electronic balance (AC1215): Sartorius, Göttingen, Germany

Electronic pipette aid: Drummond, Broomall, PA, USA

FACscan flow cytometer: Becton Dickinson Immunocytometry Systems, San Jose, CA, USA

Gel electrophoresis box: Atto Corp., Japan

Humidified incubator (Infrared CO_2 incubator): Forma Scientific, Ohio, USA

Hot plate (Dri-Block): Techne Ltd., Cambridge, UK

Laminar air flow cabinet (Holten LaminAir HB2436, Allerod, Denmark

pH meter (Radiometer PHM82 pH meter): Copenhagen, Denmark

Plate reader: Microplate autoreader, Bio-Tek Instruments Inc., VT, USA

Plate shaker: Diagnostics Corporation, UK

Semi-dry blotter: Hoefer Semiphor, Pharmacia Biotech, Herts, UK

Trans-blot electrophoresis transfer cell: Bio-Rad Laboratories, CA, USA

2.1.2 Disposables

Coverslips: Chance Propper Ltd., Warley, UK

Cytoflow tubes; Becton Dickinson Labware, NJ, USA

Glass slides: Chance Propper Ltd., Warley, UK

96 well flat-bottomed microtitre plates (Falcon): Nunc, Denmark

2% gelatin-coated culture flask: Falcoln, Lincoln Park, NJ

25 cm^2 / 75 cm^2 tissue culture flasks: Greiner Labortechnik GmbH, Frickenhausen Germany

25/50 ml starstedt tube: Sarstedt, Germany

Heparin anticoagulant tube; Sarstedt, Germany

2.1.3 Chemicals

The full names and addresses of the sources listed below are given in Appendix I

Acrylamide	Sigma
Alkaline Phosphatase Conjugate goat anti-rabbit IgG	Promega
Ammonium persulphate	Sigma
APS (ammonium persulphate)	Sigma
BCA Protein assay reagents: A + B	Pierce, Rockford, IL.
5-bromo-4-chloro-3-indolyl phosphate (BCIP) tablets	Sigma
5-BrdU labelling and detection kit 111	Boehringer Mannheim
CD14 mouse anti-human antibody	Becton Dickinson
CD45 mouse anti-human antibody	Becton Dickinson
Cell death detection ELISA^{plus} kit	Boehringer Mannheim
cNOS primary antibody (mouse anti-human IgG)	Transduction Laboratories
Dimethylformalaldehyde	Sigma
Dithiothreitol	Sigma
Dulbecco's modified eagles medium (DMEM)	Gibco
Endothelial cell growth supplement	Sigma
Endothelin-1 (ET-1) ELISA	R&D Systems
Ethylenediamine-N,N'-diacetic acid	Sigma
Foetal Calf serum	Gibco
Ficoll	Sigma

Fungizone	Gibco
Glutamine	Gibco
Glycerol	Sigma
Glycine	Sigma
H_3PO_4	Sigma
Hanks balanced solution (HBSS)	Gibco
Heparin	Gibco
HEPES	Sigma
Human microvascular endothelial cells	BioWhittaker
Hydrochloric acid	Sigma
Interleukin 1-alpha (IL-1α) ELISA	R&D Systems
Interleukin 1-beta (IL-1β) ELISA	R&D Systems
M199	Gibco
Magnesium chloride	Sigma
Marvel	Premier Beverages
2-Mercaptoethanol	Sigma
Methanol	Sigma
Microplate autoreader	Biotech Instruments
Minimal essential medium (MEM)	Gibco
N,N' -Methylene-bis-acrylamide	Sigma
Naphthethylene	Sigma
Nitro blue tetrazolium (NBT)	Promega
PBS (phosphate buffered saline)	Sigma
Penicillin	Gibco
Phenylmethylsulphonyl fluoride	Sigma
Peroxidase	Boehringer Mannheim

Potassium chloride	Sigma
Protease Inhibitor Tablets	Boehringer Mannheim
SDS (sodium dodecyl sulphate)	Sigma
SDW (sterile distilled water)	Sigma
Sodium chloride	Sigma
Streptomycin Sulphate	Gibco
Sulphanilamide	Sigma
3',3",5',5"-tetrabromophenolsupfonephthalein	Sigma
TEMED (N,N,N',N'-tetramethyl-ethylenediamine)	Sigma
Tris (Trizma Base)	Sigma
Trypan blue (0.4% solution)	Sigma
trypsin (0.05%) - EDTA (0.02%) solution	Gibco
Tumour necrosis factor -alpha (TNFα) ELISA	R&D Systems
Tween 20	Merck-Schuchardt
Vascular endothelial cell growth factor (VEGF) ELISA	R&D Systems

2.1.4 Solutions

Buffer A

10 mM HEPES, pH 7.9

1.5 mM $MgCl_2$

10 mM KCL

Before use, per 10 mls of above add:

0.5 mM DTT (Dithiothreitol)

0.5 mM PMSF (phenylmethylsulphonyl fluoride)

12% separating gel

12% acrylamide:0.25% bisacrylamide (N,N' -Methylene-bis-acrylamide)

376 mM Tris-HCL, pH 8.8

8.3 ml SDW

0.1% SDS (sodium dodecyl sulphate)

10µl TEMED

.01% APS (Ammonium persulphate)

5% stacking gel

5% acrylamide:0.1% bisacrylamide (N,N' -Methylene-bis-acrylamide)

376 mM Tris, pH 6.8

6 ml SDW

10µl TEMED

0.01% APS (Ammonium persulphate)

Electrode buffer

50 mM Tris

384 mM Glycine

0.1% SDS (sodium dodecyl sulphate)

2x Llaemmli buffer

120 mM Tris-HCL, pH 6.8

5% SDS (sodium dodecyl sulphate)

14.3 mM 2-mercaptoethanol

20% glycerol

2µl 3',3",5',5"-tetrabromophenolsupfonephthalein

Tris-buffered saline (TBS)

A stock solution of 10X TBS:

250 mM Tris-HCL, pH 7.6

1.5 M NaCl

For 1X TBS:

10X TBS diluted 1:10 with distilled H_2O.

TBST

25 mM Tris-HCL, pH 7.6

0.15 M NaCl

0.05% Tween 20

NBT (Nitro blue tetrazolium)

75 mg/ml in 70% dimethylformaldehyde

Transfer Buffer

300 ml 10X buffer

For a 10X solution:

7.266 g Trizma

33.78 g glycine

3 ml 10% SDS

Make up to a final volume of 300 ml with SDW

600 ml methanol

2100 ml distilled H_20

Blocking Solution

50ml TBS

25µl Tween (0.05%)

2.5g Marvel (original dried skimmed milk) (5%w/v)

Substrate Buffer

100 mM Tris-HCl, pH 9.5

100 mM NaCl

5 mM MgCl2

67µl NBT (nitro blue tetrazolium)/10 mls

33µl BCIP (bromochloroindolyl phosphate)/10 mls

2.1.5 Preparation and handling of reagents

All chemicals and reagents were stored according to the manufacturer's instructions. General purchase chemicals were of analytical grade (Sigma Chemical Company, St. Louis, MO, USA and BDH Chemicals Ltd., Poole, UK, unless otherwise stated). Solid chemicals were weighed using a Sartorius AC1215 electronic balance (Göttingen, Germany) and were prepared in distilled water with a conductivity of greater than 18mΩ. The pH of solutions were measured using a Radiometer PHM82 pH meter (Copenhagen, Denmark). Solutions were autoclaved within a few hours of preparation. Liquid transfer for volumes of less than and including 1 ml were carried

out using Gilson pipettes and for volumes greater than 1 ml, an electronic pipette aid (Drummond, Broomall, PA, USA) and disposable plastic pipettes were used.

2.2 Subject population

Healthy smokers and control non-smoking subjests were studied. These subjects were recruited from among hospital staff, friends and family. They ranged from 20 to 37 years of age. All were normotensive, non-diabetic and were without a history of hyperlipidaemia or a family history of premature vascular disease. They were clinically well and not taking any cardiovascular medications. Smokers were defined as those who smoked at least 20 cigarettes per day for at least 2 years (2 pack years), and had at least one cigarette in the preceeding 12 hours. Control subjects were lifelong nonsmokers.

2.3 Haemodynamic studies

2.3.1 Assesment of endothelial dependent dilatation - study protocol

The methodology described here has been used elsewhere (Celermajer *et al.*, 1992). All studies were performed in a temperature-controlled room (20°C) with the subject in the resting, supine state. The subjects right arm was comfortably immobilised in the extended position to allow constant access to the brachial artery for imaging. Brachial artery diameter and flow velocity were measured from B-mode ultrasound images using a 7.0-MHz linear array transducer ultrasound system and a standard Acuson 128XP/10 (Acuson, Mountain View, California). The artery was scanned in longitudinal section at a location in the antecubital fossa where the clearest ultrasound image was found. Surrounding soft tissue images on the images obtained were used as landmarks for follow-up scans. The centre of the artery was identified when the clearest picture of the anterior and posterior intimal layers of the brachial artery were identified. The transmit (focus) zone was set to the depth of the near wall, as the near wall "m" line (media/adventicia interface) is far more difficult to evaluate in

comparison to the far wall. Depth and gain were set to optimise lumen/arterial wall interfaces. When a satisfactory image was obtained, images were magnified using a resolution box function. Operating parameters were not changed during each study. Images were recorded on videotape for subsequent off-line analysis on the same instrument.

When a satisfactory transducer position was found, the skin was marked and the arm remained in the same position throughout the study. A baseline pre-treatment, post-no treatment, post-vitamin C and post-taurine scan were all carried out in an identical manner. The subject lay at rest for 10 minutes prior to a resting baseline scan. Increased flow was then induced by inflation of a pneumatic tourniquet placed around the forearm (distal to the scanned part of the artery) to a pressure of 240mmHg for 4.5 minutes, followed by release. A second scan was then performed continuously for 30 seconds before and 90 seconds after deflation of the cuff.

Smokers were supplememted with 2gms vitamin C/day (Roche Pharmaceuticals) and 1.5gms taurine/day (Twinlab, Ronkonkoma, N.Y.) for 5 days. A 2 week wash out period was used between supplementation of smokers with vitamin C and taurine.

2.3.2 Assesment of endothelial dependent dilatation - Data Analysis

All scans were recorded on super VHS videotape for off-line analysis. Vessel diameter measurements were made by the same observer; the results obtained compared to those obtained by an independent observer, unaware of the clinical details and stages of the experiment, and the results were averaged. Results obtained were expressed as diameter change in mm's. This methodology has been previously demonstrated to be accurate and reproducible for measuring small changes in vessel diameter (Sorensen *et al.*, 1995).

Vessel diameter was measured at a fixed distance from an anatomical marker (such as a fascial plane of a vein in cross section) using ultrasonic calipers. Measurements were

taken from the anterior to the posterior "m" line (Figure 3.1) at peak systole, incident with the peak systolic waveform on the flow velocity curve (Figure 3.2). Reactive hyperaemia measurements were taken 45-60 seconds after cuff deflation. An average of four cardiac cycles analysed for each measurement.

2.3.3 Assesment of response to the cold pressor test - study protocol

The methodology described for identification of an adequate site on the brachial artery for scanning is identical to that described in 2.3.1. All studies were performed in a temperature-controlled room (20°C) with the subject in the resting, supine state. The subjects right arm was comfortably immobilised in the extended position to allow constant access to the brachial artery for imaging. Brachial artery diameter and flow velocity were measured from B-mode ultrasound images using a 7.0-MHz linear array transducer ultrasound system and a standard Acuson 128XP/10 (Acuson, Mountain View, California). The artery was scanned in longitudinal section at a location in the antecubital fossa where the clearest ultrasound image was found. Surrounding soft tissue images on the images obtained were used as landmarks for follow-up scans. The centre of the artery was identified when the clearest picture of the anterior and posterior intimal layers of the brachial artery were identified. The transmit (focus) zone was set to the depth of the near wall, as the near wall "m" line (media/adventicia interface) is far more difficult to evaluate in comparison to the far wall. Depth and gain were set to optimise lumen/arterial wall interfaces. When a satisfactory image was obtained, images were magnified using a resolution box function. When a satisfactory transducer position was found, the skin was marked and the arm remained in the same position throughout the study. Operating parameters were not changed during each study. Images were recorded on videotape for subsequent off-line analysis on the same instrument.

The subject lay at rest for 10 minutes prior to a resting baseline scan. Baseline recordings of arterial diameter were obtained, following which the left hand and wrist were immersed in iced water for 3 minutes and then withdrawn, while the right artery was continuously imaged and recorded. An additional investigator was present in the examination room during this time to oversee accurate timing of placement and extraction of the hand in iced water and to ensure adequate coverage of the left hand and wrist with the iced water.

2.3.4 Assesment of response to the cold pressor test - Data Analysis

All scans were recorded on super VHS videotape for off-line analysis. Brachial artery diameter measurements were obtained at baseline, and at 1 and 3 minutes immersion of the contralateral hand in ice. Vessel diameter measurements were made by the same observer, the results obtained compared to those obtained by an independent observer, unaware of the clinical details and stages of the experiment. Results obtained were expressed as diameter change in mm's. Vessel diameter was measured at a fixed distance from an anatomical marker (such as a fascial plane of a vein in cross section) using ultrasonic calipers. Measurements were taken from the anterior to the posterior "m" line (Figure 3.1) at peak systole, incident with the peak systolic waveform on the flow velocity curve (Figure 3.2). An average of four cardiac cycles were analysed for each measurement.

Measurements at the time points of 1 and 3 minutes in ice were used, as these time points have been recognised as the differentiating determinants between subject groups (Corretti *et al.*, 1995; Nabel *et al.*, 1988) Results obtained were expressed as percentage change in brachial artery diameter; the change in diameter induced by the CPT was divided by the baseline diameter measurement.

2.3.5 Applanation tonometry

2.3.5.1 Arterial Applanation Tonometry (Sphygmocardiography)

Using methodology previously outlined by Kelly *et al.* (Kelly *et al.*, 1989), the pulse pressure waveform and amplitude were obtained from the left radial artery with a probe which incorporates a high-fidelity strain-gauge transducer (model TCB-500, Millar Instruments). High-fidelity pulse waves were obtained by placing the pencil-type probe perpendicularly over the point of strongest pulsation of the radial artery. The principle of applanation tonometry is based on the fact that flattening or applanation of the curved surface of a pressure containing structure (in this case the radial artery) under the probe which allows direct measurement of the arterial pulse pressure within the artery (figure 6.1). The flat sensor flattens the wall of the artery, tangential pressures are eliminated and the sensor is exposed to the pressure within the artery and records this acurately (Kelly *et al.*, 1989). The relationship between pressure waves at different arterial sites is expressed as a transfer function, where harmonic components of arterial waves at the 2 arterial sites are related in amplitude and phase as a function of frequency or harmonic (Nichols and O'Rourke,1990). A single transfer function to describe wave transmission in the upper limb has been used on numerous occasions (O'Rourke *et al.*, 1970; Lasance *et al.*, 1976; Karamanoglu *et al.*,1993; Chen *et al.*,1997), and for ease of use in the clinical setting a software programme has been introduced by O'Rourke (O'Rourke *et al.*, 1992).

Using the high-fidelity transducer, tonometry has been shown to record pressure waves which are virtually identical to those recorded intra-arterially (Kelly *et al.*, 1989). Best results were obtained with the wrist in the dorsiflexion position using a support under the wrist. Steady, gentle pressure was applied until a waveform signal appears wholly within the display. When a good waveform was found (a good waveform is consistent, large (at least 3cm on screen) and in a steady horizontal position), the probe was kept in position and a minimum of 20 consecutive pressure

waveforms were analysed and averaged as previously described (Kelly at al., 1989; Murgo *et al.*, 1980; Tanaka *et al.*, 1988). From these waveforms peripheral haemodynamic parameters such as dP/dt , the differential of the pressure wave, is obtained.

2.3.5.2 Assessment of vascular-ventricular interactions

With the use of a consistent transfer function from the ascending aorta to the radial artery, applanation tonometry allows the aortic pressure waveform to be synthesised, and evaluation of left ventricular function can take place. Direct measurements from the radial pressure wave can also be taken from the tracing, notably the differential of the pressure wave (dP/dt), the absolute value of which is thought to be of little value but necessary however for calculation of the amplitude of reflected peripheral waves from the vascular tree. When the ratio of this reflected wave is compared to the initial transit of the impulse generated by the heart, the augmentation index (AI) can be calculated. An earlier wave reflection occurs with a stiffer vessel, such as occurs with aging, and results in an increased AI.

Left ventricular properties such as ejection duration (ED) and heart rate (HR) can be derived from the aortic pressure wave. In addition, by separating the aortic wave into systolic and diastolic periods, vascular ventricular interactions at baseline and in response to a physiological stress such as the CPT, may also be determined. Mean systolic pressure and end-systolic pressure are taken as indices as left ventricular systolic load (Weber & Janicki,1971; Suga & Sagawa,1974). The Buckberg Index (subendocardial viability ratio) is the ratio of the diastolic time index (integral of pressure and time during diastole), which is an index of capacity for left ventricular perfusion through the coronary arteries (Feigl 1983; Nichols & O'Rourke, 1990; O'Rourke *et al.*, 1992)) to the tension time index (integral of pressure and time during systole), which is an index of left ventricular myocardial oxygen and blood demand (Sarnoff *et al.*, 1958)). The Buckberg Index is a measure for the propensity

for myocardial ischaemia on the basis of altered haemodynamic forces (Buckberg *et al.*, 1972a.b; O'Rourke 1982).

2.3.5.3 Measurements

Applanation tonometry was conducted in a quiet, temperature controlled room with subjects resting for 5 minutes prior to the baseline scan. The left radial pulse was used and blood pressure was measured by auscultation over the left brachial artery using a standard mercury sphygmomanometry, with diastolic pressure defined as Korotkoff phase V according to American Heart Association guidelines (Perloff at al., 1993). Baseline peripheral and central haemodynamic parameters were obtained. After a baseline scan was completed, the right hand and wrist were immersed in a bath of ice water for 3 minutes and then withdrawn, as previously described by Corretti *et al.*(Corretti *et al.*, 1995). Blood pressure measurements were taken prior to each tonometric recording, which were taken at 1 and 3 minutes in ice. Subsequent peripheral and central haemodynamic parameters were then obtained in response to the cold pressor test.

Following this, the subjects were suppemented with 1.5gms taurine/day for 5 days and haemodynamic responses at baseline conditions and responses to the cold pressor test post taurine were investigated.

2.4 Tissue culture

All tissue culture work was carried out in an aseptic manner within a laminar air flow cabinet (Holten LaminAir HB2436, Allerod, Denmark). The cabinet was turned on at least 20 minutes before use. It was sanitised using 70% (v/v) isopropyl alcohol. All equipment and reagents were also sanitised prior to their use in the cabinet.

2.4.1 Human umbilical vein endothelial cell isolation and culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins that were cannulated, perfused with Hanks' solution to remove blood and then incubated with 1% collagenase for 15 minutes @ 37 °C. After removal of collagenase the cells were cultured on 2% gelatin-coated culture flask (Falcon, Lincoln Park, NJ) in complete M199 medium supplemented with 20% FCS, penicillin (100U/ml), streptomycin sulphate (100 μg/ml), fungizone (0.25 μg/ml), heparin (16 U/ml), endothelial cell growth supplement (75 μg/ml) and 2 nM glutamine as previously described (Jaffe *et al.*, 1973). Cells were grown at 37°C in a humidified 5% CO^2 conditioned chamber.

All cells were cultured routinely in either 25 cm^2 or 75 cm^2 tissue culture flasks (Greiner Labortechnik GmbH, Frickenhausen, Germany). Flasks were cultured at 37°C and 5% CO_2/95% air in a humidified incubator (Infrared CO_2 incubator, Forma Scientific, Ohio, USA) and the culture medium was replaced every second day. Cells were passaged by trypsinization with 0.05% trypsin-0.02% ethylene diamine tetraacetic acid, followed by splitting and subculturing in 1:4 ratio when confluent monolayers were reached. Endothelial cells were identified by typical phase contrast "cobblestone" morphology and by immunofluorescence staining with von Willebrand factor antigen. In all experiments reported, HUVECs were used as individual isolates between passages 3 and 5. Twenty four hours prior to experimentation, cells were washed twice with PBS and maintained in serum-free M199 medium.

2.4.2 Human microvascular endothelial cell isolation and culture

Primary cultures of human dermal microvascular endothelial cells (HMVECs) were obtained from BioWhittaker (Niederlassung, Germany). The manufacturer supplied endothelial cell growth medium containing the following supplements:

hydrocortisone, hFGF-ß, R3-IGF, ascorbic acid, heparin, VEGF, hEGF, GA-100 and 5% (v/v) FBS. HBSS, trypsin/EDTA and trypsin neutralising solution were also supplied with the cells. The medium was changed every two days. HMVEC cell cultures are irreversibly contact inhibited and therefore were subcultured before they reached 85% confluency.

For trypsinisation, cells were rinsed twice with Hanks Balanced Salt Solution (HBSS), which was warmed up to room temperature. Medium from each flask of cells was aspirated. 3 mls of Biowhitakers trypsin/EDTA solution was added and the flasks were laid flat and left @ 30°C for 5 minutes. 3 mls of Trypsin Neutralising Solution was added to the flasks and cells were transferred to 50 ml sterile tubes. The flasks were then rinsed with 2 mls of HBSS to collect residual cells and this was added to the 50ml mixture. The cells were then centrifuged @ 220 x g for 5 minutes at room temperature, counted and diluted in 10 mls of growth medium. 5mls of this mixture was then added into a new flask and incubated @ 37 °C with 5% CO_2.
These cells have a very limited life span and usually reached senescence within five passages.

2.4.3 Cell counting

Cell suspensions were counted using an Improved Neubauer haemocytometer slide. Trypan blue dye exclusion was used to determine cell viability. 20 μl of cell suspension was added to 180 μl of trypan blue, mixed, and allowed to stand for 2 minutes. A sample of this mixture was added to the counting chamber and the cells were visualised and enumerated under a light microscope. Viable cells only were included. The four corner squares of the grid, each with a volume of 1 mm^3, were counted and the number of cells were calculated using the following equation :-

cells / ml = count x 1,000 x 10 (dilution factor) / 4

2.5 Isolation of monocytes

Approximately 21 mls of venous blood was collected from volunteers in heparin anti-coagulant tubes (Sarsedt, Wicklow, Ireland). Peripheral blood mononuclear cells were isolated under sterile conditions by way of a Ficoll layer gradient technique. Samples were taken from healthy smokers with a 2-pack year history of cigarette smoking, and from healthy volunteer controls. Blood samples were diluted with DMEM to make up to 45 mls of sample. 7 mls of diluted blood was gently layered onto 3 mls of Ficoll in 15 ml universal containers, and was centrifuged at 400 x g for 40 minutes. The buffy layer was harvested and spun at 800 x g for 5 minutes. The cell pellet was washed twice with 10 mls of DMEM, and spun at 400 x g for a further 5 minutes. Cells were resuspended in 3 mls of DMEM and counted using Trypan Blue staining. Cells were greater than 96% viable using this staining procedure.

2.5.1 Flow cytometry

Monocyte content of recovered cells was established using mouse anti-human monoclonal CD45/CD14 antibodies (Becton Dickinson, Mountain View, CA, USA) and run on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). 10μl of resuspended cells was incubated with 1 μl of FITC-labelled CD 45 mouse anti-human monoclonal antibody and a further 10μl was incubated with 1 μl of phycoerythrin-conjugated CD14 mouse anti-human monoclonal antibody for 20 minutes at 4°C. Each cell suspension was centrifuged at 300 x g for 5 minutes. The cell pellets were washed twice with phosphate buffered saline and centrifuged at 300 x g for 5 minutes. A FACscan flow cytometer was used to immediately analyse receptor expression by mean channel fluorescence. A minimum of 10,000 events were collected and analysed using Lysis 11 software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Using the FL_1 filter (≈530 nm) to detect FITC

labelled CD45 and FL_2 filter (≈565 nm) to detect phycoerythrin (RPE) labelled CD14, the forward scatter and side scatter of the cell populations were simultaneously measured and the intensity of fluorescence of individual cells was determined for each population.

The percentage of monocytes present was obtained by calculating the number of cells that expressed CD14 (specific for monocytes) and CD45 (present on nearly all leukocytes), and presenting those expressing both antibodies as a percentage of total CD 45 expression. Flow tubes used in all experiments were obtained from Becton Dickinson Labware, NJ, USA. Depending on each individual subject, a range of 3-15% of cells isolated were monocytes.

2.5.2. Preparation of monocyte conditioned medium

Using the monocytes obtained as per 2.5, cells were corrected to $2x10^6$ monocytes/ 5 mls DMEM / 0.5% foetal calf serum (FCS) / 0.1% (v/v) Penicillin/Streptomycin (P/S) solution, and were plated in flat bottomed microtitre plates (Nunc, Denmark). DMEM was then concentrated in a time (24 and 48 hours)- and concentration ($2x10^6$ monocytes)- dependent manner. Samples were obtained at both 24 and 48 hour time points and stored at -40 °C until quantitative assays were performed.

2.5.3 Preparation of MCM/HUVEC co-culture

Isolated HUVECs were made up to 4×10^5 cells/ml. A 0.5 ml cell suspension of this was added to each well of a 24 well plate, previously coated with 2% gelatin, thus plating 2×10^5 cells/well. These cells were then incubated at 37°C, 5% CO_2 for 12 hours, after which the supernatant was removed from each well and 0.25 ml of DMEM/0.25 ml MCM was added and incubated at 37°C, 5% CO_{2p} for 12 and 24 hours.

2.6 Ex-*vivo* assays

2.6.1 Nitric Oxide assay

Nitric Oxide (NO) production was determined indirectly by measuring the concentration of the stable end product nitrite in the supernatant of endothelial cells treated with MCM from smokers, non-smokers and taurine treated smokers using the Griess reaction. Griess reagents were made up in a 1:1 ratio of N-1-Naphthylethylenediamine (NED), (0.1% in H_2O) and Sulphanilamide (Sulf.), (1% in 5% H_3PO_4). NED and Sulf. were combined in equal volumes to make the reagent. 100 µl of supernatant from samples prepared as per 2.5.3. were added to a 24 well plate in duplicate. 100 µl of Griess reagent was then added and the samples were incubated for 10 minutes at room temperature. Griess reagents converted nitrite into a purple azo compound. A standard curve was prepared by adding 100 µl of DMEM to 8 wells in duplicate, then adding 100 µl of a 200 nM standard $NaNO_2$ solution followed by 7 x 1:1 dilutions giving a range of 0-20 nM of nitrite. The coloured product produced from the assay (if nitrite present) was then quantified photometrically using a microplate autoreader (Microplate Autoreader EL 311, Bio-Tek Instruments INC.) at a wavelength of 550 nm, by plotting a curve of known nitrite concentration standards against known signals (optical densities), and thus determining the concentrations of unknowns.

2.6.2 Endothelin-1 assay

ET-1 was detected using an ELISA kit for ET-1 detection (R&D Systems, UK).This assay involves the reaction of any ET-1 present in the sample with two antibodies directed against different epitopes of the ET-1 molecule. One antibody is coated onto

the surface of the wells on the microtitre plate and the other is conjugated to the enzyme horseradish peroxidase. Any ET-1 present forms a bridge between the two antibodies. Unbound material is removed by aspiration and washing, and the amount of ET-1 conjugate bound to the well is detected by a reaction with a substrate which yields a colour product proportional to the amount of conjugate (and thus ET-1 in this sample). This coloured product can then be quantified photometrically using a microplate autoreader (Microplate Autoreader EL 311, Bio-Tek Instruments INC.), Then by analysing standards of ET-1 concentration coincident with the samples, and plotting a curve of signal (optical density) versus concentration, the concentration of the unknowns are determined.

A 100µl of standard, control and supernatant were added to each well with sufficient force to ensure mixing. The reagent addition was uninterrupted and complete within 10 minutes. The plate was covered with a plate sealer and incubated at room temperature for one hour. Contents were then decanted and washed by adding 300µl of wash buffer to each well. The process was repeated five times for a total of six washes. A 100µl substrate was added to each well, the plate was covered again with a new plate sealer and incubated at room temperature for 30 minutes. A 100 µl of stop solution was added to each well in the same order as the substrate. Optical density of each well was determined after 30 minutes of addition of stop solution using a microplate reader set to 450nm. Mean absorbency values for each set of diluted standards was calculated. A standard curve was constructed by plotting the mean absorbency for each standard on the y-axis against the concentration on the x-axis and then drawing a best fit curve through the points in the graph. Concentration of each unknown sample was determined by calculating the concentration of ET-1 corresponding to the mean absorbency from the standard curve.

2.6.3. Plasma taurine quantification

14 mls of blood were obtained from each subject pre- and post- supplementation with 1.5 gms of taurine/day for 5 days. Samples were spun @ 395 x g for 20 minutes. 0.5 mls of plasma supernatant was obtained and samples were deproteinised by addition of 0.5 mls of 35% sulfosalicylic acid. Samples were then stored @ -20°C until plasma taurine was measured using high performance liquid chromatography (HPLC) by the Department of Biochemistry, Amino Acid Laboratory at The Children's Hospital, Temple St.

2.6.4 Human tumour necrosis factor α assay

TNFα was detected using an ELISA kit for cytokine detection (R&D Systems, UK).This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNFα is coated onto the surface of the wells on the microtitre plate. After addition of 50µl of assay diluent to each well, 200µl of standards or samples were pipetted into the wells. Samples were added at a volume of 200µl monocyte conditioned medium, which was conditioned by 4 x 10^5 monocytes/ 200 µl DMEM, as per 2.3.3. Any cytokine present in that volume is bound by the immobilised antibody. The reagent addition was uninterrupted and complete within 10 minutes. The plate was covered with a plate sealer and incubated at room temperature for two hours. Contents were then decanted and washed by adding 400µl of wash buffer to each well, unbound material being removed by aspiration and washing. The process was repeated twice times for a total of three washes.

200µl of an enzyme-linked polyclonal antibody specific for TNFα (TNFα conjugate) was then added to the wells. The plate was covered with a new plate sealer and incubated at room temperature for 1 hour. Contents were again decanted and washed by adding 400µl of wash buffer to each well, unbound material being removed by aspiration and washing. The process was repeated twice times for a total of three washes. The amount of cytokine bound to the well was then detected by a reaction with the substrate which yields a colour product proportional to the amount of conjugate. This coloured product can then be quantified photometrically using a microplate autoreader (Bio-Tek Instruments), by plotting a curve of known cytokine concentration standards against known signals (optical densities), and from this determining the concentrations of unknowns. A 200µl substrate was added to each well, the plate was covered again with a new plate sealer and incubated at room temperature for 20 minutes. A 50 µl of stop solution was added to each well in the same order as the substrate. Optical density of each well was determined after 30 minutes of addition of stop solution using a microplate reader set to 450nm. Mean absorbency values for each set of diluted standards was calculated. A standard curve was constructed by plotting the mean absorbency for each standard on the y-axis against the concentration on the x-axis and then drawing a best fit curve through the points in the graph. Concentration of each unknown sample was determined by calculating the concentration of TNFα corresponding to the mean absorbency from the standard curve.

2.6.5 Human interleukin-1 alpha (IL1-α) assay

IL1-α was detected using an ELISA kit for cytokine detection (R&D Systems, UK). This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL1-α is coated onto the surface of the wells on the microtitre plate. 200µl of standards or samples were pipetted into the wells. Samples were added at a volume of 200µl monocyte conditioned medium, which was conditioned by 4 x 10^5 monocytes/ 200 µl DMEM, as per 2.3.3. Any cytokine present in that volume is bound by the immobilised antibody. The reagent addition was uninterrupted and complete within 10 minutes. The plate was covered with a plate sealer and incubated at room temperature for two hours. Contents were then decanted and washed by adding 400µl of wash buffer to each well, unbound material being removed by aspiration and washing. The process was repeated twice times for a total of three washes.

200µl of an enzyme-linked polyclonal antibody specific for IL1-α (IL1-α conjugate) was then added to the wells. The plate was covered with a new plate sealer and incubated at room temperature for 1 hour. Contents were again decanted and washed by adding 400µl of wash buffer to each well, unbound material being removed by aspiration and washing. The process was repeated twice times for a total of three washes. The amount of cytokine bound to the well was then detected by a reaction with the substrate which yields a colour product proportional to the amount of conjugate. This coloured product can then be quantified photometrically using a microplate autoreader (Bio-Tek Instruments), by plotting a curve of known cytokine concentration standards against known signals (optical densities), and from this

determining the concentrations of unknowns. A 200µl substrate was added to each well, the plate was covered again with a new plate sealer and incubated at room temperature for 20 minutes. A 50 µl of stop solution was added to each well in the same order as the substrate. Optical density of each well was determined after 30 minutes of addition of stop solution using a microplate reader set to 450nm. Mean absorbency values for each set of diluted standards was calculated. A standard curve was constructed by plotting the mean absorbency for each standard on the y-axis against the concentration on the x-axis and then drawing a best fit curve through the points in the graph. Concentration of each unknown sample was determined by calculating the concentration of IL1-α corresponding to the mean absorbency from the standard curve.

2.6.6 Human interleukin-1 beta (IL1-β) assay

IL1-β was detected using an ELISA kit for cytokine detection (R&D Systems, UK). This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL1-β is coated onto the surface of the wells on the microtitre plate. 200µl of standards or samples were pipetted into the wells. Samples were added at a volume of 200µl monocyte conditioned medium, which was conditioned by 4 x 10^5 monocytes/ 200 µl DMEM, as per 2.3.3. Any cytokine present in that volume is bound by the immobilised antibody. The reagent addition was uninterrupted and complete within 10 minutes. The plate was covered with a plate sealer and incubated at room temperature for two hours. Contents were then decanted and washed by adding 400µl of wash buffer to each well, unbound material being

removed by aspiration and washing. The process was repeated twice times for a total of three washes.

200µl of an enzyme-linked polyclonal antibody specific for IL1-β (IL1-β conjugate) was then added to the wells. The plate was covered with a new plate sealer and incubated at room temperature for 1 hour. Contents were again decanted and washed by adding 400µl of wash buffer to each well, unbound material being removed by aspiration and washing. The process was repeated twice times for a total of three washes. The amount of cytokine bound to the well was then detected by a reaction with the substrate which yields a colour product proportional to the amount of conjugate. This coloured product can then be quantified photometrically using a microplate autoreader (Bio-Tek Instruments), by plotting a curve of known cytokine concentration standards against known signals (optical densities), and from this determining the concentrations of unknowns. A 200µl substrate was added to each well, the plate was covered again with a new plate sealer and incubated at room temperature for 20 minutes. A 50 µl of stop solution was added to each well in the same order as the substrate. Optical density of each well was determined after 30 minutes of addition of stop solution using a microplate reader set to 450nm. Mean absorbency values for each set of diluted standards was calculated. A standard curve was constructed by plotting the mean absorbency for each standard on the y-axis against the concentration on the x-axis and then drawing a best fit curve through the points in the graph. Concentration of each unknown sample was determined by calculating the concentration of IL1-β corresponding to the mean absorbency from the standard curve.

2.6.7 Vascular endothelial growth factor (VEGF) assay

VEGF was detected using an ELISA kit for cytokine detection (R&D Systems, UK). This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF is coated onto the surface of the wells on the microtitre plate. After addition of 50µl of assay diluent to each well, 200µl of standards or samples were pipetted into the wells. Samples were added at a volume of 200µl monocyte conditioned medium, which was conditioned by 4 x 10^5 monocytes/ 200 µl DMEM, as per 2.3.3. Any cytokine present in that volume is bound by the immobilised antibody. The reagent addition was uninterrupted and complete within 10 minutes. The plate was covered with a plate sealer and incubated at room temperature for two hours. Contents were then decanted and washed by adding 400µl of wash buffer to each well, unbound material being removed by aspiration and washing. The process was repeated twice times for a total of three washes.

200µl of an enzyme-linked polyclonal antibody specific for VEGF (VEGF conjugate) was then added to the wells. The plate was covered with a new plate sealer and incubated at room temperature for 2 hours. Contents were again decanted and washed by adding 400µl of wash buffer to each well, unbound material being removed by aspiration and washing. The process was repeated twice times for a total of three washes. The amount of cytokine bound to the well was then detected by a reaction with the substrate which yields a colour product proportional to the amount of conjugate. This coloured product can then be quantified photometrically using a microplate autoreader (Bio-Tek Instruments), by plotting a curve of known cytokine concentration standards against known signals (optical densities), and from this determining the concentrations of unknowns. A 200µl substrate was added to each

well, the plate was covered again with a new plate sealer and incubated at room temperature for 20 minutes. A 50 µl of stop solution was added to each well in the same order as the substrate. Optical density of each well was determined after 30 minutes of addition of stop solution using a microplate reader set to 450nm. Mean absorbency values for each set of diluted standards was calculated. A standard curve was constructed by plotting the mean absorbency for each standard on the y-axis against the concentration on the x-axis and then drawing a best fit curve through the points in the graph. Concentration of each unknown sample was determined by calculating the concentration of VEGF corresponding to the mean absorbency from the standard curve.

2.7 Western Blot for constitutive NO synthase (c-NOS) expression

2.7.1 Extraction of human umbilical vein endothelial cell cytosolic proteins

HUVECs were cultured as per 2.2.4, trypsinised at the 2nd passage and counted. 15 x 10^6 cells were obtained, made up to 20 mls with DMEM and divided into 4 polypropylene tubes. 1.5 mls of monocyte conditioned medium (5 x 10^5 cells/ml) from each sample group were added to the HUVEC and made up to 10 mls with DMEM. Samples were incubated @37 ^{o}C, 4% CO_2 for 6 hours. They were then centrifuge @ 1200 rpm for 10 minutes. The pellet was then transferred to an eppindorf tube, 1 ml of PBS was added and samples were spun @ 1800 rpm for 5 minutes. The pellet was resupsended in 1 ml cold Buffer A and spun @ 1800 rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in 20µl cold Buffer A, to which was added 0.1% NP-40. Samples were put on ice for 10 minutes, vortexed gently and spun down @13,000 rpm for 10 minutes. The supernatant was collected and freeze dried with liquid nitrogen and stored at -80^{o}C.

2.7.2 Determination of protein concentrations

The determination of cytosolic protein concentration was carried out using a modification of the spectrophotometric method of Lowry (Lowry *et al.*, 1951), using bovine serum albumin (BSA) as the standard. A stock solution of 30µg/ml BSA in distilled water, pH 7.2, was used to make a standard curve. Standards were prepared in the range of 50-1000 pg/ml by diluting bovine serum albumin with distilled water and were added to a 96 well plate. A portion of unknown samples were diluted in distilled water and were also added to a 96 well plate in triplicate. 10 µl of each sample or standard was added in triplicate to a 96 well plate. The BCA protein was prepared

by adding 1 ml of Reagent B to 50 mls of Reagent A. 200µl of this mixture was added to each well and the plates were incubated @ 37°C for 40 minutes and then read on a microplatereader @ 570nm. The mean absorbency value for each value was plotted against the BSA concentration for that standard. Only standards with R2 values of more than 0.98 were used. Using the equation for the line, the protein concentrations of the samples were calculated from the mean absorbency value for each sample. If the sample absorbency was not on the straight part of the curve, the samples in question were either diluted further (if their absorbency values were too high) or diluted less (if their absorbency values were too low), and samples were run again using a new standard curve until all the diluted sample protein concentrations could be accurately calculated. The true protein concentration of each sample was determined by multiplying the concentration found by the dilution factor relevant for that particular sample. All samples for each set of experiments were adjusted to the same protein concentration by dilution in PBS and stored at -80°C.

2.7.3 Sodium Dodichol Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.7.3.1 Preparation of gels

Isolated proteins were separated by SDS-PAGE. A 12% separating gel was made up and allowed to set between two glass electrophoresis plates. A 5% stacking gel was made and was immediately poured on top of the separating gel. A plastic comb was carefully inserted to avoid any bubbles, and the gel was allowed to set. The comb was then slowly removed and the clips and plastic seal were removed from the glass plates. The plates were then placed in the electrophoresis box and secured. The box was filled above the gel line with electrode buffer, ensuring no large bubbles at the

bottom of the box. The lanes were straightened with a needle and vigorously flushed with buffer using a pipette to ensure no gel was sealing the lane.

2.7.3.2 Preparation of sample

To 20 μg of cytosolic protein extract, Llaemli buffer was added to a volume of 15μl. Samples were heated up to 95°C for 5 minutes and microcentrifuged for 10 seconds @300 rpm. They were then loaded onto minigels. A solution of protein markers was carefully pipetted into the first lane and 20μl of a blank solution containing PBS:lamelli buffer (1:1) was pipetted into the second lane. The gel was run @ 100mV and 30mA until samples were out of the wells, and then increased to 60mA . Running time was about 90 minutes.

2.7.4 Gel Transfer

The gel containing the proteins was transferred onto a nitrocellulose membrane for labelling. The gel was taken off the electrophoresis apparatus and carefully laid on a transfer filter block soaked in cold transfer buffer. A sheet of nitrocellulose the same size as the gel was placed on top of the gel and a similar sized piece of filter paper was placed over that. The nitrocellulose was marked to correspond to the bottom left lane corner of the gel. A second transfer filter was placed on top of this sandwich and the whole assembly was placed in the transfer apparatus filled with cold transfer buffer. The gel was transferred using a semi-dry blotter @ 0.8mA/cm^2 for 90 minutes.

2.7.5 Blocking

To block non-specific binding of the monoclonal antibodies, the nitrocellulose was incubated for 30 minutes on a plate shaker 30 mls of blocking solution. The membrane was then washed 3 times for 10 minutes each in 50ml TBST.

2.7.6 Protein Labelling

The primary antibody (mouse anti-human IgG1 monoclonal antibody specific for constitutive nitric oxide synthase, Transduction Laboratories, Kentucky, USA) was optimally diluted 1:500 with TBST. The nitrocellulose membrane was incubated overnight at 4°C with the monoclonal antibody. The gel was then again washed 3 times for 10 minutes each in TBST and incubated with the secondary antibody (1:1000 dilution) for 2 hours and washed again 3 times. The secondary antibody, (IgG1 sheep antimouse conjugated to alkaline phosphatase, Serotec, Oxford, UK) was optimally diluted in TBST. Again the membrane was washed as before and 10 ml of substrate buffer was added to the nitrocellulose membrane. The membrane was gently shaken for 5-8 minutes until the substrate slightly changed colour. The reaction was stopped by pouring off the substrate and adding distilled water over the membrane. The nitrocellulose membrane was allowed to dry and was then examined for bands corresponding to the molecular weight of the constitutive nitric oxide synthase protein being assessed (140 kDa), known molecular weight markers being present in lane 1.

2.8 Endothelial cell turnover studies

2.8.1 Endothelial cell proliferation assay

Cellular proliferation was measured by quantitative determination of 5-bromo-2'deoxy-uridine (BrdU) incorporation during DNA synthesis in replicating cells.

Using an ELISA, 5-BrdU labelling and detection kit 111, (Boehringer Mannheim, Sussex, England), the amount of BrdU incorporated by replicating/proliferating cells was determined by labelling cells with an anti-BrdU antibody.

10µl of BrdU was added to each 96 well containing previously prepared HUVEC. After 10 hours of incubation @ 37°C, the solution was aspirated, cells were washed twice with PBS and fixed with 70% ethanol (in 0.5M HCL for 30 minutes @ -20°C). After fixation, the cells remained attached to the wells during the whole procedure described below. After a further 3 washes with PBS, samples were incubated with nucleases @ 37°C for 30 minutes to partially digest DNA and allow for accessibility of antibody to BrdU. Monoclonal anti-BrdU, labelled with peroxidase (POD) (Boerhinger Mannheim, Germany), was added for a further 30 minutes @37 °C. Finally, the POD substrate was added. POD catalyses the cleavage of the substrate, producing a coloured reaction product. The absorbence in the samples was determined with the use of a standard microtitre plate (ELISA) reader at 405 nm. The data was expressed as percentage of the extinction of control cells, using the formula: % of control = (O.D. subject group/O.D. control sample) x 100%.

2.8.2 Endothelial cell apoptosis assay.

The effect of MCM on endothelial apoptosis was quantified by measuring cytoplasmic histone-associated DNA fragments (mono- and oligonucleosome) by a cell death detection ELISA^{plus} kit (Boehringer Mannheim, Germany). HUVECs were isolated as previously described, grown to confluency and plated in 2% gelatin-coated 96-well flat-bottomed plates at a concentration of 1.5 x 10^4 cells/well (100 µl of cellular suspension /well). This was followed by treatment with 100µl MCM (@ a concentration of 2 X 10^5 monocytes/ml of medium). Duplicates of each sample were

used, and duplicates of 100µl/well of culture medium without MCM were used as a negative control. After incubation @ 37 °C in humidified 5% CO_2 for 4 hours, the microtitre plate was centrifuged @ 200 x g for 20 minutes. The supernatant (200µl) was removed by fast inversion of the microtitre plate and the cell pellet was resuspended in 200µl of lysis buffer for 30 minutes @ room temperature. The lysate was centrifuged @ 200 x g for 10 minutes, and 20µl of the supernatant (= cytoplasmic fraction) was transferred onto the streptavidin coated microtitre plate and incubated with a 80µl mixture of anti-histone-biotin and anti-DNA-peroxidase. These compounds were covered with foil, placed on a microtitre plate shaker and incubated @ room temperature for 2 hours. During this incubation, nucleosomes were captured via their histone component by anti-histone-biotin antibody, while binding to the streptavidin coated microtitre plate. Simultaneously, anti-DNA-peroxidase binds to the DNA part of the nucleosomes. After removal of the unbound antibodies, the amount of peroxidase retained in the immunocomplex was determined photometrically using a plate reader @ 405 nm. Using previously described methodology (Leist *et al.*, 1994; Leist *et al.*, 1995), control medium was taken as 100%, and % of control was obtained using the formula:

% of control = (100%) x (O.D. of MCM samples for each group-O.D. of blank)/(O.D. of control samples-O.D. of blank).

2.9 Ex-*vivo* endothelial cell studies

2.9.1 Isolation of circulating endothelial cells

CECs were isolated by the technique described by Hladovec and Rossman (Hladovec *et al.*, 1973). 9 mls of venous blood were drawn into a siliconised tube containing 1 ml 3.8% trisodium citrate (Sarstedt, Germany) and erythrocytes were removed by

centrifugation at 395 x g, 4°C for 20 minutes. 1 ml of this supernatant was aspirated and mixed with 0.2 mls of adenosine-5-diphosphate (1 mg/ml) (Boehringer Mannheim, Mannheim, Germany) and was mechanically shaken for 10 minutes prior to centrifugation at 395 x g for 20 minutes to remove platelet aggregates. The supernatant was then centrifuged at 2100 x g for 20 minutes to obtain a cell pellet. After suspension of the sediment in 0.1 ml normal saline, improved Neubauer chambers were filled with the suspension. The number of endothelial cells in the 9 large squares were counted four times and averaged. Results were expressed as the mean number of cells in the four 0.9 μl chambers. The only material present except endothelial cells were some small platelet aggregates and cellular debris. All this material was easily differentiated from endothelial cells which were as a rule 30-50 μm in diameter, being one quarter of a small square diameter (0.2 mm) in an improved Nuebauer chamber. Results were then expressed as the arithmetic mean number of cells in each 0.9 μl chamber.

2.9.2 Identification of endothelial cells

Cells were stained with fluorescene-labelled anti-human factor VIII and fluorescene-labelled low-density lipoprotein (LDL). Suspended cells were incubated for 45 minutes with a 1:5 dilution of the fluorescent antibody, and with the use of a cytospin (Shandon, UK) they were transferred to glass slides. Specific yellow-green immunofluoresence of the endothelial cells was apparent when looked at using an inverted fluorescene microscope (Nikon, Japan)

2.9.3 Phenotypic studies of endothelial cells

To detect antigens that endothelial cells express only or to a markedly greater extent on activation (ICAM-1, VCAM-1, P-selectin, E-selectin and CD 34), CECs were isolated

and made up to a 500 µl volume with PBS. ICAM-1 (Immunotech, Marseille, France) and E-Selectin (Serotec, UK) were FITC labelled. P-selectin (Becton-Dickinson, Lincoln Park, N.J.) was phycoerethrin labelled. VCAM-1 (CD 106) (Immunotech) was an unlabelled monoclonal antibody. We analysed the phenotype of these cells using direct or indirect immunofluorescent staining. To 100 µl sample, 15 µl of each antibody was added and the samples were incubated for one hour at room temperature. After 30 minutes, a secondary monoclonal antibody (IgG1(mouse anti-human) (Immunotech)) was added to VCAM-1 sample and incubated for a further 30 minutes at room temperature. Expression by CECs of FITC-labelled CD 36 (Immunotech) was also assessed. With the use of a cytospin these samples were transferred to a glass slide. After the staining procedures were completed, preparations of CECs were examined under an inverted fluorescene microscope. Positively staining cells were expressed as a percentage of total number of cells counted per field of vision on 5 different areas of the slide, and expressed as the arithmetic mean percentage of positively staining cells. Number of observations (n) refers to number of samples taken.

2.9.4 Immunohistochemical detection of circulating endothelial cell apoptosis

Apoptosis of endothelial cells was assessed with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) technique, using an In Situ Cell Death Detection kit (Boehringer Mannheim). This method is based upon enzymatic in situ labelling of apoptosis-induced DNA strand breaks. Cleavage of genomic DNA during apoptosis may yield double-stranded DNA fragments. These strand breaks can be identified by labelling the free 3-OH termini with modified nucleotides in an enzymatic reaction. Terminal deoxynucleotidyl transferase (TdT) catalyses the polymerisation of the nucleotides and is used to label the DNA strand breaks.

14 mls of venous blood were drawn into a siliconised tube containing 1 ml 3.8% trisodium citrate. The endothelial cells were isolated as previously described and were placed on a slide using a cytospin. They were then fixed with paraformaldehyde (4% in PBS , pH 7.4) for 30 minutes at room temperature and the slide was rinsed with PBS twice. Endogenous peroxidase was blocked with H_2O_2 (0.3% in methanol) for 30 minutes at room temperature. The slides were rinsed with PBS twice and 0.1% Triton X-100 (in 0.1% sodium citrate) was used to permealise the cells for 3 minutes at room temperature. The slides were again rinsed with PBS twice and 50 µl of TUNEL reaction was added on to sample. The slides were then incubated in a humidified chamber for 60 minutes @ 37°C. Slides were rinsed three times with PBS and 50 µl of converter peroxidase (POD) was added on to the sample. Slides were again incubated in a humidified chamber for 30 minutes @ 37°C. They were again rinsed three times with PBS. 100 µl of DAB-substrate solution was added on to the samples and they were incubated for 10 minutes @ room temperature. They were rinsed three times with PBS and mounted with a coverslip. Stained cells were analysed under a light microscope.

2.9.5 Immunohistochemical detection of Akt expression

14 mls of venous blood were drawn into a siliconised tube containing 1 ml 3.8% trisodium citrate. The endothelial cells were isolated as previously described and were placed on a slide using a cytospin. They were then fixed with paraformaldehyde (4% in PBS , pH 7.4) for 30 minutes at room temperature and the slide was rinsed with PBS twice. Endogenous peroxidase was blocked with H_2O_2 (0.3% in methanol) for 30 minutes at room temperature. The slides were rinsed with PBS twice and 0.1% Triton X-100 (in 0.1% sodium citrate) was used to permealise the cells for 3 minutes

at room temperature. The slides were again rinsed with PBS twice and incubated in rabbit serum for 1 hour to inhibit non-specific antibody binding. To 100µl of sample 10µl of primary antibody (goat anti-human polyclonal antibody, Santa Cruz Biotechnology) at a dilution of 1:400, giving a final concentration of 0.5µg of antibody, was added and incubated for 30 minutes at room temperature. Separate slides were prepared without the addition of the primary antibody, as a negative control. Slides were then washed 3 times with PBS and the secondary antibody (Peroxidase-conjugated rabbit anti-goat IgG; Dako, Denmark), at a dilution of 1:200 giving a final concentration of 0.32µg antibody, was added for 45 minutes. The slides were again washed 3 times with PBS. DAB-substrate solution was then added on to the samples (2 mls of reconstituted DAB substrate diluted in 15 mls H_2O_2) and they were analysed under a light microscope until a colour change was seen in some cells and H_20 was added to stop then stop the reaction. Positive staining cells, with a brown colour, were then counted.

Chapter 3

"Establishing the effects of cigarette smoking on endothelial function *in vivo*, and investigating the effects of vitamin C and taurine supplementation"

3.1 Introduction

Endothelial cells form a monolayer of metabolically active cells which play a key role in maintenance of vascular integrity. Alterations in the functional role of the endothelium is now thought to play an important pathophysiological role in atherogenesis, abnormalities in function serving as the initial pathophysiological event, and antedating plaque formation. The functional implications of endothelial dysfunction in cardiovascular disease are becoming more evident, with such dysfunction affecting vascular tone and organ perfusion, especially during stress situations where an increase in blood flow is required (Katz *et al.*, 1992; Stamler *et al.*, 1994; Wennmalm *et al.*, 1993).

A major physiological role of the endothelium is control of vasomotor tone. The serendipitous observation by Furchgott and Zawadzki in 1980 served as the principal insight into the importance of the endothelium as the mediator of regulation of basal systemic and regional tone (Furchgott & Zawadski, 1980). The endothelium possesses the ability to modulate tone by the release of vasodilator and vasoconstrictor substances, among them Endothelium Derived Relaxation Factor (EDRF) and Endothelin-1. Assessment of endothelium-dependent relaxation remains the most common method for assessing endothelial function. EDRF, now known to be NO (Palmer *et al.*, 1987; Ignarro *et al.*, 1987), is released from endothelial cells in response to an increase in blood flow. NO diffuses into the underlying smooth muscle cells, guanylate cyclase is activated and cGMP is subsequently elevated. This increased level of cGMP produces a fall in intracellular Ca^{2+} and the vessel relaxes. Inadequate production of EDRF is therefore likely to predispose to disease by facilitating vascular wall-platelet and vascular wall-monocyte interactions, and adhesion of circulating monocytes to the endothelium.

Cigarette smoking remains the most modifiable risk factor for atherogenesis. It has been previously demonstrated that smoking is associated with a dose-related impairment of endothelial-dependent dilatation in young healthy adults (Celermajer *et al.* 1993; McVeigh *et al.* 1996). The mechanisms by which smoking induces endothelial functional abnormalities are not established, but a number of possibilities have been proposed. *In vitro* studies have demonstrated that cigarette smoke extract contracts isolated porcine coronary arteries by superoxide-mediated degradation of EDRF (Murohara *et al.*, 1994). Increased production of superoxide radicals, which rapidly inactivate EDRF, is a feature characteristic of experimental models of atherosclerosis (Minor *et al.*, 1990; Ohara *et al.*, 1993). In addition, smoking is associated with a direct toxic effect on endothelial cells (Davis *et al.*, 1985; Asmussen *et al.*, 1975). Diminished NO formation may therefore be secondary to a direct downregulatory effect of smoking, as occurs with prostacyclin production (Reinders *et al.*, 1986; Nadler *et al.*, 1983). A significant inverse correlation has been shown to exist between pack years smoked and flow-dependent dilatation of the brachial artery (Celermajer *et al.*, 1993). An impaired response to acetylcholine (Heitzer *et al.*, 1996), and an impaired vasoconstrictor response to inhibition of NO (McVeigh *et al.*, 1996) by the NO inhibitor L-NMMA in long-term smokers, supports the concept that smoking is associated with impaired availability of NO.

Many studies have investigated the effects of the powerful antioxidant vitamin C on endothelial function in groups with known abnormalities of function, such as diabetics (Ting *et al.*, 1996) and those with chronic heart failure (Hornig *et al.*, 1998). When vitamin C was administered intra-arterially to smokers, to create a local forearm concentration known to inhibit superoxide-mediated lipid peroxidation (Frei *et al.*, 1989), forearm blood flow responses were markedly improved in response to acetylcholine (Heitzer *et al.*, 1996). It was therefore concluded that endothelial dysfunction in chronic smokers was mediated, at least in part, by enhanced formation of oxygen-derived free radicals. While these effects prove important to the

understanding of possible mediators of dysfunction in smokers, intra-arterial administration would not be feasible as part of regular maintenance or preventative strategies against atherogenesis. As part of further investigation into the pathogenesis of smoking and oxidative damage, Reilly *et al.* demonstrated 8-*epi*-prostaglandin (PG $F_{2\alpha}$), a stable product of lipid peroxidation *in vivo*, to be significantly decreased by oral supplementation of vitamin C of 2 gms/day for 5 days (Reilly *et al.*, 1996). As the prior intra-arterial vitamin C study (Heitzer et al, 1996) chose their dose according to intra-arterial vitamin C levels shown to inhibit lipid peroxidation, the effects of a more easily administered oral dose of vitamin C on endothelial function would be an important consideration.

As discussed in chapter 1, taurine is a semi-essential β-amino acid which is the most abundant intracellular free amino acid present in mammalian cells. The effect of taurine supplementation on endothelial dysfunction in smokers has not yet been investigated. Taurine has been proposed to act as both a direct and an indirect antioxidant; it reacts with and removes the powerful oxidising agent hypochlorous acid (HOCL) directly (McLoughlin *et al.*, 1991; Stapelton & Bloomfield, 1993), and indirectly functions as a membrane stabiliser (Tower, 1968; Read & Welty, 1963). Its protective activity, when used as a prophylactant against reperfusion injury during myocardial revascularisation (Milei *et al.*, 1992), was attributed to free radical scavenging. In animal studies, taurine has been shown to be protective against lipid-peroxidation-induced bronchiolar tissue damage (Gordon *et al.*, 1986). It is also protective against neutrophil-mediated pulmonary microvasculature injury in an animal ischaemia-reperfusion model (Barry *et al.*, 1997), also thought to be mediated through the ability of taurine to function as an antioxidant. Specifically related to cardiovascular disease, taurine has been shown to be inversely related to cardiovascular disease mortality in a world-wide epidemiology study (WHO and WHO collaborating Centres, 1996). It is of importance therefore that oral

supplementation of taurine should be investigated and compared to vitamin C supplementation. As taurine has not previously been administered to smokers, a dose required by subjects with insulin-dependent diabetes mellitus to restore plasma taurine levels to normal, 1.5 gms/day, was used (Franconi *et al.*, 1995). However, in contrast to the 90 days supplementation used in this diabetic study, a 5 day supplementation period was used, as this study chose to look at and compare the effects of short term supplementation of antioxidants on endothelial function.

The aim of this chapter was therefore to confirm the abnormal effects of cigarette smoking on endothelial function in young, healthy adults, and to investigate and compare the effects of oral supplementation of vitamin C and taurine on endothelial function, as assessed by flow-mediated dilatation.

3.2 Methodology

3.2.1 Subjects

15 healthy smokers and 15 control non-smoking subjects were studied. These subjects were recruited from among hospital staff, friends and family. All were normotensive, non-diabetic and were without a history of hyperlipidaemia or a family history of premature vascular disease. They were clinically well and not taking any cardiovascular medications. Smokers were defined as those who smoked at least 20 cigarettes per day for at least 2 years (2 pack years), and had at least one cigarette in the preceding 12 hours. Control subjects were lifelong non-smokers. Smokers were supplemented with 2 gms vitamin C/day (Roche Pharmaceuticals) and 1.5 gms taurine/day (Twinlab, Ronkonkoma, N.Y.) for 5 days. A 2 week wash out period was used between supplementation of smokers with vitamin C and taurine.

3.2.2 Study protocol

The methodology described here has been used elsewhere (Celermajer *et al.*, 1992) and is described in detail in section 2.3.1. All studies were performed in a temperature-controlled room (20°C) with the subject in the resting, supine state. Briefly, the subjects right arm was comfortably immobilised in the extended position to allow constant access to the brachial artery for imaging. Brachial artery diameter and flow velocity were measured from B-mode ultrasound images using a 7.0-MHz linear array transducer ultrasound system and a standard Acuson 128XP/10 (Acuson, Mountain View, California). The artery was scanned in longitudinal section at a location in the antecubital fossa where the clearest ultrasound image was found. Surrounding soft tissue images on the images obtained were used as landmarks for follow-up scans.

A baseline pre-treatment, post-no treatment, post-vitamin C and post-taurine scan were all carried out in an identical manner. The subject lay at rest for 10 minutes prior to a resting baseline scan. Increased flow was then induced by inflation of a tourniquet placed around the forearm to a pressure of 240 mmHg for 4.5 minutes followed by release.

3.2.3. Data Analysis

All scans were recorded on super VHS videotape for off-line analysis. Methodology used is described in detail in section 2.3.2. Briefly, vessel diameter was measured at a fixed distance from an anatomical marker (such as a fascial plane of a vein in cross section) using ultrasonic callipers. Measurements were taken from the anterior to the posterior "m" line (Figure 3.1) at peak systole, incident with the peak systolic waveform on the flow velocity curve (Figure 3.2). Reactive hyperaemia measurements were taken 45-60 seconds after cuff deflation. Results obtained were expressed as diameter change in mm's.

Descriptive statistics were expressed as mean ± SD. The control and the smoking group were compared using the two-sample *t* test. The effects of vitamin C and taurine supplementation on arterial dilatation in smokers were then compared using the paired *t* test. Statistical significance was inferred at $p \leq 0.05$.

3.2.4. Plasma taurine levels

14 mls of blood were obtained from each patient prior to and post supplementation of taurine, as described in section 2.6.3. Samples were then stored @ -20°C until plasma taurine was measured using high performance liquid chromatography (HPLC) by the Department of Biochemistry, Amino Acid Laboratory at The Children's Hospital , Temple St.

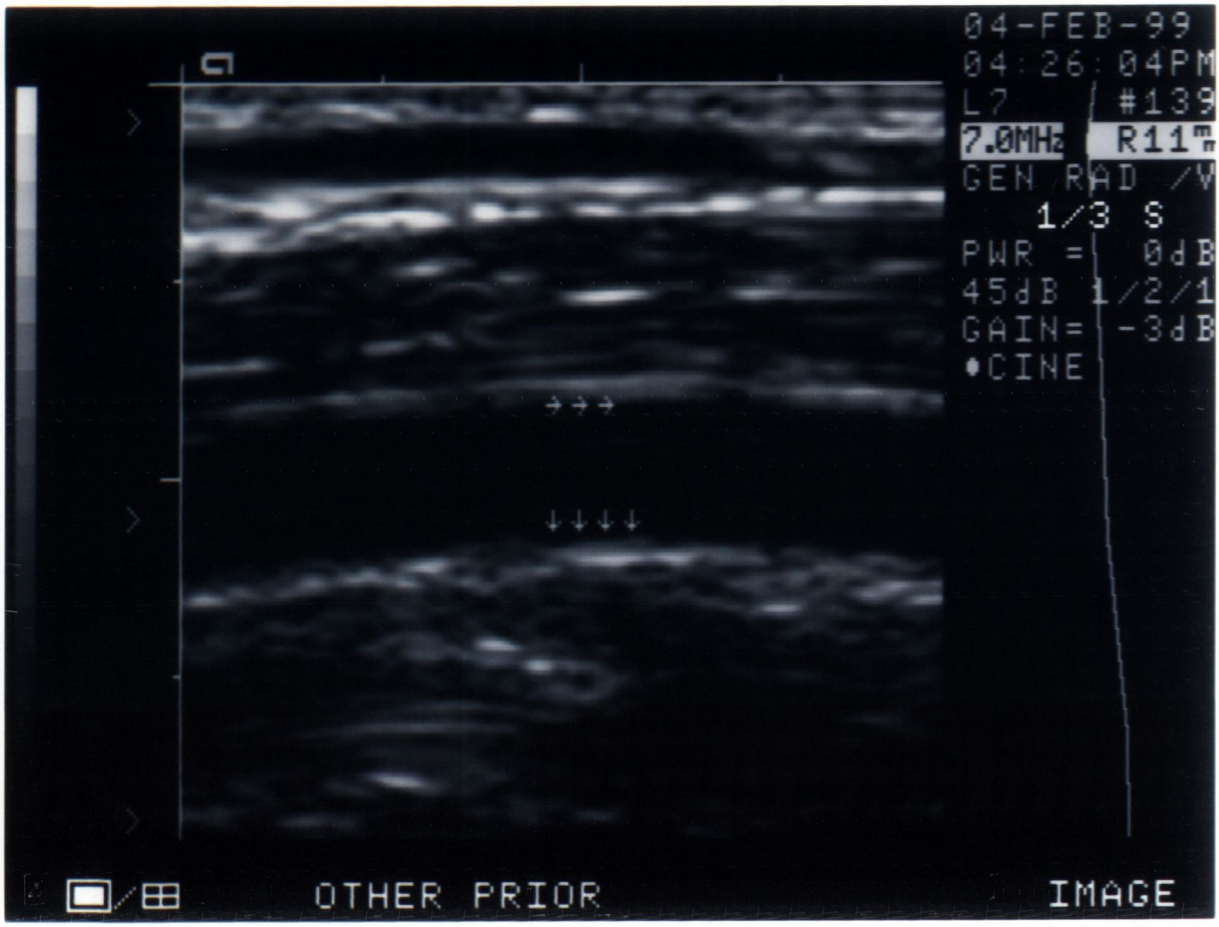


Figure 3.1

Typical B mode ultrasound image obtained from the right brachial artery of an adult subject using a resolution box function. Arterial diameter was measured from the posterior (vertical arrows) to the anterior (horizontal arrows) "m line" (interface between media and adventitia), at a fixed distance from an anatomical marker such as a vessel or a fascial plane, pre and post inflation of a blood pressure cuff.

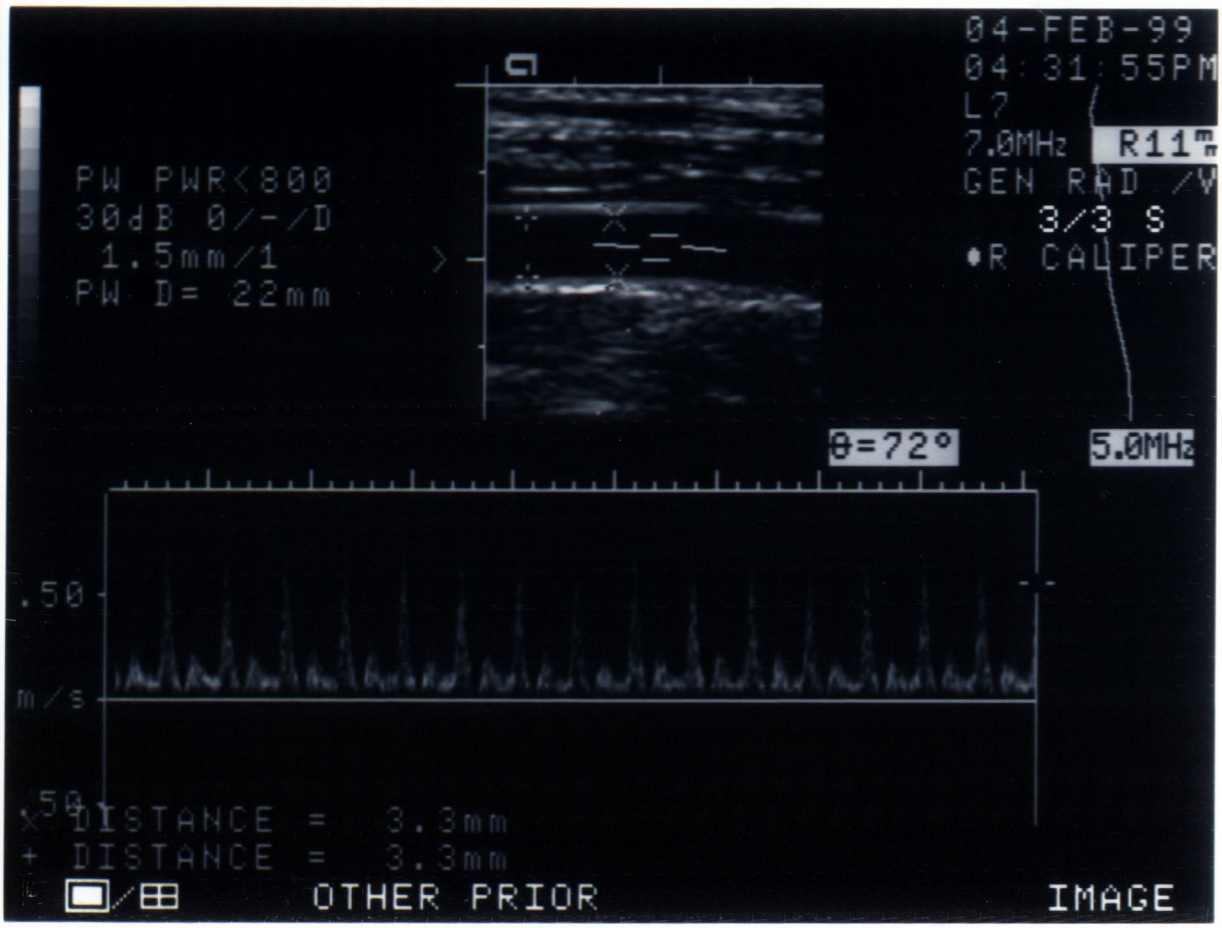


Figure 3.2

Typical B mode ultrasound image obtained from the right brachial artery of an adult subject with associated pulse wave velocity curve. Arterial diameter was measured from the posterior to the anterior "m line" at peak systole, coincidentally with the pulse wave velocity peak. At each image for analysis 2 measurements were obtained using ultrasonic callipers. An average of 4 images at the time points desired (pre- or 46-60 seconds post- cuff deflation) were obtained.

3.3 Results

The raw data for chapter 3 is presented in Appendix 11.

3.3.1 The effect of reactive hyperaemia on brachial artery diameter in smokers and non smokers.

The effect of reactive hyperaemia on brachial artery diameter in smokers (n=15) and non-smokers (n=15) was assessed using duplex ultrasonography. Measurements were obtained in mm, as the difference in arterial diameter pre-inflation and post-deflation of a blood pressure cuff to 240mmHg. Flow-mediated dilatation was observed in control subjects but this parameter was significantly impaired in smokers. Actual arterial diameter measurements in non-smokers pre- and post-stimulus were 3.39(±0.43)mm pre- and 3.7(±0.38)mm post- reactive hyperaemia, (*p=0.0007; Wilcoxon Signed Rank; Figure 3.3(a)) Those for smokers were 3.33(±0.49)mm pre- and 3.36(±0.50)mm post- reactive hyperaemia, (p=ns; Wilcoxon Signed Rank; Figure 3.3(a))

3.3.2 The effect of vitamin C supplementation in smokers on the response to reactive hyperaemia.

The effect of vitamin C supplementation in smokers (n=15) on the response to reactive hyperaemia was also assessed using duplex ultrasonography. The study was carried out pre and post supplementation with 2 gms/vitamin C/day for 5 days. It was found that oral supplementation with vitamin C had a significant effect on flow-dependent dilatation response, when compared to the pre-dilatation diameter. Actual arterial diameter measurements in smokers pre- and post- vitamin C supplementation

were 3.3(±0.49)mm pre- and 3.45(±0.48)mm post- supplementation, (*p=0.0005; Wilcoxon Signed Rank: Figure 3.3(a)).

However, the flow dependent dilatation response was not restored to that of normal controls; baseline pre-flow stimulus values between control non-smokers and post-vitamin C smokers being non-significant (p=0.53, Mann-Whitney U test), and post increased blood flow diameters being significantly different between the two groups ($p \leq 0.05$, Mann-Whitney U test: Figure 3.3(b)).

3.3.3 The effect of taurine supplementation in smokers on the response to reactive hyperaemia.

The effect of taurine supplementation in smokers (n=15) on the response to reactive hyperaemia was also investigated. The study was carried out pre and post supplementation with 1.5 gms/taurine/day for 5 days. It was found that oral supplementation with taurine significantly improved flow-mediated arterial dilatation in smokers, when compared to diameter changes obtained pre-supplementation. Actual arterial diameter measurements in smokers pre- and post- taurine supplementation were 3.33(±0.49)mm pre- and 3.7(±0.47)mm post supplementation, (*p=0.0005; Wilcoxon Signed Rank, Figure 3.3(a)).

The flow dependent dilatation response was restored to that of normal controls; baseline pre-flow stimulus values between control non-smokers and post-taurine smokers being non-significant (p=0.80, Mann-Whitney U test), and post flow stimulus diameters remaining non-significantly different, indicative of similar magnitude of responses (p=0.82, Mann-Whitney U test: Figure 3.3(b)).

3.3.4 Plasma taurine levels in non-smokers and smokers pre and post taurine supplementation were measured using HPLC at an outside institution. Plasma taurine levels were found to be significantly higher in smokers (n=22) in comparison to non-smokers (n=11): 102(±39) μmol/L vs. 51.5(±21) μmol/L: (*p≤0.0001; Students 2-sample t-test: Figure 3.4). When smokers were supplemented with taurine (n=9), there was no significant effect on plasma taurine levels; 104(±46) μmol/L pre-supplementation vs. 115(±45) μmol/L post-supplementation: (ns; Students paired t-test: Figure 3.4).

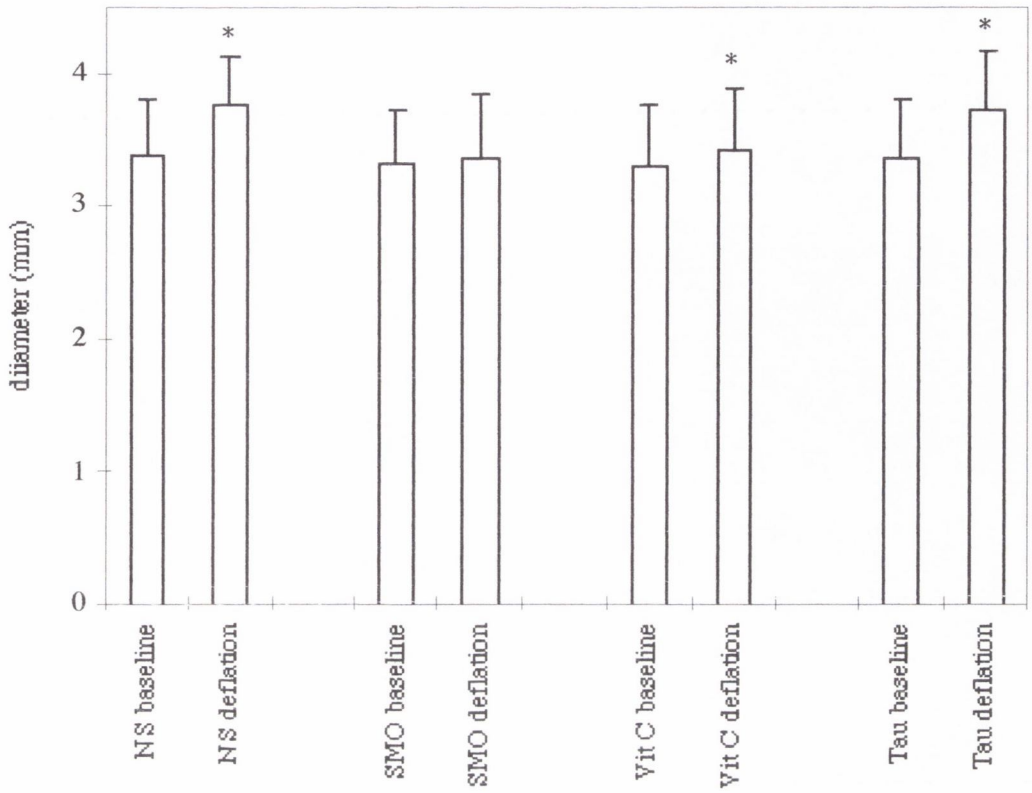


Figure 3.3 (a) The effect of an increase in arterial blood flow on endothelial-dependent dilatation: (non-smokers, n= 15; smokers, n=15; smokers-vitamin C, n=15; smokers-taurine, n=15). NS = non-smokers, SMO = smokers, deflation = diameter increase post cuff deflation. *p< 0.05 vs. corresponding baseline pre-stimulus diameters, Wilcoxon Signed Rank test.

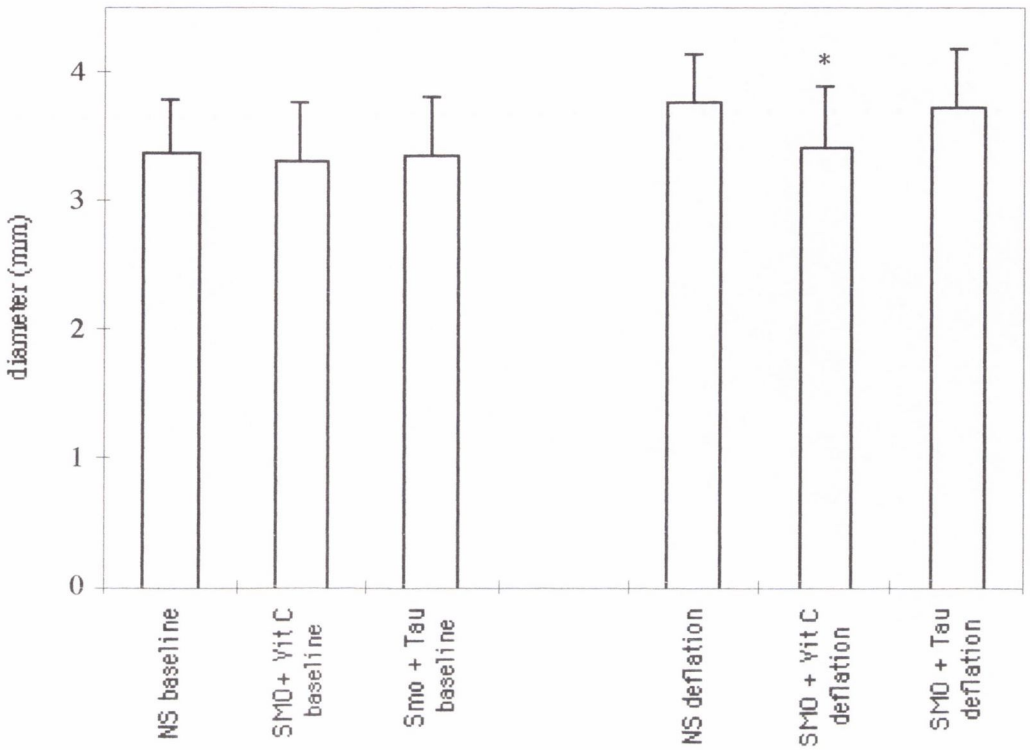


Figure 3.3 (b) The effect of an increase in arterial blood flow on endothelial-dependent dilatation in non-smoking controls compared to smokers treated with vitamin C and taurine: (non-smokers, n= 15; smokers + vitamin C, n=15; smokers +taurine, n=15). NS = non-smokers, SMO = smokers, deflation = diameter post cuff deflation. Post supplementation with vitamin C, flow induced diameter changes remain significantly different from flow induced diameter changes in non-smokers. *p≤0.05 vs. non-smokers post cuff deflation, Mann-Whitney U test.

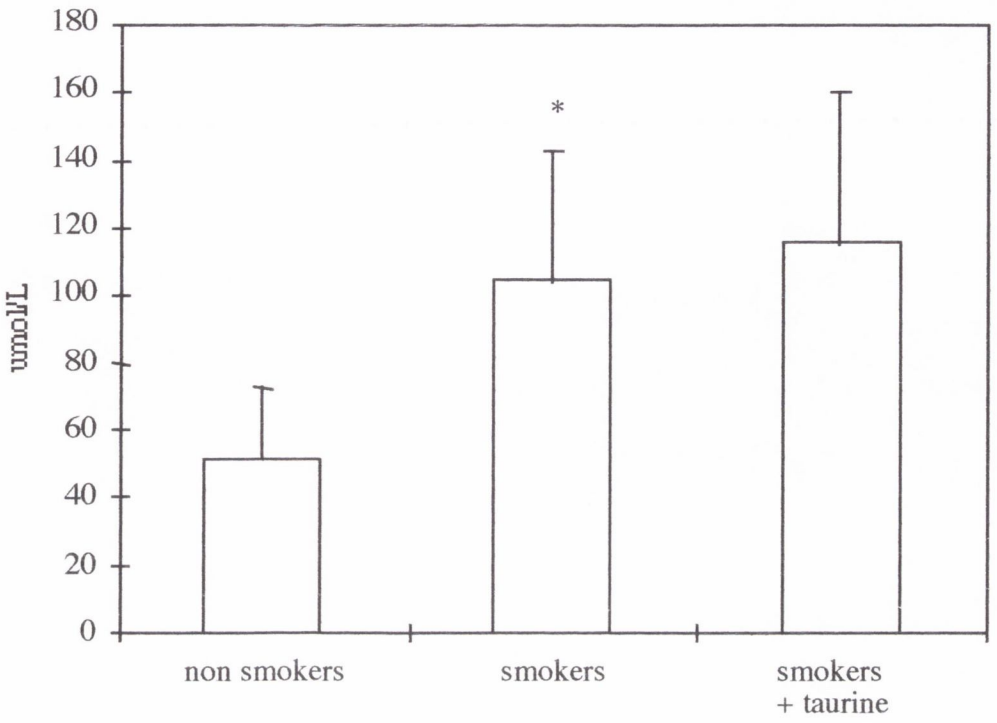


Figure 3.4 Plasma taurine levels, measured in μM/L: (non-smokers, n= 11; smokers, n=22; smokers-taurine, n=9). *$p<0.001$ vs. non-smokers, Students 2-sample t-test.

3.4 Discussion

Furchgott and Zawadzki first demonstrated that an intact endothelium is necessary for acetylcholine to induce vasodilatation, damage to or loss of the endothelium resulting in loss of relaxation (Furchgottt & Zawadzki, 1980). This observation has now extended to clinical studies assessing endothelial health. In arteries lined with healthy endothelium, an increase in blood flow causes dilatation of the vessel, a mechanism which fails with dysfunctional endothelium. This chapter demonstrates that flow-mediated endothelium dependent dilatation is impaired in healthy young smokers, results which are consistent with an association between smoking and endothelial dysfunction. These results are supported by similar observations, using the same methodology, by Celermajer *et al.*, where an average of 10% dilatation was found in control non-smokers (Celermajer *et al.*, 1993; Celermajer *et al.*, 1994; Celermajer *et al.*, 1996; Raitakari *et al.*, 1999). The reproducibility of this technique has been previously established (Sorensen et al, 1995; Corretti *et al.*, 1995). The accuracy of a 7.0 MHz linear array transducer is sufficient to allow detection of diameter changes as small as 0.1 mm. Using this same transducer, a low coefficient of variation for measurements of arterial diameter (1-3% variation between observers) and a high correlation between consecutive control measurements within a study has been found (Sorensen et al, 1995).

This chapter demonstrates that supplementation of smokers with vitamin C for 5 days, a dose known to modify levels of oxidant stress *in vivo* in smokers, does not significantly restore endothelial-dependent dilatation. In contrast, supplementation of this same group with the amino acid taurine restores endothelial function. The finding of a discrepancy between the effects of these two antioxidants may reflect the fact that a single mechanism is not solely responsible for smoking-induced endothelial damage, and may lead to further insight into the exact mediators of dysfunction.

The powerful antioxidant vitamin C has been previously shown to significantly improve endothelial function in smokers during simultaneous infusion with acetylcholine (Heitzer *et al.*, 1996). Simultaneous administration of intra-arterial vitamin C has also been demonstrated to restore about 60% of the attenuated endothelial-dependent dilatation in another cardiovascular disease risk factor group, diabetic patients (Ting *et al.*, 1996) and also in hypercholesterolaemic patients (Ting *et al.*, 1997). There are a number of possibilities as to why complete restoration is not possible with oral vitamin C supplementation. Firstly, the improvement with vitamin C after acute intra-arterial administration is most likely mediated by the antioxidant ability of this compound to scavenge excess superoxide anion in a local area, and thereby decrease NO inactivation. It is therefore possible that a higher dose of oral vitamin C may have a greater whole-body scavenging capacity, but not to the magnitude achievable with an intra-arterial dose. Indeed, the recent reports of pro-oxidant and mutagenic effects of vitamin C at lower daily doses than used in this study would suggest caution with high dose vitamin C administration (Podmore *et al.*, 1998). Secondly, if as suggested by Reilly *et al.* (Reilly *et al.*, 1996), that the dose administered is appropriate, the optimal therapeutic window for assessment may have been missed. However, these results are not likely to be explained by a short half-life (t1/2) of vitamin C as this study was undertaken 12 hours after completion of supplementation, plasma depletion of vitamin C being a first order process and t1/2 being thought to average 14.2 days (Blanchard, 1991). Thirdly, the possibility that vitamin C binds and stabilises EDRF, increasing the availability of NO by a mechanism independent of free-radical scavenging, as suggested by Ting *et al.* (Ting *et al.*, 1996), cannot be ruled out. Alternatively, it is possible that a combination of factors may contribute to endothelial dysfunction in smokers, enhanced superoxide-mediated EDRF degradation, and its theoretically possible prevention by vitamin C, may be just one of a combination of factors. Smoking-induced impaired basal release

of NO, as opposed to increased NO degradation, may represent the key mediator of dysfunction, a factor not necessarily ameliorated by vitamin C supplementation.

In contrast to vitamin C, taurine administration to young smokers restores flow-mediated endothelial-dependent dilatation to levels of control non-smokers. It is unlikely that this effect is mediated through taurine acting as a greater direct antioxidant than vitamin C, through scavenging of HOCL. It is possible, however, the indirect function of taurine as a membrane stabiliser and maintainer of homeostasis may help combat the excess oxidant load. Plasma taurine has been found to be decreased in diabetic subjects (Franconi *et al.*, 1995). However, a similar picture does not prevail in smokers. Plasma taurine levels are suprisingly high in smokers. This may be a reflection of cellular damage as increased plasma taurine levels have been reported in acute myocardial infarction (Bhatnager *et al.*, 1990). The alternative explanation of decreased uptake by stressed cells is supported by in-vitro observations with stressed enterocytes (O'Flaherty *et al.*, 1997). Taurine supplementation of smokers had no significant effect on plasma taurine concentrations, but was associated with normalisation of the flow-mediated response, which may be a reflection of increased cellular uptake. However, absence of, or an decrease in flow-mediated dilatation, could also be secondary to impaired basal release of NO. Taurine has been demonstrated to restore endothelial-dependent dilatation in hypercholesterolaemic and diabetic animal studies (Kamta *et al.*, 1996). While enhancement of LDL receptor binding may be responsible for the effects seen in the hypercholesterolaemic study, it is also possible that taurine may have a direct upregulatory effect on NO production, as a decrease in NO release is a common factor in diabetics, hypercholesterolaemics and smokers..

This chapter therefore supports previous observations that smoking is an important causative agent for vascular disease, the earliest functional changes of which can be demonstrated in young, otherwise healthy smokers. While oral supplementation of vitamin C does not have a significant positive effect on endothelial

function, short term administration of the micro-nutrient taurine restores function. The exact mechanism as to how taurine restores function is as yet unknown. This experiment, however, offers the possibility of arterial disease prophylaxis with micro-nutrient supplementation. In a world-wide epidemiological study on dietary prevention and cardiovascular disease, the so-called CARDIAC study (WHO and WHO Collaborating Centres, 1986), it was found that an average of 100 gms of fish per day was sufficient to keep coronary artery disease mortality as low as that in the Japanese race. Considering that 100 gms fish contains 300-800 mg taurine (Zhao *et al.*, 1998), dietary modification would allow for sufficient taurine intake to obtain the therapeutic levels obtained in this study. In the light of a recent epidemiological study where it was found that, despite social prohibitions, smoking trends are again on the increase in the young (Stapleton, 1998), this study offers the possibility of cardiovascular disease prevention or modification in smokers by dietary modification.

Chapter 4

"Establishing the effects of cigarette smoking on vasomotor responses to a cold pressor stimulus *in vivo*, and investigating the effects of taurine supplementation"

4.1 Introduction

The previous chapter has demonstrated abnormal responses to an increase in blood flow to be present in the arteries of young smokers. This abnormality of vasoactivity is an endothelial-dependent function. In the presence of intact endothelium, an increase in blood flow or a muscarinic agonist (e.g. acetlycholine) dilates arteries through the release of EDRF. Another such marker of vasoactivity is the response to the cold pressor test (CPT). Abnormalities of the CPT have been previously demonstrated in the brachial artery of patients with coronary artery disease (Corretti *et al.*, 1995). Multiple stepwise regression analysis in this group revealed a positive correlation between smoking status and an abnormal response.

The CPT, whereupon the subject's hand is immersed in iced-water, is a well standardised physiological stressor (Saab *et al.*, 1993; Sherwood & Turner 1992),. The physiological response to a cold stimulus is the release of noradrenaline from adrenergic nerve terminals, with subsequent contraction of smooth muscle cells (SMCs). However, Martin *et al.* have demonstrated that in the face of an intact, functioning, healthy endothelium, the smooth muscle response to a vasoconstrictor stimulus may be attenuated (Martin *et al.*, 1986). The possible mechanisms through which this occurs are many (Figure 4.1). A sympathetic response may result in an increase in blood flow, which promotes an accompanying release of EDRF from a healthy endothelium. Humoral NA can also directly stimulate α_2 receptors on endothelial cells, which are of critical importance in mediating this response and result in release of NO (Carrier & White, 1984; Cocks & Angus, 1983). Intact endothelium is also known to increase the metabolism of noradrenaline. These factors together are thought to initially overcome the adrenergic-induced vasoconstrictor effect on SMCs (Figure 4.1).

It is however possible that a similar pathophysiological process which leads to endothelial dysfunction in smokers may also affect endothelial-independent functioning, such as SMC contractility. While it is possible that restoring endothelial function in smokers, as has been demonstrated to occur with taurine supplementation, could normalise an abnormal response to the CPT in individuals with dysfunctional endothelium, this would not occur if cigarette smoking also induced abnormalities in endothelial-independent functioning. For example, smoking may directly alter the SMC response or induce downregulation of α_2 receptors on the endothelium, thereby decreasing the capacity of noradrenaline to induce EDRF release. In this case, abnormal endothelial-independent responses would not be overcome and restoration of endothelial function would not allow for a normal CPT response.

The role of this chapter was therefore to investigate the effects of the CPT on brachial artery vasoactivity, at 1 and 3 minutes in ice, on smokers and non-smokers. Considering 5 days supplementation with taurine restored to normal endothelial-dependent relaxation in smokers, this chapter also served to investigate if taurine would have an effect on the predicted abnormal CPT response of smokers.

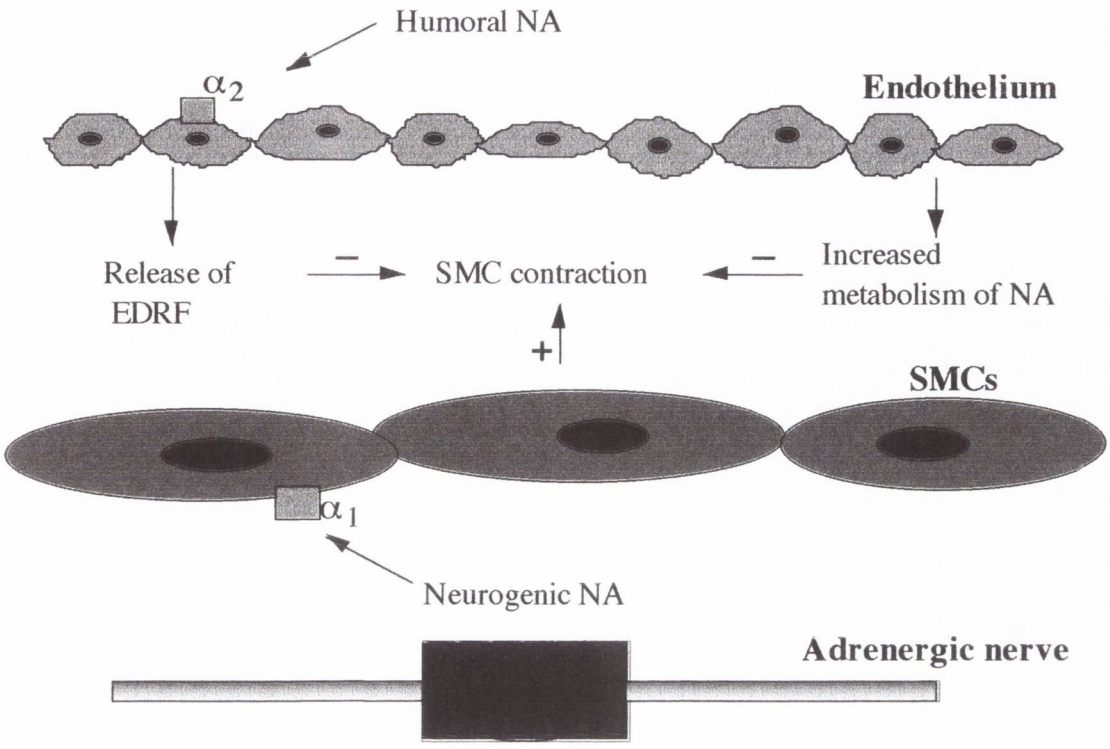


Figure 4.1: Schematic representation of the role of an intact endothelium in response to the CPT. In response to the CPT both humoral noradrenaline (NA) and adrenergic noradrenaline are released. Humoral noradrenaline stimulates α_2 receptors which can produce dilatation through the release of EDRF. An intact endothelium can also increase the metabolism of humoral NA. These factors together inhibit the neurogenic noradrenaline-induced SMC contraction through stimulation of SMC α_1 receptors.

4.1 Methodology

4.2.1 Subjects

7 healthy smokers and 6 control non-smoking subjects were studied. These subjects were recruited from among hospital staff, friends and family, and were those same subjects examined in chapter 3, being normotensive, non-diabetic and without a history of hyperlipidaemia or a family history of premature vascular disease. Smokers were defined as those who smoked at least 20 cigarettes per day for at least 2 years (2 pack years), and had at least one cigarette in the preceding 12 hours. Control subjects were lifelong non-smokers. Smokers were supplemented with 1.5 gms taurine/day (Twinlab, Ronkonkoma, N.Y.) for 5 days.

4.2.2 Study protocol

The methodology described here has been used elsewhere (Corretti *et al.*, 1995) and is described in detail in section 2.3.3. All studies were performed in a temperature-controlled room (20°C) with the subject in the resting, supine state; the subject lay at rest for 10 minutes prior to a resting baseline scan. The subjects right arm was comfortably immobilised in the extended position to allow constant access to the brachial artery for imaging. Baseline recordings of arterial diameter were obtained, following which the left hand and wrist were immersed in ice water for 3 minutes and then withdrawn, while the right artery was continuously imaged and recorded.

4.2.3. Data Analysis

This methodology is described in detail in section 2.3.4. All scans were recorded on super VHS videotape for off-line analysis. Brachial artery diameter measurements were obtained at baseline, and at 1 and 3 minutes immersion of the contralateral hand in ice. Briefly, vessel diameter was measured at a fixed distance

from an anatomical marker, incident with the peak systolic waveform on the flow velocity curve. An average of four cardiac cycles were analysed for each measurement.

Results obtained were expressed as percentage change in brachial artery diameter; the change in diameter induced by the CPT was divided by the baseline diameter measurement.

Descriptive statistics were expressed as mean ± SD. The control and the smoking group were compared at 1 and 3 minutes in ice using the two-sample *t* test. The effect of taurine supplementation on the CPT response in smokers was then compared with pre-supplementation smokers using the paired *t* test. Statistical significance was inferred at $p \leq 0.05$.

4.3 Results

The raw data for Chapter 4 is presented in Appendix 111.

4.3.1 Effect of the CPT on brachial artery diameter in smokers and non smokers at 1 minute in ice.

The effect of the CPT on brachial artery diameter in smokers and non-smokers at 1 minute in ice was assessed using duplex sonography. Measurements were obtained pre and 1 minute post placement of the contralateral hand in iced water. Results were expressed as actual arterial diameter measurements. Actual arterial diameter measurements at baseline and 1 minute post immersion of the contralateral hand in ice in control non-smokers were 3.58(±0.52)mm and 3.63(±0.52)mm respectively, and 3.67(±0.80)mm and 3.57(±0.75) in smokers. Significant arterial

vasoconstriction was observed in smokers (*$p < 0.05$, Wilcoxon Signed Rank, Figure 4.2).

4.3.2 Effect of taurine supplementation on the CPT response in smokers at 1 minute in ice.

The effect of taurine supplementation on the cold pressor response in smokers at 1 minute in ice was also investigated using duplex ultrasonography.

Actual arterial diameter measurements at baseline and 1 minute post immersion of the contralateral hand in ice in taurine-treated smokers were 3.67(±0.80)mm and 3.57(±0.75)mm pre-supplementation vs. 3.52(±0.70)mm and 3.5(±0.73) post supplementation with taurine. Supplementation with taurine prevented the smoking-induced decrease in arterial diameter in response to the CPT at 1 minute in ice; (Figure 4.2)

4.3.3 Effect of the CPT on brachial artery diameter in smokers and non smokers at 3 minutes in ice.

The effect of the CPT on brachial artery diameter in smokers and non-smokers at 3 minutes in ice was also assessed using duplex sonography. Measurements were obtained pre and 3 minutes post placement of the contralateral hand in iced water. Actual arterial diameter measurements at baseline and 3 minutes post immersion of the contralateral hand in ice in control non-smokers were 3.58(±0.52)mm and 3.45(±0.51)mm respectively, and 3.67(±0.80)mm and 3.40(±0.77) in smokers. A significant decrease in arterial diameter, indicating arterial constriction, was observed in both control and smoking subjects at this time point of 3 mintes in iced water in comparison to pre immersion: (*$p < 0.05$, Wilcoxon Signed Rank, Figure 4.3).

4.3.4 Effect of taurine supplementation on the CPT response in smokers at 3 minutes in ice.

Actual arterial diameter measurements at baseline and 3 minutes post immersion of the contralateral hand in ice in taurine-treated smokers were 3.67(±0.80)mm and 3.40(±0.77)mm pre-supplementation vs. 3.52(±0.70)mm and 3.4(±0.79) post supplementation with taurine. Taurine supplementation in smokers did not prevent the significant decrease in arterial diameter in response to the CPT at 3 minutes in ice. (Figure 4.3).

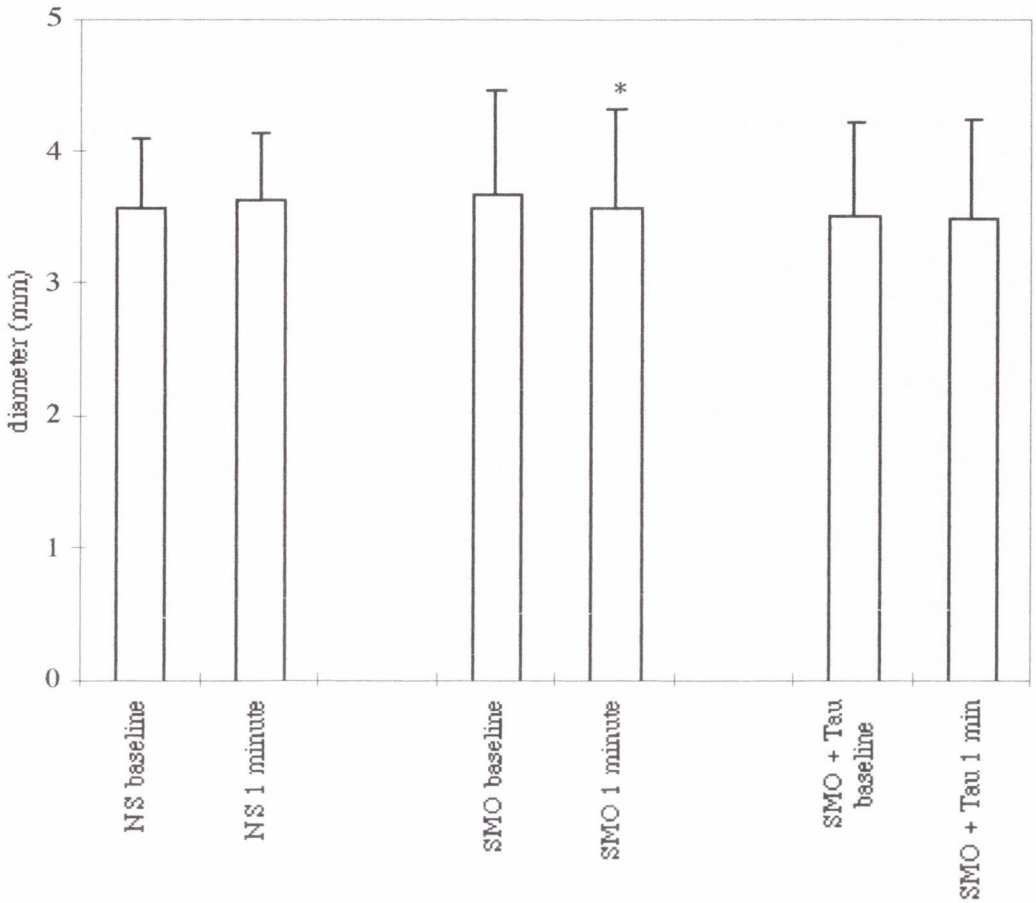


Figure 4.2 Brachial artery diameter changes in response to cold pressor stimulation of 1 minute immersion of the contralateral hand in ice: (non-smokers, n= 6; smokers, n=7; smokers-taurine, n=6). NS = non smokers; SMO = smokers. *$p<0.05$ vs. corresponding baseline value, Wilcoxon Signed Rank.

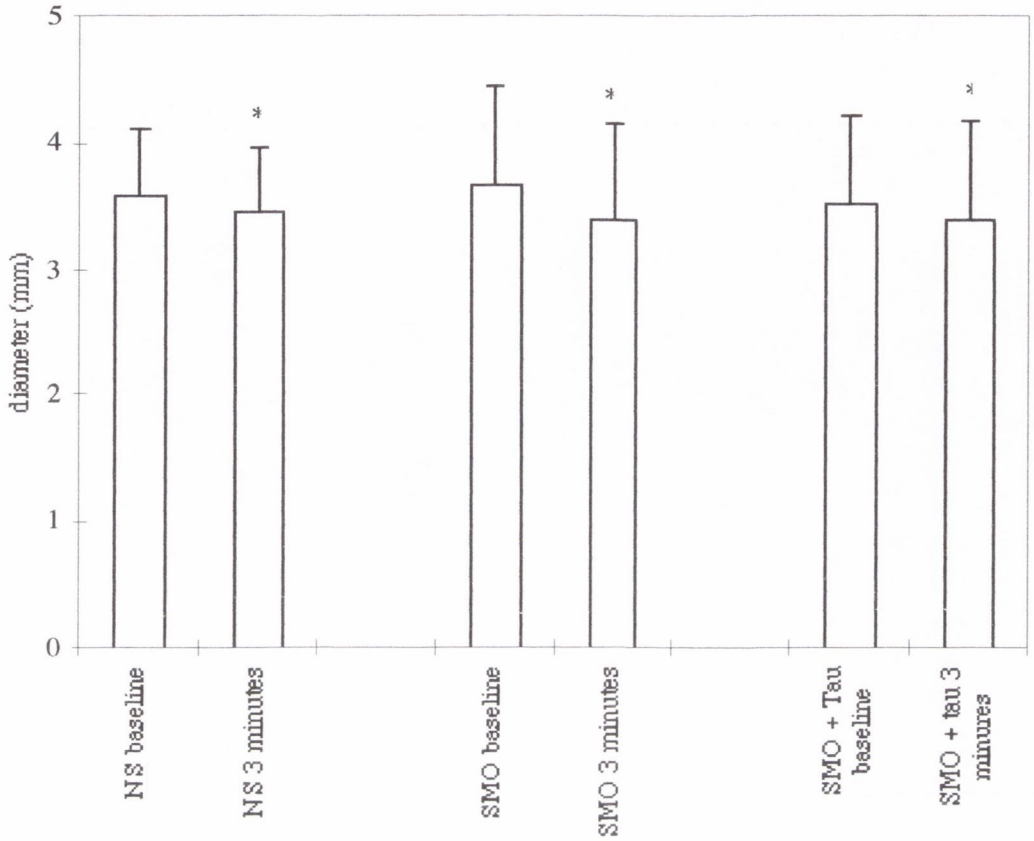


Figure 4.3 Brachial artery diameter changes in response to cold pressor stimulation of 3 minutes immersion of the contralateral hand in ice: (non-smokers, n= 6; smokers, n=7; smokers-taurine, n=6). NS = non smokers; SMO = smokers. *$p<0.05$ vs. corresponding baseline value, Wilcoxon Signed Rank.

4.4 Discussion

This chapter demonstrates that in the presence of healthy endothelium from non-smokers the CPT induces dilatation at 1 minute in ice, while the same stressor induces vasoconstriction in the brachial artery of smokers at this time point. Exposure to a cold pressor stress for 3 minutes induces vasoconstriction in both subject groups. Supplementation of smokers with taurine significantly reduces the vasoconstrictor effect of the CPT at 1 minute in ice, but it does not restore the response to that of controls.

These results are supported by similar observations in healthy controls where a vasodilatatory effect on the brachial artery was found at 1 minute in ice, the opposite to which occurred at later time points (Corretti *et al.*, 1995). The CPT has been shown to dilate normal coronary arteries at 1 minute in ice, but it constricts atherosclerotic vessels in proportion to disease severity (Nitenberg *et al.*, 1997; Zeiher *et al.*, 1991). Cold pressor vasoactivity appears to depend on endothelial-dependent vasodilatory function, but also on adrenergic stimulation of the peripheral vessels. The abnormal vasoconstrictor response seen in the brachial arteries of smokers could therefore reflect endothelial-dependent abnormalities, endothelial-independent abnormalities, or both.

There are many possible explanations for the deviation in results obtained between smokers and non-smokers. Firstly, it has been previously shown that a functional intact endothelium can override vascular smooth muscle contractility in response to vasoconstrictor agonists such as NA. In the previous chapter it was demonstrated that taurine can restore endothelial-dependent function to normal; results obtained here indicate that restoration of endothelial-function is not sufficient to normalise the response to CPT. This may suggest that the role of α_2 endothelial

receptors in mediating the initial dilatory CPT response may be pivotal. Cigarette smoking may induce downregulation of these receptors, as is known to occur with ageing (Zeigler *et al.*, 1976), and in accordance with previously demonstrated decreased β-adrenoceptor densities noted in smokers (Laustiola *et al.*, 1991). α_2 receptor function may not be restored by taurine supplementation.

The findings obtained here may suggest that smoking-induced changes in the vessel wall may not be limited to the endothelium and may also involve vascular SMC abnormalities. Endothelial-independent SMC abnormalities in smokers is a possible consideration for abnormalities observed here, considering recent observations of SMC dysfunction in adults with risk factors for atherosclerosis, independent of impaired endothelial function (Adams *et al.*, 1998). It is therefore possible that functional abnormalities may occur in the SMC, such as decreased cGMP-induced relaxation of SMCs or decreased activity of guanylate cyclase, long before structural changes such as SMC replication and plaque formation occur. Therefore, even in the face of restored function to the endothelium, with subsequent release of NO, the vasodilatory SMC function may be impaired.

It is also possible that cigarettes smokers may have altered catecholamine sensitivity, considering smoking is associated with an increased adrenergic discharge (Cryer *et al.*, 1976) and decreased clearance of catecholamines (Grassi *et al.*, 1994), thereby inducing vascular SMC hypersensitivity and failure of vessel dilatation. If this is so, a vasoconstrictor response to the CPT is not so much a reflection of SMC responsiveness, but rather the magnitude of the adrenergic stimulus and response. This hypothesis would be in keeping with an explanation for accelerated atherosclerosis in conditions associated with elevated cathecholamine concentrations, due to involvement of catecholamines in passage of LDL into arterial walls, and thereby accelerating the atherosclerotic process (Born, 1991).

Results obtained at 3 minutes in ice did not differ between subject groups. These findings are supported by previous studies in normal subjects and those with proven atherosclerotic coronary artery disease, where vasoconstriction of the brachial artery was observed in both groups at this time point. Vasoconstriction at 3 minutes is likely to be due to the fact that the initial protective effect of the endothelium is overwhelmed after a time of 60 to 90 seconds (Nabel *et al.*, 1988), after which time adrenergic vasoconstrictor signals override. Vasoconstriction may also be enhanced at this time by pain receptor stimulation.

While endothelial dysfunction may play a central role in precipitating alterations in vasomotion in smokers, a similar pathological process may also be affecting SMC function. The vasoconstrictor response to the CPT of the brachial arteries of smokers at the crucial time point of 1 minute in ice mimics the responses of diseased atherosclerotic arteries (Nabel *et al.*, 1988; Corretti *et al.*, 1995). This chapter therefore demonstrates that smoking results in abnormal vasoactive responses to physiological stimuli in young people. These abnormal responses are both endothelial dependent and independent, and they follow a pattern previously identified in subjects with proven atherosclerotic disease. While taurine can restore endothelial-dependent functioning, the same micro-nutrient dosage does not restore endothelial-independent abnormalities.

Chapter 5

"Investigating the effect of cigarette smoking on peripheral and central arterial haemodynamic interactions"

5.1 Introduction

The importance of the endothelium in regulating vascular tone by modulating vascular smooth muscle activity was pioneered by Furchgott and Zawadzki (Furchgott *et al.*, 1980). The presence of intact, functional endothelium can minimise the work power loss required to maintain blood flow, and by controlling smooth muscle activity the endothelium has a major role in determining vascular lumen diameter and corresponding changes in peripheral vascular resistance. The endothelium is recognised as having a dynamic nature due, in part, to the influence of its ever-changing haemodynamic environment, to which it must respond. However, the actual response of endothelial cells to physiological stresses may, in turn, influence the haemodynamics and interaction of the whole cardiovascular system.

Arterial applanation tonometry (sphygmocardiography) permits evaluation of the dynamic interaction of the left ventricle and the arterial system by analysis of the peripheral pulse pressure waveform. With the use of a single generalised transfer function (a consistent relationship between pressure waves at different sites), the ascending aortic pressure waveform can be synthesised and the effects of vasoactive stimuli or agents, such as nitroglycerin (Kelly *et al.*, 1990; Takazawa *et al.*, 1995), on central arterial haemodynamics can be determined.

Chapter 3 of this thesis revealed that cigarettes smokers have abnormal peripheral responses to increased blood flow and to cold pressor stimulation. It would appear that an abnormal response to increased blood flow is due to abnormalities in endothelial functioning, which is also responsible, at least in part, for an abnormal response to the CPT. The evaluation of ventricular-vascular interactions by applanation tonometry has been increasingly reported in the literature in recent years. The cold pressor test (CPT), first described over 60 years ago (Hines & Brown 1936), has frequently been used as a model of systemic physiological stress on the cardiovascular system (Nabel *et al.*, 1988; Corretti *et al.*, 1995). It activates

endothelial cells and results in increased serum markers of adhesion markers (Buemi *et al.*, 1997), and induces abnormal vasoactive responses in the coronary circulation in hypertension (Antony *et al.*, 1994) and hypercholesterolaemia (Dubois-Rande *et al.*, 1995). The effect of the CPT on haemodynamic parameters which access ventricular-vascular interactions in smokers and non-smokers are unknown.

The amino acid taurine plays a role in cardiovascular protection. Taurine restores endothelial function, as reported in chapter 3, but its biological cardiovascular protective properties involve a wide variety of functions, which suggest that supplementation in smokers might significantly alter responses to the CPT. Firstly, taurine is also known to have a beneficial effect on resting sympathetic nerve tone in humans (Mizushima *et al.*, 1997) and chronic taurine administration to rats prevents stress-induced haemodynamic and plasma catecholamine changes (Yamamoto *et al.*, 1985). Taurine has been shown to reduce both systolic and diastolic blood pressure in humans with baseline hypertension, and it also decreases adrenaline levels to those of normotensives (Fujita *et al.*, 1987). Secondly, taurines effect on cardiac function, both under experimental and clinical conditions, suggest potential benefits to the stressed heart. Taurine offsets the calcium paradox-induced heart failure; that is, the increase permeability of cell membranes to calcium ion, resulting in cellular damage after calcium-free perfusion (Schaffer *et al.*, 1981). It has beneficial effects in the treatment against heart failure (Azuma *et al.*, 1984) and it reverses arrhythmias associated with toxic doses of adrenaline or digitalis glycosides (Read *et al.*, 1963).

Having established that endothelial abnormalities are present in smokers, the aim of this chapter was to examine the effect of smoking on vascular-ventricular haemodynamic interactions and to investigate if taurine supplementation of smokers had any effect on these parameters. This was a 3-step process: step (1), examination of peripheral and central haemodynamic parameters in smokers and non smoking controls; step (2), examination of the responses of smokers and non smokers to the

physiological CPT, while in step (3) the effect of taurine supplementation on baseline parameter and the response to the cold pressor test was investigated.

5.2 Methodology

5.2.1 Subjects

8 smokers and 11 non-smoking controls aged 20 to 34 years of age were studied. They were recruited from among hospital staff, friends and family. All subjects were normotensive, non-diabetic and were without a history of hyperlipidaemia or a family history of premature vascular disease. They were clinically well and not taking any cardiovascular medications. Smokers were defined as those who smoked at least 20 cigarettes per day for at least 2 years (2 pack years), and had at least one cigarette in the preceding 12 hours. Control subjects were lifelong non-smokers.

5.2.1 Study protocol

This methodology is described in detail in 2.3.5. Applanation tonometry was conducted in a quiet, temperature controlled room with subjects resting for 5 minutes prior to the baseline scan. The left radial pulse was used and blood pressure was measured by auscultation over the left brachial artery using a standard mercury sphygmomanometry, with diastolic pressure defined as Korotkoff phase V according to American Heart Association guidelines (Perloff at al., 1993). After a baseline scan was completed, the right hand and wrist were immersed in a bath of ice water for 3 minutes and then withdrawn, as previously described by Corretti *et al.*(Corretti *et al.*, 1995). Blood pressure measurements were taken prior to each tonometric recording, which were taken at 1 and 3 minutes in ice.

Smokers were supplemented with 1.5 mgs taurine/day for 5 days, and baseline and cold pressor test responses were repeated.

Descriptive statistics were expressed as mean ± SD. The control and the smoking group were compared using the two-sample t test. The effects of taurine

supplementation on haemodynamic parameters in smokers were then compared using the paired *t* test. Statistical significance was inferred at $p \leq 0.05$.

In situations where clinically important changes were obtained and were found to be outside the significant $p \leq 0.05$ range, the "effect size", a measure of the magnitude and direction of the effect under investigation was used. The effect size is based upon the statistical distribution of the values obtained in a population study, and translates changes in health status measurements into a standard unit of comparison and assesses the magnitude and meaning of changes in health status (Kazis *et al.*, 1989; Heavy & McGee, 1998). An effect size of 0.2 or less is designated as small, 0.5 is medium and 0.8 or greater is large (Cohen, 1977). It is calculated as follows:

Effect size (Kazis *et al.*, 1989)

$$A - B / C$$

where A= mean at time 2

B= mean at time 1

C= standard deviation at time 1

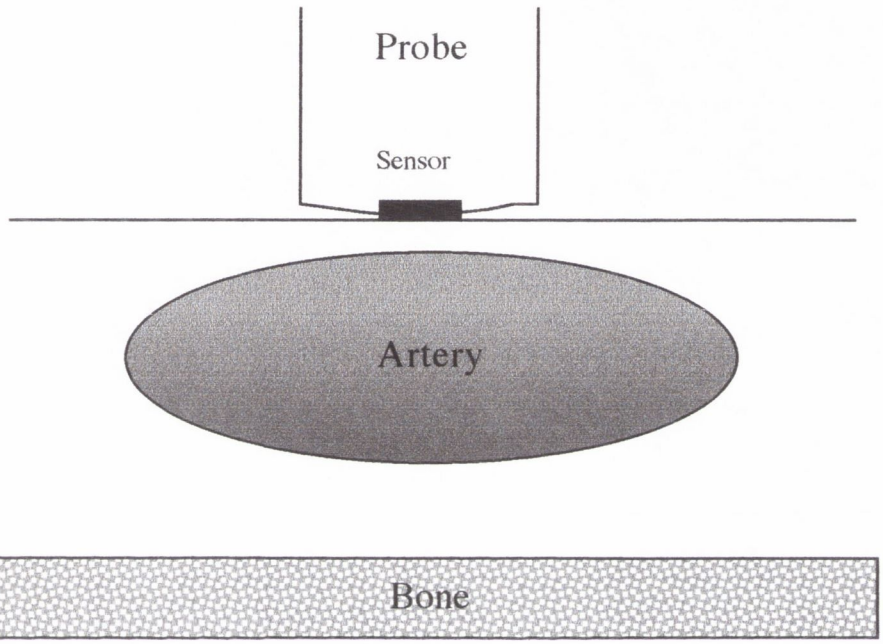


Figure 5.1 The theory of applanation tonometry.

The tonometric probe flattens the wall of the artery, tangential pressures are eliminated and the sensor is exposed to the pressures within the artery. Complete accuracy is only ensured when the tissue behind the artery (the radius bone in this study) prevents the artery from moving backwards so that its anterior wall may be applanated.

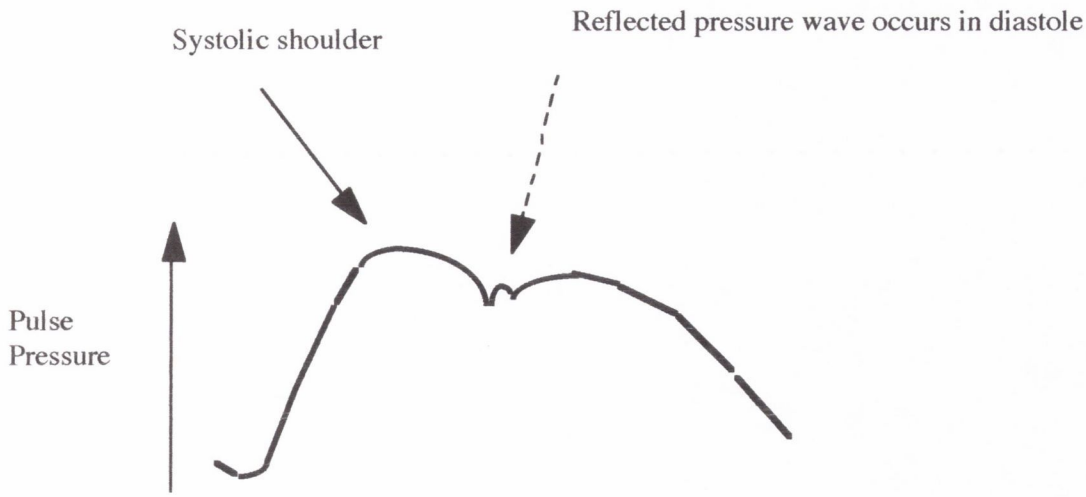


Figure 5.2 Schematic illustration of pressure flow waves in normal adolescent subjects. The systolic peak is not followed by a later systolic pressure rise, but a diastolic peak is apparent which signifies peripheral wave reflection from compliant vessels.

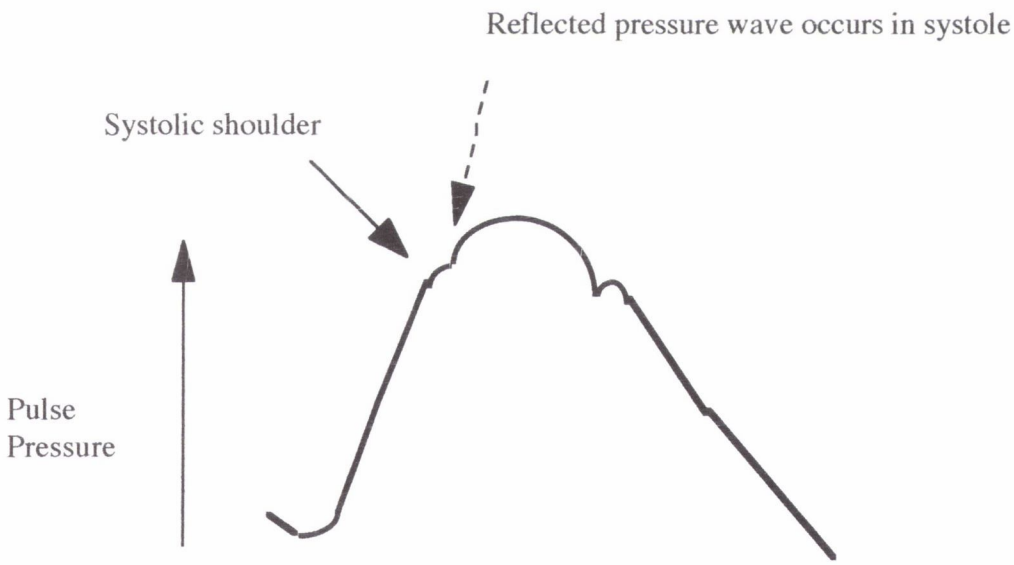


Figure 5.3 Schematic illustration of pressure flow waves in ageing subjects. With advancing age and increasing arterial stiffness, reflected waves arrive earlier and the pressure wave rises from the systolic shoulder to a later systolic peak.

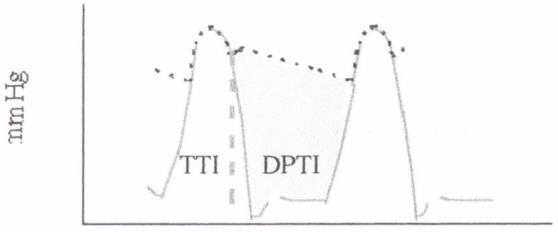
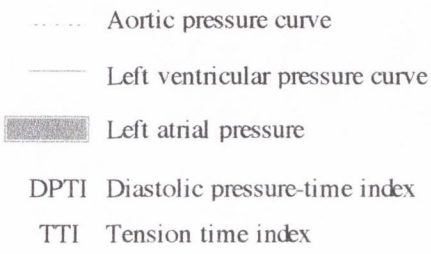


figure 5.4(a)



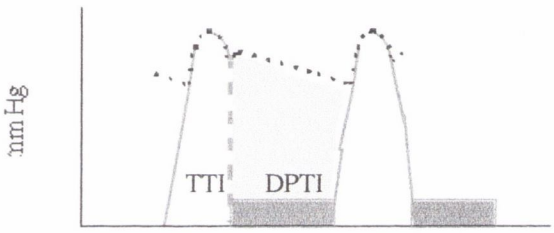


figure 5.4(b)

Figure 5.4(a) Superimposed left ventricular and aortic blood pressure waveforms. The stippled area was used to calculate the diastolic pressure-time index and the tension time index.

Figure 5.4(b) The pressure gradient between the aorta and the left atrium determine the diastolic pressure-time index.

5.3 Results

5.3.1 Baseline peripheral haemodynamic parameters (Table 5.1 and 5.2)

There was no difference between dP/dt of smokers and non smokers at baseline (figure 5.5). Taurine supplementation of smokers had no effect on baseline dP/dt.

5.3.2 Baseline central haemodynamic parameters (Table 5.3 and 5.4).

Baseline heart rate was significantly higher in smokers than non smokers: 80.63(±11.57)bpm versus 59.1(±11.35)bpm: (*p=.001; Students 2-sample t-test: figure 5.6). Taurine treatment of smokers had no effect on heart rate :75(±10)bpm versus 77(±10)bpm. Likewise, ejection duration, or time of the cardiac cycle spent in systole, was significantly greater in the smoking group: 41(±5)% versus 32.5(±5)% in the non smoking group: (*p<0.002;Students 2-sample t-test: figure 5.7), with taurine having no effect: 39(±3)% versus 40(±3)%.

Indices of left ventricular load, mean and end systolic pressures were significantly greater in the smokers. Mean systolic pressure was 103(± 8.6)mmHg in smokers versus 94(±7.5)mmHg in non smoking controls: (*p<0.05;Students 2-sample t-test: figure 5.8). Likewise, end systolic pressure was greater in smokers than non smokers: 104(±11)mmHg versus 91.2(±7)mmHg: (*p≤0.05; Students 2-sample t-test: figure 5.9) and mean diastolic pressure was also higher in smokers than in the control non smoking group: 89(±9)mmHg versus 79(±6) mmHg: (*p<0.05; Students

2-sample t-test: figure 5.10). Importantly, taurine supplementation had an effect on baseline mean systolic and diastolic pressures. Mean systolic pressure increased after treatment: 101(±13)mmHg pre-supplementation versus 111(±14)mmHg post-supplementation, as did end systolic pressure: 101(±16) pre-supplementation versus 112(±17) post taurine supplementation. Again, taurine also increased mean diastolic aortic pressure: 84(±14)mmHg versus 96(±14)mmHg. While numerically these increases in pressure post-treatment are outside accepted "p" values for significance, such clinically important changes are emphasised by the effect size. The effect size of change in mean systolic pressure and end systolic pressure being 0.85 and 0.69 respectively, and the change in diastolic pressure being 0.9.

Central augmentation index was significantly greater in smokers at baseline than non smokers: 134(±32)% versus 100(±20)%: (*$p<0.05$; Students 2-sample t-test: figure 5.11). Suprisingly, taurine supplementation had no effect on this parameter: 144(±20)% versus 132(±14)%.

A measure of subendocardial viability, the Buckberg Index, is significantly lower in smokers versus control non smokers at baseline: 131.5(±28)% versus 189(±44)%: (*$p<0.005$; Students 2-sample t-test: figure 5.12); taurine had no effect on this index: 133(±20)% versus 132(±9)%:

5.3.3 Effects of the Cold Pressor Test on peripheral hemodynamic parameters (Table 5.5 and 5.6).

In response to the CPT, d*P*/d*t* was significantly decreased in non smokers at 1 and 3 minutes in ice when compared to baseline levels: 549(±92)mmHg/sec and 549(±99)mmHg/sec, respectively, versus 697(±59)mmHg/sec: (*$p<0.05$; Students paired t-test: figure 5.13).This phenomenon did not occur with smokers in whom there was a non significant increase in dP/dt at 1 minute in ice and no change at 3 minutes in ice: 658(±88)mmHg/sec and 594(±99)mmHg/sec, respectively, versus 599(±57)mmHg/sec. However, when the degree of change from baseline to 1 and 3 minutes in ice was investigated, it was found that the direction of response in smokers was the opposite to that of non smokers, with smokers having a significant increase in their d*P*/d*t* in comparison to non smokers who had a decrease at 1 minute in ice: 66(±111)mmHg/sec versus -165(±63)mmHg/sec: (*$p<0.005$; Students 2-sample t-test: figure 5.14). Taurine treatment of smokers resulted in a non-significant change in the direction of response of smokers at 1 minute in ice: 767(±167) at 1 minute and 726(±267) at 3 minutes and a baseline value of 687(±133) pre treatment, versus 685(±159) and 739(±93) at 1 and 3 minutes post treatment and a baseline value of 698(±85); (figure 5.15). The degree of change in response to the CPT is slightly (non significantly) modified after taurine supplementation with the increase in dP/dt in response to 1 minute in ice being reduced: 81(±133)mmHg/sec pre-supplementation versus 55(±20)mmHg/sec post-supplementation (figure 5.16).

5.3.4 Effects of the Cold Pressor Test on central hemodynamic parameters (Table 5.7 and 5.8).

The CPT had no effect on central haemodynamic timing parameters. There was no change in heart rate or ejection duration in either subject group in response to the stimulus. Taurine supplementation had no effect on these timing parameters.

Indices of left ventricular load, mean systolic pressure and end systolic pressure were increased in both smokers and non smokers at 1 and 3 minutes in ice in response to the CPT. In non smokers mean systolic pressure increased from 91(±5)mmHg at baseline to 107(±16)mmHg and 110(±18), at 1 and 3 minutes in ice respectively: (*$p<0.05$; Students paired t-test), and in smokers from 103(±8)mmHg at baseline to 111(±9)mmHg and 111(±7)mmHg at 1 and 3 minutes in ice: (*$p<0.05$; Students paired t-test: figure 5.17). End systolic pressure increased in both groups in response to the CPT, but only significantly so in non smokers: 88(±6)mmHg at baseline versus 107(±18)mmHg and 110(±20) at 1 and 3 minutes in ice: (*$p<0.05$; Students paired t-test: figure 5.18). Taurine supplementation of smokers failed to affect the increase in left ventricular load in response to the CPT.

Mean diastolic pressure was significantly increased in both subject groups in response to the CPT. Smokers increased from 90(±9)mmHg at baseline to 98(±8)mmHg and 99(±8)mmHg, and non smokers from 79(±9)mmHg to 93(±12)mmHg and

94(±12)mmHg at 1 and 3 minutes respectively (*$p<0.05$; Students paired t-test: figure 5.19). Supplementation with taurine somewhat diminished the increase in diastolic pressure in response to the CPT: an increase from baseline of 26(±20)mmHg pre-treatment versus 15(±6)mmHg post-treatment at 1 minute in ice, and an increase of 26(±20)mmHg pre-treatment versus 17(±9)mmHg post-treatment at 3 minutes in ice. This effect failed to reach significance. In response to the CPT, overall mean central blood pressure changes increased at 1 minute in ice by 18(±19)mm Hg in smokers and by 12(±4)mm Hg in non smokers, and at 3 minutes by 17(±18)mm Hg in smokers and 11(±6)mm Hg in non smokers (figure 5.20). This greater increment in smokers was somewhat diminished post taurine supplementation: an increase of 13(±8)mm Hg post treatment versus 22(±21)mm Hg pre treatment at 1 minute, and an increase of 14(±10)mm Hg post treatment versus 22(±21)mm Hg pre-treatment; (figure 5.21).

The cold pressor test had no effect on central augmentation index (CAI) in smokers. In contrast, this stress resulted in an increase in CAI in non smokers at 1 and 3 minutes, and may represent a lesser magnitude of vasoconstriction at baseline. However, these values failed to reach significance (figure 5.22). Taurine supplementation had no effect on these values.

Non smokers had a non-significant increase in subendocardial viability (Buckberg Index) at 1 and 3 minutes in ice. The CPT had no effect on the Buckberg Index in treated and untreated smokers (figure 5.23).

Table 5.1 Baseline Peripheral Haemodynamic Parameters

	Smokers (n=8)	Non smokers (n=11)
d*P*/d*t* (mmHg/sec)	583.37±70.5	643±99

Data are mean±SD

Table 5.2 Effect of Taurine on Baseline Peripheral Haemodynamic Parameters

	Smokers (n=5)	Non smokers (n=5)
d*P*/d*t* (mmHg/sec)	686.4±133	698±85

Data are mean±SD

Table 5.3 Baseline Central Haemodynamic Parameters

	Smoker (n=8)	Non smoker (n=11)	
Timing Data			
Heart Rate (bpm)	80.63±11.57	59.1±11.35	*
Ejection Duration (% time in systole)	41±4	32.5±5	*
Pressure Data			
Mean Systolic pressure (mmHg)	102.77±8.8	94.7±7.5	*
End systolic pressure (mmHg)	104±11	92.8±8.66	*
Mean Diastolic Pressure (mmHg)	89±9	79.9±6	*
Buckberg Index (%)	131.5±28	189±43	*
Central Augment. Index (%)	134±32	100±20	*

Data are mean±SD

*=p≤0.05 vs. smokers using Students 2-sample t-test.

Table 5.4 Effect of Taurine on Baseline Central Haemodynamic Parameters

	Smoker (n=5)	Smoker + taurine (n=5)
Timing Data		
Heart Rate (bpm)	75±10	77±9
Ejection Duration (% time in systole)	39±4	40±3
Pressure Data		
Mean Systolic pressure (mmHg)	101±13	112±15 @
End systolic pressure (mmHg)	101±16	112±17 Ψ
Mean Diastolic Pressure (mmHg)	84±14	97±15 @
Buckberg Index (%)	133±20	132±8
Central Augment. Index (%)	144±20	132±14

Data are mean±SD

@ = Effect size ≥ 0.8 ; Ψ = Effect size = 0.69

Table 5.5 Effect of the Cold Pressor Test on Peripheral Haemodynamic Parameters

	Smokers (n=8)			Non smoker (n=5)		
	b/line	1 min	3 min	b/line	1 min	3 min
dP/dt (mmHg)	599±57	658±88	594±99	697±59	549±92 *	549±99 * ns
delta dP/dt (baseline to 1 min ice; mmHg)		66±111	29±182		-165±63 †	-109±186 ns

Data are mean±SD

*= p≤0.05 vs. baseline value for that subject population using Students paired t-test.

†=p≤0.05 vs. smoker at that time point using 2 sample t-test.

ns= not significant

Table 5.6 Effect of Taurine supplementation on Cold Pressor Test-induced on Peripheral Haemodynamic Parameters

	Smokers (n=5)			Smokers +Taurine (n=5)		
	b/line	1 min	3 min	b/line	1 min	3 min
dP/dt (mmHg)	686±133	767±167	726±99	698±85	685±159	739±93
					ns	ns
delta dP/dt (baseline to 1 min ice; mmHg)		80±133	39±225		55±20	65±69
					ns	ns

Data are mean±SD

ns= not significant

Table 5.7 Effect of the Cold Pressor Test on Central haemodymnamic parameters

	Smokers (n=8)			Non smokers (n=5)		
	b/line	1 min	3 min	b/line	1 min	3 min
Timing indices						
Heart Rate (bpm)	81±12	80±11 ns	79±13 ns	59±14	59±19 ns	59±15 ns
Ejection Duration (% time in systole)	41±5	41±4 ns	40±5 ns	33±6	31±8 ns	32±6 ns
Pressure indices						
Mean systolic pressure (mmHg)	103±8	111±9 *	111±7 *	91±5	107±16 *	110±18 *
End systolic pressure (mmHg)	104±12	111±11 ns	112±9 ns	88±6	107±18 *	110±18 *
Mean diastolic pressure (mmHg)	90±9	98±8 *	99±8 *	79±9	93±12 *	94±12 *
Buckberg Index (%)	129±29	134±31 ns	124±22 ns	189±55	222±90 ns	203±61 ns
Central Augmentation Index (%)	136±34	134±30 ns	136±17 ns	100±20	124±32 ns	119±25 ns

Data are mean ±SD; *=p≤0.05 vs. baseline value for that subject group.

Table 5.8 Effect of Taurine supplementation on Cold Pressor Test-induced Central Haemodynamic changes

	Smokers (n=5)			Smokers + Taurine (n=5)		
	b/line	1 min	3 min	b/line	1 min	3 min
Timing indices						
Heart Rate (bpm)	75±10	80±9 ns	75±8 ns	77±9	81±12 ns	70±12 ns
Ejection Duration (% time in systole)	39±4	42±3 ns	39±4 ns	40±3	41±6 ns	40±5 ns
Pressure indices						
Mean systolic pressure (mmHg)	101±13	121±9	124±8	111±14	125±15 ns	127±14 ns
End systolic pressure (mmHg)	101±16	121±6 ns	124±6 ns	112±17	125±17 ns	127±15 ns
Mean diastolic pressure (mmHg)	84±14	105±8 ns	108±10 ns	97±14	108±13 ns	110±14 ns
Buckberg Index (%)	133±20	121±19 ns	131±20 ns	132±9	127±27 ns	133±30 ns
Central Augmentation Index (%)	144±20	136±34 ns	138±6 ns	132±14	140±13 ns	143±13 ns

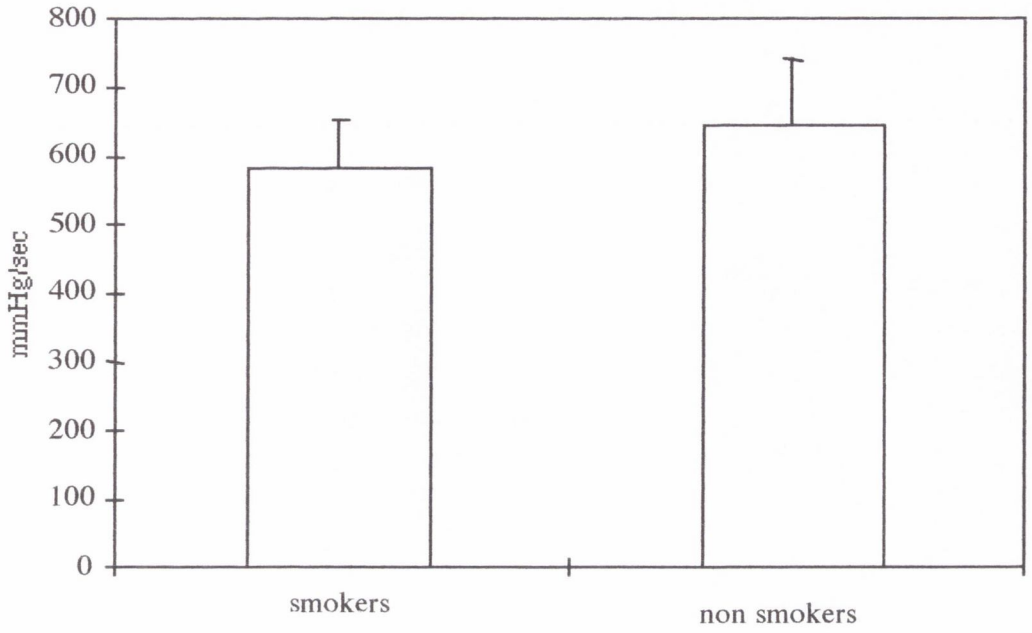


Figure 5.5 Baseline dP/dt
(smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

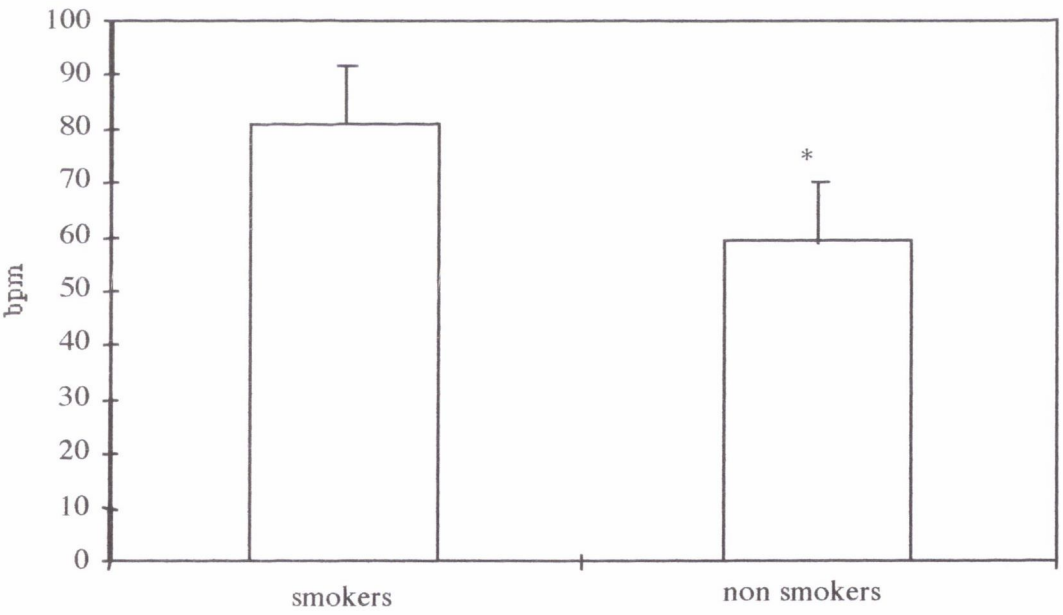


Figure 5.6 Baseline heart rate. *=p<0.05 vs. smokers (Student's 2 sample t-test)
(smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

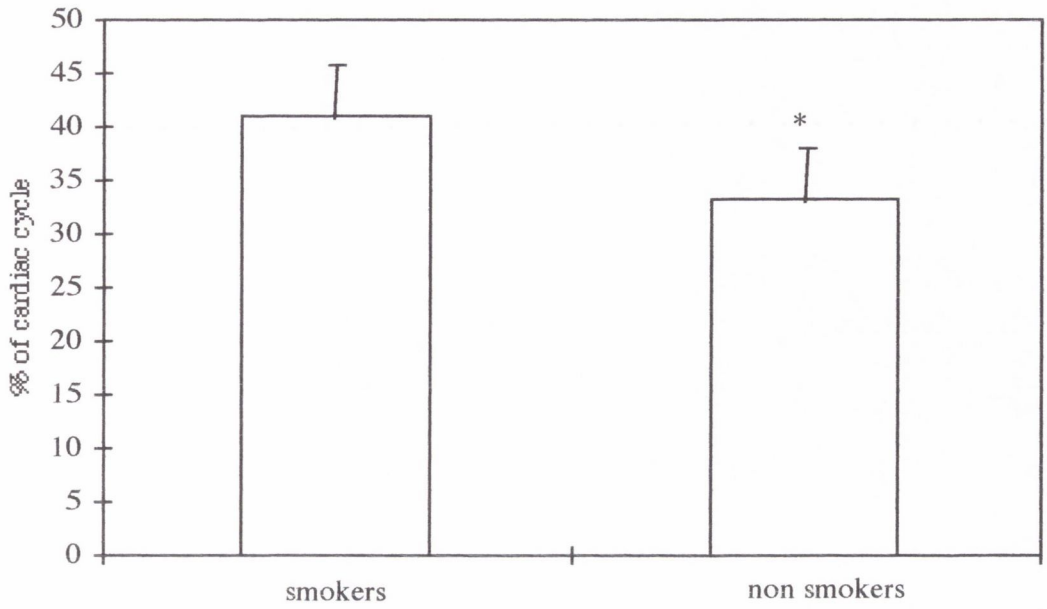


Figure 5.7 Baseline Ejection Duration; *=p<0.05 vs. smokers, Student's 2 sample t-test. (smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

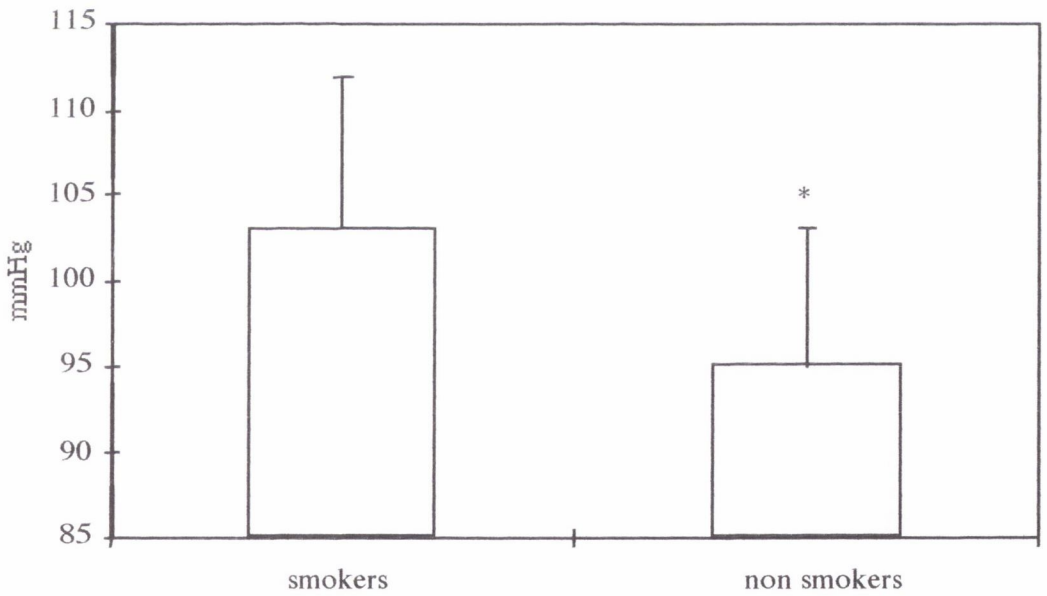


Figure 5.8 Baseline Mean Systolic Pressure; *=p<0.05 vs. smokers, Student's 2 sample t-test. (smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

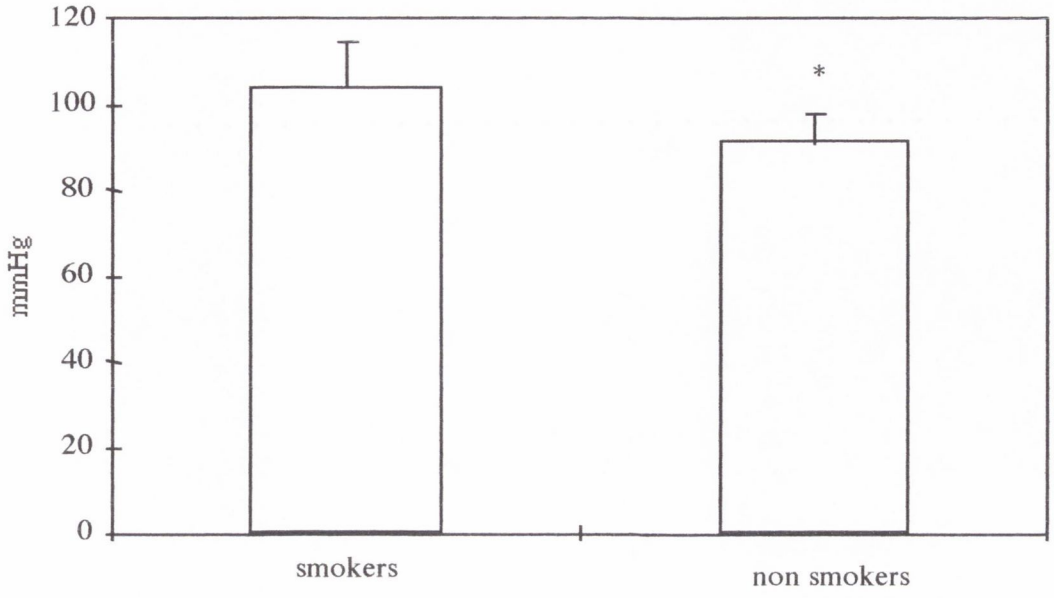


Figure 5.9 Baseline End Systolic Pressure; *=p<0.05 vs, smokers, Student's 2 sample t-test. (smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

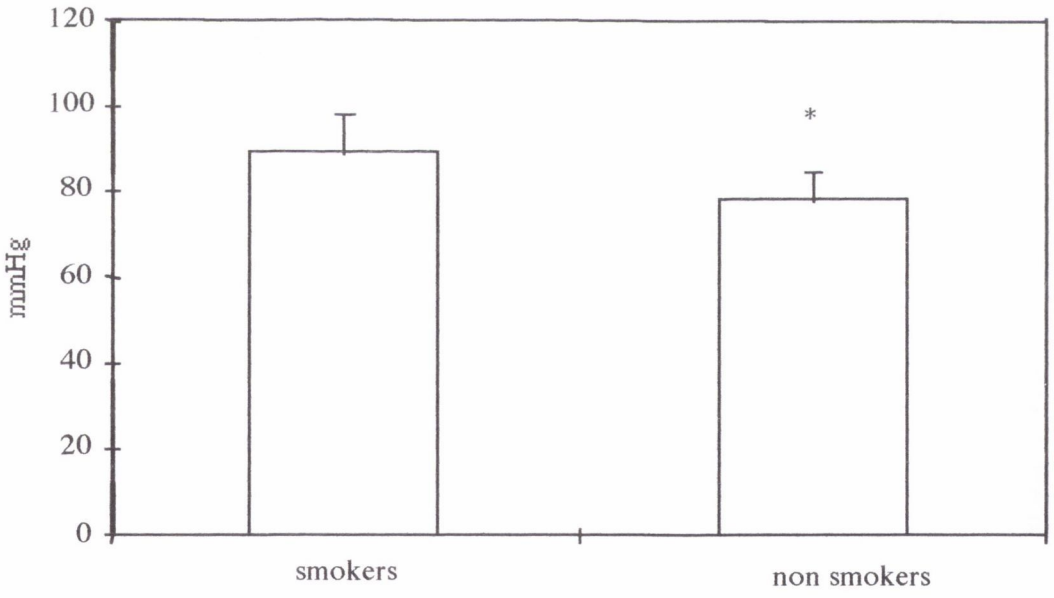


Figure 5.10 Baseline Mean Diastolic Pressure; *=p<0.05 vs. smokers, Student's 2 sample t-test. (smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

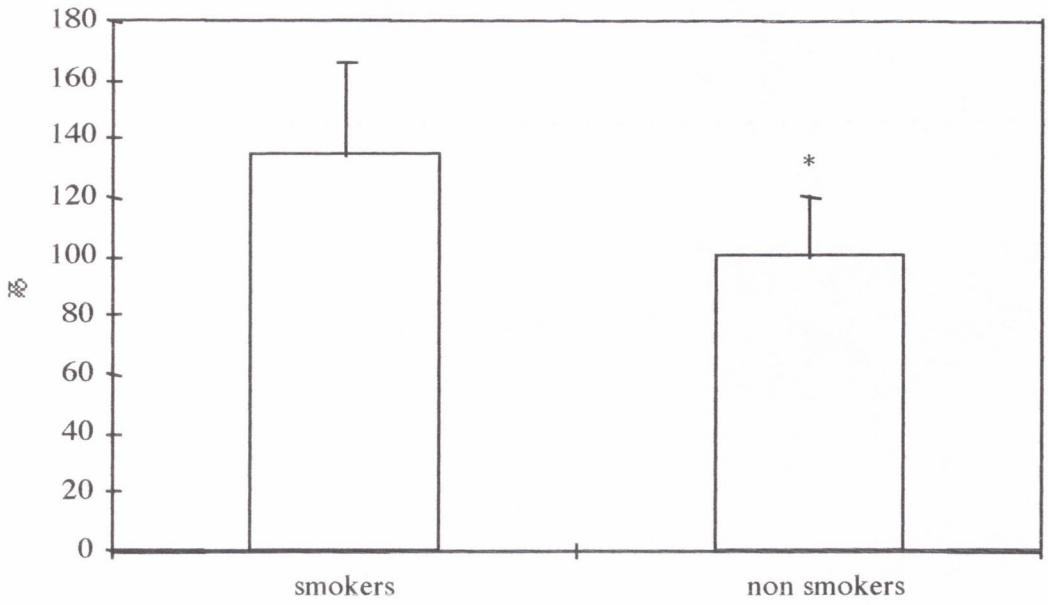


Figure 5.11; Baseline Central Augmentation Index; *=p<0.05 vs. smokers, Student's 2 sample t-test. (smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

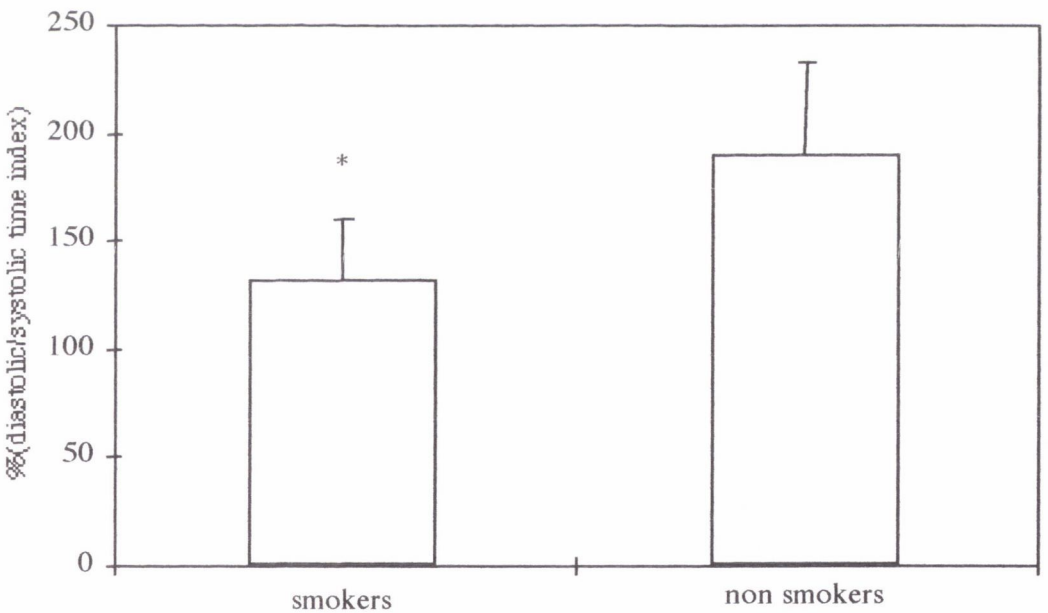


Figure 5.12 Baseline Buckberg Index; *=p<0.05 vs. non smokers, Student's 2 sample t-test. (smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

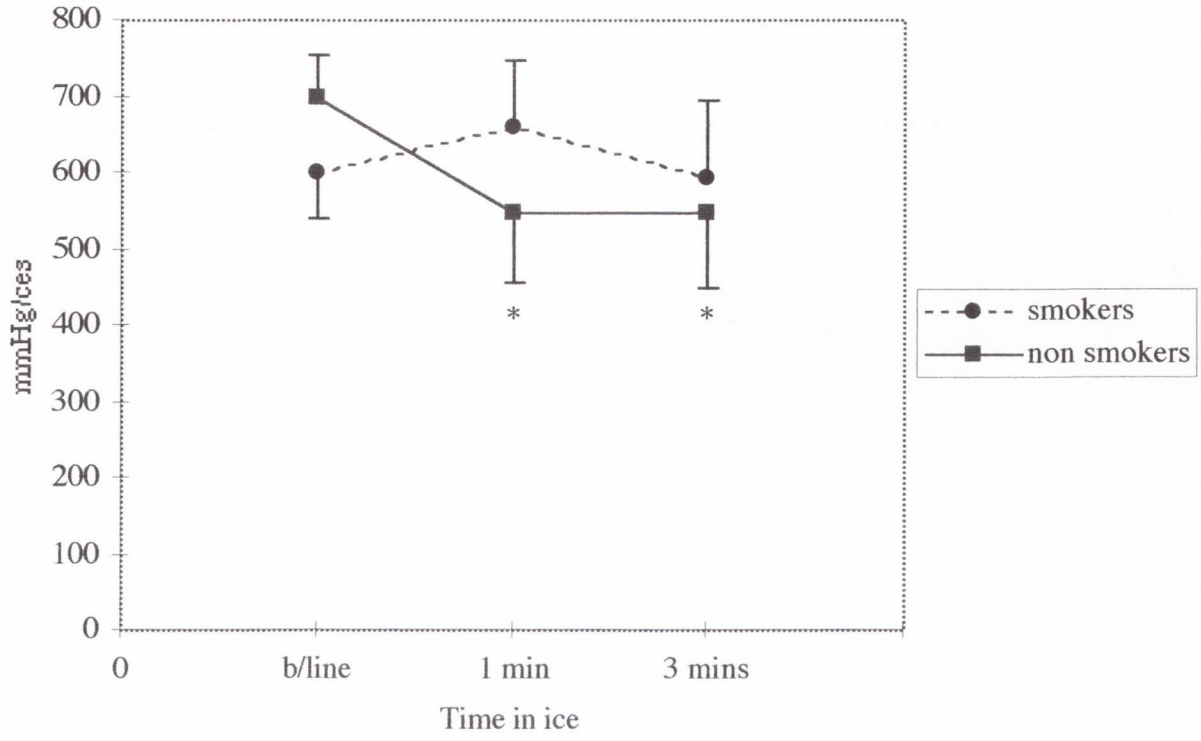


Figure 5.13 dP/dt changes in response to the CPT; *=p<0.05 vs. baseline, Student's paired t-test. (smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

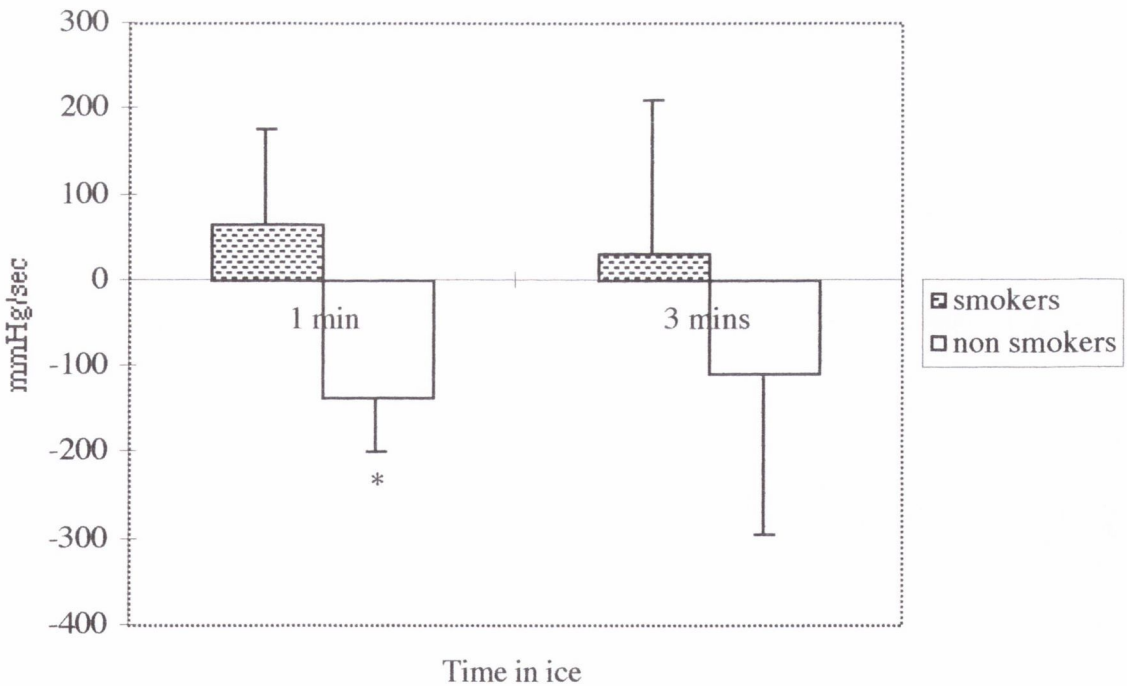


Figure 5.14 Delta dP/dt in response to CPT; *=p<0.05 vs. smokers, Student's 2 sample t-test. (smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

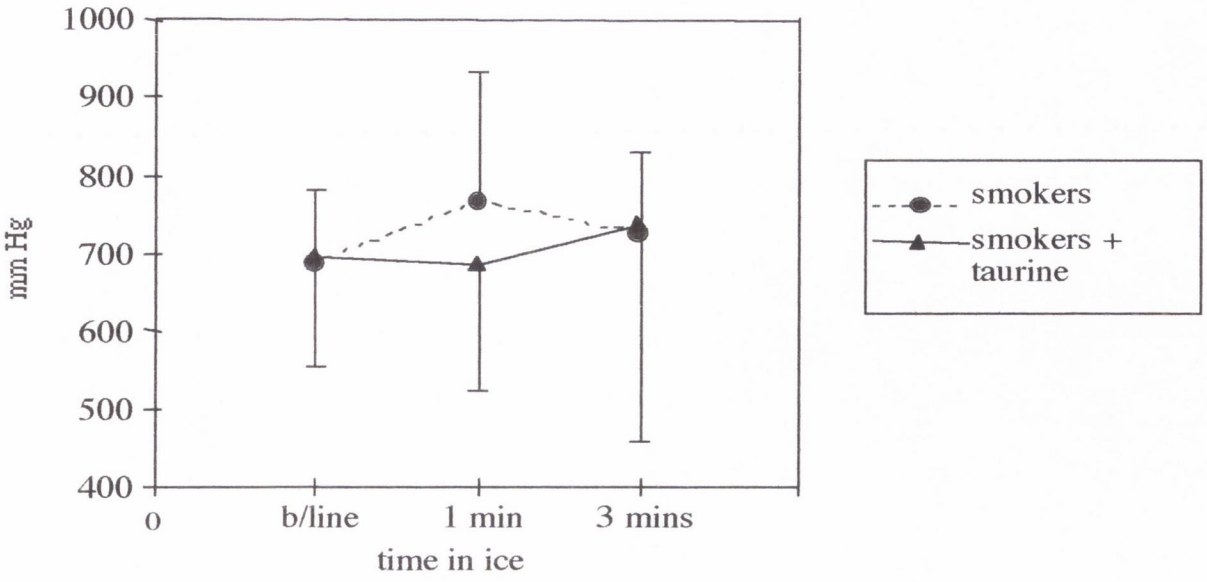


Figure 5.15 Effect of Taurine on dP/dt in response to the CPT (smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

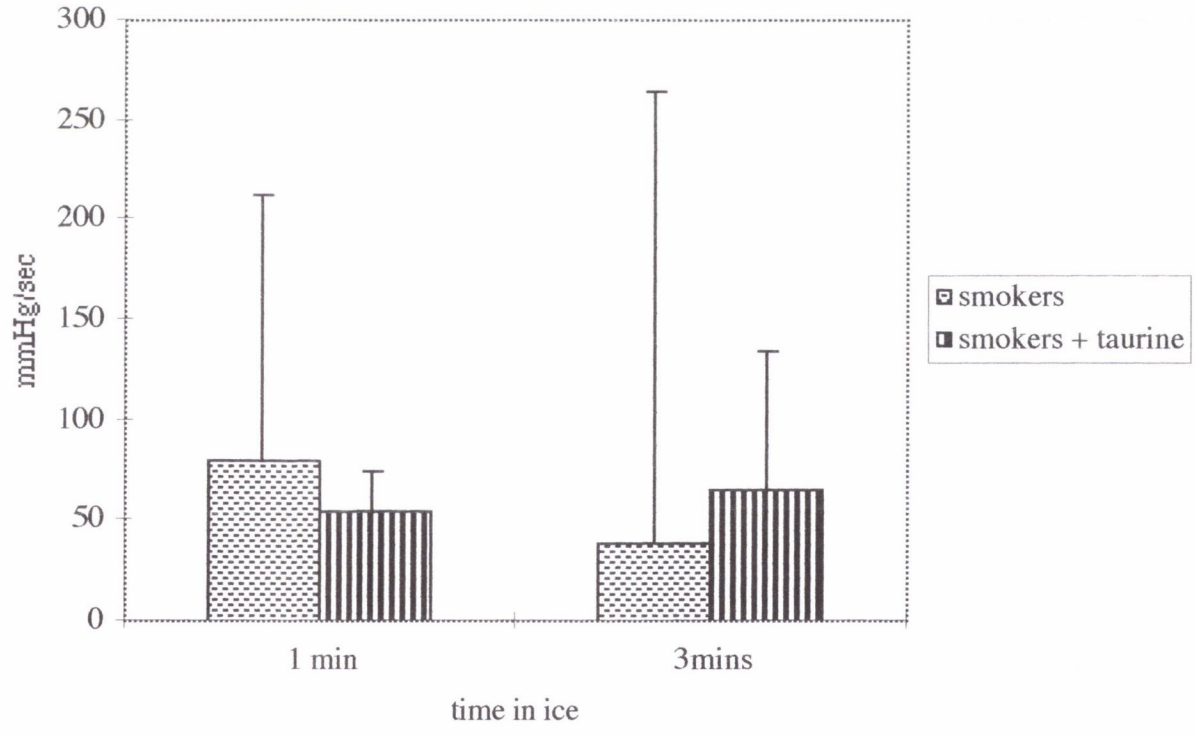


Figure 5.16 Effect of taurine on delta dP/dt in response to the CPT (smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

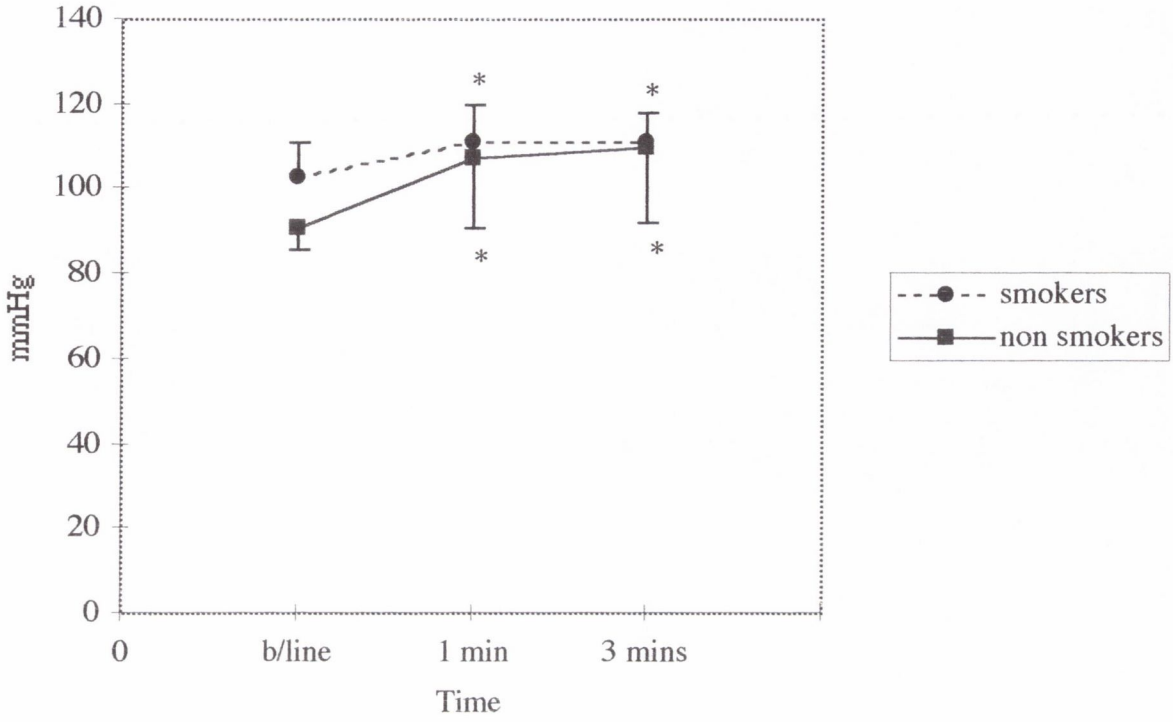


Figure 5.17 Mean systolic pressure changes in response to the CPT; *=p<0.05 vs. baseline, Student's 2 sample t-test. (smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

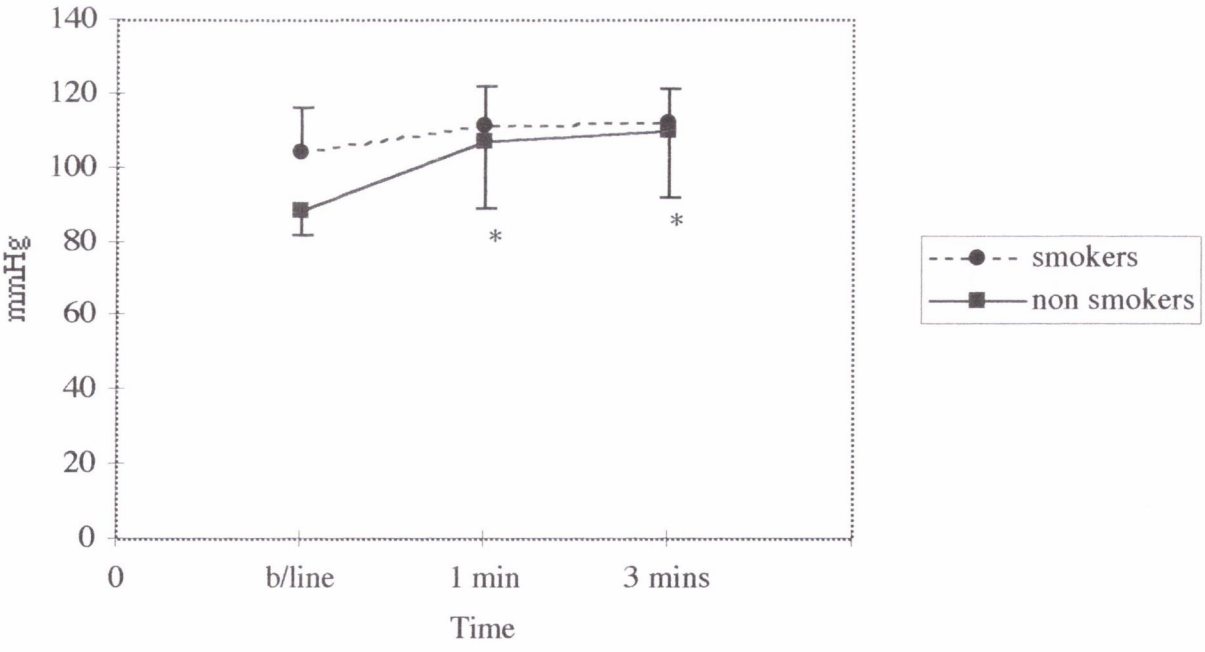


Figure 5.18 End Systolic Pressure changes in response to the CPT; *-p<0.05 vs. baseline, Student's 2 sample t-test (smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

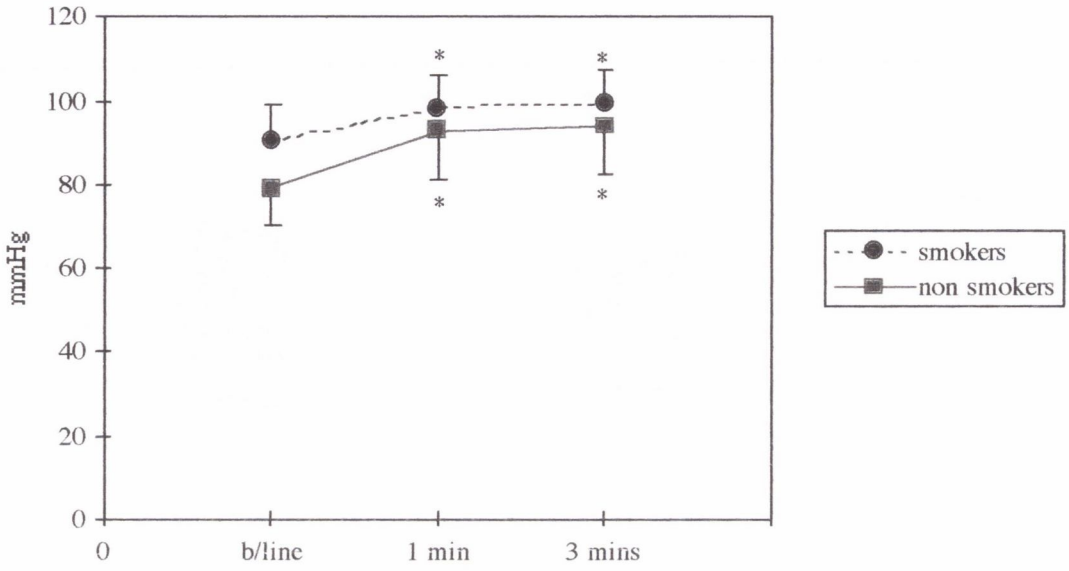


Figure 5.19 Mean diastolic pressure changes in response to the CPT; *p≤ 0.05 vs. baseline, Student's paired t-test (smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

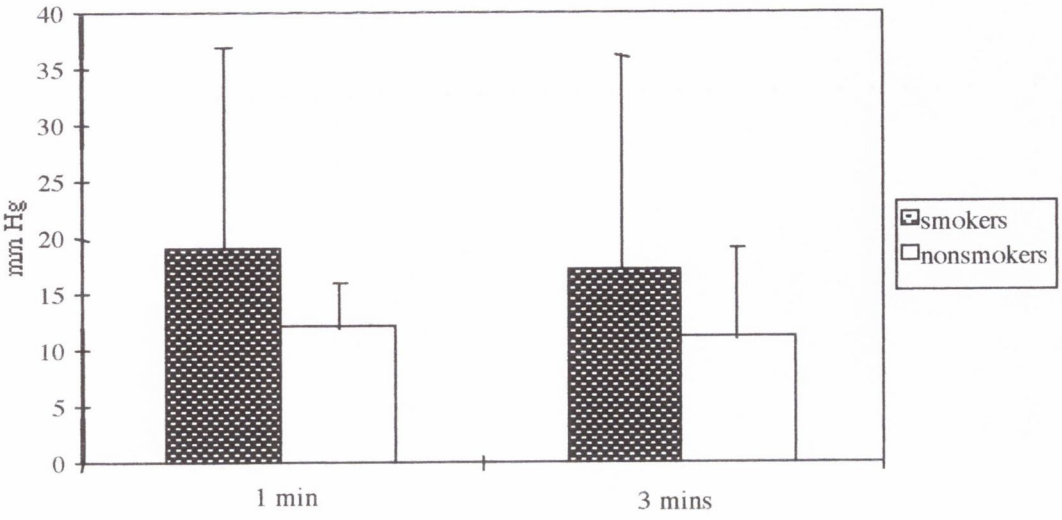


Figure 5.20 Effect of CPT on mean central blood pressure changes
(smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

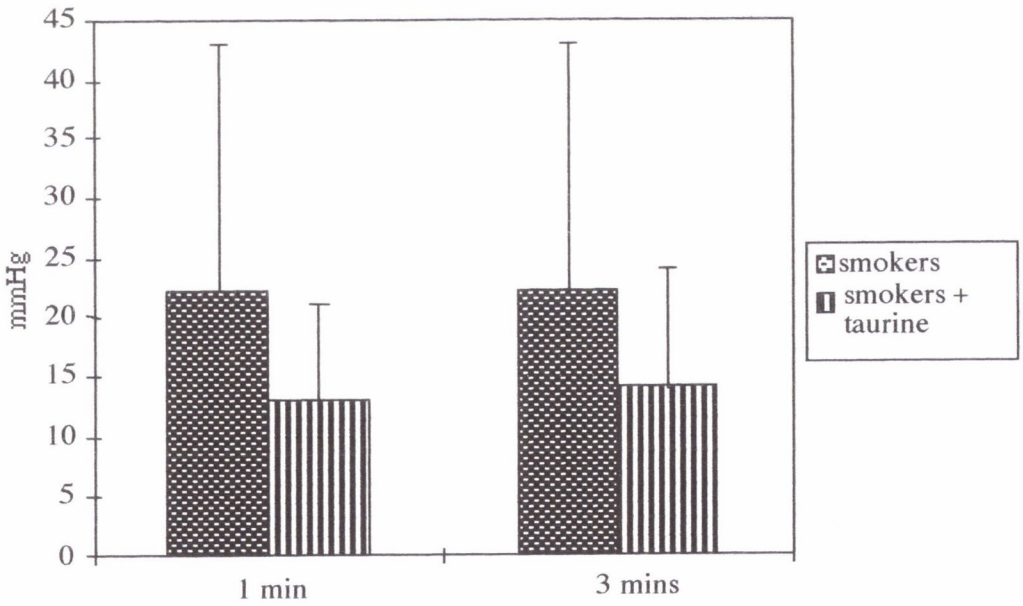


Figure 5 21 Effect of Taurine on CPT-induced mean central pressure changes.
(smokers, n=8; non smokers, n=11; smokers +taurine, n=6)

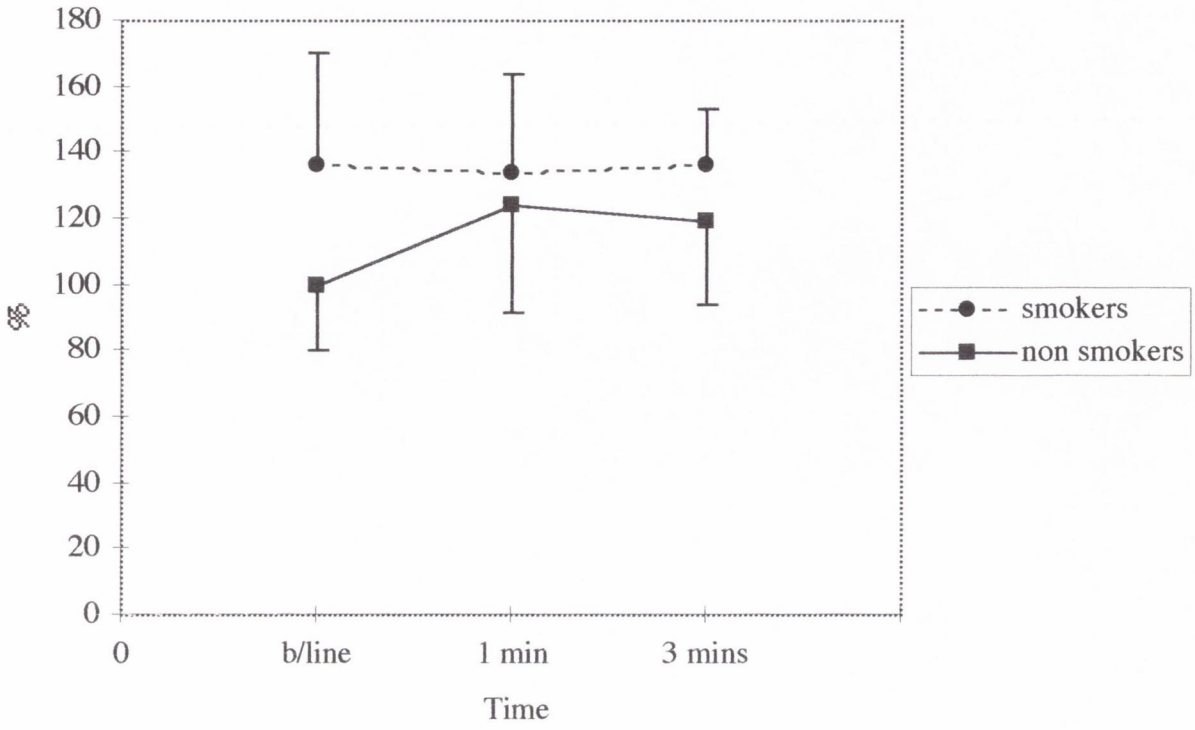


Figure 5.22 Effect of the CPT on Central Augmentation Index

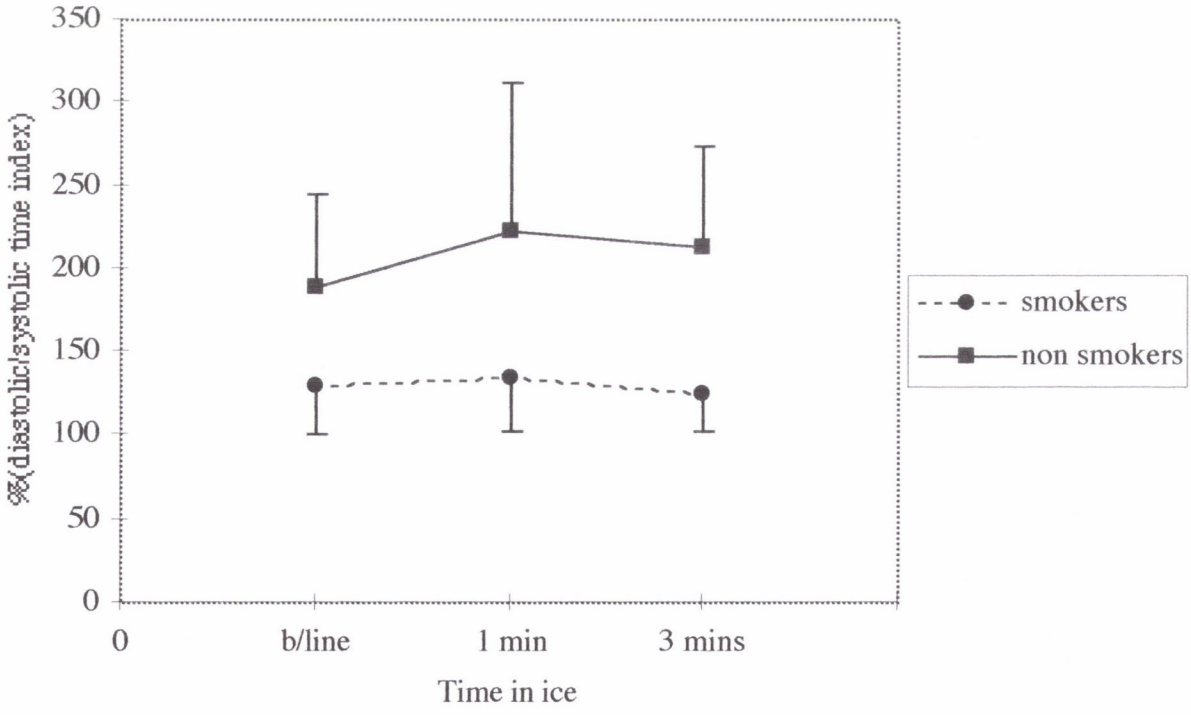


Figure 5.23 Effect of the CPT on the Buckberg Index

5.4 Discussion

The endothelium is a structurally simple yet functionally complex tissue whose integrity is essential for the health of the vessel wall. This monolayer of polygonal cells occupies a critically strategic position between body and blood, and regulates vascular mechanics from a cellular level. Endothelial injury is a central feature of cardiovascular disease induced by cigarette smoking. It is likely that smoking results in impairment of the regulation of systemic as well as regional haemodynamics. Many studies have looked at the baseline cardiac (Stork *et al.*, 1992), peripheral macrovascular (Morecraft *et al.*, 1994) and peripheral microvascular (Hashimoto *et al.*, 1994, Tur *et al.*, 1992) responses to smoking. However, simultaneous ventricular-vascular interactions in response to smoking have only recently become an area of study (Mahmud & Feely, 1999).

The aim of this chapter was to determine the dynamic interaction of the left ventricle with the arterial system of smokers at baseline and in response to a physiological cold pressor stimulus, and to investigate the effects of taurine supplementation of smokers on these parameters. Cigarette smokers are known to have dysfunctional endothelium but the repercussions from this on the whole cardiovascular tree is unknown. This chapter confirms that the interaction of the arterial system with the left ventricle in smokers at baseline is significantly different to controls. The evoked response to a physiological stress is also modified.

5.4.1: Baseline peripheral and central haemodynamic parameters.

Cigarette smoking has an adverse effect on cardiovascular interactions at baseline. As regards timing parameters, heart rate is significantly higher in smokers, as has been previously demonstrated (Hansen *et al.*, 1996). Heart rate is thought to be inversely related to left ventricular ejection time (ejection duration) (O'Rourke, 1997), as the period of time spent in the cardiac cycle is less. Although the relative proportion of

time spent in systole in much greater in smokers, simple calculations would demonstrate that, in fact, absolute time spent in systole is less: eg, at a heart rate of 60 beats per minute, time spent in diastole is approximately 0.68 seconds and that spent in systole in 3.2 seconds; at a hert rate of 80 beats per minute the time spent in diastole is 0.45 seconds and that spent in systole is 3.2 seconds.

This subendocardial viability index, the Buckberg Index, is the ratio of the aortic diastolic pressure-time integral to the aortic systolic pressure-time integral; that is, the ratio of the haemodynamic capacity for supply to the myocardial oxygen demand. The aortic pressure-time index is an acceptable substitute for the ventricular pressure-time index (Buckberg, 1972). The same could not be said for peripheral arterial derived indices where amplification of the pressure wave occurs and identification of the incisura is uncertain (the incisura is necessary for calculation of the diastolic pressure time index which assumes that the pressure falls from the incisura and rises instantaneously from the aortic wave foot) (figure 5.3(a)). One of the main determinations of this index is therefore the ejection duration, which is increased in smokers, and so the diastolic time index is particularly low in persons with short diastolic periods. In addition, the myocardial oxygen demand is increased due to increased systolic pressures. It is therefore fitting that the subendocardial viability ratio is significantly decreased in smokers.

Abnormal timing parameters, at baseline conditions in young smokers, are further compounded by the fact that indices of left ventricular systolic load, such as mean systolic and end systolic pressures are significantly higher in smokers. Left ventricular load is the pressure generated in the left ventricle and ascending aorta during ventricular ejection, and is dependent upon two factors: one is a steady mean arterial pressure, and the other is an additional pressure that the ventricle needs to generate during systole to overcome the pulsatile nature of ejection into the ascending aorta (O'Rourke, 1976). Pressure indices can represent ventricular load as ventricular ejection and myocardial oxygen demand are dependent upon the pressure against

which the heart pumps. Peripheral wave reflection may also increase left ventricular load by increasing aortic pressure in late systole. Mean and end systolic pressure values obtained in this experiment in smokers are in keeping with previous systolic pressure measurements by Hansen (Hansen *et al.*, 1996), indicating a greater haemodynamic load on the myocardium in smokers. An increase in left ventricular load requires a corresponding increase in myocardial blood flow. The result of this is a considerable decrease in myocardial blood flow reserve. This phenomenon is a normal occurrence associated with ageing (Czernin *et al.*, 1993), and is of course accentuated by the tachycardia reported in smokers.

Stork *et al.* have previously observed that cigarette smoking significantly affects left ventricular diastolic function, independently of its role as a risk factor for coronary atherosclerosis (Stork *et al.*, 1992). Abnormalities of diastolic function may result in delayed ventricular relaxation with a consequent decrease in left ventricular filling throughout diastole. It is of interest to note that individuals with diastolic dysfunction have longer ejection durations, with shorter diastolic periods (Nichols & O'Rourke, 1997), as was found in smokers. Using applanation tonometry it was found that central mean diastolic pressure was significantly elevated in smokers. This result may reflect a compensatory mechanism whereby an increase in pressure rise in the ascending aorta during diastole may increase coronary blood flow to compensate for the shorter duration of diastole, as both the pressure gradient between the aorta and the left atrium, and the duration of diastole determine coronary blood flow. This finding is also consistent with a shorter diastolic period, where a gradual decrease in aortic pressure fails to occur during ventricular diastole.

The differences in central and peripheral haemodynamics between smokers and non-smokers in terms of pressure and derived indices of ventricular load under resting and stressed conditions are consistent with reports in the literature. The changes observed in the pressure wave form are also consistent with the haemodynamic theory in that one would anticipate both increased magnitude and

speed of reflected waveforms from a constricted peripheral bed. This should in turn result in an increase in augmentation index in the smokers, as has been found in this study.

Changes in pulse wave velocity and arterial distensability result in major differences in arterial pressure wave contours. These changes are due to differences in the timing and energy content (intensity) of pulse wave reflections from peripheral sites, in particular the lower limbs. Normally, in healthy adolescents, peripheral wave relection to the ascending aorta occurs immediately after ventricular ejection, i.e. the augmentation in the ascending aorta caused by the reflected wave occurs in diastole and not systole. Wave reflection in diastole is thought to be advantageous, as this delayed pressure rise in diastole increases coronary blood flow (figure 5.2). With advancing age or arterial disease, arteries become less compliant (i.e. more stiffer) and pulse wave velocity increases. This results in an earlier return of reflected waves to the ascending aorta during ventricular ejection. Unfortunately, such timing is derimental as the reflected wave increases systolic pressure and ventricular afterload (figure 5.3) This timing of reflected waves has been described as the degree of augmentation or augmentation index, i.e. the relative height of the first systolic peak onthe arterial pressure wave contour (due to the transit of impulse generated by the heart) compared to the second systolic peak (due to the return of the reflected wave). The augmentation index (AI) can be calculated using applanation tonometry from the derived central aortic pressure waveform. The first systolic shoulder in the waveform is equivilant to peak systolic flow in the vessel. Using the differentail of the pressure wave (dP/dt) the foot of the wave after the shoulder can be identified to determine the amplitude of the second reflected wave and this can be compared to the pulse pressure to obtain the AI In the study group examined in this chapter, the AI was significantly higher in the cigarette smokers, implying that peripheral reflected waves arrive earlier than in the non smoking control group. This greater degree of reflection in smokers is due to increased generation of reflected waves from the vasoconstricted

microvasculature, decreased compliance (resulting in increased wave velocity), or a combination of both factors. Increased wave reflection early in the cardiac cycle (i.e. increased A.I.) also increases myocardial load. This is in keeping with an increase in mean and end systolic pressures, reflecting an increased left ventricular load.

5.4.2: The effect of the CPT on peripheral and central haemodynamic parameters

Using applanation tonometry, the magnitude of the peripheral response and the resultant dynamic interaction of the left ventricle with the arterial system to the CPT is assessed. The effect of the CPT on peripheral vasoactivity has been described in chapter 3 and a paradoxical dilatation of conduit vessels of non-smokers at 1 minute in ice was found. The magnitude of the peripheral response, as well as ventricular-vascular interactions, to the CPT is of interest. The response of smokers to the CPT is consistent with the responses seen earlier in the conduit vessel, an increase in blood pressure occurring at both the 1 and 3 minute time period. In the non-smokers the response to ice at 1 minute must be one of microvascular vasoconstriction as an increase in blood pressure is again observed. As might be anticipated, the magnitude of blood pressure increase in the smokers is less than that seen with non-smokers. These differences in magnitude of blood pressure change, those of the non-smoker being far greater than those of the smoker, are in keeping with higher resting tone in the smokers. This is possibly as a result of a decrease in basal release of NO, or a reflection of decreased constitutive NO synthase (cNOS) expression in the smoker.

The CPT had no effect on central haemodynamic timing parameters. The fact that there was no change in HR in response to the CPT is in agreement with some previous CPT studies (Covesh *et al.*, 1992; Frank *et al.*, 1994) and in disagreement with others (McLean *et al.*, 1991; Dubois-Rande *et al.*, 1995). Many of these studies referred to do not state exactly how the pulse rate was calculated. HR calculation in this experiment was not manually performed but the sensitive tonometric sensor

calculated and averaged the rate over 20 cycles per reading. In addition, investigators who have examined the effects of adrenoceptor blockade on the CPT responses have found that coronary (Mudge *et al.*, 1976) and peripheral vascular (Frank *et al.*, 1994) constriction are noradrenergically mediated. The increase in HR observed in other studies may represent adrenaline release. Because the investigator was known to the subject population in this study group, to whom the experimental procedure was previously explained, an adrenergically-mediated increase in HR may have been prevented. Similarly, because left ventricular ejection time is closely related to HR, no change in ED was observed.

The differential of the pressure wave (dP/dt), used to determine A.I., is significantly decreased in non smokers in response to the CPT. At 1 minute in ice, conduit vessels are known to dilate in non smokers. However, by 3 minutes in ice they are constricted. While dilatation at 1 minute may allow for increased absorption of reflected waves, this phenomenon of dilatation cannot explain decreased dP/dt at 3 minutes. The decrease in dP/dt cannot reflect a decrease in downstream pressure build-up as previous studies on digital skin temperature and laser doppler flux have shown microvascular vasoconstriction to occur (Pollock *et al.*, 1993; Koman *et al.*, 1995; Frank & Raja, 1994; Hausberg *et al.*, 1997). However, conduit artery blood flow decreases at 1 and 3 minutes in ice. It is possible that the initial conduit vasodilatatory effect is a protective mechanism by the endothelium to a physiological stress, indicating an adaptive response to protect the ventricle from a potential sudden increase in load. It is conceivable that signalling between the microcirculation and macrocirculation occurs to induce dilatation to absorb the impact of microvascular vasoconstriction and resultant pressure changes.

In the case of the smoker, absolute values of dP/dt are increased, although not significantly so, at 1 minute in ice. There is no change at 3 minutes in ice. This increase at 1 minute would signify an increase in amplitude of peripheral wave reflection. As the microvasculature is known to immediately constrict in response to

the CPT and at 1 minute in ice the macrovasculature is known to constrict in smokers, there is an increase in the amplitude of peripheral wave reflection, as demonstrated by an increased dP/dt. If the magnitude of change, at 1 minute in ice, of smokers and non smokers is examined, there is a significant difference in the direction of response to the CPT: smokers have an overall increase in dP/dt whereas non smokers have a decrease, reflecting an increase and a decrease, respectively, of amplitude of peripheral wave reflection. At 3 minutes in ice, while there is still an apparent difference in the magnitude of response between the two groups, these results are not significantly different, perhaps reflecting a degree of accommodation to the stress.

5.4.3: The effect of the taurine supplementation on peripheral and central haemodynamic parameters

The most surprising finding in this study is the paradoxical changes in blood pressure in response to taurine supplementation. In experimental animal models of hypertension (Yamamoto *et al.* 1985; Sato *et al.*, 1987; Nakagawa *et al.*, 1994; Ji *et al.*, 1995) taurine has been shown to reduce blood pressure. In human studies of taurine supplementation to borderline hypertensive males, at a greater dosage of taurine (6 gms/day for 7 days) (Fujita *et al.*, 1987) baseline systolic and diastolic pressures were also reduced. In this study, 1.5 gms taurine/day for 5 days resulted in a clinically important increase in blood pressure measurements. Interestingly, the effect on central AI is not commensurate with the observed increase in blood pressure, taurine having no effect on central AI. AI, a marker of early wave reflection and arterial stiffness would be expected to rise with an increase in blood pressure. If taurine treatment prevented this increase, findings would point towards a beneficial effect of taurine on large vessel compliance.

It is possible that the intense vasoactive effects of smoking on both the macro- and microcirculation masks a differential response of the endothelium in the two vascular beds. The changes reported earlier in flow-mediated dilatation responses are a

reflection of basal NO bioavailability in the conduit vessel. In the microvasculature the endothelium may be activated by the pro-inflammatory properties of cigarette smoking. Such activation would result in an increased synthesis of inducible NO synthase (iNOS) with a resultant tendency towards smooth muscle relaxation. Taurine has been shown to protect the microvasculature against inflammatory cell attack (Wang *et al.*, 1996; Barry *et al.*, 1997). If the same protection pertains in the smokers the net effect on the microvasculature of taurine supplementation would be one of increased vasoconstriction.

5.4.4: Discussion summary

It would therefore appear that baseline haemodynamics are upset in the cigarette smoker, both centrally and peripherally. Both baseline abnormalities and the adverse responses of smokers to a physiological stress signifies, even at this preliminary stage, abnormalities in ventricular-vascular interactions. Cigarette smoking is known to have a deleterious effect on endothelial functioning at a localised level, but this study highlights the effect of this risk factor on the whole mechanical system of the cardiovascular tree. It recognises how smoking induced damage to the endothelium, and perhaps also to the myocardial cell, can upset complex haemodynamic negative feedback mechanisms with a resultant increase in myocardial workload and a decreased capacity for coronary perfusion.

The results also suggest that the pro-inflammatory activities of cigarette smoking may interact to offset some of the adrenergic and other pressor-mediated vasoconstrictor effects. Modulation of such inflammatory response with treatment would be desirable in terms of decreased propensity for tissue damage. However, the further increase in vasoconstriction would result in an increase in resting blood pressure. The interaction between the monocyte (an inflammatory cell) and the different endothelial beds may well be the key to this problem.

The important unexpected observations obtained from this study needing further explanation include an increase in blood pressure in non-smokers at 1 minute in ice and a lesser magnitude of blood pressure response in smokers, perhaps representing a higher resting vascular tone, possibly due to decreased availability of NO (perhaps cNOS). A paradoxical increase in blood pressure in response to taurine treatment, unparalleled by absence of a corresponding increase AI, were also unexpected findings and may be explained by differential functions of, and effects of taurine on, macro- and microvasculature.

Chapter 6

"Establishing a role for the monocyte in mediating endothelial dysfunction"

6.1 Introduction

The endothelium is the key determinant of vascular tone. It works as a powerful endocrine organ, the effector of which is the smooth muscle cell (SMC) which relaxes and contracts in response to endothelial-derived vasomotor substances, resulting in vessel relaxation or contraction. In the previous chapter, abnormalities of endothelial-dependent functions were demonstrated in young smokers. While the discovery by Furchgott and Zawadzki of the importance of the endothelial role in vascular relaxation proved fundamental to the understanding of cardiovascular physiology (Furchgott & Zawadzki, 1980), the development and progression of cardiovascular disease has also established a role for endothelial-derived vasoconstrictors and vasodilators. Although various endothelial-derived relaxing factors are released, endothelial-derived NO is the chief physiological regulator of basal vascular tone (Chu *et al.*, 1991; Lerman & Burnett, 1992). Using the non-essential amino acid L-Arginine as a substrate, NO is synthesised by the endothelium through oxidation of the guanidino nitrogen of arginine by constitutive nitric oxide synthase (c-NOS). Taurine supplementation of smokers restored endothelial function, as described by loss of flow-dependent dilatation. Taurine supplementation also had a significant effect on the response of smokers to the cold pressor test. These responses were not however normalised.

Fundamental to absolute regulation of cardiovascular tone, the endothelium also produces a peptide, endothelin-1 (ET-1), which results in sustained contraction of arterial and venous smooth muscle in vitro. ET-1 is a potent vasoconstrictor and growth promoter which has a short plasma half life but has a prolonged onset and duration of action (Clarke *et al.*, 1989). It is unclear whether endothelin-1 plays a role in basal physiological function, but the presence of NO decreases its release (Mitaychi *et al.*, 1989). While the physiological significance of ET-1 is well recognised in proven atherosclerotic disease (Lerman *et al.*, 1991), hypertension (Shihiri *et al.*, 1990; Kohno *et al.*, 1990; Saito *et al.*, 1990), hyperlipidaemia (Arendt *et al.*, 1990)

and congestive heart failure (Clavell *et al.*, 1993), the potential role it may play in mediating dysfunction, and exactly how it is upregulated in young cigarette smokers, is unknown.

Based on other studies (Celermajeur *et al.*, 1996) and observations in the previous chapter, it is likely that the abnormal response to an increase in blood flow in smokers is due to a decrease in NO availability. It is also possible that the abnormal responses of the endothelium of smokers to physiological stimuli may involve an increase in ET-1 production from the endothelium. As the response of the endothelium of smokers to an increase in blood flow post taurine supplementation was one of dilatation, it is therefore likely that taurine has an effect on endothelial NO, and also perhaps ET-1, production.

The exact components in cigarette smoke which have the potential to mediate these effects are too vast to target. In addition, it has been previously demonstrated that the endothelium is more likely to be injured as a result of inflammatory cell involvement (Weber *et al.*, 1996, Lehr *et al.*, 1993). Initially shown in animal studies (Gerrity, 1981), it is now well accepted that in areas susceptible to atherosclerosis, lesion formation is preceded by monocyte adherence. Unstimulated monocytes are known to be minimally cytotoxic to human umbilical vein endothelial cells (HUVECs) (Peri *et al.*, 1990), but cigarette smoking has been shown to significantly alter circulating monocyte function (Dovgan *et al.*, 1994). During early enhanced monocyte-endothelial interactions, prior to lesion formation, monocytes may play a role in modifying endothelial function through the release of soluble mediators because of close contact. An important relationship between growth factors, cytokines and endothelial dysfunction (defined as decreased endothelial dependent relaxation) has been demonstrated (Lefer & Ma, 1993), and so it is possible that cigarette smoking in young healthy adults without proven arterial disease may have an adverse effect on monocyte functioning. This in turn may lead to abnormalities of endothelial functioning, with resultant downregulation of NO and upregulation of ET-1. Based on

findings reported in chapter 3 of taurine supplementation restoring FMD in smokers, it is likely that taurine supplementation of smokers may upregulate NO production, and also perhaps downregulate ET-1 production from the endothelium. It is possible that this proposition may be mediated through the inflammatory cell, the monocyte.

The aims of this chapter therefore were 1) to investigate the effects of smokers and non-smokers on endothelial NO and ET-1 production, using the monocyte as a mediator; 2) to explore the effects of monocytes isolated from taurine-treated smokers on endothelial NO and ET-1 production; 3) to investigate a potential mechanism by which monocytes may affect endothelial NO production.

6.2 Methods

6.2.1 Subjects

Monocytes were isolated from 10 smokers pre and post supplementation with taurine, and from 9 non-smokers, aged 23 to 36 years of age. Both subject groups fitted the criteria as described in 2.2. Blood samples were obtained from smokers and non smokers on day 1. Smokers were then supplemented with 1.5 gms taurine/day (Twinlab, Ronkonkoma, N.Y.) for 5 days, and a further sample was taken on day 6.

6.2.2 Isolation of peripheral blood monocytes

This methodology is described in detail in section 2.5. Briefly, peripheral blood mononuclear cells were isolated under sterile conditions by means of a Ficoll layer gradient technique. Percentage monocytes were assessed using the Simultest Leucogate CD45/CD14 antibody and run on the FACScan (Becton Dickinson, Mountain View, CA, USA), as per 2.5.1. The FL_1 filter ($\approx$ 530 nm) was used to detect FITC labelled CD45 and the FL_2 filter ($\approx$ 565 nm) to detect phycoerythrin

(RPE) labelled CD14. A minimum number of 10, 000 events were collected and analysed on the software Lysis 11.

6.2.3 Preparation of monocyte conditioned medium

Using the monocytes obtained as per 6.2.2, cells were corrected to $2x10^6$ monocytes/ 5 mls DMEM / 0.5% foetal calf serum (FCS) / 0.1% (v/v) Penicillin/Streptomycin (P/S) solution, and were plated in flat bottomed microtitre plates (Nunc, Denmark). DMEM was then concentrated in a time (24 and 48 hours)- and concentration ($2x10^6$ monocytes)- dependent manner. Samples were obtained at both 24 and 48 hour time points and stored at -40 °C until quantitative assays were performed.

6.2.4 Isolation of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins and cultured as per 2.4.1. Endothelial cells were identified by typical phase contrast "cobblestone" morphology and by immunofluorescence staining with von Willebrand factor antigen. In all experiments reported, HUVECs were used as individual isolates between passages 3 and 5. Twenty four hours prior to experimentation, cells were washed twice with PBS and maintained in serum-free M199 medium.

6.2.5 Preparation of MCM/HUVEC co-culture

Isolated HUVECs were made up to 4×10^5 cells/ml. A 0.5 ml cell suspension of this was added to each well of a 24 well plate, previously coated with 2% gelatin, thus plating 2×10^5 cells/well. These cells were then incubated at 37°C, 5% CO^2 for 12 hours, after which the supernatant was removed from each well and 0.25 ml of

DMEM/0.25 ml MCM was added and incubated at 37°C, 5% CO^2 for 12 and 24 hours.

6.2.6 Nitric Oxide assay

Nitric Oxide (NO) production was determined indirectly by measuring the concentration of the stable end product nitrite in the supernatant of endothelial cells treated with MCM from smokers, non-smokers and taurine treated smokers using the Griess reaction. This methodology is described in detail in section 2.6.1. 100 µl of supernatant samples were added to a 24 well plate in duplicate. 100 µl of Griess reagent was then added and the samples were incubated for 10 minutes at room temperature. Griess reagents converted nitrite into a purple azo compound. A standard curve was prepared by adding 100 µl of DMEM to 8 wells in duplicate, then adding 100 µl of a 200 nM standard NaNO2 solution followed by 7 x 1:1 dilutions giving a range of 0-20 nM of nitrite. The coloured product produced from the assay (if nitrite present) was then quantified photometrically using a microplate autoreader (Microplate Autoreader EL 311, Bio-Tek Instruments INC.) at a wavelength of 550 nm, by plotting a curve of known nitrite concentration standards against known signals (optical densities), and thus determining the concentrations of unknowns.

6.2.7 Endothelin-1 assay

ET-1 was detected using an ELISA kit for ET-1 detection (R&D Systems). This methodology is described in detail in section 2.6.2. This assay involves the reaction of any ET-1 present in the sample with two antibodies directed against different epitopes of the ET-1 molecule. One antibody is coated onto the surface of the wells on the microtitre plate and the other is conjugated to the enzyme horseradish peroxidase. Any ET-1 present forms a bridge between the two antibodies. Unbound

material is removed by aspiration and washing, and the amount of ET-1 conjugate bound to the well is detected by a reaction with a substrate which yields a colour product proportional to the amount of conjugate. This coloured product can then be quantified photometrically using a microplate autoreader (Bio-Tek Instruments), by plotting a curve of known ET-1 concentration standards against known signals (optical densities), and thus determining the concentrations of unknowns.

6.2.8 Western Blot for constitutive NO synthase (c-NOS) expression.

The methods used are described in detail in Section 2.7. Briefly, HUVECs were cultured, trypsinised at the 2nd passage, incubated with MCM from each sample group for 6 hours and then endothelial cytosolic proteins were extracted. The BCA assay was then used to photometrically quantify the cytoplasmic protein content. The proteins were separated using SDS-PAGE and the gel was transferred to a nitrocellulose membrane for labelling using a semi-dry blotter. To block non-specific binding of the monoclonal antibody, the nitrocellulose was incubated with blocking buffer. It was then incubated with the primary antibody, a mouse anti-human IgG1 monoclonal antibody specific for cNOS (Transduction Laboratories, Lexington, KY, USA) overnight. The secondary antibody, IgG1 antimouse conjugated to alkaline phosphatase, was then added and incubated for 2 hours. Finally, the gel was incubated with substrate buffer until the colour developed. The reaction was stopped and the nitrocellulose membrane was allowed to dry. This was then examined for bands corresponding to the molecular weight marker of 140 kDa, specific for cNOS. Results obtained were then quantified using densitometry.

6.3 Results

6.3.1 The effect of monocyte conditioned medium from smokers, non smokers and taurine supplemented smokers on endotheial NO production: medium conditioned with monocytes for 24 hours and co-incubated with HUVEC for 24 hours.

The effect of monocyte-conditioned medium from smokers and non-smokers on endothelial NO production, using medium conditioned with monocytes for 24 hours, and co-incubated with HUVEC for 24 hours, was measured using the Griess reaction.

Monocytes isolated from smokers (n=10) had a down-regulatory effect on endothelial NO production in comparison to non-smokers (n=9): 0.002(±.003) nM nitrite (smokers) versus 0.114(±0.02) nM nitrite (non smokers): (*p=0.0004; Students 2-sample t-test: figure 6.1). The effect of taurine supplementation of smokers on endothelial NO production was also measured. Again, medium was conditioned with monocytes for 24 hours, and co-incubated with HUVEC for 24 hours. Monocytes isolated from smokers treated with taurine (n=10) upregulated endothelial NO production in comparison to pre-treatment: 0.187(±.022) nM nitrite obtained post supplementation with taurine versus 0.002(±0.003) nM nitrite obtained pre supplementation: (*p≤0.0001; Students paired t-test: figure 6.1).

6.3.2 The effect of monocyte conditioned medium from smokers, non smokers and taurine supplemented smokers on endotheial NO production: medium conditioned with monocytes for 48 hours and co-incubated with HUVEC for 24 hours.

The effect of monocyte-conditioned medium from smokers and non-smokers on endothelial NO production, using medium conditioned with monocytes for 48

hours, and co-incubated with HUVEC for 24 hours was also measured using the Griess reaction. Monocytes isolated from smokers (n=10) had a down-regulatory effect on endothelial NO production in comparison to non-smokers (n=9): 0.007(±.011) nM nitrite (smokers) versus 0.099(±0.05) nM nitrite (non smokers): (*p=0.02; Students 2-sample t-test: figure 6.2). The effect of taurine supplementation of smokers on endothelial NO production was also measured. Medium was conditioned with monocytes from smokers (n=10) and taurine-treated smokers (n=10) for 48 hours and co-incubated with HUVEC for 24 hours. Monocytes isolated from smokers treated with taurine upregulated endothelial NO production in comparison to pre treatment: 0.164(±.04) nM nitrite (post supplementation) versus 0.007(±0.01) nM nitrite (pre treatment): (*p≤0.0001; Students paired t-test: figure 6.2).

6.3.3 The effect of monocyte conditioned medium from smokers, non smokers and taurine supplemented smokers on endotheial NO production: medium conditioned with monocytes for 24 hours and co-incubated with HUVEC for 48 hours.

The effect of monocyte-conditioned medium from smokers and non-smokers on endothelial NO production using medium conditioned with monocytes for 24 hours, and co-incubated with HUVEC for 48 hours was then measured. Monocytes isolated from smokers (n=10) had a down-regulatory effect on endothelial NO production in comparison to non-smokers (n=9): 0.014(±.018) nM nitrite versus 0.183(±0.03) nM nitrite: (*p≤0.0001; Students 2-sample t-test: figure 6.3). Medium conditioned for 24 hours with monocytes isolated from smokers treated with taurine, and co-incubated with HUVEC for 48 hours upregulated endothelial NO production in comparison to MCM from the same subjects pre treatment: 0.39(±.09) nM nitrite post

supplementation versus 0.014(±0.018) nM nitrite pre treatment: (*p≤0.0001; Students paired t-test: figure 6.3).

6.3.4 The effect of monocyte conditioned medium from smokers, non smokers and taurine supplemented smokers on endotheial NO production: medium conditioned with monocytes for 48 hours and co-incubated with HUVEC for 48 hours.

The effect of monocyte-conditioned medium from smokers (n=10) and non-smokers (n=9) on endothelial NO production using medium conditioned with monocytes for 48 hours, and co-incubated with HUVEC for 48 hours again demonstrated monocytes isolated from smokers to have a down-regulatory effect on endothelial NO production in comparison to non-smokers: 0.017(±.015) nM nitrite versus 0.228(±0.07) nM nitrite: (*p=0.022; Students 2-sample t-test: figure 6.4). The effect of monocyte-conditioned medium from these same smokers treated with taurine (n=10), again conditioned with monocytes for 48 hours and co-incubated with HUVEC for 48 hours, once again demonstrated that taurine upregulates endothelial NO production in comparison to pre-treatment: 0.44(±.09) nM nitrite post supplementation with taurine versus 0.017(±0.015) nM nitrite pre supplementation: (*p≤0.0001; Students paired t-test: figure 6.4).

6.3.5 The effect of monocyte conditioned medium from smokers, non smokers and taurine supplemented smokers on endotheial ET-1 production: medium conditioned with monocytes for 24 hours and co-incubated with HUVEC for 24 hours.

The effect of monocyte-conditioned medium from smokers and non-smokers on endothelial ET-1 production, with medium conditioned by monocytes for 24 hours and co-incubated with HUVEC for 24 hours was measured using an ELISA test.

Monocytes isolated from smokers upregulated endothelial ET-1 production in comparison to non-smokers: 68.54(±8.1) ρg/ml versus 32.9(±3.9) ρg/ml: (*p=0.004; Students 2-sample t-test: figure 6.5). The effect of monocyte-conditioned medium from these same smokers treated with taurine on endothelial ET-1 production, the medium conditioned with monocytes for 24 hours and co-incubated with HUVEC for 24 hours, demonstrated that monocytes isolated from smokers treated with taurine downregulated endothelial ET-1 production in comparison to pre-treatment: 31.3(±6.5) ρg/ml post treatment versus 68.5(±8.14) ρg/ml pre supplementation: (*p=0.01; Students paired t-test: figure 6.5).

6.3.6 The effect of monocyte conditioned medium from smokers, non smokers and taurine supplemented smokers on endotheial ET-1 production: medium conditioned with monocytes for 24 hours and co-incubated with HUVEC for 48 hours.

The effect of monocyte-conditioned medium from smokers (n=10) and non-smokers (n=9) on endothelial ET-1 production, with medium conditioned with monocytes for 24 hours, and co-incubated with HUVEC for 48 hours was also investigated using an ELISA test. Monocytes isolated from smokers upregulated endothelial ET-1 production in comparison to non-smokers: 61.96(±11.2) ρg/ml (in smokers) versus 37.2(±5.5) ρg/ml (in non smokers): (*p=0.0026; Students 2-sample t-test: figure 6.6). The effect of monocyte-conditioned medium from these same smokers treated with taurine (n=10) on endothelial ET-1 production was then investigated, the medium conditioned with monocytes for 24 hours, and co-incubated with HUVEC for 48 hours. Monocytes isolated from smokers treated with taurine downregulated endothelial ET-1 production in comparison to pre-treatment: 33.4(±4)

ρg/ml post supplementation versus 61.96(±11.28) ρg/ml pre treatment: (*p=0.0066; Students paired t-test: figure 6.6).

6.3.7 To investigate the effect of MCM from smokers, non-smokers and smokers treated with taurine on endothelial c-NOS expression, a western blot to demonstrate cNOS protein expression was carried out. Medium was conditioned with monocytes for 24 hours, as per 2.5.2. HUVECs were cultured as per 2.2.4. They were then cultured with MCM, as per 2.7.1. Briefly, 15 x 10^6 HUVECs were obtained, made up to 20 mls with DMEM, and divided into 4 polypropylene tubes. To each tube, 1.5 mls of MCM (5 x 10^5 cells/ml) were added to each tube and made up to 10 mls with DMEM. Samples were incubated @ 37 ^{0}C, 4% CO_2 for 6 hours. A subsequent western blot was performed as per 2.7.

MCM of non-smokers and taurine treated smokers upregulated cNOS expression in HUVECs in comparison to MCM of smokers. (see figure 6.7).

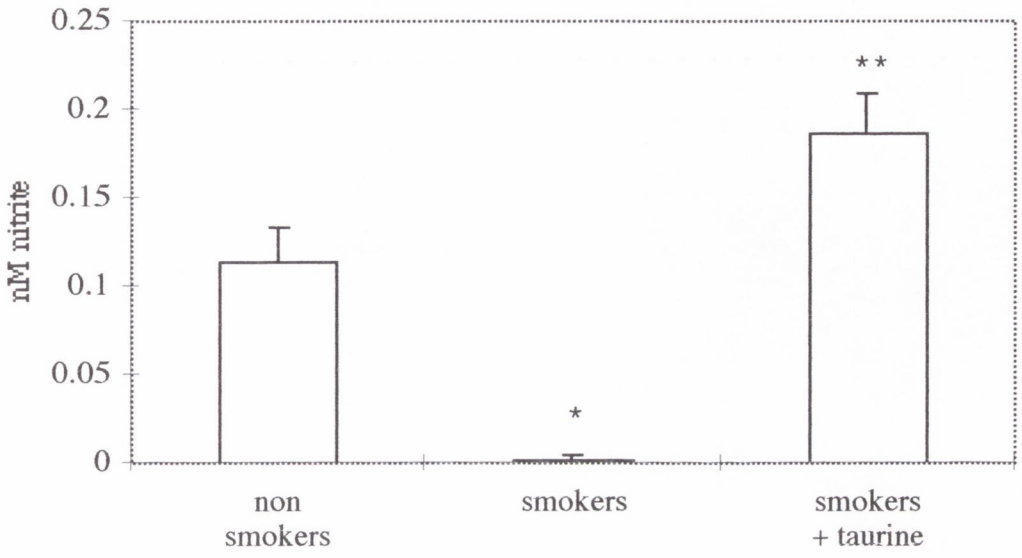


Figure 6.1 The effect of medium conditioned with monocytes from smokers (n=10), non smokers (n=9) and taurine treated smokers (n=10) for 24 hours, and co-incubated with HUVEC for 24 hours, on endothelial NO release, as assessed by nitrite production: *p=0.0004 vs. non-smokers, Students 2-sample t-test; **p≤0.0001 vs. post treatment of same group, Students paired t-test.

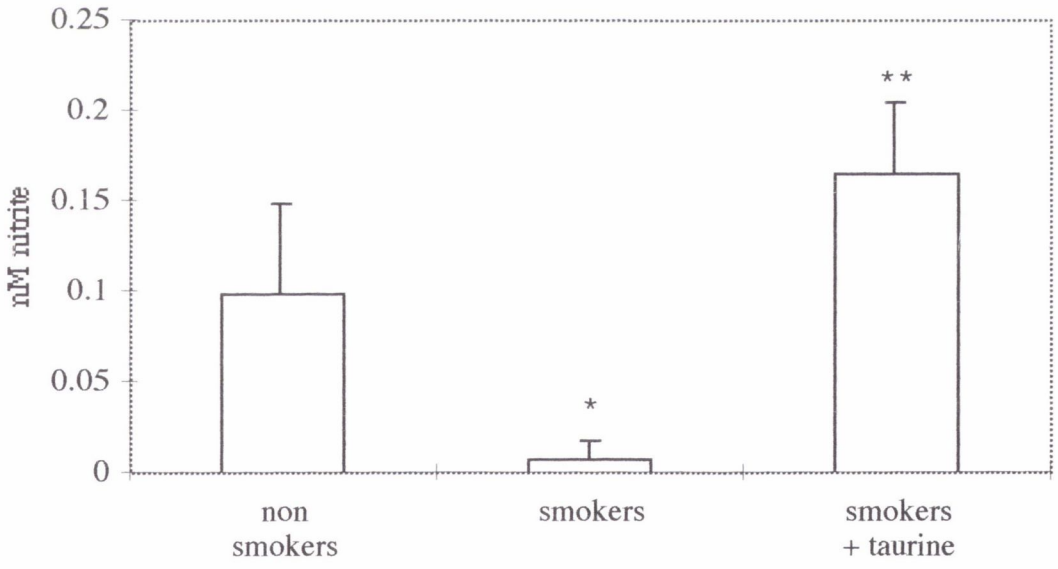


Figure 6.2 The effect of medium conditioned with monocytes from smokers (n=10), non smokers (n=9) and taurine treated smokers (n=10) for 48 hours, and co-incubated with HUVEC for 24 hours, on endothelial NO release, as assessed by nitrite production: *p=0.02 vs. non-smokers, Students 2-sample t-test; **p≤0.0001 vs. post treatment of same group, Students paired t-test.

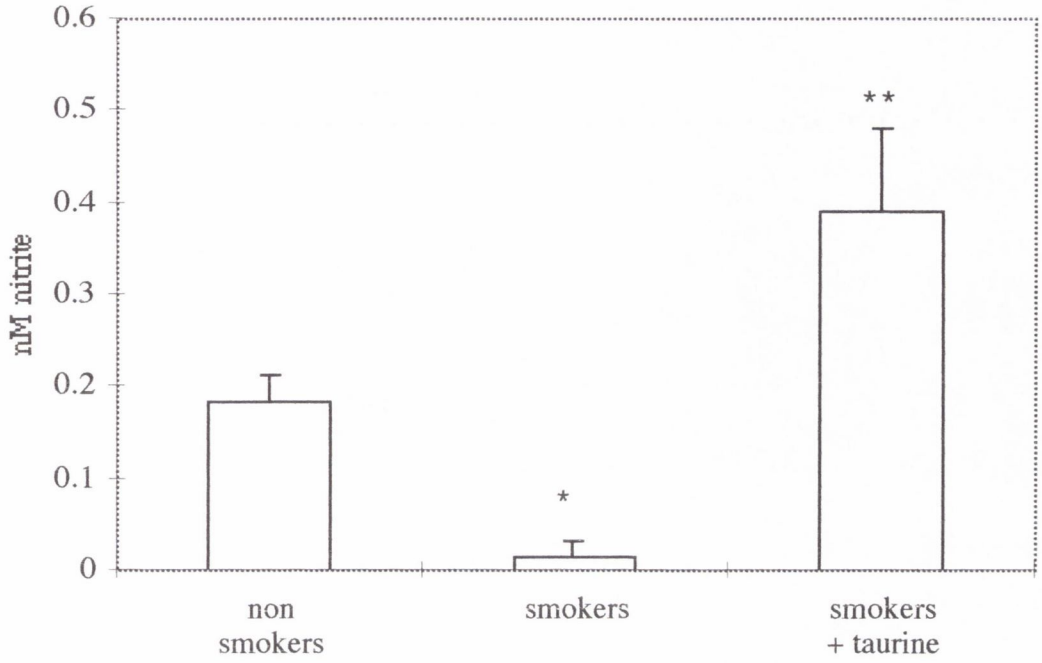


Figure 6.3 The effect of medium conditioned with monocytes from smokers (n=10), non smokers (n=9) and taurine treated smokers (n=10) for 24 hours, and co-incubated with HUVEC for 48 hours, on endothelial NO release, as assessed by nitrite production: *p≤0.0001vs. non-smokers, Students 2-sample t-test; **p≤0.0001 vs. post treatment of same group, Students paired t-test.

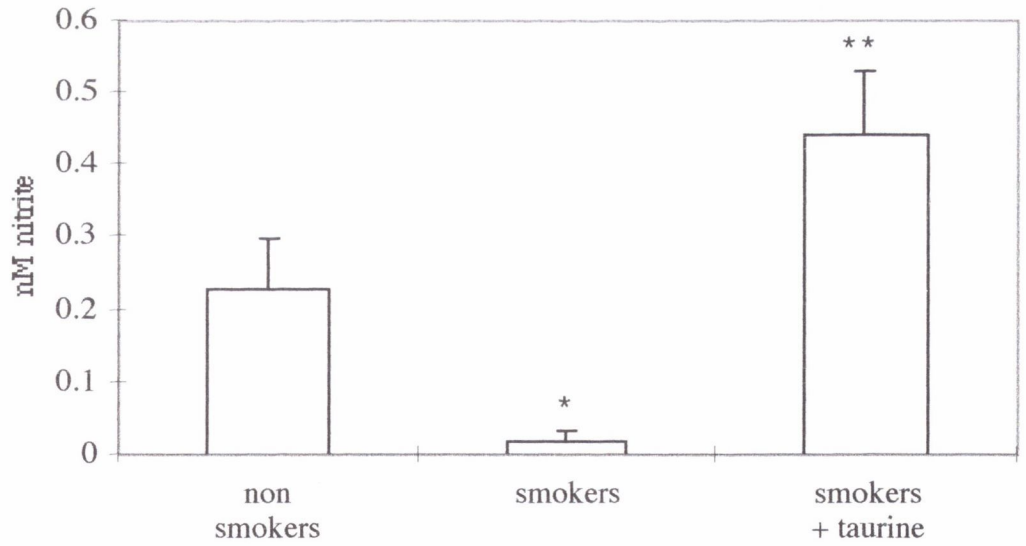


Figure 6.4 The effect of medium conditioned with monocytes from smokers (n=10), non smokers (n=9) and taurine treated smokers (n=10) for 48 hours, and co-incubated with HUVEC for 48 hours, on endothelial NO release, as assessed by nitrite production: *p=0.022 vs. non-smokers, Students 2-sample t-test; **p≤0.0001 vs. post treatment of same group, Students paired t-test.

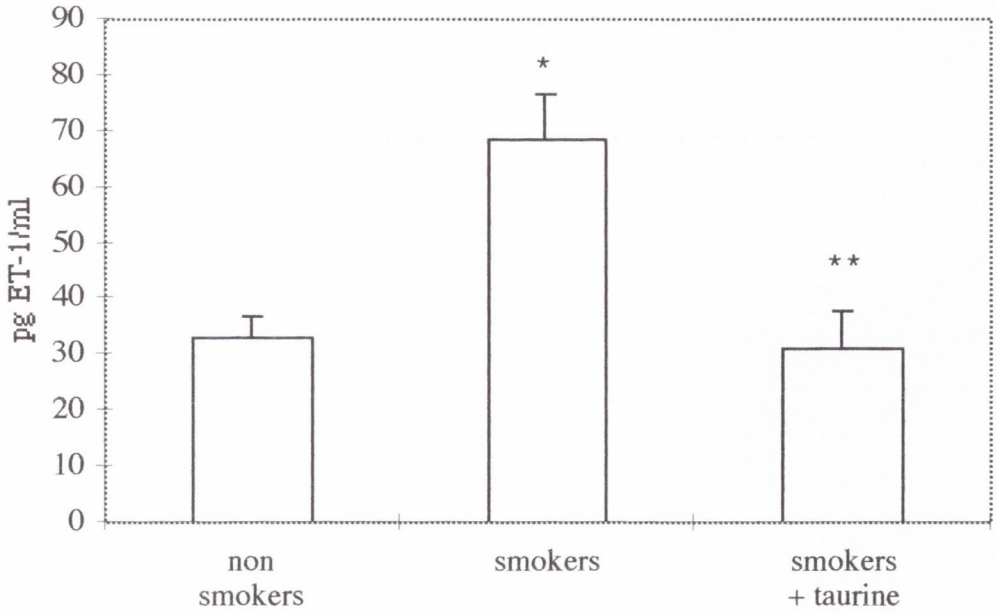


Figure 6.5 The effect of medium conditioned with monocytes from smokers (n=10), non smokers (n=9) and taurine treated smokers (n=10) for 24 hours, and co-incubated with HUVEC for 24 hours, on endothelial ET-1 release: *p=0.004 vs. non- smokers, Students 2-sample t-test; **p=0.01 vs. post treatment of same group, Students paired t-test.

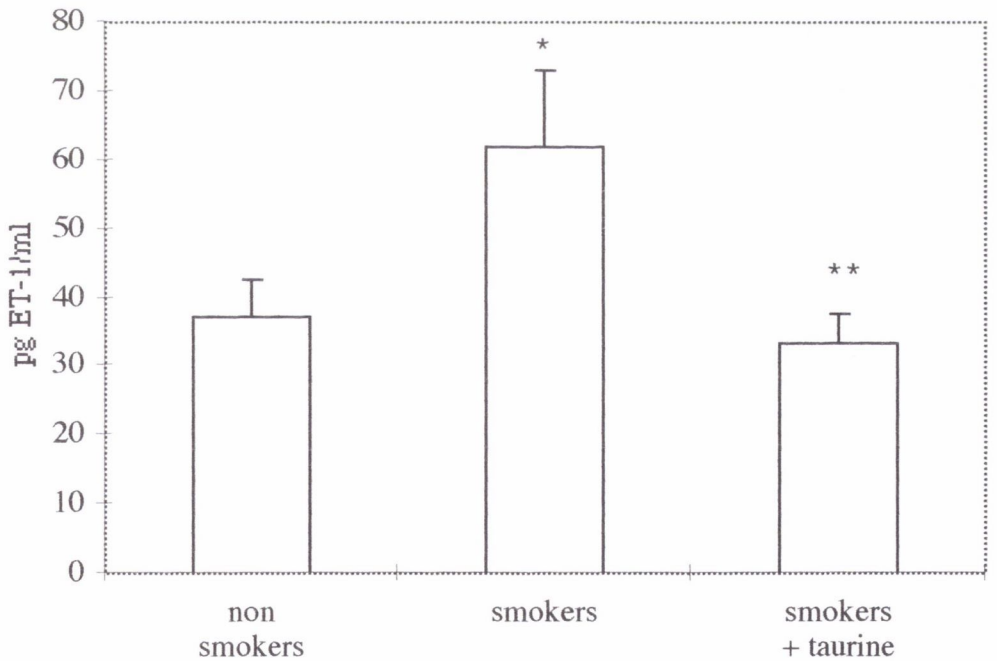


Figure 6.6 The effect of medium conditioned with monocytes from smokers (n=10), non smokers (n=9) and taurine treated smokers (n=10) for 24 hours, and co-incubated with HUVEC for 48 hours, on endothelial ET-1 release: *p=0.0026 vs. non-smokers, Students 2-sample t-test; **p=0.0026 vs. post treatment of same group, Students paired t-test.

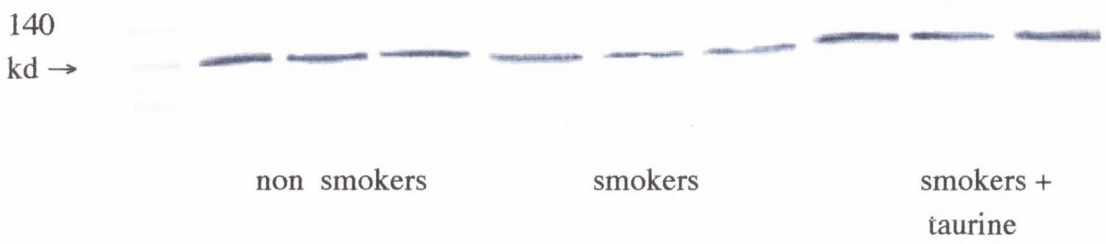


Figure 6.7 Photograph of a western blot depicting cNOS expression on HUVEC after co-incubation with MCM from non-smokers (first 3 lanes), smokers (second 3 lanes) and these same smokers treated with taurine (last 3 lanes).

6.4 Discussion

Flow-mediated endothelial dependent dilatation is impaired in young smokers and in subjects with known risk factors for atherogenesis, and enhanced monocyte-endothelial interactions are implicated in the pathogenesis of this disease process. The aim of these experiments, was to investigate the role for the monocyte in endothelial dysfunction. It was hypothesised that monocytes from smokers release soluble mediators which in turn affect the production of NO and ET-1, and that this effect would be attenuated by 5 days supplementation with taurine at a dose of 1.5 gms/day for 5 days. After 24 and 48 hours of incubation with monocyte-conditioned medium of smokers, non-smokes and taurine-treated smokers, endothelial production of NO and ET-1 was measured.

Results obtained here provide evidence that monocyte functioning and its interaction with the endothelium is abnormal in smokers. It is demonstrated that conditioned medium of monocytes from young healthy smokers significantly attenuates NO release and upregulates ET-1 release from normal endothelium. Taurine supplementation of smokers restores levels of NO and ET-1 production back to non-smoking control levels. Furthermore, the changes seen in NO release are mirrored by the expression of cNOS, the constitutive form of NO synthase which regulates basal NO generation.

Recent intense research has focused on the effect of smoking on monocyte adherence to the endothelium, an event which promotes the pathway in which the initial smoke-induced inflammatory response in atherogenesis occurs. However, endothelial dysfunction antedates plaque formation and results here would suggest that the monocyte plays a role in mediating the initial physiological dysfunction. From these results it could be argued that the release of soluble mediators, and not the actual adhesion and subendothelial migration of monocytes, is the key factor in mediating the initial endothelial functional abnormalities; monocyte adhesion being required to

initiate anatomical abnormalities and to provide close proximity of the endothelium to the released soluble mediators, allowing for functional abnormalities to continue. It is of interest to note that in patients with proven atherosclerosis, monocyte adhesiveness was been shown to be unrelated to the severity of their disease, but to the presence of risk factors, which include smoking (Duplaa *et al.*, 1993). Cigarette smoking therefore has an obvious effect on pathological monocyte adhesion and plaque formation, but the severity of arterial disease must be governed by factors other than the anatomical alignment of the monocyte. It is possible that soluble mediators, such as those released into the circulation directly from the cigarette smoke or from cellular mediators affected by the smokers, such as circulating monocytes, may have an effect on disease severity; unstimulated monocytes having no cytotoxic effect on cultured endothelial cells (Peri *et al.*, 1990).

Having established that MCM of smokers downregulates endothelial NO release, a potential mechanism by which this effect took place was investigated. Theoretically, it is possible that the decreased amount detected may be due to increased breakdown or decreased production. Under certain conditions, activated monocytes have been shown to generate partially reduced reactive oxygen species (Munn & Armstrong, 1993; Dukes at al., 1993). An increase in superoxide or hydrogen peroxide could therefore increase NO breakdown; however, it has previously been discovered that reactive oxygen species do not to play a role in mediating the inhibitory effects of monocytes on NO release (Marczin *et al.*, 1996).

Basal endothelial NO production is regulated by the enzyme constitutive nitric oxide synthase (cNOS). Current perspectives on decreased NO availability, the primary process involved in the pathogenesis of endothelial dysfunction, point towards a reduction in NO production (Wever *et al.*, 1998). A rate-limiting concentration of extracellular L-arginine, the cNOS substrate, is an unlikely cause for this decrease in NO as intracellular concentrations of L-arginine far exceed the K_m value of cNOS (Arnal *et al.*, 1995), and addition of L-arginine to monocyte-

endothelial cultures does not increase NO production (Marczin *et al.*, 1996). We therefore evaluated basal expression of cNOS, and results obtained show that it is downregulation of cNOS expression that is responsible for the decreased endothelial NO production of the endothelium in response to MCM of smokers. This observation is supported by a recent study in atherosclerotic disease patients where a decrease in NO production was found to be secondary to a decrease in cNOS expression (Oemar *et al.*, 1998).

Cigarette smoking has been shown to increase plasma ET-1 levels, establishing it as a potential mediator of vasoconstriction and a risk factor for atherosclerosis. However, the exact mediator responsible for the stimulatory effect of ET-1 production is unknown. Nicotine has been ruled out (Goerre *et al.*, 1995), but other smoke components, either by directly acting on the endothelium or by modifying blood components have not been identified.

Results obtained here show that MCM of smokers upregulates ET-1 production from normal endothelium. There are many possible ways in which the monocyte may play a role in modifying endothelial ET-1 production. In normally functioning endothelium, it has been demonstrated that hypoxic conditions upregulate ET-1 expression and secretion (Kourembanas *et al.*, 1991). However, adequate NO production can modify this response at the level of transcription (Kourembanas *et al.*, 1993). These studies are of interest in the setting of the cigarette smoking environment, where the endothelium is dysfunctional and NO production is reduced. An hypoxic-induced increase in ET-1 expression could therefore result in a greater baseline ET-1 secretion. It is possible that MCM of smokers may create a more hypoxic endothelial environment which may directly, or indirectly by upregulating cytokine production, affect ET-1 release. Monokines from MCM may also have a indirect effect on endothelial ET-1 release by directly downregulating NO.

Taurine supplementation of smokers has a significant effect on monocyte functioning. While conditioned medium of monocytes isolated from smokers reduced

endothelial NO production, treatment of these same smokers with taurine significantly attenuated this inhibitory effect. In addition, the upregulatory effect of conditioned medium of monocytes on endothelial ET-1 production was abrogated with taurine supplementation. Results obtained here show that treating smokers with taurine increased NO production through upregulation of cNOS expression, which was mediated through modification of monocyte function.

In vitro studies have shown that taurine is released into the extracellular medium by human leukocytes (Grisham *et al.*, 1984; Thomas *et al.*, 1983; Test *et al.*, 1984). Therefore, it could be argued that taurine supplementation may increase taurine release by monocytes into the extracellular medium. This is theory could upregulate cNOS by regulating intracellular Ca^{2+} fluctuations, increases in intracellular Ca^{2+} being required for cNOS expression. However, results reported in chapter X in this thesis disprove this theory, as plasma taurine levels are higher in the smoking group, and preliminary studies would point towards a decreased intracellular uptake of taurine by monocytes and neutrophils of smokers from extracellular medium. The effects of smokers and non-smokers MCM on endothelial NO and ET-1 production, and the subsequent effects of taurine treatment on these parameters, are more likely to be explained by differences in monocyte-derived secretory products and the modification thereof.

Results reported in this chapter provide a role for the monocyte in modifying endothelial release of NO and ET-1. These results may therefore offer a possible contributory role for the monocyte in mediating endothelial dysfunction, as described by loss of flow-mediated dilatation. However, a role of the monocyte in mediating the response of smokers to the cold pressor test may also be possible. Downregulation of cNOS by MCM of smokers could potentially play a role in preventing the initial stimulus-induced $\alpha 2$-mediated NO release. Once supplemented by taurine, however, NO release should allow for return of a normal response, unless the $\alpha 2$ receptor is in

some way modified by smoking also. In addition, experimental evidence has shown there to be an upregulatory effect of ET-1 on noradrenaline and adrenaline release (Wilkes & Boarder, 1991; Sone *et al.*, 1991; Yang *et al.*, 1990). This interaction may play a role in the cold pressor response of smokers, if baseline ET-1 is increased in an hypoxic environment and unchecked by NO, and the cold pressor-induced increase in circulating catecholamines is increased further still in comparison to non-smokers by a baseline stimulatory effect of ET-1. Once supplemented with taurine for 5 days, endothelial NO and ET-1 production are restored to control non-smoking levels. While freshly isolated monocytes from taurine treated smokers can successfully decrease cultured endothelial ET-1 levels back to those of controls, this modification may take longer in the *in vivo* situation. This may be because NO modifies ET-1 at the transcription level and therefore the inhibitory effect of NO on ET-1 production may not yet be in full effect. In the *in vivo* situation, endothelial cells in which modification at the transcription level is not yet fully complete, may produce higher levels of MCM-induced ET-1. Therefore, increased ET-1 may still be resulting in increased amounts of circulating catecholamines, thereby preventing the return of the cold pressor response to normal.

It is evident from this chapter that monocyte plays a role in the initial stages of smoking-induced arterial disease that antedate plaque formation, that is, endothelial dysfunction. Soluble mediators released from monocytes modulate endothelial production of vasoactive agents, which are responsible for control of vessel tone and optimal perfusion. Modification by soluble mediators of monocytes of the chief regulator of vascular tone, NO, is at the level of enzyme expression. Supplementation of smokers with the amino acid taurine can normalise the effects of monocytes on endothelial production of vasoactive agents. It is therefore evident from this chapter that modification of monocyte functioning is necessary in arterial disease management. Restoration of endothelial NO production by taurine is due to an increase in cNOS expression. This finding offers hope for both preventative and management strategies

in endothelial dysfunction patients and also those with proven arterial disease, where a decrease in NO is secondary to a decrease in cNOS expression (Oemar *et al.*, 1998).

Chapter 7

"Establishing a role for an interaction between monocyte-conditioned medium and the microvasculature in mediating abnormalities in haemodynamic parameters in smokers"

7.1 Introduction

The previous chapter has demonstrated discrepancies to be present in baseline central haemodynamic parameters between young smokers and control non-smokers. Considering that peripheral vascular resistance plays a central role in blood pressure determination, arteriolar resistance vessels acting as a source of pressure dissipation, it is possible that the microvasculature may play a role in mediating the observed haemodynamics.

The microvasculature is a regulatory environment distinct from that of the macrovasculature. While supplementation of smokers with taurine increased central blood pressure parameters, isolation of monocytes from smokers treated with taurine induced an increase in NO production from the macrovasculature. The same, however, may not be true for the effect on the microvasculature. Heterogeneity among endothelial cell populations is substantial, and site-specific NO generation is known to occur (Geiger *et al.*, 1997). It is therefore possible that discrepancies in baseline blood pressure haemodynamics between young smokers and controls may be explained by differences in microvascular NO production.

The aim of this chapter was therefore to investigate the effect of smoking, through the medium of monocyte-conditioned medium (MCM), on microvascular endothelial NO production as an explanation for the observed discrepancies in blood pressure between smokers, non-smokers and taurine-treated smokers.

7.2 Methodology

7.2.1 Subjects

Monocytes were isolated from 5 smokers pre and post supplementation with taurine, and from 5 non-smokers, aged 23 to 36 years of age. These subjects were those recruited in the prior studies, as described in 2.2. Smokers were supplemented with 1.5 gms taurine/day (Twinlab, Ronkonkoma, N.Y.) for 5 days. Blood samples were taken on day 1 in smokers and non smokers and on day 6 post taurine supplementation in smokers also.

7.2.2 Isolation of peripheral blood monocytes

Monocytes were isolated as described in 2.5. Briefly, peripheral blood mononuclear cells were isolated from healthy volunteer controls under sterile conditions by means of a Ficoll layer gradient technique. Percentage monocytes was assessed using the Simultest Leucogate CD45/CD14 antibody and run on the FACScan (Becton Dickinson, Mountain View, CA, USA) using the FL_1 filter ($\approx$ 530 nm) to detect FITC labelled CD45 and the FL_2 filter ($\approx$ 565 nm) to detect phycoerythrin (RPE) labelled CD14. A minimum number of 10, 000 events were collected and analysed on the software Lysis 11.

7.2.3 Preparation of monocyte conditioned medium

Using the monocytes obtained as per 7.2.2, cells were corrected to $2x10^6$ monocytes/ 5 mls DMEM / 0.5% foetal calf serum (FCS) / 0.1% (v/v) Penicillin/Streptomycin (P/S) solution, and were plated in flat bottomed microtitre plates (Nunc, Denmark). DMEM was then concentrated in a time (24 and 48 hours)- and concentration ($2x10^6$ monocytes)- dependent manner. Samples were obtained at both 24 and 48 hour time points and stored at -40 °C until quantitative assays were performed.

7.2.4 Cultivation of human microvascular endothelial cells (HMVECs).

HMVECs were routinely cultured as per 2.4.2. Briefly, primary cultures of HMVECs were obtained from BioWhittaker (Niederlassung, Germany). The manufacturer supplied endothelial cell growth medium containing the following supplements: hydrocortisone, hFGF-ß, R3-IGF, ascorbic acid, heparin, VEGF, hEGF, GA-100 and 5% (v/v) FBS. HBSS, trypsin/EDTA and trypsin neutralising solution were also supplied with the cells. The medium was changed every two days. HMVEC cell cultures are irreversibly contact inhibited and therefore were subcultured before they reached 85% confluency.

7.2.5 Preparation of HMVECs for NO measurement

HMVECs were prepared. 4 x 10^5 cells/ml were counted. 500µl of cell suspension was added into each well of the 24-well plate (12 mls total), to give a concentration of 2 x 10^5 cells/well. These cells were incubated for 6 hours @ 37^0C, 5% CO_2. The supernatant was then removed and a mixture of 100µl MCM and 500µl medium was added and incubated for 24 hours @ 37^0C, 5% CO_2. The supernatant was then removed at 24 hours and NO was measured.

7.2.6 Nitric Oxide assay

This methodology is described in detail in 2.6.1. Briefly, nitric oxide production was determined indirectly by measuring concentration of the stable end product nitrite in the supernatant of endothelial cells treated with MCM from smokers, non-smokers and taurine treated smokers using the Griess reaction. Briefly, 100 µl of supernatant sample was added to a 24 well plate in duplicate and 100 µl Griess reagent

was then added and the samples were incubated for 10 minutes at room temperature. A standard curve was prepared and the coloured product produced from the assay (if nitrite present) was then quantified photometrically using a microplate autoreader (BioTech Instruments) at a wavelength of 550 nm, by plotting a curve of known nitrite concentration standards against known signals (optical densities), and from this determining the concentrations of unknowns.

7.3 Results

7.3.1 The effect of monocyte conditioned medium from smokers, non smokers and taurine treated smokers on microvascular NO production: medium conditioned with monocytes for 24 hours and co-incubated with HMVECs for 24 hours.

The effect of monocyte-conditioned medium from smokers (n=8) and non-smokers (n=8) on microvascular NO production, using medium conditioned with monocytes for 24 hours and co-incubated with HMVECs for 24 hours was investigated and measured using the Griess reaction. Monocytes isolated from smokers had an up-regulatory effect on microvascular endothelial NO production in comparison to non-smokers: 0.048(±.03) nm nitrite (smokers) versus 0.015(±0.014) nm nitrite (non smokers): (*p=0.02; Students 2-sample t-test: figure 7.1). The effect of monocyte-conditioned medium from smokers and these same smokers treated with taurine (n=7) on microvascular NO production was then investigated. The medium was again conditioned with monocytes for 24 hours, and co-incubated with HMVECs for 24 hours. Monocytes isolated from smokers treated with taurine had a down-regulatory effect on endothelial NO production in comparison to baseline smokers: 0.016(±.009) nm nitrite post treatment versus 0.05(±0.023) nm nitrite pre-treatment: (*p= 0.046; Students 2-sample t-test: figure 7.1).

7.3.2 The effect of monocyte conditioned medium from smokers, non smokers and taurine treated smokers on microvascular NO production: medium conditioned with monocytes for 24 hours and co-incubated with HMVECs for 48 hours.

The effect of monocyte-conditioned medium from smokers and non-smokers on microvascular NO production using medium conditioned with monocytes for 24 hours, and co-incubated with HMVECs for 48 hours was then measured. Monocytes isolated from smokers (n=8) had an up-regulatory effect on endothelial NO production in comparison to non-smokers (n=8): 0.017(±.003) nm nitrite in smokers versus 0.003(±0.003) nm nitrite in non smokers: (*p= 0.0002; Students 2-sample t-test: figure 7.2). The effect of monocyte-conditioned medium from smokers and the same smokers treated with taurine (n=7) on microvascular NO production was subsequently investigated using medium conditioned with monocytes for 24 hours, and co-incubated with HMVECs for 48 hours. Monocytes isolated from smokers treated with taurine had a down-regulatory effect on endothelial NO production in comparison to baseline effects of smokers: 0.010(±.003) nm nitrite post supplementation versus 0.02(±0.004) nm nitrite pre-treatment: (*p= 0.03; Students 2-sample t-test: figure 7.2).

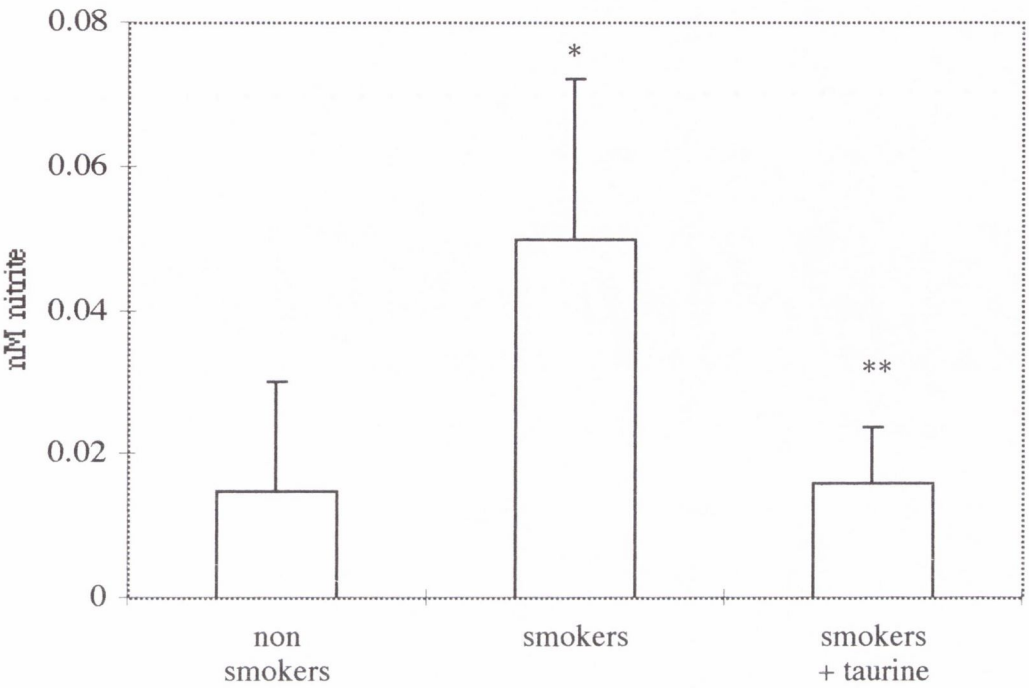


Figure 7.1 The effect of medium conditioned with monocytes for 24 hours from non smokers (n=8), smokers (n=8) and the same smokers treated with taurine (n=7) on microvascular endothelial NO production, after 24 hours incubation,: *p=0.02 vs. non-smokers, Students 2-sample t-test; **p= 0.046 vs. smokers, Students 2-sample t-test.

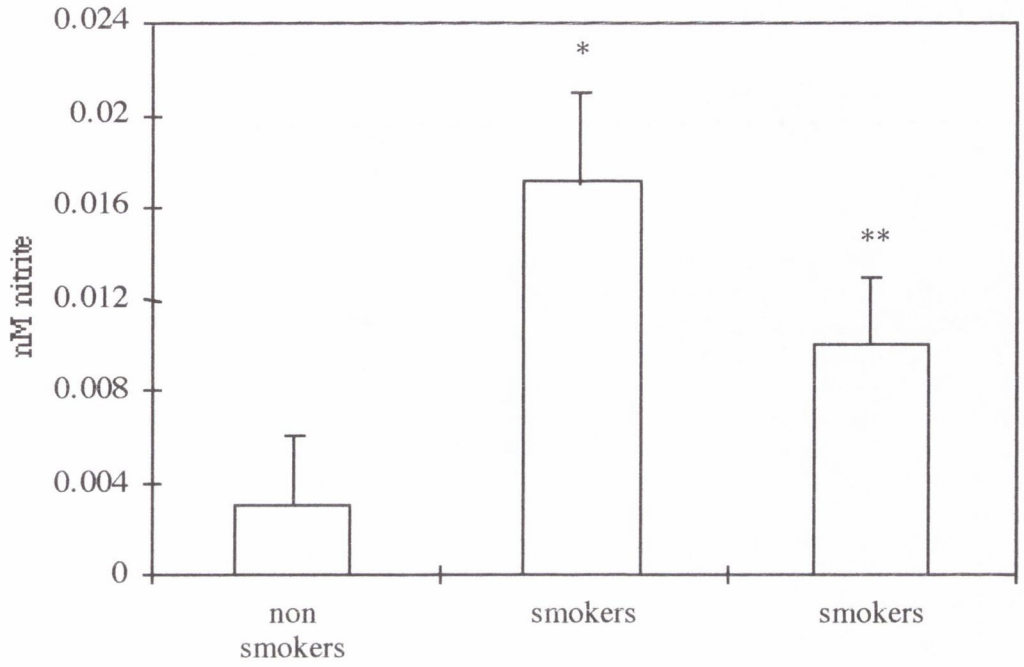


Figure 7.2 The effect of medium conditioned with monocytes for 24 hours from non smokers (n=8), smokers (n=8) and the same smokers treated with taurine (n=7) on microvascular endothelial NO production, after 48 hours incubation,: *p= 0.0002 vs. non-smokers, Students 2-sample t-test; **p= 0.03 vs. smokers, Students 2-sample t-test.

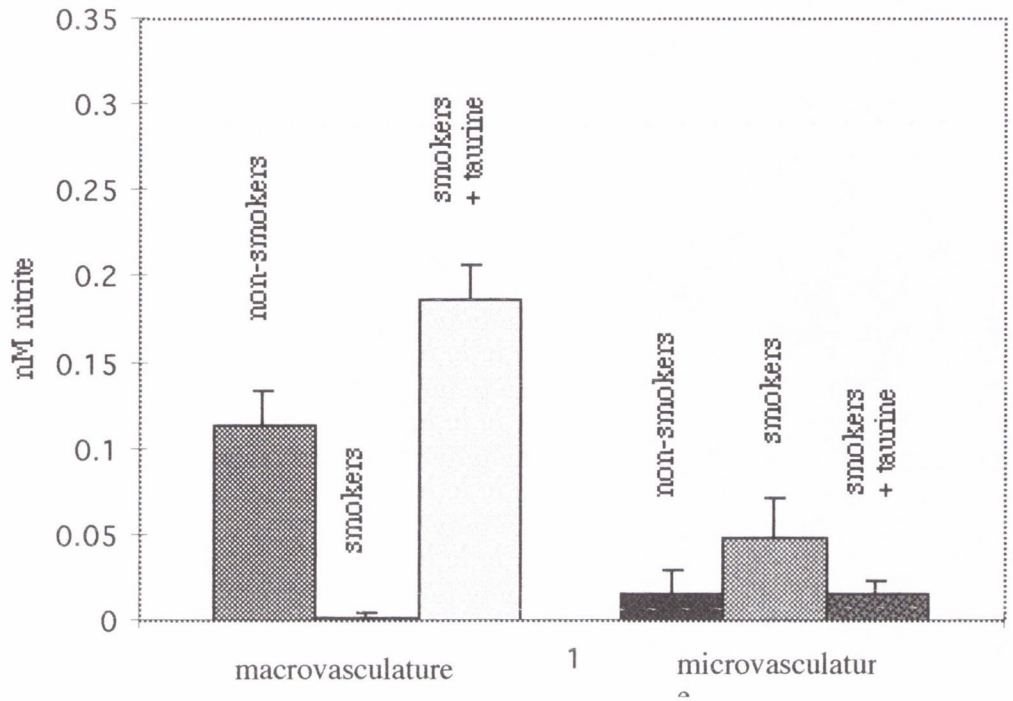


Figure 7.3 The effect of medium conditioned with monocytes for 24 hours from non smokers (n=8), smokers (n=8) and the same smokers treated with taurine (n=7) on macrovascular and microvascular endothelial NO production, after 24 hours incubation.

7.4 Discussion

In response to incubation of microvascular cells with MCM from smokers, an upregulation in NO was observed in comparison to incubation with MCM from controls. Monocytes isolated from smokers supplemented with taurine induced a significant downregulation in NO produced from the microvascular endothelium to the level of control non-smokers. These results are in contradistinction to those obtained from co-incubation with macrovascular cells.

Interestingly, macrovascular NO production in response to co-incubation with MCM from non-smokers and smokers treated with taurine is an average of 10 times higher than NO levels obtained from the microvasculature. This is supported by previous findings of differential NO production by micro- and macrovascular cells (Geiger *et al.*, 1997). In contrast to this, macrovascular NO production post-incubation with MCM of smokers was an average of 5 times lower than that produced from the microvasculature (see Figure 7.3).

These different responses by micro- and macrovascular endothelium to the same set of stimuli is not entirely surprising. Structural and functional heterogeneity's between micro- and macrovasculatures and between vasculatures of different organs and sites have been reported. These include differences in morphology, nutritional and metabolic pathways, receptor expression, eicosanoid synthesis and most importantly, NO release. *In vivo* differential responses to an infusion of endotoxin by the systemic and pulmonary vasculature, namely systemic vasodilatation and pulmonary circulation vasoconstriction have been demonstrated. Significant differences with respect to nitrite release by NO synthesis between rat micro- and macrovascular responses to dual stimulation with cytokines has been demonstrated (Geiger *et al.*, 1997). It was suggested that differential responses may have evolved so as to facilitate leukocyte-endothelial cell interactions in the slower moving microvasculature whilst minimising

risk of injury to the host. An association between smoking-induced inflammatory changes in inflammatory cells and microvascular endothelial dysfunction is increasingly being recognised.

The impact of inflammatory changes in the microvasculature on cardiovascular disease have recently been reviewed by Hansen. Smoking is a recognised risk factor in which inflammatory mediators such as reactive oxygen species, TNFα and inducible NO have been incriminated (Hansen, 1998). In response to smoking, an inflammatory response may be incurred which results in an increase in NO production through upregulation of an inflammatory mediator, such as iNOS. (Hansen, 1998). The microvasculature may also be sensitive to circulating catecholamines, raised levels being known to occur in smokers (Baer & Radichevich, 1985; Cryer *et al.*, 1976) and smokers also known to have increased vascular resistance in the extremities (Morecroft *et al.*, 1994). It is therefore possible that the haemodynamic responses of the microvasculature of smokers may be a result of antagonistic effects between the constrictive effects of catecholamines and a dilatory effect of inflammatory-induced NO. The increased vasoconstrictive effect seen in smokers as compared to non-smokers may therefore be due to an overriding effect of catecholamines. Taurine supplementation is known to decrease the inflammatory response in response to cytokines (Bedrosian *et al.*, 1991; Stapelton *et al.*, 1998), and in doing so may induce a decrease in iNOS expression to normal control levels, with a subsequent decrease in NO levels. After taurine supplementation, the inflammatory effect may therefore be dampened, with a resultant even greater effect of the adrenergic-induced constriction. This in turn may explain an increase in blood pressure post taurine supplementation identified in chapter 6.

An alternative explanation for the finding of increased blood pressure changes in smokers supplemented with taurine is suggested by recent reports of paradoxical acute increases in blood pressure in smokers in response to glyceral trinitrate. As

reported in the present studies, the release of NO from the macrovascular endothelium is greater than that from the microvascular endothelium, by a 10-fold order of magnitude. Furthermore, taurine supplementation restores this release from near zero levels to normal. The effect of such changes in macrovascular release in the macrovasculature of NO on the down-stream responses in smokers may well be similar to that seen with exogenous NO. The resultant increase in vasoconstrictor tone may explain the increases in blood pressure seen in smokers after short-term supplementation with near physiological doses of taurine.

This chapter therefore demonstrates that differential NO production exists between the macro-and microvasculature. In addition, differential NO production exists between MCM of smokers, non-smokers and taurine-treated smokers co-incubated with microvascular cells. These differences in microvascular NO production may be explained by the effect of various cytokines on endothelial NO production, the most likely candidate being the inflammatory-induced iNOS. The vasoactive state in the microvasculature may be controlled by the response to released catecholamines; the inflammatory-mediated NO being protective to the endothelium, but being downregulated post-taurine supplementation. These differences in microvascular endothelial NO production may in turn explain unexpected blood pressure haemodynamic parameters obtained in smokers and smokers treated with taurine as demonstrated in the prior chapter.

Chapter 8

"Investigating a potential mediator of the effects of monocyte-conditioned medium on endothelial function"

8.1 Introduction

Monocyte interactions with the endothelium represents one of the initial steps in the response to injury hypothesis of atherogenesis. Results from the previous chapter would indicate that monocytes have an effect on physiological endothelial functioning *in vitro*, abnormalities known to occur prior to plaque formation. Adhesion of monocytes to endothelial cells, and monocyte-derived secretory products from conditioned medium, can down-regulate steady-state endothelial NO production (Marczin *et al.*, 1996). The prior chapter showed this effect to be increased in smokers in comparison to non-smoking controls, and it also demonstrated that a decrease in cNOS expression mediates the inhibitory effects of monocytes on NO release.

Insult to the endothelial lining is a critical event in the genesis of tissue remodelling, vascular leak syndrome and atherogenesis, all of which involve leukocyte participation and cytokine release. Cytokines are multipotent mediators of inflammation and immunity that can affect key functions of endothelial cells. There is growing evidence to suggest that cytokines are involved in mediating monocyte-induced atheromatous lesion initiation and progression. The involvement of monocyte-derived soluble mediators have been implicated in the atheromatous disease process on many occasions. Cytokines such as tumour necrosis factor alpha (TNFα) and interleukin-1 alpha (IL-1α) are present in atheromatous tissue (Barath *et al.*, 1990; Moyer at al., 1991). In addition, TNFα and IL-1 have been shown to upregulate monocyte chemotactic protein-1 (MCP-1) expression on endothelial cells (Cushing *et al.*, 1990), a chemokine which increases expression of adhesion molecules on endothelial cells to facilitate monocyte adhesion and subsequent plaque formation. On exposure to monokines such as IL-1β , IL-1α and TNFα, endothelial cells undergo morphological modification and alterations in function, such as increased expression of adhesion

molecules and cytokine production (Pober, 1988; Mantovani & Dejana, 1989). Focusing on the endothelial function of dilatation in response to an increase in blood flow, animal studies have demonstrated inhibition of EDRF release by TNFα from carotid arteries (Aoki *et al.*, 1989) and isolated perfused hearts (Lefer *et al.*, 1990). This TNFα-induced reduction in cNOS was shown to be mediated by an increase in cNOS mRNA degradation (Yoshizumi *et al.*, 1993). These established interactions of monocytes and their derived secretory products with the endothelium therefore suggests that cytokines, such as TNFα, IL-1β and IL-1α may be responsible for the downregulatory effect of MCM from smokers on endothelial NO production.

However, another such cytokine, whose functions of endothelial mitogenesis and monocyte pro-migration are most explicitly investigated in the context of angiogenesis, also has the potential of being a player in monocyte-induced endothelial dysfunction. The effects of Vascular Endothelial Growth Factor (VEGF) on endothelial cell biology have more recently been shown to extend beyond mitogenesis and migration to involve a physiological endothelial protective role. This concept of a maintenance role for VEGF has been increasingly proposed (Peters *et al.*, 1993; Jakeman *et al.*, 1992; Alon *et al.*, 1995) and animal experiments have directly demonstrated that VEGF induces relaxation of isolated canine coronary arterioles, which can be abolished by the NO inhibitor L-NMMA (Ku *et al.*, 1993). However, the role that VEGF may play in smoking-induced arterial disease is not yet known, and the potential for VEGF as a monocyte-derived secretory factor in smokers has not yet been elucidated.

An argument for monocytes from smokers secreting more VEGF than non-smokers can be proposed. It is well documented that hypoxia (an environment indigenous to the circulation of smokers) is a potent inducer of VEGF production, both *in vitro* and *in vivo* (Minchenenko *et al.*, 1994; Namiki *et al.*, 1995; Aiello *et al.*,

1995), and VEGF production by the monocyte-derived macrophage is also increased in such conditions (Harmey *et al.*, 1998). A combination of a dysfunctional endothelium and hypoxic conditions may have an effect of upregulating monocyte VEGF production in smokers. An inverse relationship between NO and VEGF regulation has been described (Tsurumi *et al.*, 1997), with VEGF resulting in decreased release of NO from the endothelium. It is also of interest to note that taurine has previously been demonstrated to up-regulate NO production from monocytes (Watson *et al.*, 1995). In this regulatory negative feedback environment, to which VEGF and endothelial NO production are exposed, a potential role for monocyte-derived VEGF is investigated.

The aim of this set of experiments was to: 1) examine the amount of the cytokines- TNFα, IL-1β, IL-1α and VEGF -which could potentially have an effect on endothelial NO production, in the MCM of smokers and non-smokers, and to correspond these levels to the pattern of NO release from the endothelium; 2) examine the effect of taurine supplementation on monocyte cytokine production, and to correspond these levels to the pattern of NO release from the endothelium post taurine supplementation.

8.2 Methods

8.2.1. Subjects

Monocytes were isolated from 8 smokers pre and post supplementation with taurine, and from 7 non-smokers. Both subject groups fitted the criteria as described in 2.2. Smokers were supplemented with 1.5 gms taurine/day (Twinlab, Ronkonkoma, N.Y.) for 5 days. Blood samples were taken on day 1 in smokers and non smokers, and on day 6 post taurine supplementation in smokers also.

8.2.2 Isolation of peripheral blood monocytes

Monocytes were isolated as outlined as described in detail in Section 2.5. Briefly, peripheral blood mononuclear cells were isolated by means of a Ficoll layer gradient technique. Cells were counted using Trypan Blue staining and were greater than 96% viability using this staining procedure. Percentage monocytes was assessed using the Simultest Leucogate CD45/CD14 antibody and run on the FACScan (Becton Dickinson, Mountain View, CA, USA) using the FL_1 filter ($\approx$ 530 nm) to detect FITC labelled CD45 and the FL_2 filter ($\approx$ 565 nm) to detect phycoerythrin (RPE) labelled CD14. A minimum number of 10, 000 events were collected and analysed on the software Lysis 11.

8.2.3 Preparation of monocyte conditioned medium

Monocyte conditioned medium was prepared as outlined in Section 2.5.2. Briefly, cells were plated in flat bottomed microtitre plates (Nunc, Denmark) at a certain concentration, and DMEM was then concentrated in a time (24 hours)- and concentration ($2x10^6$ monocytes)- dependent manner. Samples were obtained at 24 the hour time point and stored at -40 °C until quantitative assays were performed.

8.2.5 Cytokine assay

TNFα, IL-1α, IL-1 β and VEGF were detected using ELISA kits for cytokine detection (R&D Systems), as per 2.6.4, 2.6.5, 2.6.6 and 2.6.7. These assays employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for the cytokine is coated onto the surface of the wells on the microtitre plate. Samples and standards are pipetted into the wells, at a volume of 200μl MCM, which was conditioned by 4 x 10^5 monocytes/ 200 μl DMEM. Any cytokine present in that volume is bound by the immobilised antibody. Unbound material is removed by aspiration and washing, and an enzyme-linked polyclonal antibody specific for the particular cytokine is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added. The amount of cytokine bound to the well is detected by a reaction with the substrate which yields a colour product proportional to the amount of conjugate. This coloured product can then be quantified photometrically using a microplate autoreader (Bio-Tek Instruments), by plotting a curve of known cytokine concentration standards against known signals (optical densities), and from this determining the concentrations of unknowns.

8.3 Results

8.3.1 The TNFα content in MCM of smokers, non smokers and taurine supplemented smokers.

The TNFα content of MCM of smokers (n=8), non-smokers (n=7) and taurine supplemented smokers (n=8) was measured using an ELISA kit. MCM of smokers

did not contain a significantly greater amount of TNFα than that of non-smokers: 83(±41) vs. 62(±9) pg/ml supernatant; (figure 8.1). Also, supplementation of smokers with taurine did not have an effect on TNFα levels in MCM: 83.4(±41) pg/ml supernatant pre-treatment vs. 82.5(±40) pg/ml supernatant post supplementation; (figure 8.1.).

8.3.2 The IL-1α content in MCM of smokers, non smokers and taurine supplemented smokers.

The IL-1α content of MCM of smokers (n=8), non-smokers (n=7) and taurine supplemented smokers (n=8) was subsequently measured using an ELISA kit specific for IL-1α. MCM of smokers did not contain a significantly different amount of IL-1α than that of non-smokers: 4.2(±1.9) vs. 5.7(±1.3) pg/ml supernatant; Figure 8.2. However, supplementating smokers with taurine significantly reduced IL-1α levels in MCM: 4.2(±1.8) pg/ml supernatant pre supplementation vs. 1.7(±1.2) pg/ml supernatant post supplementation: (*$p<0.05$; Students paired t-test: figure 8.2).

8.3.3 The IL-1β content in MCM of smokers, non smokers and taurine supplemented smokers.

In a similar fashion, the IL-1β content of MCM of smokers (n=8), non-smokers (n=7) and taurine supplemented smokers (n=8) was measured using an ELISA. Once again, MCM of smokers did not contain a significantly different amount of IL-1β than that of non-smokers: 12.9(±15) vs. 20.3(±4) pg/ml supernatant; (figure

8.3). Likewise, supplementation of smokers with taurine did not have an effect on IL-1β levels in MCM: 12.9(±15) vs. 28(±27) pg/ml supernatant; (figure 8.3).

8.3.4 The VEGF content in MCM of smokers, non smokers and taurine supplemented smokers.

The VEGF content of MCM of smokers (n=10), non-smokers (n=8) and taurine supplemented smokers (n=10) was also estimated using an ELISA kit, specific for VEGF. MCM of smokers did contain a significantly greater amount of VEGF than that of non-smokers: 192(±250) in smokers MCM vs. 19(±95) pg/ml supernatant in MCM of non smokers; ($p<0.05$; Students 2 sample t-test: figure 8.3). Post supplementation of smokers with taurine, no effect on VEGF levels in MCM was seen: 192(±250) pg/ml supernatant pre treatment vs. 145(±152) pg/ml supernatant post treatment, (figure 8.4).

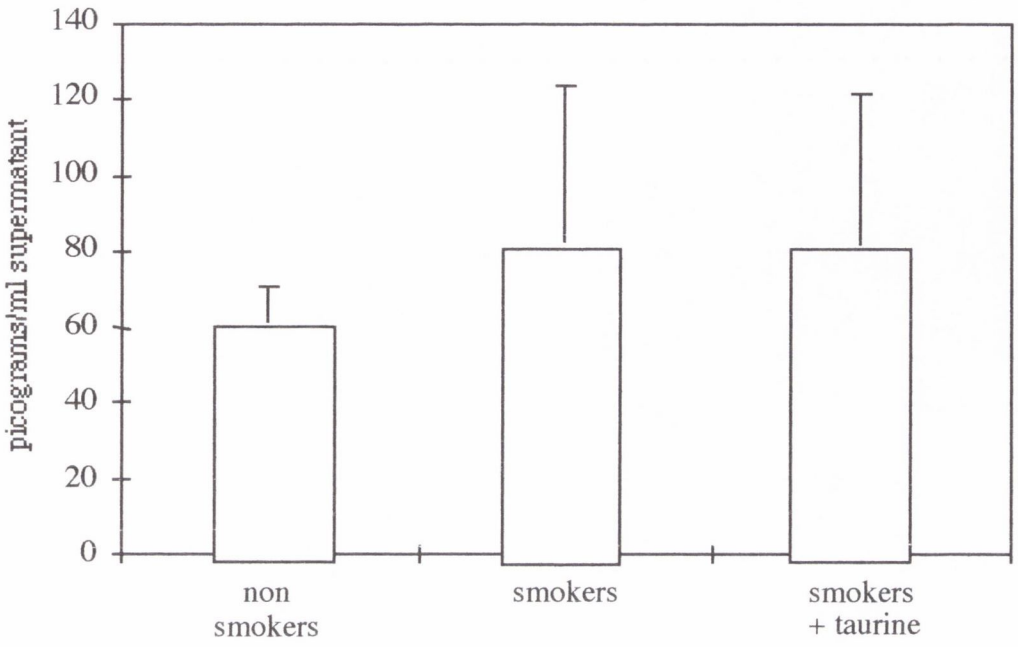


Figure 8.1 TNFα content in MCM of non-smokers (n=7), smokers (n=8) and smokers supplemented with taurine (n=8), as assessed by ELISA technique.

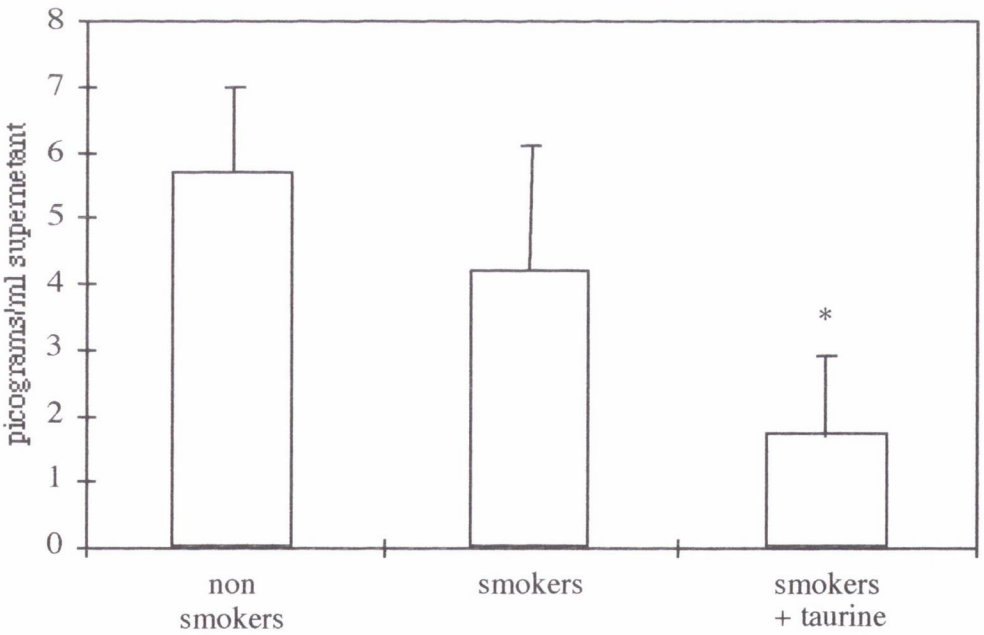


Figure 8.2 IL-1α content in MCM of non-smokers (n=7), smokers (n=8) and smokers supplemented with taurine (n=8), as assessed by ELISA technique: *$p<0.05$ vs. smokers; Students paired t-test.

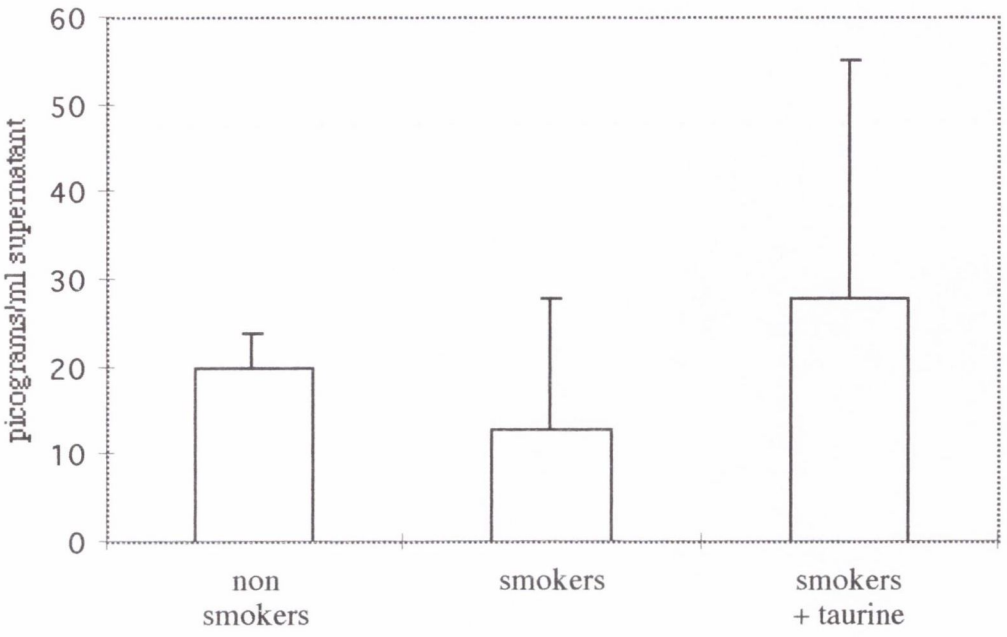


Figure 8.3 IL-1β content in MCM of non-smokers (n=7), smokers (n=8) and taurine treated smokers (n=8), as assessed by ELISA technique.

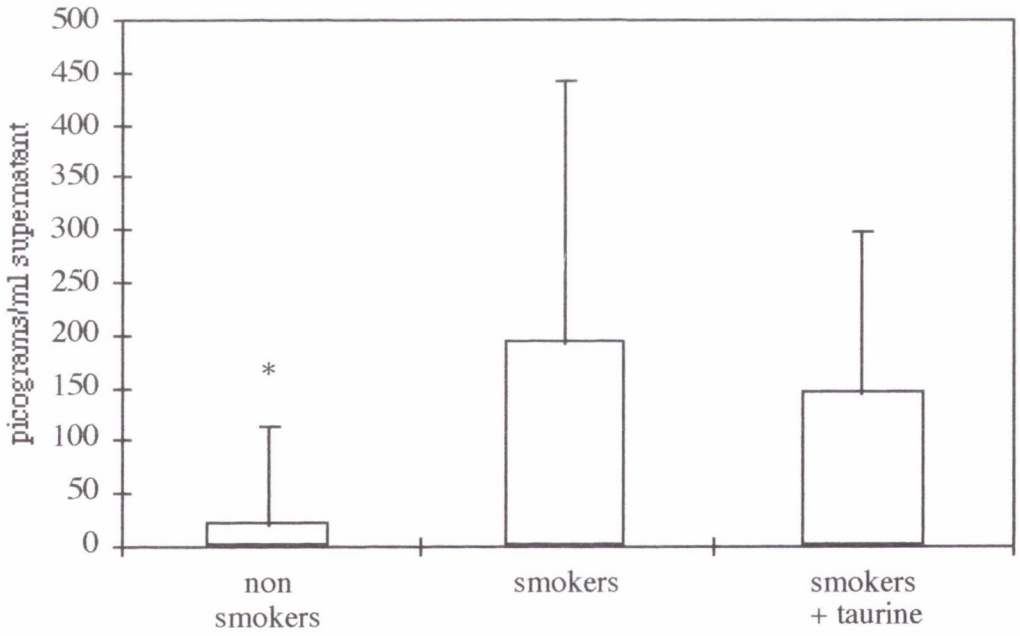


Figure 8.4 VEGF content in MCM of non-smokers (n=8), smokers (n=10) and taurine supplemented smokers (n=10), as assessed by ELISA technique: *$p<0.05$ vs. smokers; Students 2-sample t-test.

8.4 Discussion

MCM from smokers downregulates endothelial NO production by downregulating c-NOS expression. Cytokines which previously have been demonstrated to exert a modulatory effect on endothelial functioning were investigated in this chapter and each was found not to be solely responsible for the changes observed.

The amount of TNFα in MCM of smokers, while greater than that of non-smokers, was not found to be significantly so. This finding is in keeping with a previous study where a neutralising antibody against TNFα failed to augment NO release from endothelial/monocyte co-cultures (Marczin *et al.*, 1996). IL-1α or IL-1β concentration in the MCM of smokers and non-smokers also did not significantly differ. Based on these observations, it is likely that one single cytokine is not responsible for the effects seen. It has been previously postulated that IL-1β is the first in a set of cytokines released by monocytes (Marczin *et al.*, 1996). If this were to hold true in this set of experiments and if cytokines are to be held responsible for the differences in NO production observed, a point must arise in the cascade series when a single, or group of cytokines, released into the medium must differ between the two groups. It may therefore be considered that, in addition to the normal cascade of monocyte-induced cytokines released, cigarette smoking, through one or many of its various components, may have an additional direct upregulatory effect on cytokine release from monocytes, extrinsic to the cascade pathway, which independently affects endothelial NO release.

Isolation of monocytes from smokers treated with taurine had a direct upregulatory effect on endothelial c-NOS expression. The exact mediator(s)

responsible for this was not identified. Taurine supplementation significantly decreased IL-1α release from monocytes of smokers treated with taurine. This, while not solely responsible for the effects on endothelial NO, is of clinical importance as IL-1α has been shown to be a key cytokine in vascular smooth muscle proliferation in plaque formation and progression (Moyer *et al.* 1991; Porreca *et al.* 1993), and demonstrates a potential role for taurine supplementation in modifying progression of arterial disease. It is possible, therefore, that taurine may be exerting its beneficial effect on endothelial NO production through an as-yet undefined cytokine. It is also possible that upregulation of cNOS may occur through a direct effect of taurine in the MCM on the endothelium, as taurine is known to upregulate intracellular calcium, and preliminary data in the thesis would suggest that taurine release from monocytes into medium is greater in smokers.

The growing evidence that cytokines participate as autocrine or paracrine mediators in atherogenesis, and recent perceptions of VEGF having a role as an endogenous regulatory factor in maintaining or promoting endothelial integrity, provided for ample interest in the effect of smoking on monocyte release of VEGF; results which theoretically could deviate in either direction, and may or may not provide an explanation for MCM-induced downregulation of NO in smokers.

Monocytes from smokers were found to produce significantly higher amounts of VEGF than non-smoking controls. The increased release of VEGF, the most potent angiogenic molecule known, from smokers' monocytes is clearly of importance given the recent demonstration of increased angiogenesis within the atherosclerotic plaque (Moulton *et al.*, 1999). The lack of effect of taurine on monocyte release of VEGF is somewhat of a surprise given the previously demonstrated capacity of taurine to upregulate endothelial NO, and considering the interaction between endothelial VEGF and NO release, with increased NO release acting in a negative feedback loop to decrease VEGF (Tsurumi *et al.*, 1997). The fact that the amino acid taurine does not

reduce MCM-derived VEGF levels back to those of control non-smokers, provides evidence that the modulatory role of taurine in upregulating cNOS expression is not associated with restoration of VEGF levels. In fact, VEGF is thought to upregulate NO production by increasing intracellular calcium (Tilton *et al.*, 1997); the amino acid taurine may also work in such a manner, but initiation by modifiable receptors, such as Flk-1, and subsequent tyrosine phosphorylation of phospholipase C (as required for NO release by VEGF (Tilton *et al.*, 1997)) are not necessary. Thus, it seems, taurine upregulation of cNOS is mediated through a pathway independent of VEGF. While a direct connection between the mode of action of taurine and VEGF is not elucidated, the fact that one can upregulate NO in the face of soluble medium from cigarette smokers, and the other failed to do so, is an interesting finding which may be of clinical relevance. Increasing numbers of *in vitro* and *in vivo* animal studies have looked at the effects of VEGF on myocardial blood flow (Hariawala *et al.*, 1996), on haemodynamics and cardiac performance (Yang *et al.*, 1996), and more recently the effects of intracoronary VEGF delivery is being investigated (Lopez *et al.*, 1997). In the clinical setting of atherosclerosis, where the intravascular environment provides for continued endothelial insult, the amino acid taurine may play an important role as undeterred NO upregulator.

This chapter, therefore, reveals there to be a complex interaction between MCM, endothelial cells and NO production. An important relationship between cytokines and endothelial dysfunction has been outlined many times, but the exact mediators of physiological dysfunction, as defined by a decrease in NO production, are unidentified. The previous chapter pointed towards monocyte-derived soluble mediators as having a role in mediating dysfunction, but this does not appear to occur through one solitary, or a known cascade of, cytokines. Results obtained here clearly demonstrate that downregulation of neither the commonly cited cytokines or VEGF are responsible for the maintenance of HUVEC cNOS expression in face of exposure to monocyte conditioned medium from smokers supplemented with taurine.

A complex arrangement of cytokine pathways, an as-yet unidentified cytokine, or simply a non-cytokine related mediator which has a direct effect on intracellular calcium and cNOS expression and which in turn can be modulated by taurine, may represent possibilities for potential mediators of the effects of monocyte-conditioned medium on endothelial NO release.

Chapter 9

"Establishing a role for the monocyte in mediating alterations in endothelial turnover"

9.1 Introduction

A direct interaction between cigarette smoking and the endothelium has been previously suggested to morphologically alter the vascular endothelium (Booyse *et al.*, 1981), and results from this thesis demonstrate that smoking directly activates and desquamates endothelial cells. An increase in the number of CECs is characteristic of non-denuding endothelial injury if the cells are replaced by spreading of, or an increase in the turnover of, adjacent cells. However, if the rate of cell desquamation and death is not accompanied by a corresponding increase in cell proliferation, endothelial barrier function may be altered.

Control of endothelial cell death and proliferation is a tightly controlled physiological process under normal conditions (Folkman & Haudenschild, 1980; Gimbone, 1976; Maciag, 1984). The first section of this thesis demonstrates there to be disturbed endothelial physiology in smokers, and an increase in the number of desquamated circulating endothelial cells. Many attempts have been made to characterise the modulators of endothelial proliferation and migration. Monocyte/macrophage derived factors have been the main focus in control of these processes (Polverini *et al.*, 1977; Martin *et al.*, 1971; Ooi *et al.*, 1983; Mantovani & Dejana, 1989), and may play an important role in control of endothelial proliferation in smokers. In addition, results obtained in this thesis also demonstrate there to be an increase in the amount of VEGF in the conditioned medium of monocytes from smokers. VEGF, or vascular permeability factor (VPF), first described as a tumour-secreted protein (Senger *et al.*, 1983; Senger *et al.*, 1986), is a potent stimulus for increasing vascular permeability. It has been observed that normal, unstimulated monocytes from healthy humans inhibit endothelial proliferation, and a designated "monocyte-derived endothelial inhibitory factor" has been introduced to describe a not-as-yet identified monocyte-derived factor which inhibits endothelial growth (Vilette *et al.*, 1990).

A role for monocytes in inducing endothelial cell apoptosis has yet to be elucidated. However, cytotoxic factors released from monocytes can induce apoptosis in target cells (Old, 1986; Selmaj *et al.*, 1991). A protein known as SKT factor, also released by monocytes, has been shown to induce apoptosis in tumour cells (Ghibelli. et al, 1994). In the case of endothelial cells, NO has of recent times been shown to play a significant role in preventing endothelial apoptosis. While very high levels of NO induce endothelial apoptosis, physiological NO inhibits TNF-α induced apoptosis, a process thought to be mediated through stimulation of cGMP pathway (Shen *et al.*, 1998). Other experiments have demonstrated that NO can inhibit shear stress induced endothelial apoptosis (Hermann *et al.*, 1997; DeMeester *et al.*, 1998). As this thesis demonstrates that monocytes of smokers downregulates endothelial NO production, it is possible that MCM from smokers may induce endothelial cell apoptosis.

The amino acid taurine has been shown to upregulate endothelial cNOS expression, and hence NO production, previously described in this thesis. An upregulation of NO, which in turn upregulates the cGMP process, may therefore protect against apoptosis. Taurine has also been demonstrated in this thesis to decrease the number of activated and apoptotic desquamated endothelial cells. It is therefore feasible that taurine supplementation may have a beneficiary effect on endothelial barrier function.

The aim of this chapter was to investigate if MCM of smokers has an effect on endothelial barrier function. The effect of MCM of smokers, non-smokers and smokers treated with taurine on endothelial cell apoptosis and proliferation was therefore investigated.

9.2 Methods

9.2.1. Subjects

Monocytes were isolated from smokers (n=8) pre and post supplementation with taurine, and from non-smokers (n=8). All subjects fitted the criteria as outlined in 2.2. Smokers were supplemented with 1.5 gms taurine/day (Twinlab, Ronkonkoma, N.Y.) for 5 days.

9.2.2 Isolation of peripheral blood monocytes

Monocytes were isolated as outlined as described in detail in Section 2.5. Briefly, peripheral blood mononuclear cells were isolated by means of a Ficoll layer gradient technique. Cells were counted using Trypan Blue staining and were greater than 96% viability using this staining procedure. Percentage monocytes was assessed using the Simultest Leucogate CD45/CD14 antibody and run on the FACScan (Becton Dickinson, Mountain View, CA, USA) using the FL_1 filter ($\approx$ 530 nm) to detect FITC labelled CD45 and the FL_2 filter ($\approx$ 565 nm) to detect phycoerythrin (RPE) labelled CD14. A minimum number of 10, 000 events were collected and analysed on the software Lysis 11.

9.2.3 Preparation of monocyte conditioned medium

Monocyte conditioned medium was prepared as outlined in Section 2.5.2. Briefly, cells were plated in flat bottomed microtitre plates (Nunc, Denmark) at a concentration of $2x10^6$ monocytes/ 5 mls DMEM / 0.5% foetal calf serum (FCS) / 0.1% (v/v) Penicillin/Streptomycin (P/S) solution, and DMEM was then concentrated in a time (24)- and concentration ($2x10^6$ monocytes)- dependent manner. Samples were obtained after 24 hours incubation and stored at -40°C until quantitative assays were performed.

9.2.5 Preparation of HUVECs

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins and cultured as per 2.4.1. Briefly, after removal of collagenase the cells were cultured on 2% gelatin-coated culture flask in complete M199 medium supplemented with 20% FCS, penicillin (100U/ml), streptomycin sulphate (100 μg/ml), fungizone (0.25 μg/ml), heparin (16 U/ml), endothelial cell growth supplement (75 μg/ml) and 2 nM glutamine as previously described (Jaffe *et al.*, 1973). Cells were grown at 37°C in a humidified 5% CO^2 conditioned chamber, passaged by trypsinization with 0.05% trypsin-0.02% ethylene diamine tetraacetic acid, followed by splitting and subculturing in 1:4 ratio when confluent monolayers were reached.

9.2.6 Preparation of endothelial cells for proliferation assay

Isolated HUVECs were made up to a volume of 1x 10^5 cells/ml, and 100μl cell suspension of this was added to each well of a 96 well plate, previously coated with 2% gelatin, thus plating 1 x 10^4 cells/well. These cells were then allowed a 12 hour equilibration period in a medium containing 3% FCS @ 37oC in a humidified 5% CO2.

The supernatant was then aspirated and 100μl of DMEM together with 100μl MCM (@ a concentration of 2 X 10^5 monocytes/ml of medium) were added and incubated at 37°C, 5% CO^2 for 24 hours. Triplicates of MCM samples from non smokers, smokers and these same smokers treated with taurine were used, and triplicates of 100μl/well of culture medium without MCM were used as a negative control.

9.2.7 Endothelial cell proliferation assay.

The methodology for this is described in detail in 2.8.1. Briefly, cellular proliferation was measured by quantitative determination of 5-bromo-2'deoxy-uridine (BrdU) incorporation during DNA synthesis in replicating cells. Using an ELISA (5-BrdU labelling and detection kit 111(Boehringer Mannheim, Sussex, England), the amount of BrdU incorporated by replicating/proliferating cells was determined by labelling cells with an anti-BrdU antibody.

9.2.8 Endothelial cell apoptosis assay.

The methodology for this section is described in detail in 2.8.2. Briefly, the effect of MCM on endothelial apoptosis was quantified by measuring cytoplasmic histone-associated DNA fragments (macro-and oligonucleosome) by a cell death detection ELISA^{plus} kit (Boehringer, Mannheim, Germany). HUVECs were isolated and treated with MCM, after which the supernatant was removed and the cell pellet was resuspended in lysis buffer. The lysate was centrifuged and the supernatant (= cytoplasmic fraction) was transferred onto the streptavidin-coated microtitre plate and incubated with a mixture of anti-histone-biotin and anti-DNA-peroxidase. During this incubation, nucleosomes were captured via their histone component by anti-histone-biotin antibody, while binding to the streptavidin coated microtitre plate. Simultaneously, anti-DNA-peroxidase binds to the DNA part of the nucleosomes. After removal of the unbound antibodies, the amount of peroxidase retained in the immunocomplex was determined photometrically using a plate reader @ 405 nm.

9.3 Results

9.3.1 The effect of MCM from smokers, non smokers and taurine treated smokers on HUVEC proliferation.

The effect of MCM from non-smokers (n=8), smokers (n=8) and taurine treated smokers (n=8) on HUVEC proliferation was accomplished by quantitative determination of BrdU incorporation during DNA synthesis after incubation of the HUVEC with MCM from the 3 subject groups for 24 hours. DMEM was used as control medium. Taking control medium to induce 100% proliferation, monocyte-conditioned medium from non-smokers resulted in a decrease of proliferation to 85(±10) % in comparison to control medium, (*$p<0.005$; Students 2-sample t-test: figure 9.1). MCM from smokers also resulted in a decrease of proliferation to 63(±14) % in comparison to control medium, (*$p<0.002$; Students 2-sample t-test: figure 9.1). Comparing the effects of MCM from smokers and non-smokers on HUVEC proliferation, MCM from smokers resulted in a significantly less endothelial cell proliferation in comparison to MCM from non-smokers; 63(±14) % proliferation of endothelial cells when incubated with MCM from smokers vs. 85(±10) % proliferation of endothelial cells when incubated with MCM from non smokers; (*$p<0.004$; Students 2-sample t-test: figure 9.1). The effect of taurine supplementation of smokers on HUVEC proliferation revealed that MCM of taurine treated smokers significantly increased endothelial proliferation in comparison to MCM from smokers pre-supplementation; 95(±14) % proliferation of endothelial cells post supplementation with taurine vs. 63(±14) % proliferation of endothelial cells, pre supplementation; (*$p<0.002$; Students paired t-test: figure 9.1).

9.3.2 The effect of MCM from smokers, non smokers and taurine treated smokers on HUVEC apoptosis.

The effect of MCM from non-smokers on HUVEC apoptosis was investigated using a cell death detection ELISA kit. HUVECs were incubated with MCM from the 3 subject groups (n=8 per group) for 24 hours. DMEM was again used as a control medium. Taking control medium to induce 100% apoptosis, MCM from non-smokers resulted in a decrease in apoptosis to 49(±114) %, in comparison to the control medium. This was not significant. MCM from smokers resulted in an increase in apoptosis to 508(±191) %, in comparison to the control medium; (*p= 0.003; Students 2-sample t-test: figure 9.2). When the effects of MCM from smokers and non-smokers on HUVEC apoptosis were compared, MCM of smokers were found to significantly increases apoptosis of HUVEC in comparison to MCM of non-smokers; 507(±191) vs. 49(±114)%, (*p= 0.0001; Students 2-sample t-test: figure 9.2).

The effect of taurine supplementation of smokers on MCM-induced endothelial apoptosis was then investigated. MCM of taurine-treated smokers significantly reduced the apoptosis of HUVEC in comparison to MCM of smokers pre-supplementation; 257(±113) vs. 507(±191)%, (*p= 0.0009; Students paired t-test: figure 9.2).

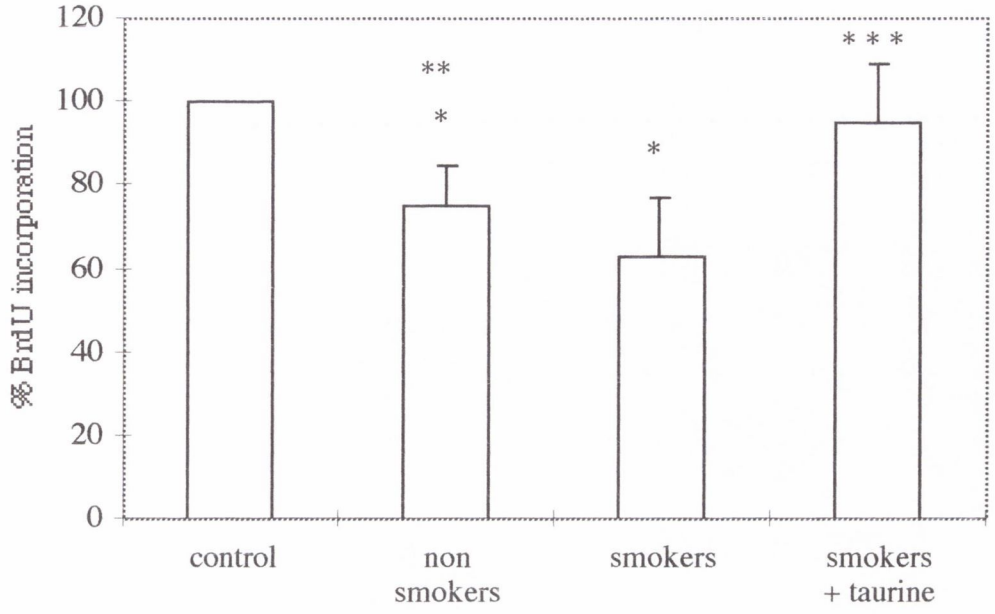


Figure 9.1 The effect of medium conditioned with monocytes from non smokers (n=8), smokers (n=8) and the same smokers treated with taurine (n=8) on endothelial proliferation after co-incubation with HUVEC for 24 hours,: *p≤0.005 vs.control medium, Students 2-sample t-test; **p<0.004 vs. smokers, Students 2-sample t-test ;***p= 0.002 vs. pre-treatment of same group, Students paired t-test.

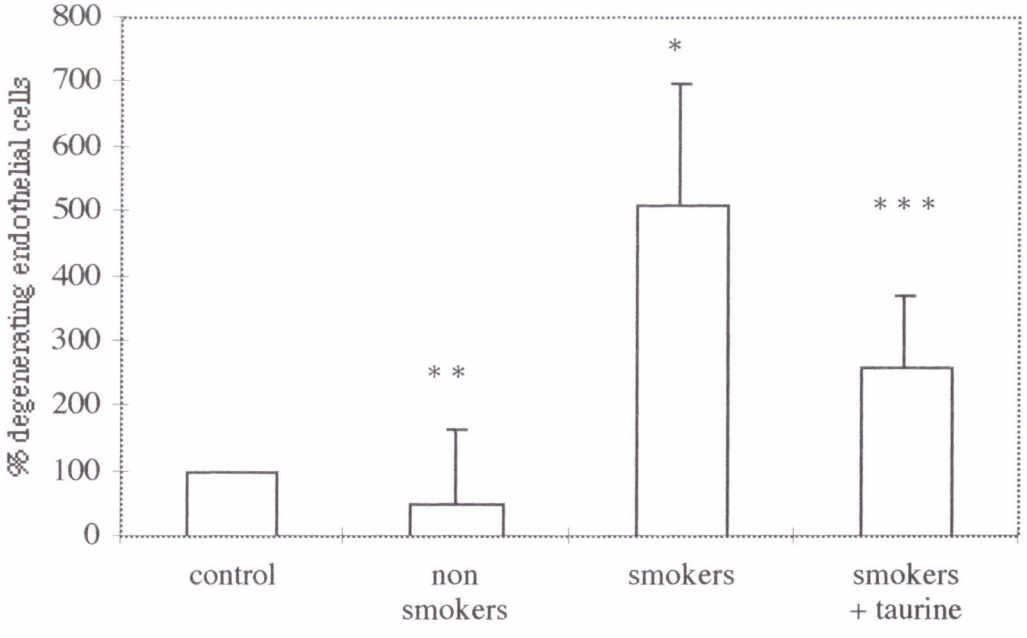


Figure 9.2 The effect of medium conditioned with monocytes from non smokers (n=8), smokers (n=8) and the same smokers treated with taurine (n=8) on endothelial apoptosis after co-incubation with HUVEC for 24 hours,: *p= 0.003 vs. control medium, Students 2-sample t-test; **p= 0.0001 vs. smokers, Students 2-sample t-test; ***= 0.0009 vs. pre-treatment of same group, Students paired t-test.

9.4 Discussion

Functional alterations in the endothelium have the potential for significant pathological sequelae because the endothelium regulates vasoreactivity, thrombogenesis and vascular permeability. The aim of this set of experiments was to investigate the effect of MCM from smokers, non-smokers and taurine supplemented smokers on endothelial proliferation and apoptosis.

Results obtained in this chapter demonstrate that incubation of HUVECs with MCM from both non-smokers and smokers significantly inhibits endothelial proliferation when compared to control medium. MCM from smokers has a significantly greater inhibitory effect on HUVEC proliferation than non-smokers. Supplementing smokers with taurine significantly decreases this inhibitory effect on endothelial proliferation.

It has been previously demonstrated that MCM from control normal subjects, with no arterial disease risk factors, has an inhibitory effect on endothelial proliferation (Dosquet *et al.*, 1994; Vilette *et al.*, 1990), thereby validating the observations of the effects of non-smokers obtained here. However, while this same group did not find monocytes of diabetics to have a greater downregulatory effect on endothelial proliferation, results in this chapter demonstrate monocytes of smokers to significantly do so. The greater inhibitory effect of MCM of smokers on endothelial proliferation may be explained by a dysequilibrium between normal stimulatory and inhibitory factors for endothelial proliferation, monocytes and macrophages being known to produce many growth promoting and growth inhibiting cytokines. The greater inhibitory effect may be secondary to a complicated network of smoking-induced stimulation of inhibitory cytokines. Three such endothelial growth inhibitory cytokines, TNFα, IL-1α, and IL-1β (Schweigerer *et al.*, 1987; Norika *et al.*, 1987)

are unlikely to be solely responsible, as results in this thesis demonstrate that levels of these cytokines are not significantly elevated in smokers as compared to non-smokers. While many of the cytokines producing an inhibitory effect on endothelial growth are preferentially released by activated monocytes, one inhibitory factor, MECIF, is released by monocytes under basal conditions (Vilette *et al.*, 1990) and may be responsible for the baseline inhibitory effect of monocytes from non-smokers. It is possible that cigarette smoking may cause direct upregulation of baseline monocyte production of MECIF. This in turn may be downregulated by taurine, as MECIF production is a reversible time-dependent process involving protein synthesis, and taurine has been shown to interfere with the action of a number of proteins, and may be taurine-dependent phenomenon involve modification of proteins (Huxtible, 1992).

Whatever the exact mediator(s) of endothelial proliferation inhibition is, it is a soluble cytokine released in small amounts by monocytes of normal subjects, and to a greater degree by smokers. It is a soluble mediator, the levels of which may in turn be reduced, or the effects of which are antagonised, by taurine supplementation. Interestingly, it cannot be argued that the nicotine component of cigarette smoke is responsible for effects seen, as direct incubation of endothelial cells with physiological, post-smoking levels of nicotine has been found to have a stimulatory effect on endothelial DNA synthesis and proliferation (Villablanca, 1998). While the over-riding effects of circulating monocytes or nicotine on endothelial proliferation is debatable, it would seem reasonable that the presence of monocytes within the vascular wall, and subsequent release of their soluble factors, would have a more direct impact on endothelial proliferation.

This chapter also demonstrates that MCM of non-smokers decreases the detection of endothelial DNA fragments in comparison to control medium alone. This indicates that MCM of non-smokers prevents against apoptosis of endothelial cells in comparison to medium alone. On the other hand, treating HUVECs with MCM of smokers results in increased detection of endothelial DNA fragments in comparison to

control medium, and in comparison to non-smokers. Therefore, MCM of smokers significantly increases endothelial cell apoptosis in comparison to control medium and MCM of non-smokers. Treatment of these same smokers with taurine significantly reduces the degree of MCM-induced apoptosis in comparison to pre-treatment.

It would therefore appear that soluble factors released by monocytes from non-smokers prevents against apoptosis of endothelial cells in comparison to control medium, while factors produced by monocytes of smokers induce apoptosis. Taurine decreases endothelial apoptosis, but not to the level of that of non-smokers.

There are at least 3 possible mechanisms by which MCM may have an effect on endothelial apoptosis. Firstly, monocytes from smokers may directly produce soluble apoptosis-inducing cytokines. While TNFα is a well known apoptosis-inducing cytokine, the likelihood of this cytokine being solely responsible for the apoptotic effects is small, as this thesis has demonstrated smokers not to have significantly greater TNFa levels than non-smokers. While the possibility that other cytokines, or factors such as SKT factor, are released in the MCM of smokers, it is necessary for monocytes of non-smokers to secrete an anti-apoptotic factor into the medium to reduce the level of apoptosis induced by medium alone. This effect is most likey explained by monocyte growth-promoting agents not present in medium alone.

Secondly, monocytes may have an effect on endothelial apoptosis through modification of NO production. NO has been shown to play a significant role in endothelial apoptosis, physiological levels of NO inhibiting TNFα-mediated endothelial apoptosis (Dimmeler *et al.*, 1997; Shen *et al.*, 1998; DeMeester *et al.*, 1998). It has been previously demonstrated in this thesis that monocytes of smokers downregulates endothelial NO production, and that this is restored with taurine supplementation. Restoration of endothelial NO production may therefore play a role in the preventative and initiative processes of apoptosis.

Thirdly, a direct association between endothelial cell activation and apoptosis has been proposed in chapter 3, with activation of an endothelial cell priming the cell for, or initiating the process of, apoptosis. As endothelial cells of smokers were found to be more activated than those of non-smokers, and as recent evidence suggests that inflammation within the vasculature is associated with NF-kappa B activation and subsequent apoptosis of endothelial cells (von Albertini *et al.*, 1998), it is possible that MCM may induce inflammation and activation of endothelial cells, predisposing them to apoptosis. Taurine may subsequently protect against endothelial apoptosis, possibly by preventing activation of endothelial cells, or by directly inhibiting this process, as demonstrated by Wang *et al.*, (Wang *et al.*, 1996).

The tightly controlled processes of apoptosis and proliferation, responsible in normal cell populations for maintenance of stable cell numbers, are adversely affected by soluble factors in the monocyte conditioned medium of smokers. Subendothelial migration and localisation of monocytes is one of the earliest events in atheromatous plaque formation. It has been previously demonstrated in this thesis that the monocyte plays a role in endothelial dysfunction in smokers by altering the production of NO and ET-1. This chapter also demonstrates that the monocyte plays a role in priming the endothelium for lesion formation by decreasing barrier function and predisposing to denuding injury.

Chapter 10

"Investigating a direct effect of cigarette smoking on endothelial cell desquamation"

10.1 Introduction

The endothelium is a structurally simple yet functionally complex tissue which forms a confluent monolayer lining the intimal surface of all blood vessels and is therefore situated at the vital interface between circulating blood and tissue. Intact endothelial cell metabolism and functional activity play a vital role in the maintenance of vascular integrity. Recent reports suggest that alterations in the mechanical, chemical or humoral environment may cause functional and structural endothelial disturbances and eventually lead to endothelial cell death (Chen *et al.*, 1997; Meredith *et al.*, 1993). Endothelial cell death may occur in a programmed (apoptosis) or unprogrammed (necrosis) fashion. Generally, apoptosis is a controlled programmed event which occurs without membrane lysis, thereby avoiding surrounding tissue injury (Golstein *et al.*, 1991). It is a genetically controlled process of cell suicide characterised by cell shrinkage, nuclear condensation and DNA fragmentation, the cytoplasmic membrane remaining intact during the initial stage. However, in the case of the endothelial cells, apoptosis may result in organ dysfunction due to loss of barrier function as a result of reduced size and altered shape of the apoptotic endothelial cells leading to distortion of the monolayer (Wang *et al.*, 1996; Buchman *et al.*, 1993; Abello *et al.*, 1994).

There is increasing evidence that apoptosis of vascular endothelial cells plays a significant role in atherosclerotic and inflammatory lesion formation (Alvarez *et al.*, 1997; Takeya *et al.*, 1997; Dzau *et al.*, 1997; Dimmeler *et al.*, 1998). Vascular endothelial cells require attachment to the extracellular matrix (ECM) and to each other for proper growth, function and survival. It has been recently suggested that a chemical or mechanical stress to an endothelial cell can result in either of two events: the endothelial cell may stretch over the ECM and repair itself, or it may detach itself, lose contact with the ECM, subsequently change its shape and die by apoptosis (Chen *et al.*, 1997; Ruoslahti, 1997). Loss of this anchorage results in disruption of the

ECM resulting in "anoikis", or apoptosis from loss of anchorage, with cell shape governing whether a cells lives or dies. Such loss of anchorage would result in migration of the apoptotic endothelial cells into the circulation to be engulfed by circulating monocytes.

Surprisingly, endothelial cells are detectable in the circulation under healthy and diseased states associated with vascular injury, such as myocardial infarction and angina (Hladovec *et al.*, 1978; Bouvier *et al.*, 1970), cytomegalovirus infection (Grefte *et al.*, 1993; Percivalle *et al.*, 1993), endotoxinaemia (Gerrity *et al.*, 1976), and sickle cell anaemia (Solovey *et al.*, 1997). Acute smoking is also known to increase the number of CECs (Davis *et al.*, 1985), but it is not as yet known whether chronic cigarette smoking in young people is associated with an increase in CECs. Neither, as yet, have these cells been identified as apoptotic.

The role of cell shape in governing and suppression of anoikis is now recognised (Frisch & Rouslahti, 1997; Re et al, 1994), but the mechanism by which cell shape may govern apoptosis is unknown. It has been demonstrated that in addition to controlling DNA proliferation and metabolism, call shape may also represent a signal as to whether a process of self-destruction is activated (Re *et al.*, 1994), more rounded cells being susceptible to apoptosis, and those forced to spread out resisting apoptosis (Ruoslahti *et al.*, 1997; Chen *et al.*, 1997). In addition to cell shape, anoikis is also controlled by cell-cell contact inhibition and by mediators such as focal adhesion kinase and Akt (Frisch *et al.*, 1996; Khwaja *et al.*, 1997; Kennedy *et al.*, 1997). Akt, a serine/threonine kinase, also known as protein kinase or Rac kinase has recently been shown to play a role in matrix adhesion and integrin-mediated signal transduction (Franke *et al.*, 1997; Khwaja *et al.*, 1997; Kennedy *et al.*, 1997). The regulation of cell survival by Akt involves phosphorylation of a critical serine residue on Bad, an apoptotic promoter which, upon phosphorylation dissociates from Bcl-x_1.

Bad normally functions by blocking activity of Bcl-x_l. Once freed from Bad by Akt, Bcl-x_l can resume its activity as a suppresser of cell death.

Solovey *et al.* have recently demonstrated that many desquamated endothelial cells in sickle cell anaemia are activated (Solovey *et al.*, 1997). This may suggest that some CECs are protected from apoptosis. Cigarette smoking is associated with upregulation of activation markers *in vitro* (Kalra *et al.*, 1994), and circulating levels of soluble markers have more recently been used to indicate the degree of systemic endothelial activation (Wayne *et al.*, 1997; de Caterina *et al.*, 1997). Endothelial activation itself is a prerequisite for arterial disease and assessment of activation of CECs may provide useful material for the study of this event. The effect of chronic smoking in young people on surface phenotype was also investigated in isolated CECs.

In vitro cytokine and oxidant-mediated endothelial activation and apoptosis has been shown to be prevented, or at least ameliorated, by taurine.(Wang *et al.*, 1996). The aim of this chapter was to investigate the effect of smoking on endothelial desquamation, apoptosis and activation, and also to examine the effects of taurine supplementation on these parameters.

10.2 Methods

10.2.1 Subjects

CECs were isolated from 8 smokers and 11 non-smoking controls, who fit the criteria as outlined in 2.2. Smokers were supplemented with 1.5 gms taurine/day (Twinlab, Ronkonkoma, N.Y.) for 5 days. The post supplementation sample was taken on the sixth day.

In addition, CECs from 2 smokers and 2 non-smokers, whose endothelial cells had been previously isolated for apoptosis detection, were again isolated and expression of the anti-apoptotic signal Akt was investigated

10.2.2 Isolation of circulating endothelial cells.

CECs were isolated by the technique described by Hladovec and Rossman (Hladovec *et al.*, 1973), as outlined in 2.9.1. Briefly, 9 mls of venous blood were drawn and erythrocytes were removed by centrifugation at 395 x g, 4°C for 20 minutes. 1 ml of this supernatant was aspirated and mixed with 0.2 mls of adenosine-5-diphosphate (1 mg/ml) (Boehringer Mannheim, Mannheim, Germany) and was mechanically shaken for 10 minutes prior to centrifugation at 395 x g for 20 minutes to remove platelet aggregates. The supernatant was then centrifuged at 2100 x g for 20 minutes to obtain a cell pellet. After suspension of the sediment in 0.1 ml normal saline, improved Neubauer chambers were filled with the suspension. The number of endothelial cells in the 9 large squares were counted four times and averaged. Results were expressed as the mean number of cells in the four 0.9 µl chambers.

Results were then expressed as the arithmetic mean number of cells in each 0.9 µl chamber. Statistical analysis was performed using the Data Desk 5.1 software package on a Macintosh LC630. Comparisons were made between smokers and non-smokers using unpaired two-tailed Students t-test, where variances were not assumed equal,

and between smokers pre and post treatment using a paired Students t-test. Results were considered significant when $p<0.05$.

10.2.3 Identification of endothelial cells.

On 2 occasions, cells were stained with fluorescein-labelled anti-human factor VIII and fluorescein-labelled low-density lipoprotein (LDL). Suspended cells were incubated for 45 minutes with a 1:5 dilution of the fluorescent antibody, and with the use of a cytospin (Shandon, UK) they were transferred to glass slides. Specific yellow-green immunofluoresence of the endothelial cells was apparent when looked at using an inverted fluorescene microscope (Nikon, Japan)

10.2.4 Phenotypic studies of endothelial cells.

This methodology is described in detail in 2.9.3. Briefly, to detect antigens that endothelial cells express only or to a markedly greater extent on activation (ICAM-1, VCAM-1, P-selectin, E-selectin and CD 34), CECs were isolated and assessment of their phenotype of these cells was carried out using direct or indirect immunofluorescent staining. Expression by CECs of FITC-labelled CD 36 (Immunotech) was also assessed. With the use of a cytospin these samples were transferred to a glass slide. After the staining procedures were completed, preparations of CECs were examined under an inverted fluorescene microscope. Positively staining cells were expressed as a percentage of total number of cells counted per field of vision on 5 different areas of the slide, and expressed as the arithmetic mean percentage of positively staining cells. Number of observations (n) refers to number of samples taken.

10.2.5 Immunohistochemical detection of circulating endothelial cell apoptosis.

Apoptosis of endothelial cells was assessed with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) technique, using an In Situ Cell Death Detection kit (Boehringer Mannheim), as described in detail in 2.9.4. This method is based upon enzymatic in situ labelling of apoptosis-induced DNA strand breaks. Cleavage of genomic DNA during apoptosis may yield double-stranded DNA fragments. These strand breaks can be identified by labelling the free 3-OH termini with modified nucleotides in an enzymatic reaction. Terminal deoxynucleotidyl transferase catalyses the polymerisation of the nucleotides and is used to label the DNA strand breaks. Incorporated fluorescein is detected with anti-fluorescein antibody fragments.

10.2.6 Immunohistochemical detection of Akt expression.

This methodology is described in detail in 2.9.5. Briefly, the endothelial cells were isolated as previously described (2.9.1) and were placed on a slide using a cytospin. They were then fixed with paraformaldehyde (4% in PBS , pH 7.4) for 30 minutes at room temperature, endogenous peroxidase was blocked with H_2O_2 (0.3% in methanol) and 0.1% Triton X-10 (in 0.1% sodium citrate) was used to permealise the cells for 3 minutes at room temperature. The slides were incubated in rabbit serum for 1 hour to inhibit non-specific antibody binding. To 100μl of sample, 10μl of primary antibody (goat anti-human polyclonal antibody, Santa Cruz Biotechnology) at a dilution of 1:400, giving a final concentration of 0.5μg of antibody, was added and incubated for 30 minutes at room temperature, and a secondary antibody (Peroxidase-conjugated rabbit anti-goat IgG; Dako, Denmark), at a dilution of 1:200 giving a final concentration of 0.32μg antibody, was later added for 45 minutes. DAB-substrate solution was then added on to the samples (2 mls of reconstituted DAB substrate

diluted in 15 mls H_2O_2) and they were analysed under a light microscope until a colour change was seen in some cells and H_20 was added to stop then stop the reaction. Positive staining cells, with a brown colour, were then counted.

10.2.7 Assessment of circulating endothelial cell shape

The shape of cells isolated were objectively assessed as rounded or elongated/spindle-shaped. Rounded cells were defined as those whose maximum diameter did not exceed any other diameter of that cell by more than a factor of 2. Spindle shaped cells were those whose maximum diameter was greater than 3 times any other diameter of that cell.

10.3 Results

10.3.1 Quantitative studies. (table 10.3.1)

Isolated cells stained positively for von Willebrand Factor and for Low Density Lipoprotein (LDL), identifying these cells as endothelial cells on a separate occasion to counting. The number of CECs present was then counted using a Neubauer chamber. The number of CECs per 0.9μl chamber of the 8 smokers was significantly greater (mean ± SD, 3.3 ± 1.43 cells per 0.9μl chamber) than those of non-smokers (0.93 ± 0.42 cells per 0.9 μl chamber). Peripheral blood from these same smokers after 5 days of treatment with taurine contained a significantly lower number of CECs (1.11 ± 0.59 cells per 0.9 μl chamber).

10.3.2 Phenotypic studies (table 10.3.2)

CECs were then examined for expression of specific adhesion markers that appear on activated endothelial cells. The percentage of CECs with a surface expression of ICAM-1 (63± 13 vs. 42±12), VCAM-1 (66±8 vs. 35±7), P-Selectin (66±12 vs. 35±8), E-Selectin (63±10 vs. 29±11) and CD 34 (54±7 vs. 28±5) was

significantly greater among smokers than non-smoking controls. The percentage expression of CD36 by endothelial cells, an antigen expressed only by endothelial cells of microvascular origin, was also examined. Over half (56.6±8 %) of CECs in samples from smokers were positive for CD36, which was significantly higher than those CECs of non-smokers samples (31±5 %). Activation markers did not discriminate between cell shape. Taurine supplementation of smokers did not have an effect on EC activation. Representative cell staining samples are shown in Figure 10.1

10.3.3 Detection of apoptosis.

Histochemical staining disclosed evidence of apoptosis in 9% of isolated cells counted in non smokers (n=3) and in 11% of cells counted in smokers (n=3). Light microscopy revealed that cell membranes of isolated cells were intact, and cell membrane blebbing was also apparent. Representative cell staining samples are shown in Figure 10.3

10.3.4 Akt expression

Histochemical staining disclosed evidence of positive staining for Akt expression in approximately 5% of isolated elongated cells in both smokers (n=2) and non smokers (n=2). More rounded-shaped cells did not express Akt. Representative cell staining samples are shown in Figure 10.4

10.3.5 Cell shape of isolated cells

The majority of isolated CECs were spindle in shape in all isolated populations (>70%).

Subjects	No. of Samples	Circulating Endothelial Cells § cells/0.9 µl chamber / cells/mm^3	
Non smokers	11	0.93±0.42	/ 1.03±0.47
Smokers	5	3.29±1.43 *	/ 3.66±1.59 *
Smokers + Taurine	7	1.11±0.59 †	/ 1.23±0.65 †

Table 10.3.1 Quantification of Circulating Endothelial Cells

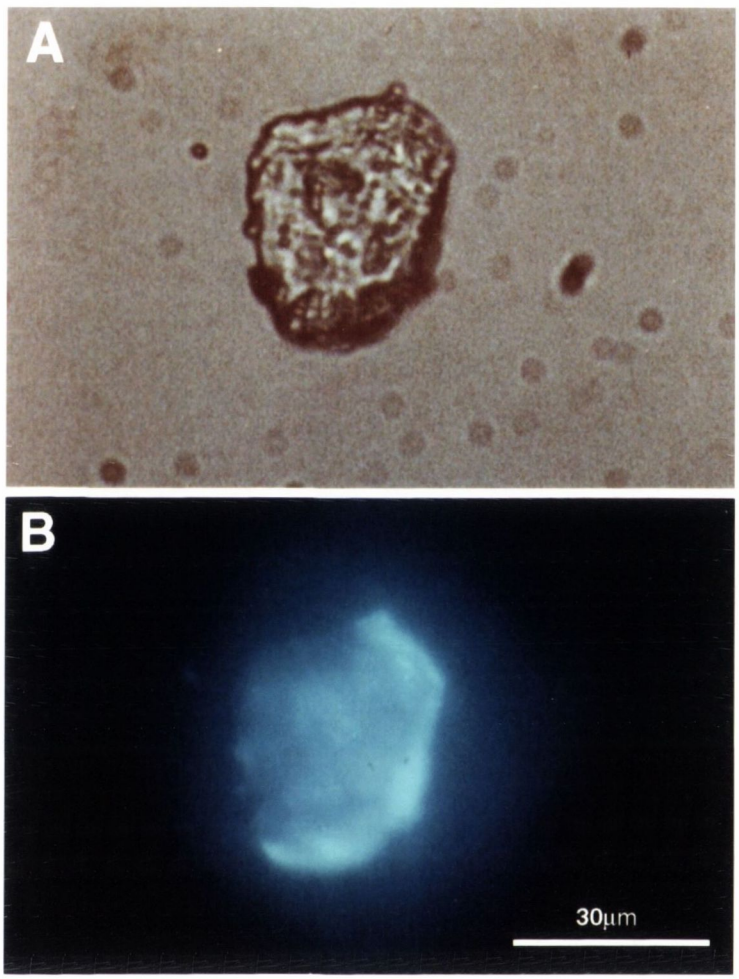
The table shows mean values (±SD; number of cells/0.9µl chamber) of isolated circulating endothelial cells. There was a significantly greater number isolated from cigarette smokers compared to non smokers (* p=0.02; students 2 sample t-test). This number was significantly decreased after supplementation of smokers with taurine (†=p=0.006; students paired t-test).

Variable	ICAM-1	VCAM-1	E-selectin	P-Selectin	CD34	CD36
Control subjects						
Value (%)	42±12	35±7	29±11	35±8	25±15	28±5
No. of subjects	6	6	6	5	3	3
Smokers						
Value (%)	63±13	66±8	63±10	66±12	64±16	54±7
No. of subjects	7	7	7	6	6	6
p value *	0.02	0.0001	0.0003	0.0008	0.03	0.0025

Table 10.3.2 Phenotype of Circulating Endothelial Cells

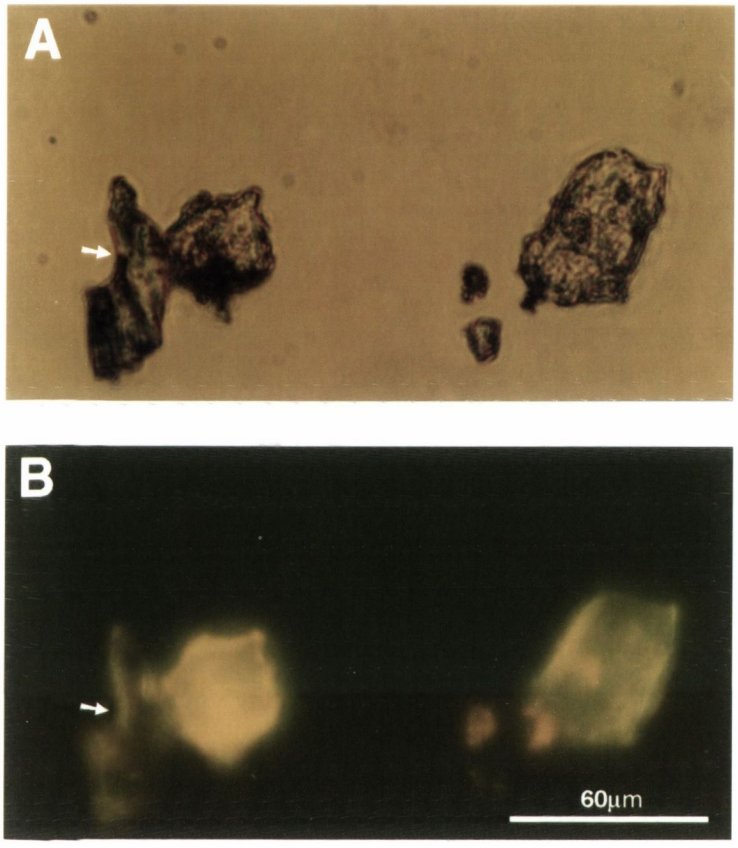
This table shows mean values (±SD; % of expression) of activation marker positive isolated circulating endothelial cells.

* p values are for the comparison between smokers and non-smokers



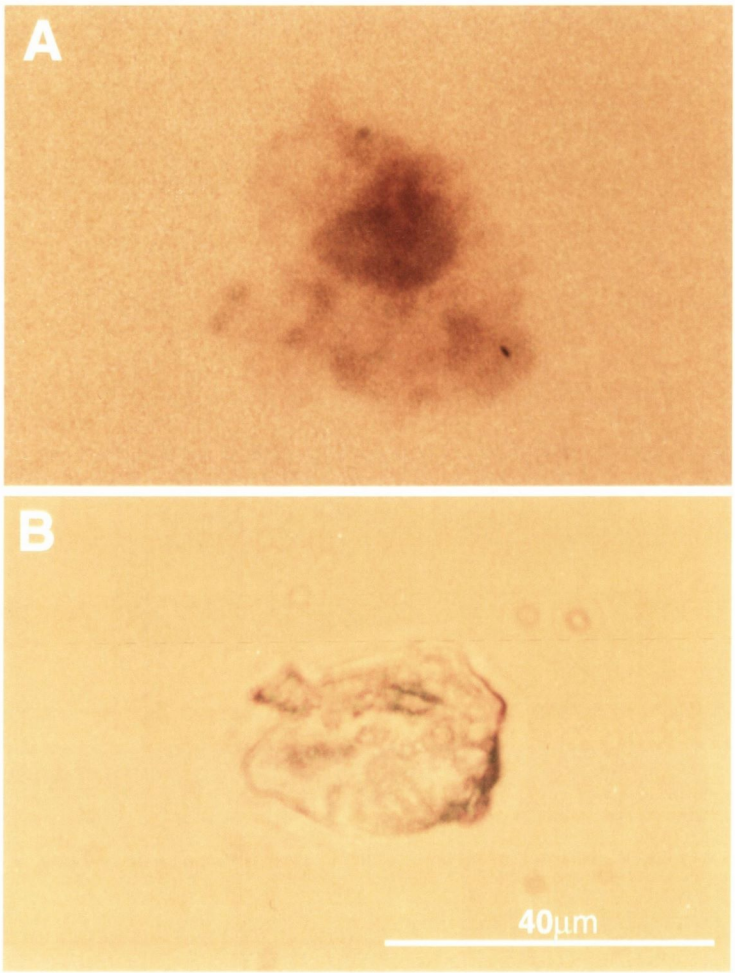
Figs 10.1a and 10.1b

Examples of rounded CECs without (a) and with (b) immunofluorescent staining for activation markers; ICAM-1 expression is being demonstrated in this example.



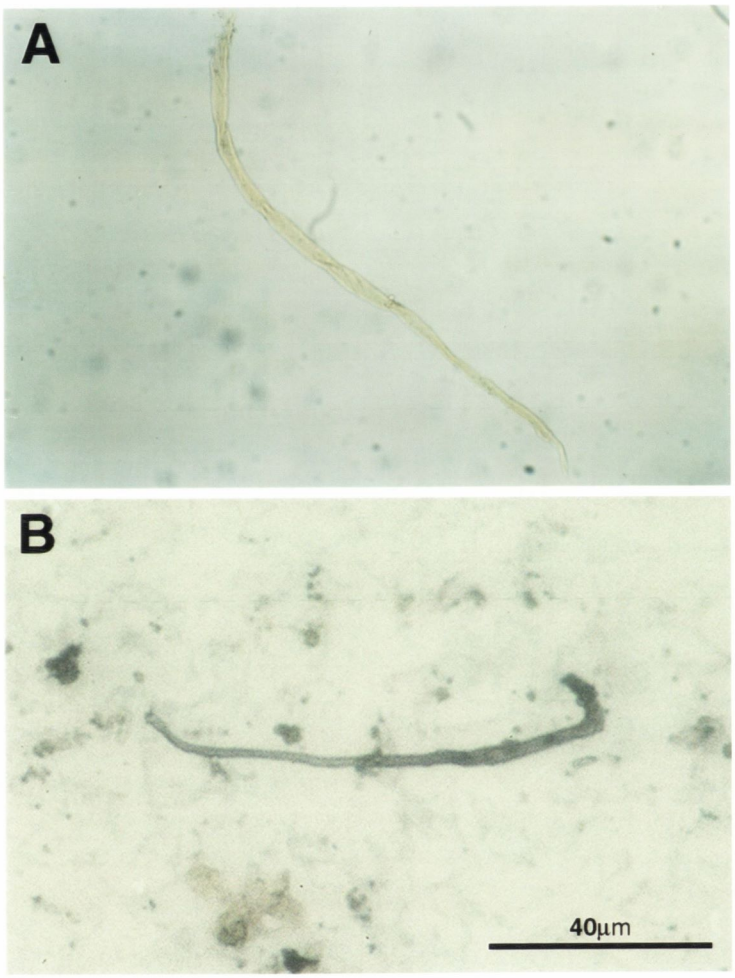
Figs 10.2a and 10.2b

Examples of spindle-shaped CECs (arrowed) lying adjacent to rounded CECs, without (a) and with (b) immunofluorescent staining for markers of activation; ICAM-1 is being demonstrated in this example.



Figs 10.3(a) and 10.3(b)

Representative example of a rounded CEC staining positive with terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) stain, indicative of apoptosis (a), and a control cell demonstrating absence of staining (b).



Figs 10.4(a) and 10.4(b).

A representative example of a spindle-shaped CEC staining positive with indirect staining for Akt expression (a), and a spindle-shaped CEC demonstrating level of background staining in absence of primary antibody (b).

10.4 Discussion

The exact components of cigarette smoke implicated in the pathogenesis of atheromatous disease are not known, nor is the exact mechanism by which smoking-related endothelial damage occurs. Smoking may lead to arterial disease by perturbing endothelial barrier function either by increasing expression of adhesion receptors thereby facilitating transmigration of inflammatory cells, especially monocytes, or by denuding endothelial surfaces by increased desquamation and endothelial cell death. These concepts were examined by assessing the extent of endothelial cell activation, desquamation and apoptosis and it was found that chronic cigarette smoking in young people indeed leads to increased activation and desquamation of endothelial cells. A minority of these cells stain positively for apoptosis. Taurine supplementation of smokers reduced the total number of CECs.

10.4.1 Endothelial cell desquamation

Using methodology described by Hladovec and Rossman, and previously used by other groups (Prerovsky and Hladovac, 1989; Davis *et al.*, 1995) the data has shown that chronic cigarette smoking results in increased numbers of CECs. It is not clear from these studies, given that the majority of cells are not apoptotic, why cells are dislodged into the circulation. If the apoptotic process has not been initiated in these cells, why did they detach and migrate into the circulation? It is of interest that the majority of CECs are spindle shaped. Change in endothelial cell morphology is regarded as important in altering endothelial barrier function. The first detectable changes in endothelial cells on exposure to cytokines is reported to be a change in shape from cobblestone to spindle (Nelimarkka *et al.*, 1997; Horrevoets *et al.*, 1999), a striking visual finding noted here also. Perhaps such spindle cells may be more readily detached when exposed to adverse haemodynamic and local environmental

conditions as exist in smokers. Smokers are known to have abnormal haemodynamics (Meade *et al.*, 1987; Nowak *et al.*, 1987; Benowitz *et al.*, 1991) and these abnormalities antedate structural changes in the arterial wall by many years. It is possible that the increase in CECs may reflect physical dislodgement of cells from vessels due to endothelial cell injury at times of haemodynamic or oxidant challenge. It is also possible, as has been proposed by Nerem, that the vascular endothelium of arterial disease patients may in fact be functioning normally (that is, within normal physiological limits) and that the fault lies with the environment in which the cell finds itself, and not the cell itself (Nerem *et al.*, 1993). The cell then responds "normally" to this abnormal environment.

10.4.2 Endothelial cell apoptosis.

These experiments revealed that CECs in both smokers and non-smokers are committed to death through apoptosis. Approximately 10% of isolated CECs in both groups stained positively for apoptosis. Also, CECs are detached but membrane integrity is preserved and cells continue to express activation markers. In the early stages of apoptosis cell membrane integrity is preserved (Ortiz *et al.*, 1996) whereas a permeable membrane is a feature of a necrotic or late-stage apoptotic cell. Other morphological features of apoptosis, such as cell membrane blebbing, were also identified. There has been much interest of late in controlled or selective death of endothelial cells. A view held by Raff is "just as a cell seems to need signals from other cells in order to proliferate, so it needs signals from other cells in order to survive; in their absence, the cell kills itself by activating an intrinsic suicide programme" (Raff, 1992). Growth, function and survival of endothelial cells is dependent on the ECM. This dependence on substrate attachment is known as anchorage dependence; apoptosis from loss of anchorage being coined "anoikis" (Frisch *et al.*, 1994). Integrins act as the mechanical link between the ECM and the endothelium, but they also control signalling pathways for growth and survival.

Changes in integrin signalling is one school of thought as to how the apoptotic switch of endothelial cells is mediated (Boudreau *et al.*, 1995; Meredith *et al.*, 1993). Recent work by Chen *et al.*, have proposed that endothelial cells can alter their shape in response to instructions from the ECM, and that cell shape may govern whether the endothelial cell lives or dies (Chen *et al.*, 1997; Re *et al.*, 1994). In response to either a chemical or a mechanical stimulus, endothelial cells normally extend and spread themselves out over a large surface area, survive and proliferate. In contrast, cells that lose contact with the ECM become more rounded and apoptose; retraction and rounding up being a well recognised feature of apoptosis (Bellamy *et al.*, 1995). The observation that the ECM is required as a cell survival factor to prevent programmed cell death in capillary endothelial cells (Meredith *et al.*, 1993), and Chen's suggestions that living cells can filter the same set of chemical inputs (activation of integrin and growth factor receptor signalling) to produce different functional outputs (growth versus apoptosis) as a result of local mechanical deformation of the cell, may help explain our observation that most of the CECs isolated were typical spindle or polygonal shaped cells, but more rounded, contracted type cells were also present. Promotion of cytoskeletal proteins and actin polymerisation by endothelial cells are necessary for flattening and survival of the cell, without which they undergo actin polymerisation, obtain a round shape and die by apoptosis (Fabio *et al.*, 1994).

It is possible that the rounded CECs seen are those who failed to organise actin microfilaments and died. It is also conceivable that the environment within which endothelial cells of smokers are found is one of repeated vasoconstriction (Gerlis, 1955; Nowak *et al.*, 1987) and loss of vasodilatation thereby preventing or decreasing the stimulus for endothelial cell spreading, with resultant apoptosis, as it is known that endothelial cells die when grown in conditions that prevent cell spreading *in vitro* (Ingber *et al.*, 1990; Inger *et al.*, 1992; Meredith *et al.*, 1993). It is also possible that there is a direct connection between EC activation and EC death by apoptosis. A key concept of apoptosis is that which follows from the observation that all types of

damage can initiate apoptosis, and cells do not die as a result of a specific insult. Once the cell senses these insults it signals a self-execution process. It may therefore follow that activation of the endothelial cell, from whatever source, or indeed the actual stimulus for activation itself, may have a role in initiating EC death. This direct relationship is seen in the case of neutrophil apoptosis (Kettritz *et al.*, 1997) and even more so in the case of lymphocyte apoptosis, where the degree of apoptosis correlates with the state of activation of the immune system (Gougeon *et al.*, 1996; Jaleco *et al.*, 1994).

Results obtained here demonstrate that a small percentage of spindle-shaped circulating endothelial cells from both smokers and non smokers express Akt. Prevention of apoptosis by Akt expression in elongated circulating endothelial cells is therefore only possible in a small number of cells. This is in keeping with recent observations that Akt protein levels are repressed in endothelial cells undergoing anoikis (Fujio & Walsh, 1999). Activation of genetically controlled cell death is under the control of environmental signals such as growth factors (Duke & Cohen, 1986; Koury & Bondurant 1990; Williams *et al.*, 1990) and cytokines (Colotta et al, 1992; Colotta *et al.*, 1993). Growth factors and cytokines act by activating a particular receptor which in turn activates signal transduction pathways involved in proliferation and survival. Akt mediates survival signals transduced by serum and growth factors (Kennedy *et al.*, 1997). A possible explanation for results obtained is that once in the circulation the integrin-mediated signalling cascade from the extracellular matrix to FAK would be lost. Signalling from growth factors, through integrins which induce Akt expression, would no longer occur and may therefore perhaps result in decreased expression of Akt in endothelial cells which circulate, the 5% perhaps representing the more recently desquamated cells.

It is also possible that apoptosis prevention in elongated spindle cells may be due to continued expression of other, or a combination of, protein receptors, as serum-mediated inhibition of apoptosis is through multiple signalling pathways,

inhibition by Akt being similar to that of Insulin Like Growth Factor-1 (IGF-1), insulin and Platelet Derived Growth Factor (PDGF) (Harrington *et al.*, 1994). The prevention of apoptosis in circulating endothelial cells may therefore be due to expression of a protein receptor which lasts on the circulating cell for a longer time than Akt.

On the other hand, it could be argued that it is not totally impossible for Akt to mediate apoptosis prevention. If only 10% of CECs stained positively for apoptosis and only 5% of cells stained positively for Akt, 85% of CECs are not staining for either. Perhaps loss of Akt expression allows for the overrule of cell death promoters, cells loose their elongated shape and become more rounded, and apoptosis signals are put into action. Apoptotic staining, however, may not be detected straight away.

This chapter therefore reveals that while the concept of Akt expression on elongated endothelial cells signalling an inhibitory process of self-destruction is not conceptually incorrect, expression only occurring on elongated cells, not all of such spindle-type cells express this protein. The transition from a proliferating to an apoptotic cell, or from a spindle to a rounded cell, is therefore not one which is clearly defined, nor does it appear to be mediated by a single signalling pathway.

10.4.3 Endothelial response to taurine

This study also revealed that supplementing smokers with taurine decreased the number of CECs. There are a number of possible explanation as to why this may be so. Firstly, it has been previously demonstrated that taurine can prevent endothelial cell apoptosis *in vitro*, (Wang *et al.*, 1996). Secondly, as reported in chapter 4, supplementing smokers with taurine upregulates constitutive nitric oxide synthesis in HUVEC, a role for NO in protection from macrovascular endothelial apoptosis being identified (Ceneviva *et al.*, 1998; DeMeester *et al.*, 1998; Shen *et al.*, 1998; Fiorucci *et al.*, 1999; Suschek *et al.*, 1999). Supplementing smokers also leads to restoration of

endothelial function, as discussed in chapter 3, and may return a normal environment to the endothelial cell. Thirdly, it is also possible that taurine may exert its beneficial effects in preventing endothelial cell apoptosis by aiding integrin function. Integrins function as signalling receptors and can regulate many intracellular signalling pathways, including intracellular calcium fluctuations. If, through some mechanical of chemical stress, ECM interactions (mediated by the integrins) are precluded, taurine may aid integrin functioning by regulating intracellular calcium fluctuations, thereby allowing the ECM to continue its role as a cell survival factor. Supplementing smokers with taurine did not have an effect on EC activation. Considering the fact that EC activation type 11 takes some time to cause an effect *via* gene transcription, 5 days supplementation may not have been long enough to exert an effect. However, it is important to note that the total number of CECs was reduced with taurine supplementation. This is likely consistent with a decrease in an inflammatory response to smoking, and may be similar to that identified in sickle cell disease where an increase in CECs was observed during an inflammatory sickle cell crisis (Solovey et al, 1997).

10.4.4 Endothelial cell activation

It is likely that the endothelial cells were activated while in situ, as suggested to occur in sickle cell anaemia (Solovey *et al.*, 1997). It is conceivable how the membrane of these cells would still express activation markers for some time after desquamation, as programmed cell death is a complex process which most likely began while the endothelial cells were *in situ* and continued when the cell became detached. Two stages of endothelial cell activation exist (Bach *et al.*, 1995); the first, or type 1, does not require *de novo* protein synthesis and occurs rapidly. However, endothelial cell activation type 11 requires some time to cause an effect *via* gene transcription and protein synthesis. Some of the genes involved are those required for

adhesion molecule formation, further supporting the concept that CECs are activated whilst in situ.

There are a number of possible mechanisms by which endothelial cells become activated: through aqueous or non-aqueous cigarette smoke components, or through oxidant- or monocyte-mediated activation. However, identification of the mechanism of activation is beyond the scope of this chapter, although recent evidence would suggest that endothelial cell activation involves a central pathophysiological process of a common intracellular control mechanism through the activation of transcription factors, including nuclear factor κB (Baldwin *et al.*, 1996; Hunt &Jurd, 1998).

Investigation of endothelial expression of CD34, an antigen which is expressed on haematopoietic progenitor cells (Katz *et al.*, 1985) and activated endothelial cells (Fina *et al.*, 1990; Nicholoff *et al.*, 1991) is of particular interest. CD 34 functions as an adhesion molecule and has been shown to be concentrated on the surface of vascular endothelial cells in the region where membrane processes interdigitate between endothelial cells (Fina *et al.*, 1990). This suggests that CD34 might be involved in adhesion or migration of endothelial cells. However, not all endothelial cells are CD34 positive. The endothelium of most large arteries and veins are CD34(-), while capillary endothelium of most tissues are CD34(+) (Fina *et al.*, 1990). Expression of this antigen was significantly increased in smokers. This data is supported by the finding that more than 50% of CECs from smokers were CD36 positive, demonstrating that CECs in smokers tended to be microvascular in origin. As pointed out by Solovey *et al.*, we cannot say with certainty that CD36 negative cells are not microvascular in origin as dermal microvessels have been identified as CD 36 negative (Petzelbauer *et al.*, 1993). The fact that cigarette smoking has an adverse effect on microvascular turnover is of extreme importance in view of the fact that these microvascular endothelial cells are known to have a limited replicative capacity. It is reasonable to suggest, therefore, that the process of accelerated

microvascular cell death may in time lead to denuded microvasculature and eventual capillary obliteration. This concept of altered vascular architecture secondary to increased microvascular turnover has been previously proposed in diabetics (Mizutani *et al.*, 1996). In cigarette smokers it may lead to an increased risk of vasoocclusive disease and may contribute to the abnormal haemodynamic responses observed.

10.4.5 Summary

Based on the prevalence of cardiovascular disease in the western world today, and the consistently high incidence of smoking amongst young people, this chapter investigated a potential mechanism by which cigarette smoking may predispose to cardiovascular disease. Chronic smoking results in an increased number of CECs. Smoking also results in increased activation of endothelial cells, which most likely occurs whilst *in situ*. Endothelial cell activation is necessary for leukocyte transfer across the vascular endothelium, and is also associated with other endothelial phenotypic changes leading to increased permeability (Horvath *et al.*, 1988), increased production of the t-PA inhibitor, plasminogen activator inhibitor-1 (Loskutoff *et al.*, 1977), of endothelin (Yanagisawa *et al.*, 1988) and of tissue factor (Weis *et al.*, 1991). It is also associated with loss of vascular integrity, cytokine production and upregulation of HLA molecules (Hunt & Jurd, 1998).

Although these responses may be part of a normal vascular immunological response, they may also contribute to the observed dysfunction in this subject group. We have also found that CECs change their shape and die by apoptosis. Apoptosis of endothelial cells contributes to vascular pathology and is a possible mechanism by which barrier function may be reduced. In addition, once vascular endothelial cells become apoptotic they also become pro-coagulant (Bombeli *et al.*, 1997). It is tempting to speculate that cigarette smoking, through inducing endothelial activation, desquamation and death by apoptosis, may promote abnormalities in function and lead

to areas of exposure to the extracellular matrix, particularly in the microvasculature where turnover of cells is high. The amino acid taurine can prevent this increase in circulating endothelial cells, and may have an important role in endothelial cell functioning and survival.

Chapter 11

General Discussion

Cardiovascular disease remains the greatest cause of morbidity and mortality world-wide. According to the most recent American Heart Association data "..nearly one in five cardiovascular disease deaths is attributable to smoking....and studies show that 43% of American children are exposed to environmental tobacco smoke in the home..." (American Heart Association, 1998). This modern "tobacco epidemic" shows no signs of abating notwithstanding the vigorous health promotion programmes, tough-minded public health policies and legislation aimed at curbing the attractiveness of the habit. The dismal picture seen with primary prevention is mirrored by the experience with secondary prevention programmes, where only 4% of the general smoking population heed advice and successfully eschew the habit.

Cardiovascular function and clinical outcome have been extensively examined in cigarette smokers. Most of such studies have focused on the ability of anti-oxidants to modify either function or outcome, large scale long term dietary supplement studies or invasive provocative haemodynamic studies being used. The former, as reported earlier, have met with, at best, equivocal results. The latter studies have a number of disadvantages, not least of which is the invasive nature of the provocation tests and the unphysiological doses of the antioxidants employed. Usually only one function, for example flow-mediated dilatation, is assessed and the impact of any isolated malfunction on integrated cardiovascular physiology has been ignored. In this thesis, a multifunctional evaluation of cardiovascular physiology was adopted to overcome these limitations.

In addition to the examination of both central and peripheral haemodynamics and their effect on ventricular function, attention was also addressed to the barrier function of the endothelium. The maintenance of endothelial permeability is clearly integral to the normal haemodynamic functioning of the circulation, but it also has the capacity to influence the health of tissues and organs through either the inflammatory or the thrombotic process. Activation of the endothelium can result in the migration of cytotoxic inflammatory cells into the wall of the large vessels or through the wall of

microvessels into adjacent tissues. Thrombosis in either the macro- or microvasculature may ultimately result in tissue or organ damage, or destruction as a result of obstruction to blood flow. Endothelial barrier function is also critically dependent upon the ability to balance cell loss by cell replacement.

Cigarette smoking contains a myriad of components, at least 4,000, capable of directly affecting endothelial health and function. However, this thesis addresses an indirect pathway, that of the monocyte-endothelial cell interaction, for a number of reasons. Cigarette smoking is known to activate inflammatory cells and the interaction between the monocyte and endothelium is regarded as a critical early step in the atheromatous process. However, its role in initiating or contributing to the physiological dysfunction of the endothelium is an important question not yet answered. This novel approach to assessing mediators of endothelial dysfunction uses the circulating monocyte as a representative of the environment to which the endothelium of a cigarette smoker is exposed. In addition, the endothelium's regulation of vasomotor tone is critically dependent on the bioavailability of NO, as indeed is control of endothelial permeability. The effect of cigarette smoking on NO release from endothelial cells is also a major focus of this thesis.

The agent chosen for study, taurine, has been shown not only to be protective of endothelium against direct attack with toxins such as high levels of glucose and sodium arsenite, but also to modulate the interactions between different inflammatory cells and the endothelium. In addition, taking into consideration the results of multicenter large scale clinical trials of nutrient supplementation on cardiovascular disease prevention, it appears that investigation of nutritional approaches to disease prevention should focus on dietary manipulation rather than high level antioxidant supplementation. This thesis therefore also investigated the effects of a nutritional approach, using the amino acid taurine, on offsetting smoking-induced adverse effects.

The studies reported in this thesis confirm many previous observations on the effects of cigarette smoking on the cardiovascular system, even in otherwise healthy adults, most notably the increases in resting heart rate, systolic and diastolic pressure and the loss of flow mediated dilatation in conduit vessels. The cause of the increase in heart rate was not examined but has previously been explained on the basis of increased circulating catecholamines and decreased oxygen carrying power of the blood. Heart rate has previously been thought to be inversely related to left ventricular ejection time. However, the use of applanation tonometry measurements clearly indicates that the percentage time spent in systole is significantly greater in smokers. The Buckberg Index, as outlined in chapter 6, is the ratio of the aortic diastolic pressure time integral to the aortic systolic pressure time integral and is thus the ratio of haemodynamic capacity for supply to the myocardial oxygen demand. The changes seen in this index in smokers demonstrate an important impairment of cardiac potential. Ventricular work load is critically dependent on blood pressure, which is elevated in young cigarette smokers. However, ventricular work load is also adversely affected by the magnitude and speed of wave reflection from the periphery, which in turn is also influenced by conduit vessel wall function. Because of increased wave reflection and decreased blood vessel wall compliance in cigarette smokers, left ventricular load is increased and indices relating to coronary perfusion are decreased. Similar derangements of macro- and microvascular function must also occur in the coronary circulation, thus further compromising myocardial perfusion. Therefore, even in the absence of mural disease and structural narrowing of the coronary circulation, the increase in ventricular load and decrease in perfusion will result in adverse consequences for cardiac myocyte function.

The primary purpose of conduit vessel dilatation in response to a stressful stimulus is to initially uncouple or distance the ventricle from the sudden increase in load which results from the induced increase in peripheral resistance. The increase in the differential of the pulse wave (dP/dt) in smokers in response to the physiological

stress of cold is indicative of the loss of this important adaptive response. Smoking-induced changes in endothelial regulatory function, thus, have a capacity to disturb and disrupt integrated cardiovascular physiology.

The haemodynamic results observed in smokers post short-term taurine supplementation are intriguing. The restoration of a normal response to increased flow in conduit vessels is consistent with experimental findings of the ability of this amino acid to upregulate expression of cNOS, the enzyme which plays a pivotal role in the regulation of vascular tone. The incomplete restoration of the initial vasodilator response to the cold pressor stress confirms an important influence of cigarette smoking's adrenergically mediated impact on smooth muscle contractility. The relative failure of taurine in this context may be a reflection of the dose used. However, taurine still has the ability to favourably affect ventricular-arterial interactions, in that responses in central dP/dt to the cold pressor test in smokers supplemented with taurine approached that of non-smokers.

The most unexpected finding in the studies reported in this thesis is the significant increase in blood pressure in smokers after only 5 days supplementation with taurine. This is unexpected for a number of reasons. The increase in blood pressure seen in smokers is primarily due to adrenergically mediated vasoconstriction in the peripheral circulation. Taurine has previously been demonstrated to have potent anti-sympathetic actions both experimentally and clinically. There have been no reports of taurine induced vasoconstriction at these doses. Taurine induced increases in blood pressure in a large animal model have only been seen with an intra-venous bolus injection of taurine at doses twenty fold greater than those used in this study (Egan, 1999). The blood pressure increases in smokers post taurine supplementation are even more surprising when viewed against the background of the restoration of flow dependent dilatation in the same study group. This vasodilatory response to increased flow is believed to be critically dependent on the ability of the enzyme cNOS to increase the release of NO. This problem was addressed in this thesis by a series of

experiments where macro- and microendothelial cells were exposed to MCM from non-smokers, smokers and taurine supplemented smokers. The findings in terms of the expression of cNOS and the release of NO when macrovascular cells were exposed to MCM were entirely consistent with the observed *in vivo* changes in flow mediated dilatation, an NO-dependent phenomenon. Taurine reversed the downregulation of cNOS expression and the reduced NO release in smokers.

However, the findings were not the same in terms of NO release when microvascular cells were employed under similar conditions. In these studies, larger amounts of NO was released from the endothelium when co-cultured with MCM from smokers in comparison to non-smokers. In contrast to the macrovascular studies, taurine supplemented smokers' MCM induced a reduction in NO release. These results may suggest that smoking induces a pro-inflammatory state in the microcirculation, with resultant release of inflammatory-induced NO. All cells are conditioned by the dynamics of the micro-environment in which they function, and it is likely that both qualitative and quantitative differences in the release of NO from endothelial cells of different sizes and origins in response to inflammatory cytokines are present. When viewed in this light, the blood pressure responses seen with taurine supplementation may be explainable. One might anticipate that the conditioned response of the endothelium in the slower moving microcirculation to pro-inflammatory stimuli would be one of vasodilatation, the hallmark physiological response to inflammation. If this is the case then the vasomotor tone in cigarette smokers is the result of the balance between the adrenergically-mediated vasoconstrictor response and the inflammatory vasodilatory tendencies. Taurine modulation of the inflammatory response would thus result in an increase in blood pressure. These findings also emphasise how failure to ablate the ongoing inflammatory response in the microvasculature may go unchecked, if treatment of large vessel function, and the restoration to normal of such parameters, is the main focus of concern.

The directional role changes in NO release from endothelial cells of different origins in response to MCM suggests a role for the monocyte in the maintenance of endothelial health and function. This is further supported by the *in vitro* studies of endothelial cell proliferation where the previously observed inhibitory effect of MCM from normal subjects was confirmed. The even greater degree of inhibition seen with exposure to smokers' MCM, coupled with the observed significant increase in apoptosis, would suggest deleterious consequences if similar effects were to be obtained *in vivo*. It has been suggested that the endothelium, normally quiescent with a slow turnover, has a limited replicative capacity. An increase in turnover with exhaustion of the replicative response has been implicated in the genesis of capillary rarefaction with resultant decrease in the size of the functional microvasculature with a concomitant increase in the fixed peripheral vascular resistance. Whilst taurine supplementation does not normalise the proliferation-apoptosis responses in these studies, there is a significant improvement. Such an effect, if it should pertain *in vivo*, points to a potential benefit of this amino acid in vasculopathies, especially where excess adrenergically-mediated increases in vascular tone are not a major factor.

The attempt to identify a specific soluble mediator responsible for the NO mediated effects was unsuccessful. Whilst excess release of a number of known pro-inflammatory cytokines from monocytes isolated from smokers was identified, taurine did not significantly downregulate the mediators. However, the identification of excessive release of these mediators, which have been implicated in plaque formation, expansion and instability, is clearly of importance. This information would indicate that at least some mechanisms of atherogenesis are shared by smoking and the dyslipidaemic process.

The new observation of increased VEGF release from smokers' MCM and the endothelial cell proliferation and apoptotic effects appear contradictory; VEGF being a potent pro-angiogenic mediator. Furthermore, cigarette smoking has been demonstrated to be potently pro-angiogenic in placental blood vessels (Pfarrer et al.,

1999). However, the effects of smoking *in vivo* may be mediated by hypoxia. This angiogenic factor may have potential adverse effects on the microvasculature by increasing the total amount of circulatory bed against which the heart must pump, which in turn increases ventricular workload. In addition, considering recent observations of an association between angiogenesis and the atheromatous plaque (McCarthy *et al.*, 1999), where an increase in neovascularisation in plaques was found to render them more liable to instability and thromboembolic sequelae, the significance of raised levels of VEGF production from monocytes of smokers is even greater.

The studies on isolated CECs confirm the investigative potential of these *ex vivo* cells. The fact that such cells are detected in an apparently healthy non-smoking population raises important questions. Are such findings simply a reflection of the normal cell turnover necessary to maintain the viability and integrity of the endothelium, or are they a manifestation of ongoing endothelial damage? The fact that many of these cells express markers of activation suggests the latter. The increased number of cells and the increased expression of activation markers, which underlie the inflammatory process, permit the inference to be drawn that cigarette smoking is a potent inflammatory stimulus. Based on the mechanism of endothelial activation, it is likely that these cells were activated whilst *in situ.* The fact that the isolated cells are derived from both the macro- and the microvasculature is in keeping with the haemodynamic observations recorded in both conduit vessels and the microvasculature. Interestingly, 50% of CECs from smokers are of microvasculature origin. As only 30% of CECs from non-smokers are of microvasculature origin, this finding may be of importance when the concept of capillary rarefaction is considered, a process well-known in hypertensive subjects which may possibly play a role in increasing peripheral resistance. Indeed, recent studies on capillary rarefaction suggest that reduction in capillary density is caused by structural absence of capillaries rather than functional nonperfusion (Antonis *et al.*, 1999).

It would appear that at least some of these cells are programmed to die through apoptosis. Only 10% of CECs stained positive for apoptosis. The anti-apoptotic signal Akt is not responsible for the absence of apoptosis staining in the majority of these cells. This absence is surprising when, theoretically, cell detachment is rapidly thought to initiate cell suicide. The majority of apoptotic cells may be ingested onsite by adjacent phagocytes, but the large number of increased circulating non-apoptotic activated cells favours the theory that activation results in cell death. These cells may be activated to perform specific, specialised tasks and then programmed to die with the least risk of damage to adjacent cells. It would therefore follow that modulation of the activation process is the key to limitation of end-organ damage and the preservation of functional integrity of the cardiovascular system.

The total number of CECs desquamated is increased in smokers, and decreased by taurine supplementation. The fact that taurine supplementation can decrease the number of CECs offers a number of possibilities as to how these cells are initially desquamated. Either directly through its anti-apoptotic properties or indirectly through upregulation of NO in the macrovasculature, taurine may aid in decreasing the number of apoptotic cells. Taurine may also restore a normal haemodynamic environment to macrovasculature, thereby decreasing a stimulus for desquamation.

These studies therefore suggest the existence of a continuum from health to physiological dysfunction and pathobiological process in cigarette smokers, likely leading to the setting of clinical disease. This thesis confirms the ability of physiological measurements to identify an at-risk population. It supports the use of multidimensional physiological evaluation of the cardiovascular system, and advocates the use of non-invasive techniques such as applanation tonometry and duplex ultrasonography in the clinical setting. Whilst the ability of dietary manipulation has exciting potential, the multifaceted nature of a tobacco attack on the cardiovascular system indicates that this approach alone will not be sufficient for disease prevention. The adverse effects of cigarette smoking on the cardiovascular system can only be

avoided by eschewing tobacco. The application of these techniques and strategies to other risk states for cardiovascular disease, such as hyperlipidaemia and hyperglycaemia, and even ex-smokers should prove to be of interest.

Appendix I

Suppliers	Addresses
Becton Dickinson	Erembodegem, Belgium
BioWhittaker	Berkshire, UK
Boehringer Mannheim	Mannhein, Germany
Gibco	Paisley, UK
Merck-Schuchardt	Munich, Germany
Pierce	Rockford, IL, USA
Premier Beverages	Stafford, UK
Promega Corporation	Madison, WI, USA
R & D Systems	Minneapolis, MN, USA
Roche Pharmaceutical	East Sussex, UK
Sigma	St. Louis, MO, USA
Transduction Laboratories	Lexington, KY, USA
Twinlab	Ronkonkoma, NY

Appendix II

Raw Data from Chapter 3

Non smokers: Flow-dependent dilatation

Baseline (mm)	*Post cuff deflation (mm)*	*Diameter difference (mm)*
3.05	3.8	.75
3.1	3.4	.3
3.7	4	.3
2.9	3.2	.3
3.6	3.9	.3
2.9	3.3	.4
4	4.3	.3
4	4.3	.3
3	3.35	.35
3.6	3.9	.3
3	3.5	.5
4	4.3	.3
3.65	3.95	.3
2.9	3.35	.45
3.5	3.95	.45

Mean values

Baseline (mm)	*Post cuff deflation (mm)*	*Diameter difference (mm)*
3.39 ± 0.43	3.76 ± 0.38	0.37 ± 0.12

Smokers: Flow-dependent dilatation

Baseline (mm)	*Post cuff deflation (mm)*	*Diameter difference (mm)*
3.6	3.65	.05
3.7	3.7	0
2.7	2.7	0
3.7	3.7	0
4.0	4.0	0
2.9	3.0	.1
3.7	3.7	0
2.8	2.8	0
3.5	3.53	.03
3.7	3.73	.03
4.1	4.13	.03
2.7	2.8	.1
3.0	3.05	.05
3.2	3.15	-.05
2.7	3.73	.03

Mean values

Baseline (mm)	*Post cuff deflation (mm)*	*Diameter difference (mm)*
3.33 ± 0.49	3.36 ± 0.5	0.025 ± 0.03

Smokers and Vitamin C supplementation: Flow-dependent dilatation

Baseline (mm)	*Post cuff deflation (mm)*	*Diameter difference (mm)*
3.65	3.7	.05
3.6	3.8	.2
2.7	2.85	.15
3.7	3.85	.15
3.9	4.0	.1
2.9	3.0	.1
3.6	3.8	.2
2.8	2.9	.1
3.45	3.55	.1
3.65	3.75	.1
4.0	4.1	.1
2.7	2.8	.1
3.05	3.15	.1
3.25	3.35	.1
2.7	2.8	.1

Mean values

Baseline (mm)	*Post Vitamin C (mm)*	*Diameter difference (mm)*
3.31 ± 0.46	3.42 ± 0.47	0.12 ± 0.04

Smokers and Taurine supplementation: Flow-dependent dilatation

Baseline (mm)	*Post cuff deflation (mm)*	*Diameter difference (mm)*
3.65	3.95	.3
3.7	4.1	.4
3.9	4.3	.4
3.8	4.2	.4
2.75	3.05	.3
2.95	3.25	.3
3.7	4	.3
4.0	4.4	.4
3.5	3.9	.4
3.75	4.05	.3
2.9	3.2	.3
2.75	3.15	.4
3.0	3.4	.4
3.3	3.65	.35
2.8	3.2	.4

Mean values

Baseline (mm)	*Post cuff deflation (mm)*	*Diameter difference (mm)*
3.36 ± 0.46	3.72 ± 0.46	0.36 ± 0.05

Appendix III

Raw Data from Chapter 4

Non smokers: Cold Pressor Test

Baseline (mm)	*1 minute in ice (mm)*	*3 minutes in ice (mm)*
2.9	2.9	2.8
3.05	3.2	3.0
4	4.1	3.8
4.2	4.25	4.2
3.5	3.5	3.4
3.8	3.8	3.5

Mean values

Baseline (mm)	*1 minute in ice (mm)*	*3 minutes in ice (mm)*
3.58 ± 0.52	3.63 ± 0.52	3.45 ± 0.51

Smokers: Cold Pressor Test

Baseline (mm)	*1 minute in ice (mm)*	*3 minutes in ice (mm)*
3.8	3.7	3.5
3.4	3.3	3.1
3.0	2.95	2.7
5	4.75	4.75
4	4	3.9
2.5	2.45	2.45
4	3.85	3.5

Mean values

Baseline (mm)	*1 minute in ice (mm)*	*3 minutes in ice (mm)*
3.67 ± 0.80	3.57 ± 0.75	3.4 ± 0.77

Smokers + Taurine Supplementation: Cold Pressor Test

Baseline (mm)	*1 minute in ice (mm)*	*3 minutes in ice (mm)*
3.8	3.7	3.7
3.3	3.25	3.15
3.0	3	2.8
4.45	4.5	4.4
4	4.05	4
2.55	2.5	2.3

Mean values

Baseline (mm)	*1 minute in ice (mm)*	*3 minutes in ice (mm)*
3.52 ± 0.70	3.5 ± 0.73	3.4 ± 0.79

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