SELENOPROTEIN FUNCTION AND EXPRESSION IN HUMAN ENDOTHELIUM

BY

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DECLARATION OF ORIGINALITY

I declare that the composition of this thesis and the work presented herein is my own. Work performed by others as part of collaborative studies are indicated in the text.

Susan Mary Miller

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ABSTRACT

Atherosclerotic cardiovascular disease is the principle cause of mortality in man in Western society. Endothelial dysfunction caused by reactive oxidant species favours atherogenesis. The trace element selenium is thought to contribute to the prevention of such damage through the expression of intracellular or extracellular selenoproteins. Whilst the glutathione peroxidases may have an antioxidant role in the endothelium, other selenoproteins may also be important.

In this thesis the selenoproteins expressed by endothelial cells (isolated from various vascular beds and different species) and the modification of their expression through changes in selenium supply and activation of second messenger pathways were studied. The ability of sodium selenite and selenomethionine supplementation to prevent oxidative damage in cultured endothelial cells was also examined.

The present study has confirmed the expression of cytoplasmic glutathione peroxidase (cyGPX) and phospholipid hydroperoxide glutathione peroxidase (PHGPX) in human umbilical vein endothelial cells (HUVEC). However, thioredoxin reductase (TR) was found to be the predominant selenoprotein expressed, accounting for approximately 43% of the total intracellular [^{75}Se]-labelled proteins. No extracellular selenoproteins were synthesised by HUVEC.

The overall pattern of selenoprotein expression in endothelial cells isolated from different species and from various human tissues showed considerable variation, though differences were less pronounced when comparing the endothelial cells isolated from various human vascular beds and the human endothelial cell line EAhy926. For example, human coronary arterial endothelial cells which provide an ideal model to study atherosclerosis have a very similar selenoprotein profile to that of HUVEC.

The expression of TR, cyGPX and PHGPX and other unidentified selenoproteins in HUVEC was modified in response to the phorbol ester, phorbol-12-myristate 13-acetate (PMA). A 48 hr incubation with PMA significantly decreased expression of both TR and PHGPX to

approximately half of that found in untreated cells. The expression of cyGPX was increased approximately two-fold by PMA treatment. A brief exposure to PMA (one minute) produced similar effects on the expression of these selenoproteins after a 48 hr lag-period. These effects of PMA could be attenuated by the protein kinase C inhibitor, GF109203X. The calcium ionophore A23187 also modified selenoprotein expression, in particular increasing the expression of TR. However, these effects were only apparent after a 60 minute exposure, becoming maximal after 35 hr, after which time cells began to detach. These observations suggest that the A23187 effect may result from toxicity rather than activation of the calcium signalling pathway. Endothelial cells isolated from the human coronary artery responded to PMA in a similar manner to HUVEC. In contrast, bovine aortic endothelial cells showed no observable changes in selenoprotein expression in the presence of PMA.

HUVEC grown in selenium-deficient culture medium and then supplemented with sodium selenite for 48 hr were less susceptible to oxidative damage from tert-butylhydroperoxide (t-BuOOH) compared to cells not supplemented with sodium selenite or cells in which sodium selenite was introduced at the same time as t-BuOOH. It was also observed that sodium selenite increased TR mass and TR, cyGPX and PHGPX activity at concentrations which protected HUVEC from damage by t-BuOOH. These results are consistent with the protective effect of selenium being due to the modification of selenoprotein expression and activity rather than a direct effect of sodium selenite. Selenomethionine was much less potent than sodium selenite at inducing protection from oxidative damage.

In conclusion, human endothelial cells express very high levels of TR, a selenoprotein whose expression can be modified by selenium supply, PKC activation and oxidative stress. Since TR, under certain circumstances is more potent than GPX's at detoxifying harmful peroxides, TR maybe an important factor in the ability of selenium to protect endothelial cells from oxidative damage, thereby limiting the development of atherosclerosis.

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ABBREVIATIONS

A23187	calcium ionophore, A23187
AC	adenylate cyclase
ACh	acetylcholine
ANOVA	analysis of variance
apoB	apolipoprotein B
ATP	adenosine triphosphate
BAEC	bovine aortic endothelial cells
BH_4	6(R)-5,6,7,8-tetrahydrobiopterin
BK	bradykinin
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
cGMP	guanosine 3', 5'- cyclic monophosphate
CPSR	control processed serum replacement
CSF	colony-stimulating factor
CV	coefficient of variation
cyGPX	cytoplasmic glutathione peroxidase
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
DTT	dithiothreitol
EBSS	Earle's balanced salt solution
EBM-2	endothelial basal medium-2
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor
EDTA	ethylenediaminetetraacetic acid

EGM	endothelial growth medium
EGM-2	endothelial growth medium-2
EGPX	extracellular glutathione peroxidase
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
FABP	fatty acid-binding protein
FAD	flavin adenine dinucleotide
FADPH	flavin adenine dinucleotide phosphate
FBS	foetal bovine serum
FMN	flavin mononucleotide
FCS	foetal calf serum
FGF-2	fibroblast growth factor-2
GC	guanylate cyclase
GF109203X	bisindolymale I, hydrochloride
giGPX	gastrointestinal glutathione peroxidase
GM-CSF	granulocyte macrophage-CSF
GPX	glutathione peroxidase
GSH	reduced glutathione
GSSG	glutathione disulphide
15-HPETE	15(S)-hydroperoxyeicosatetraenoic acid
HAT	hypoxanthine, aminopterin, thymidine
HBSS	Hank's balanced salt solution
HEPES	n-[hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HCAEC	human coronary artery endothelial cells
HCl	hydrochloric acid
HNE	4-hydroxynonenal
H_2O_2	hydrogen peroxide
hr	hour

HUAEC	human umbilical artery endothelial cells
HUVEC	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule-1
IDI	iodothyronine deiodinase
IL-2	interleukin-2
IP_3	inositol (1, 4, 5) trisphosphate
kDa	kilodalton
LDH	lactate dehydrogenase
LDL	low density lipoprotein
M199	medium 199
MBq	mega Becquerel
MCP-1	monocyte chemotactic protein-1
M-CSF	macrophage colony stimulating factor
MDA	malondialdehyde
min	minute
mRNA	messenger ribonucleic acid
mU	milli units
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NO_2^-	nitrite
NOS	nitric oxide synthase
NSB	non-specific binding
O_2^-	superoxide anion
OH^-	hydroxide
oxLDL	oxidised low density lipoprotein
PAEC	porcine aortic endothelial cells
PAF	platelet activating factor
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor

PDI	protein disulphide isomerase
PEG	polyethylene glycol
PGH_2	prostaglandin H_2
PGI_2	prostacyclin
PHGPX	phospholipid hydroperoxide glutathione peroxidase
PIP_2	phosphatidylinositol 4, 5 bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
PLC	phospholipase C
plGPX	plasma glutathione peroxidase
PMA	phorbol 12-mystrate 13-acetate
PUFA	polyunsaturated fatty acid
ROOH	lipid hydroperoxide
ROS	reactive oxygen species
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
Se	selenium
SECIS	selenocysteine insertion sequence
SEM	standard error of the mean
sGC	soluble guanylate cyclase
SPS	selenoprotein synthetase
TBARS	thiobarbituric reactive substances
TEMED	NNN'N-tetramethylethylenediamine
TGFβ	transforming growth factor β
TNB	5'-thionitrobenzoic acid
TNFα	tumour necrosis factor-alpha
TR	thioredoxin reductase

Tris	2-amino-2-hydroxymethyl propane-1,3-diol
t-BuOOH	tert-butylhydroperoxide
TXA_2	thromboxane A_2
VCAM-1	vascular cell adhesion molecule-1
vWF	von Willebrand Factor

CHAPTER ONE INTRODUCTION

1.1 ATHEROSCLEROSIS

1.1.1 Introduction

Atherosclerosis (Gr. "porridge-like hardening") is a highly complex vascular disease process which has been described as the principle cause of the pathogenesis of myocardial and cerebral infarction, gangrene and loss of function of the extremities (Ross, 1993a). It is the leading cause of morbidity and mortality in the United States, Europe, and much of Asia (Ross, 1999). Extensive research over the past 20 years has aimed to elucidate the cellular and molecular mechanisms involved in the formation, development and progression of the atheromatous lesion with resultant clinical sequelae in order to establish therapeutic agents for the prevention and treatment of atherosclerosis. Whilst a number of concepts prove controversial and many details have not been clarified the mechanisms involved in the development of the atheromatous lesion have been outlined.

1.1.2 'Response to injury' hypothesis

The most widely accepted model of atherogenesis is derived from the 'response-to-injury' hypothesis which was originally formulated by Ross *et al.* (Ross and Glomset, 1973) and has since been substantially tested and modified (Ross, 1993a; Ross, 1993b). The theory proposes that injury to the endothelium (for example, by local disturbances of blood flow at certain branch points of the arterial tree) coupled with major risk factors such as hypercholesterolemia, hypertension, cigarette smoking, and microbal infections can provoke a protective, inflammatory-fibroproliferative response. In excess this response results in a series of compensatory cellular and molecular events leading to the formation and development of the atherosclerotic lesion. This response is the result of multiple interactions of three different circulatory components-monocytes, T-lymphocytes and platelets together with the endothelium and smooth muscle of the arterial wall.

1.1.3 The lesions of atherosclerosis

The lesions of atherosclerosis are principally found in large and medium-sized elastic and muscular arteries such as the femoral artery, cerebral arteries, aorta and coronary arteries. Lesions rarely occur in fine vessels (arterioles and capillaries) although the pathophysiological consequences of atherosclerosis have been demonstrated in the human coronary microcirculation (Zeiher *et al.*, 1991). In addition to the different theories concerning the etiology of atherosclerosis different forms of classification of atherosclerotic lesions exist. The more classical nomenclature of atherosclerosis; that is the fatty streak, intermediate lesion and fibrous plaque (Ross, 1995) has been superseded by 'Stary's classification' (Yutani *et al.*, 1999). The 'Stary classification' is a universally accepted classification which embraces the more recent morphological and biochemical details of the processes involved in atherosclerosis.

a) Classical nomenclature

Under the classical nomenclature atherosclerotic lesions were arbitrarily divided into three categories (Table 1.01). The initial lesion is the fatty streak which is characterized by the presence of lipid laden foam cells together with T-lymphocytes in the arterial intima. In this early stage of atherosclerosis there is an absence of gross morphological change within the vessel wall. McGill *et al.* by 'mapping' lesions at different stages of development were able to demonstrate that not all fatty streak lesions will progress to fibrous plaques (McGill, 1984). It appears that within time some fatty streaks will develop into more advanced lesions whilst others stabilise or alternatively regress. In the event of their progression, the inflammatory response stimulates the proliferation and migration of smooth muscle cells into the area of inflammation which, together with macrophages, form the intermediate lesion. This can progress to thickening of the arterial wall which is compensated for by a gradual dilation referred to as 'remodelling' with little effect on the lumen diameter (Ross, 1999). These lesions can develop into the fibrous plaque referred to as a 'complicated' or 'advanced' lesion. At some point the artery can no longer compensate by dilation, and remodelling leads to the occlusion of the artery. The fibrous

Table 1.01. Classical nomenclature describing the progression of atherosclerotic lesions.

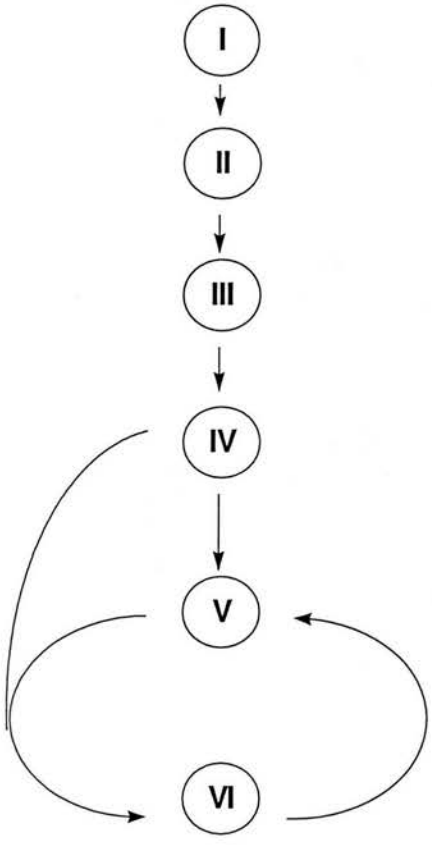
nomenclature	main histology
fatty streak	comprises lipid laden macrophages (foam cells) and T-lymphocytes
intermediate lesion (fibrofatty lesion)	comprises lipid-filled macrophages and T-lymphocytes smooth muscle cell migration into the area of inflammation and smooth muscle cell proliferation
fibrous plaque (complicated/advanced lesion)	comprises the fibrous cap (smooth muscle cells and connective tissue) and necrotic core (smooth muscle cells, macrophages associated with lipid and necrotic material)

plaque consists of a fibrous cap made up of smooth muscle cells and dense connective tissue, an underlying cellular layer, and a deeper necrotic core which comprises a mixture of leukocytes, lipid and debris. Calcification, necrosis, haemorrhage, ulceration or thrombosis can complicate the lesion further, producing the clinical manifestations of atherosclerosis sometimes with fatal clinical consequences (Fuster *et al.*, 1992). Each stage of atherosclerosis can potentially be reversed either by removal of the injurious agent or by treatment of the inflammatory and fibroproliferative processes (Ross, 1993a).

b) Stary's classification

Stary's classification characterises atherosclerotic lesions by categorising the process of lesion progression into five phases each being defined by its histological characteristics (table 1.02) (Stary *et al.*, 1995). Briefly, the initial type I lesion consists of macrophage-derived foam cells containing lipid droplets. The location of these and subsequent lesions is more evident in locations such as branches, bifurcations and curvatures which are subject to alterations in blood flow (Ross, 1999). These so-called 'adaptive thickenings' do not cause substantial morphological alteration and are present at constant locations in everyone from birth. Type II lesions (formerly designated 'fatty streaks') consist of layers of macrophage foam cells and smooth muscle cells with intracellular lipid droplets. The type III lesion represents an intermediate stage between the type II and type IV lesion. In addition to the lipid-laden cells pertaining to the type II lesion diffuse collections of extracellular lipid pools occur which serve as the immediate precursor to the core of extracellular lipid that characterises the type IV lesion. Further lesion progression occurs generally around the fourth decade of life. The lipid core may now also contain thick layers of fibrous connective tissue (type V lesion) and have the potential to rupture. Surface defects, hematoma and thrombotic deposits are also evident (type VI lesion). When type V lesions are largely calcified they are classified as typeVb whilst those which consist mainly of fibrous connective tissue with little or no accumulated lipid or calcium are referred to as

Table 1.02. Starys classification of atherosclerotic lesions. The flow diagram indicates the progression of human atherosclerotic lesions. The Roman numerals represent the different classification of lesion as defined in the left hand column and the direction of the arrows indicate the sequence in which characteristic morphologies may change Adapted from Stary *et al.* (Stary *et al.*, 1995).

nomenclature & main histology	**sequence in progression**
type I (initial lesion) isolated macrophage foam cells	
type II (fatty streak lesion) mainly intracellular lipid accumulation	
type III (intermediate) lesion type II changes & small extracellular lipid pools	
type IV (atheroma) lesion type II changes & core of extracellular lipid	
type V (fibroatheroma) lesion lipid core & fibrotic layer, or multiple lipid cores & fibrotic layers, or mainly calcific, or mainly fibrotic	
type VI (complicated) lesion fissure or rupture hematoma-hemorrhage, thrombus	

type Vc lesions. Figure 1.01 illustrates the characteristic features that may occur at a constant arterial location.

1.1.4 The cellular interactions of atherosclerosis

During the process of atherogenesis a complex series of interactions between different cells, growth factors, cytokines, and other small molecules occur. Perhaps one of the earliest events in atherogenesis is a change in the endothelial surface phenotype. At specific arterial sites the expression of molecules on the endothelium, responsible for the adherence, accumulation and migration of monocytes and T-lymphocytes, markedly increases. Adhesion molecules include selectins (e.g. L- E- and P-selectin), intracellular adhesion molecules (e.g. intracellular adhesion molecule-1 (ICAM-1)) and vascular-cell adhesion molecules (e.g. vascular cell adhesion molecule-1 (VCAM-1)) (Gimbrone, 1995; Ross, 1999). The expression of some vascular adhesion molecules such as ICAM-1 is in response to shear stress (Ross, 1999). The endothelial adhesion molecules act as receptors for glyconjugates and integrins present on monocytes and T-lymphocytes allowing the adherence of these leukocytes to the endothelial surface. An increase in permeability in the endothelium follows, mediated by oxidised low density lipoprotein (oxLDL), monocyte chemotactic protein-1 (MCP-1), interleukin-8, platelet derived growth factor (PDGF), macrophage-colony stimulating factor (M-CSF) and osteopontin which aids the transmigration of these inflammatory cells into the sub-endothelial intima by penetrating the endothelial junctions. These factors work in conjunction with chemoattractant properties of molecules such as MCP-1, oxLDL and osteopontin, which are synthesised by the endothelium, smooth muscle and monocytes and attract leukocytes to the arterial wall.

The presence of macrophages and T lymphocytes in the intima may alter the gene expression of several growth-regulatory molecules and cytokines which can potentially activate the endothelium, smooth muscle and other components of the developing lesion. Fatty streak

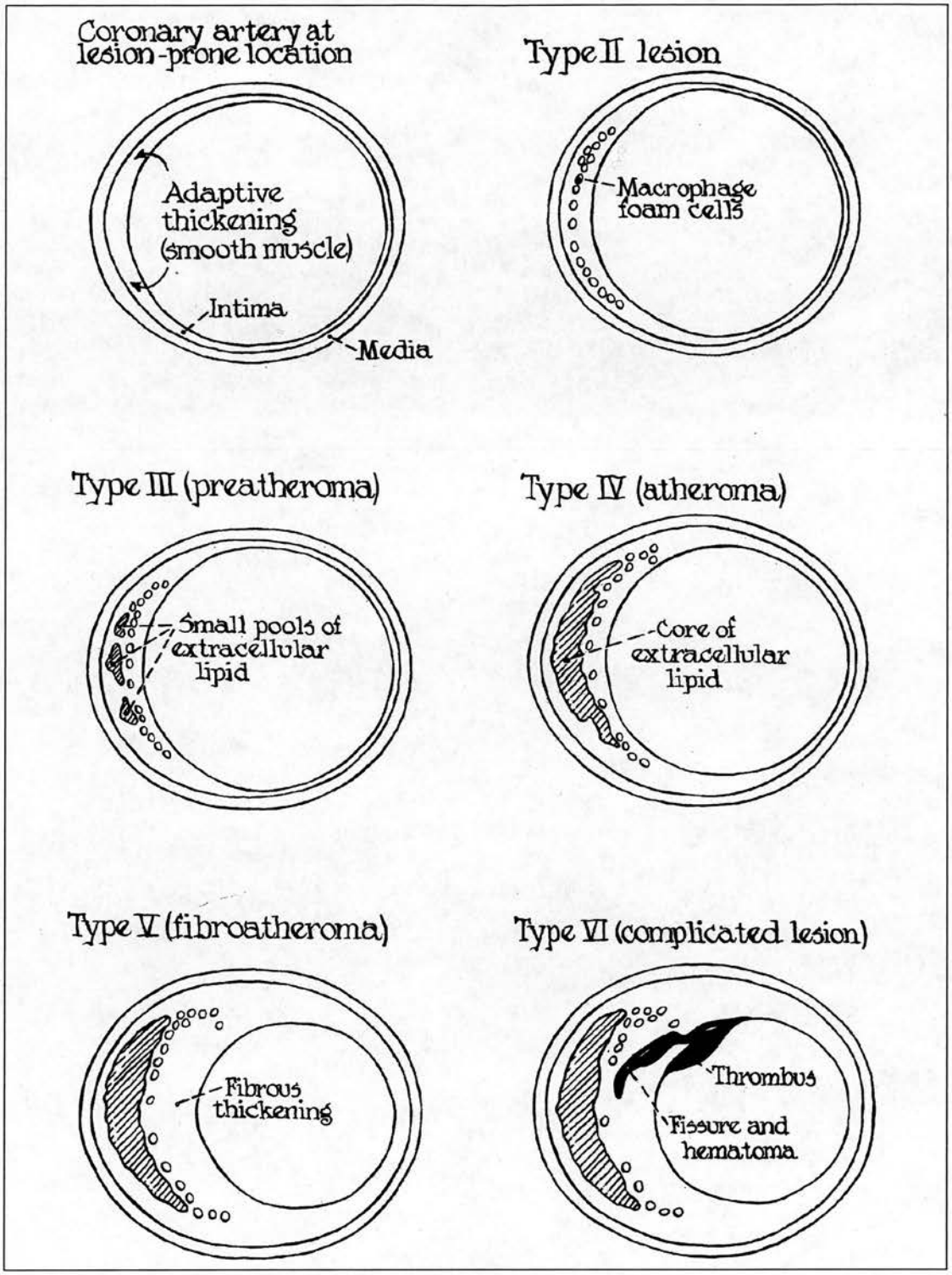


Figure 1.01. Schematic representation of cross-sections of identical, most proximal part of six left anterior descending arteries showing the sequence of atherosclerotic lesion types according to Starys classification. Identical morphologies may be found on other lesion-prone parts of the coronary and other arteries. From Stary *et al.* (Stary *et al.*, 1995).

formation involves the migration of smooth muscle cells into the intima, mediated by PDGF, fibroblast growth factor-2 (FGF-2) and transforming growth factor β (TGFβ). T lymphocyte activation is stimulated by tumor necrosis factor alpha (TNFα), interleukin-2 (IL-2), granulocyte macrophage-colony stimulating factor (GM-CSF) whilst foam cells develop through the action of oxLDL, M-CSF, TNFα, and IL-2. Platelet adherence and aggregation is also a feature of the fatty streak and is mediated by integrins, P-selectin, fibrin, thromboxane A_2, tissue factor and the factors which mediate leukocyte adherence and migration. Platelets adhere to the endothelium, exposed collagen and macrophages where upon activation they release their granules containing cytokines, growth factors and thrombin, all of which can mediate smooth muscle cell and monocyte proliferation and migration.

The development of the fatty streak into more advanced complicated lesions of atherosclerosis involves the formation of a fibrous plaque which forms as a result of the increase in PDGF, TGFβ, IL-1, TNFα and osteopontin and a decrease in connective tissue degradation. Evidence suggests that the smooth muscle cells present in the lesion have changed from a contractile phenotype which responds to nitric oxide (NO) and prostacyclin (PGI_2) to a synthetic phenotype which responds in an autocrine manner to PDGF as well as other factors to enhance proliferation and release extracellular matrix which progresses the lesion into a fibrous plaque. The continued entry of macrophages mediated by M-CSF, MCP-1 and oxLDL causes the fibrous cap, which encloses the fibrous plaque, to expand and possibly rupture.

1.2 THE ENDOTHELIUM

1.2.1 Structure and function

Arterial vessels display a layered structure of tissues and cells (figure 1.02). The outermost layer (tunica adventitia) comprises connective tissue rich in elastic and collagen fibres whilst the middle layer (tunica media) consists of connective tissue and smooth muscle cells. Adjacent to the lumen is the tunica intima consisting of lamina propria and a basement membrane upon which lies the endothelium.

The vascular endothelium is a continuous monolayer of cells of mesoblastic origin that lines the inner surface of all blood vessels providing a barrier between blood and the underlying vessel. Once regarded as a quiescent layer of cells, research since the late 1970s has identified the endothelium as a highly dynamic organ with numerous endocrine, paracrine and autocrine functions. Resulting from its strategic positioning at the interface between the blood and tunica intima the endothelium has a vital role in the maintenance of vascular homeostasis. It is involved in the regulation of vascular tone, and growth as well as modulating inflammation and coagulation. The principal functions of the endothelium are listed in table 1.03.

1.2.2 Endothelium-derived factors

The endothelium synthesises several factors including; nitric oxide (NO) (alternatively referred to as endothelium-derived relaxing factor), prostacyclin (PGI_2), endothelium-derived hyperpolarizing factor (EDHF), endothelin-1, thromboxane A_2 (TXA_2), superoxide anion (O_2^-) and possibly prostaglandin H_2 (PGH_2). These factors modulate the functions of adjacent cells including smooth muscle cells and platelets as well as the endothelial cells from which they were derived.

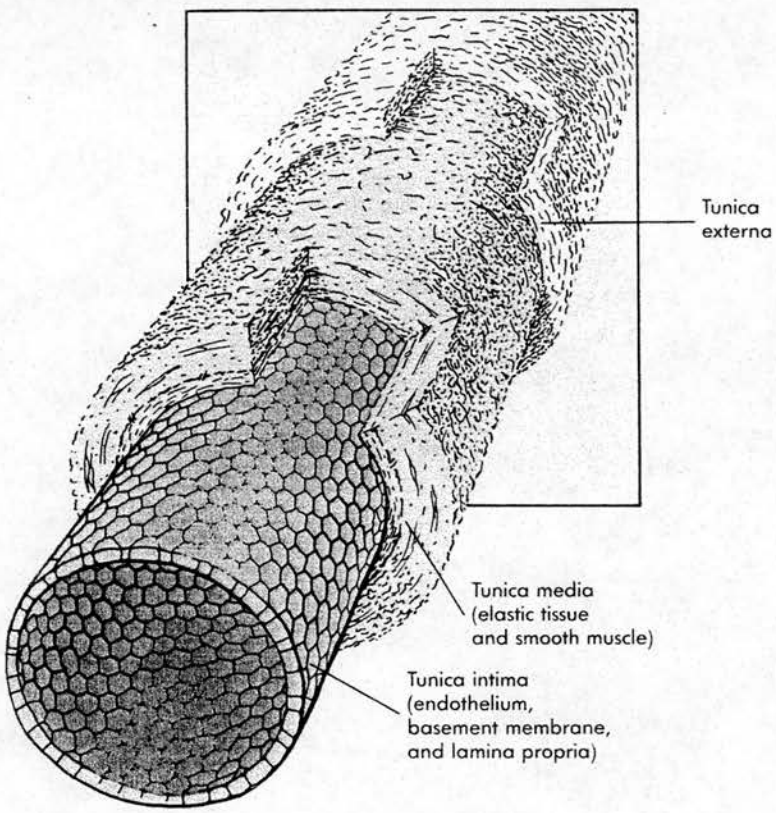


Figure 1.02. Structure of the arterial vessel wall. From Carola *et al.* (Carola *et al.*, 1992).

Table 1.03 Functions of the vascular endothelium. EDHF, endothelium-derived hyperpolarizing factor; ET-1, endothelin-1; NO, nitric oxide; O_2^-, superoxide anion; PDGF, platelet-derived growth factor; PGH_2, prostaglandin H_2; PGI_2, prostacyclin; TNFα, tissue necrosis factor alpha.

Functions of the vascular endothelium

- Regulation of vascular tone through release of vasoactive compounds, e.g. NO, PGI_2, EDHF, ET-1, O_2^-, PGH_2
- Provision of a selective permeability barrier
- Provision of a non-adherent surface for leukocytes and platelets
- Formation and secretion of a variety of growth-regulatory molecules and cytokines, e.g. PDGF and TNFα
- Modification of plasma-derived compounds e.g. oxidation of lipoprotein
- Formation and maintenance of the extracellular matrix e.g. basement membrane, collagen, elastic fibres and proteoglycans
- Maintenance of a non-thrombogenic surface through the formation of molecules such as ectoADPase, PGI_2, thrombomodulin and heparin sulphate
- Provision of anticoagulant and procoagulant properties through the production of molecules such as NO and PGI_2

Endothelium-derived factors are secreted from the endothelium in response to autonomic and sensory nerves (e.g. acetylcholine (ACh), noradrenalin, adenosine triphosphate (ATP) and substance P), circulating hormones (e.g. catecholamines, vaspressin, angiotensin II, insulin), products of coagulation (e.g. serotonin, adenosine diphosphate (ADP), thrombin), autocoids produced by endothelial or smooth muscle cells (e.g. bradykinin, ADP, ATP, angiotensins, ET-1) and also to changes in shear stress.

NO, PGI_2 and EDHF are the major endothelium-derived vasodilators (figure 1.03). NO is discussed in detail in section 1.2.3c. Although primarily produced by the vascular endothelial cell, the actions of PGI_2 (a member of the prostaglandin family), are mainly detected through specific receptors expressed on the vascular smooth muscle cell. PGI_2-receptors are coupled to adenylate cyclase to elevate cyclic AMP levels in vascular smooth muscle which in turn stimulates ATP-sensitive K^+ channels to cause hyperpolarization of the cell membrane and thus inhibit contraction. The contractile machinery is further inhibited by the cAMP-induced extrusion of Ca^{2+} from the smooth muscle cell cytosol. PGI_2 can facilitate the release of NO from the endothelium, whilst, NO potentiates the action of PGI_2 indirectly through the action of cGMP which inhibits cAMP-phosphodiesterase thus prolonging the half-life of cAMP (Mombouli and Vanhoutte, 1999).

EDHF is a diffusible endothelium-dependent vasodilator the identity of which is presently controversial. There is some evidence that it is synthesised through the cytochrome P-450 pathway (Campbell *et al.*, 1996). Hyperpolarization results from the opening of K^+ channels in the smooth muscle which then inhibits vasoconstriction through several mechanisms which includes closure of voltage-sensitive Ca^{2+}-channels, impairment of receptor-dependent activation of phospholipase C (PLC) and the subsequent Ca^{2+} release from intracellular stores and a decrease in the Ca^{2+} sensitivity of the contractile proteins. It has not been possible to study its relevance *in vivo*.

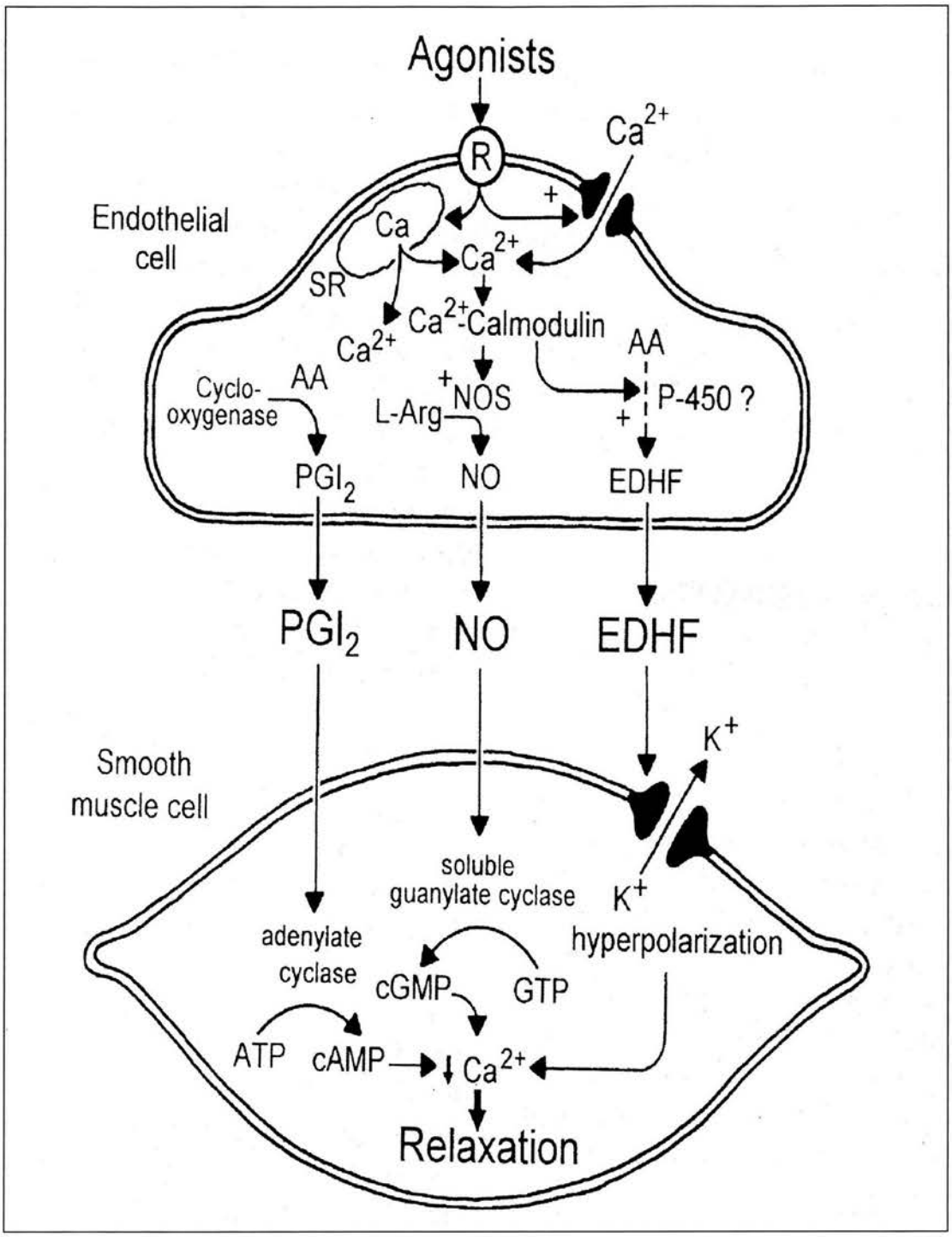


Figure 1.03. A schematic representation summarising the release of endothelium-derived relaxing factors and their effects on vascular smooth muscle cells. ACh, acetylcholine; AA, arachidonic acid; A23187, calcium ionophore A23187; ATP, adenosine triphosphate; BK, bradykinin; B2, bradykinin B2 receptor; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophophate; EDHF, endothelium-derived hyperpolarizing factor; GTP guanosine triphosphate K^+, potassium channel; L-arg, L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; PGI_2, prostacyclin; P450, cytochrome P450 monoxygenase; SR, sarcoplasmic reticulum. The broken lines indicate the action of either an inhibitor or an antagonist. From Mombouli and Vanhoutte (Mombouli and Vanhoutte, 1999).

Both PGH_2 and TXA_2 are products of arachidonic acid metabolism through the cyclooxygenase pathway. Both act on the endoperoxide/ thromboxane receptors on the smooth muscle cell surface to induce vasoconstriction. The major metabolite of arachidonic acid metabolism is PGI_2 and therefore under normal circumstances vasoconstriction is masked by PGI_2, NO and EDHF.

Reactive oxygen species (ROS) are released in response to shear stress and some agonists e.g. bradykinin (Mombouli and Vanhoutte, 1999). These ROS can inactivate NO favouring vasoconstriction. ROS may also mediate vasoconstriction by facilitating the mobilisation of Ca^{2+} in vascular smooth muscle cells and/or by enhancement of Ca^{2+} sensitivity of contractile elements.

ET-1 is potent vasoconstrictor, secreted in response to thrombin, interleukin-1, TGFβ, platelet products and neurohormones such as catecholamines and vasopressin. However its synthesis is inhibited by both the basal and stimulated production of NO through guanosine 3', 5'-cyclic monophosphate (cGMP) (Boulanger and Lüscher, 1991).

Endothelium-derived vasoactive substances also possess angiogenic and/or smooth muscle cell growth modulating properties. Under physiological conditions the endothelium provides a non-adhesive, anticoagulant and anti-thrombotic surface. For example EDHF prevents the deposition of platelets on the endothelial cell surface whilst PGI_2 and NO inhibit platelet aggregation thus inhibiting the platelet aggregation induced by shear stress.

1.2.3 Second messenger systems in the endothelial cell

a) Introduction

The numerous functions of the endothelium are regulated by a variety of endogenous and exogenous chemical stimuli, as well as mechanical stimuli. These bioactive substances or mechanical forces transduce signals from the extracellular milleu through specific receptor-

mediated processes and second messenger systems which transform extracellular signals into intracellular effects. A number of bioactive compounds have been identified which interact with endothelial cells initiating a second messenger response. The chemical stimuli include growth factors, neurotransmitters, cytokines, kinins, inflammatory mediators and agonists such as thrombin, adenine nucleotides, bradykinin (BK), ET-1, and histamine.

In the endothelial cell, several signal transduction pathways have been identified. Of the many pathways described in the endothelium those relevant to this thesis include the phospholipase C (PLC)/ inositol phosphate/ protein kinase C (PKC) pathway (Pollock, Wreggett and Irvine, 1988; Tran, Proulx and Chan, 1994), the guanylate cyclase/ cyclic GMP pathway (Schmidt, Mayer and Kukovetz, 1989), and the adenylate cyclase/ cyclic AMP pathway, all of which are coupled to G-protein linked receptors.

b) The phospholipase C/ inositol phosphate/ protein kinase C pathway

One of the principle signalling pathways of the endothelium is the PLC/ inositol phosphate/ PKC pathway (Natarajan, 1995) (figure 1.04). The agonist-receptor interaction activates PLC via a G-protein linked to the receptor. The activation of PLC in turn hydrolyses phosphatidylinositol 4,5 bisphosphate (PIP_2) generating inositol (1,4,5) trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 acting as a second messenger subsequently stimulates the release of Ca^{2+} from intracellular calcium stores which in turn stimulates a number of cellular processes via Ca^{2+}receptor proteins (for example, calmodulin) or Ca^{2+}-activated kinases. DAG activates PKC (Natarajan, 1995). Mackie *et al.* concluded that PKC has a central role in endothelial cell function as endothelial cells possess a large number of PKC substrates and exhibit high PKC activity (Mackie *et al.*, 1986). There are many reported functions which require the PLC/ inositol phosphate/ PKC system in the endothelium. These include the release of NO, stimulated by BK (Freay *et al.*, 1989; Graier, Schmidt and Kukovetz, 1992) and the generation and release of super oxide

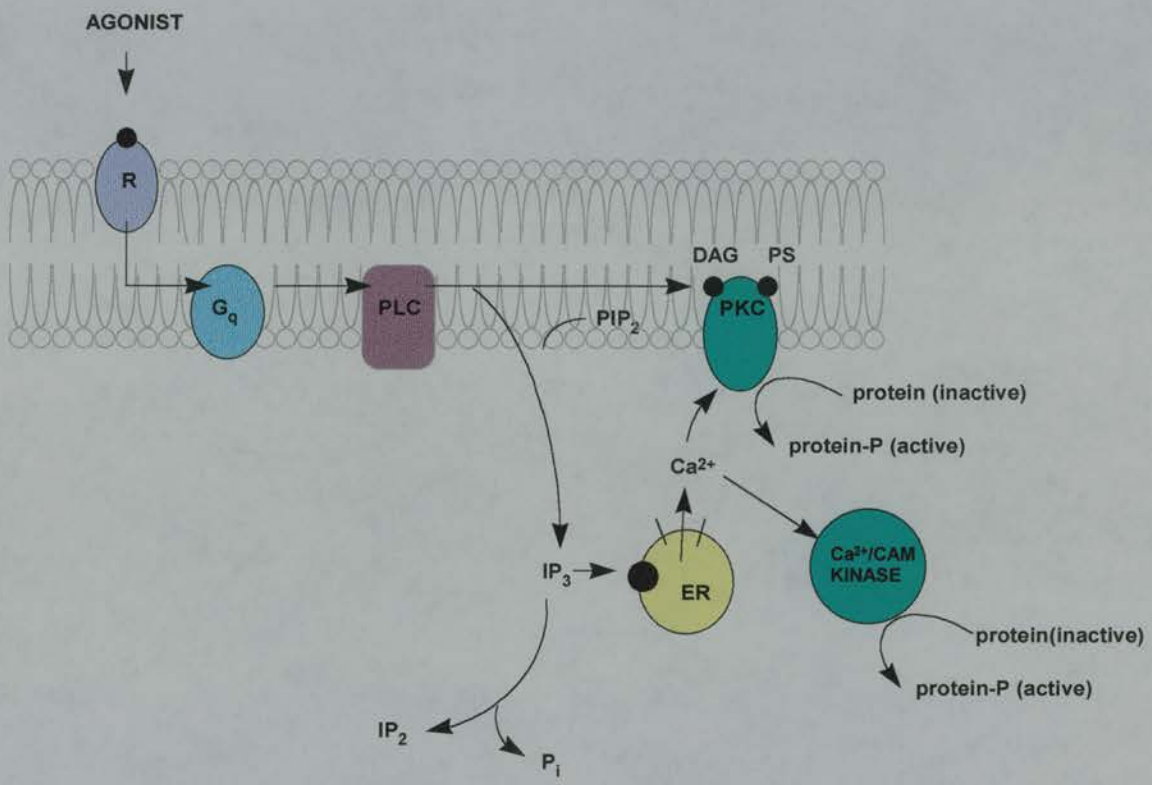


Figure 1.04. The phospholipase C/ inositol phosphate/ protein kinase C pathway. CAM, calmodulin; DAG, diacylglycerol, ER, endoplasmic reticulum; IP_2, IP_3, inositol (1, 4, 5) trisphosphate; R, receptor; P_i, phosphate; PIP_2, phosphatidylinositol 4,5 bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PS, phosphatidylserine. Adapted from Voet and Voet (Voet and Voet, 1990).

anions (Matsubara and Ziff, 1986). α-Thrombin also stimulates an increase in PIP_2 hydrolysis and intracellular Ca^{2+} mobilisation in human endothelial cells (Garcia and Natarajan, 1992).

c) The guanylate cyclase/ cyclic GMP pathway

Second messenger signalling in the endothelium is also mediated through the guanylate cyclase/ cyclic GMP pathway. The binding of various vasoactive substances (for example ACh, BK, angiotensin II and histamine) activates specific G-protein-coupled cell surface receptors which increases intracellular Ca^{2+} concentration via the PLC pathway in the manner described above. The intracellular signalling pathway for NO is mediated through two different G-protein linked pathways. The pertussis toxin-sensitive G-protein (either referred to as $G\alpha_i$ or Gi_2) mediates one whilst the second is mediated by the toxin-insensitive G-protein (Gq) pathway (figure 1.05). The increased cytosolic Ca^{2+} binds and activates calmodulin, which, in turn, activates nitric oxide synthesis by stimulation of endothelial nitric oxide synthase (eNOS). eNOS is expressed constitutively and exists in the membrane-bound particulate fraction of endothelial cells. It uses L-arginine as a substrate and molecular oxygen as a co-substrate. The mechanism of this reaction involves a five-electron oxidation of the terminal guanadine-nitrogen of L-arginine, resulting in the production of NO and the co-product, L-citrulline. The cofactors required for this reaction include flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), nicotinamide adenine dinucleotide phosphate (NADPH) and 6(R)-5,6,7,8-tetrahydrobiopterin (BH_4) (Förstermann *et al.*, 1994, Moncada, Palmer and Higgs, 1991). eNOS contains an oxidase and reductase domain. Upon the Ca^{2+}/ calmodulin binding, eNOS accepts electrons from NADPH at the reductase domain, which are shuttled by the flavin moiety to the oxidised domain where they are used to reduce molecular oxygen thereby providing a source of oxidation of L-arginine (Dart and Chin-Dusting, 1999).

Even in the absence of an external stimulus the intracellular Ca^{2+} concentration is such that NO is continuously produced maintaining a basal release of NO (Mano *et al.*, 1996). The stimulus for

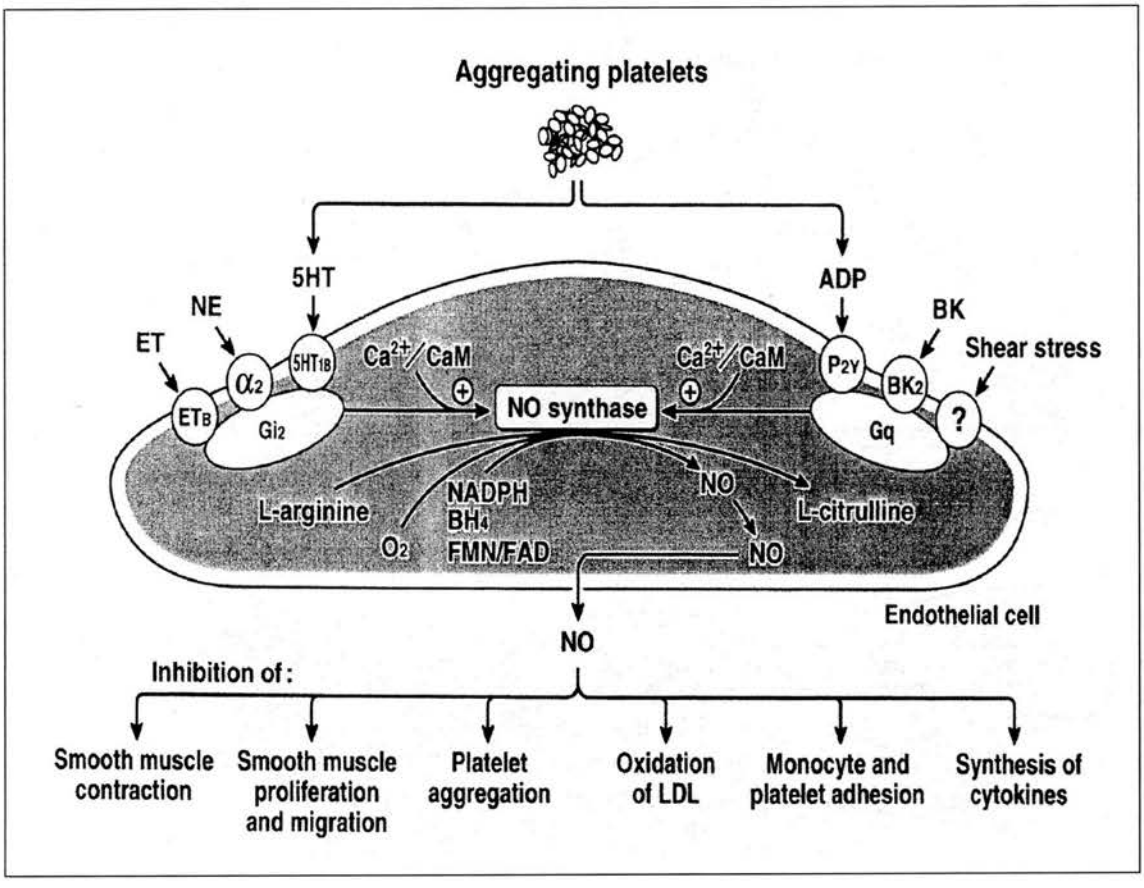


Figure 1.05. The intracellular signalling pathways for nitric oxide in the endothelium and the anti-atherogeneic actions of nitric oxide. α_2, α_2-adrenoreceptor, 5HT, serotonin; $5HT_{1B}$, $5HT_{1B}$-seretonergic receptor, ADP, adenosine diphosphate; BH_4, tetrahydrobiopterin; BK, bradykinin, CaM, calmodulin; ET, endothelin; LDL, low density lipoprotein; NE, noradrenalin; NO, nitric oxide; O_2, superoxide anion, P_{2Y}, P_{2Y}-purinergic receptor, ?, mechanoreceptor to sense changes in shear stress. From Shimokawa (Shimokawa, 1999).

the continuous, basal activation of eNOS which occurs at resting levels of intracellular Ca^{2+} is thought to be provided by shear stress, which provides an effective and sensitive system for the localised control of vascular tone.

NO has a half-life of milliseconds and activates soluble guanylate cyclase (sGC) present in the endothelium and vascular smooth muscle. Due to its lipophilic nature NO can diffuse into the underlying vascular smooth muscle to mediate vascular relaxation by the following mechanism (Shimokawa, 1999). NO binds to the heme component of sGC to enhance the formation of cGMP which through stimulation of cGMP-dependent protein kinase (PKG) inhibits vascular contractility (Lincoln, Komalavilas and Cornwall, 1994). NO has also been shown to activate charybdotoxin-sensitive, calcium-dependent potassium channels thereby inducing hyperpolarization in vascular smooth muscle cells.

NO, in addition to regulating vascular tone also inhibits platelet aggregation and the expression of adhesion molecules via the generation of cGMP. NO also impairs growth of smooth muscle cells (Britten, Zeiher and Schächinger, 1999). The ability of NO to alter the expression of genes which encode endothelial proteins has also been demonstrated (De Caterina *et al.*, 1995).

Stimulants of NO release (ATP, A23187, and BK) have been shown to increase intracellular cGMP in bovine aortic endothelial cells (Schmidt, Mayer and Kukovetz, 1989) probably by the direct activation of sGC present in the endothelial cell. Endothelial cells possess particulate GC, the activation of which by atrial natriuretic peptide, brain natriuretic peptide and C-type natriuretic peptide produces cGMP which activates PKG and inhibits the synthesis of ET-1 (Inagami, Naruse and Hoover, 1995).

d) The adenylate cyclase/ cyclic AMP pathway

The mechanism of receptor-mediated activation/ inhibition of adenylate cyclase is shown in figure 1.06.

In the endothelium, protein kinase A (PKA) mediates most of the effects of cAMP. The activation of PKA subsequently regulates cell activity by specific protein phosphorylation of serine/ threonine residues, Adenylate cyclase in the endothelium regulates thrombomodulin and tissue factor activity, the inhibition of endothelin through PGI_2, the mediation of the effects of cyclic strain, regulation of the plasma membrane calcium pump gene expression, endothelial cell permeability and the regulation of tissue plasminogen activator and plasminogen activator inhibitor expression (Manolopoulos, Samet and Lelkes, 1995). Receptors in the endothelium that are linked to the adenylate cyclase pathway include the β-adrenoreceptor, adenosine A2 purinoceptors, histamine and calcitonin gene-related peptide receptors (McKewan *et al.*, 1990). Forskolin directly activates adenylate cyclase in all endothelial cell types although its effects vary between endothelial cells isolated from different vascular beds (Manolopoulos, Samet and Lelkes, 1995).

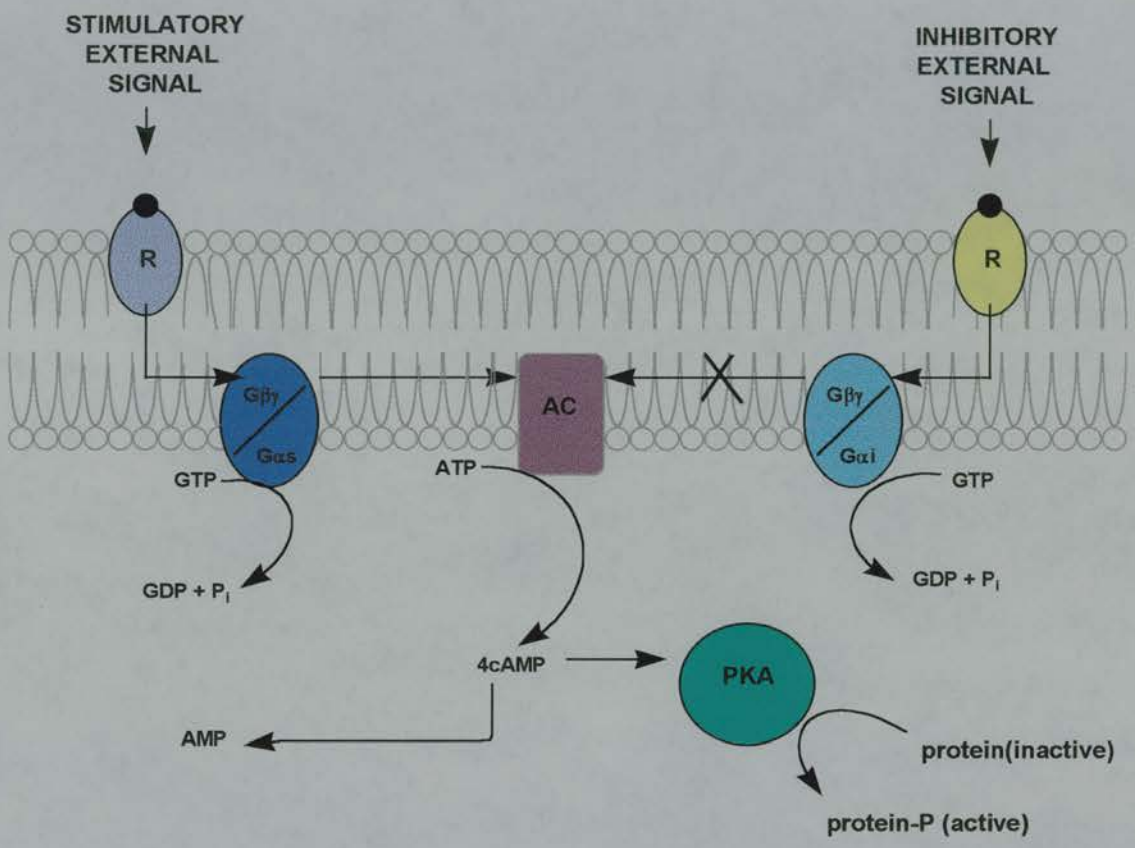


Figure 1.06. The adenylate cyclase/ cyclic AMP pathway. The interaction of the agonist with the stimulatory receptor stimulates the exchange of GDP for GTP on the α-subunit of inactive heterotrimeric G_s protein. The G protein α-subunit: GTP complex, dissociates from the Gβγ complex and activates adenylate cyclase (AC) to convert ATP to cAMP. The inherent GTPase activity of the α-subunit hydrolyses GTP to GDP and P_i leading to the dissociation of the G protein α subunit from AC and its reassociation with the Gβγ complex reverting back to the inactive state. cAMP activates protein kinase A (PKA) which catalyses the phosphorylation of various cellular proteins. The binding of an agonist to the inhibitory receptor triggers an almost identical chain of events with the exception that the association of GTP with the α-subunit of the G_i protein which leads to the inhibition of AC. Adapted from Voet and Voet (Voet and Voet, 1990).

1.3 ENDOTHELIAL DYSFUNCTION IN ATHEROSCLEROSIS

1.3.1 Introduction

Under physiological conditions the endothelium generally plays an inhibitory role; inhibiting smooth muscle cell contraction, monocyte adhesion, oxidation of low density lipoproteins, platelet aggregation and adherence, synthesis of inflammatory cytokines, vascular smooth muscle proliferation and thrombosis (figure 1.05, pg 18) (Glasser, Selwyn and Ganz, 1996; Shimokawa, 1999). However the endothelium plays an important role in the pathophysiology of several vascular diseases. The proposal that endothelial denudation provides a primary step in atherosclerosis was initially published by Ross and Glomset in 1973 following several pathophysiological observations in humans and animals (Ross and Glomset, 1973). Endothelial denudation is now more appropriately referred to as endothelial dysfunction i.e. 'the shut-down or inactivation of certain intrinsic anti-atherogenic mechanisms' (Busse and Fleming, 1996). There is considerable evidence that endothelial dysfunction is important in the etiology of atherosclerosis by either initiating and/or sustaining atherogenesis.

Due to its anatomical position the endothelial surface provides a primary target for attack from damaging stimuli. There are a number of risk factors which either alone or in combination can cause 'endothelial dysfunction'. Fluid shear stress has been strongly implicated in the changes to endothelial function, as atherosclerotic lesions are focal and localised to areas such as branches, bifurcations and regions of arterial narrowing where there are disturbances in the pattern of blood flow (Mano *et al.*, 1996). Changes in flow can alter the expression of genes which have elements in their promoter regions which can respond to such variations. Low shear stress for example has been shown to increase the expression of ICAM-1, PDGF-B chain, and tissue factor which all promote endothelial dysfunction (Ross, 1999). Fluid shear stress may be coupled to one or more other risk factors to promote endothelial dysfunction, such as elevated and modified low density lipoprotein; free radicals caused by cigarette smoke; ageing; menopause: hypertension; ischemia; diabetes mellitus; elevated plasma homocysteine

levels and infectious organisms such as Herpes virus or *Chlamydia pneumoniae* (Ross, 1999; Shimokawa, 1999).

1.3.2 Endothelial dysfunction as a primary event in atherosclerosis

Endothelial dysfunction is observed prior to the development of overt atheroma which may suggest a principal role for endothelial dysfunction in the initiation of atherogenesis. There is substantial evidence in support of this hypothesis. Several studies have demonstrated that endothelial dysfunction precedes the detection of atherosclerosis using either angiography or intravascular ultrasound (Ludmer *et al.*, 1986; Mano *et al.*, 1996). For example, in arterial segments prone to atherogenesis, such as branching points, a paradoxical vasoconstriction in response to acetylcholine is observed at a stage when there is no angiographic evidence of atherosclerosis (McLenachan, Vita and Fish, 1990). However some controversy exists as to whether the methods used in these studies are sensitive enough to detect the partial intimal lesions that may only be observed microscopically at post-mortem (Mano *et al.*, 1996).

Further evidence that endothelial dysfunction may culminate in the lesions of atherosclerosis has been shown using experimental animal models with impairment of endothelial function. For example, a chronic reduction of NO bioavailability through the inhibition of NOS was shown to accelerate the progression of atherosclerosis, inducing neointimal formation (Cayette *et al.*, 1994). Also, eNOS knock-out mice have been shown to develop typical atherosclerotic lesions following adventitial vessel wall injury, whereas wild-type mice do not (Moroi *et al.*, 1998).

Clinical data shows that endothelial dysfunction is detectable in patients with a family history of atherosclerosis prior to the manifestation of atherogenesis (Busse and Fleming, 1996). Also, the impairment of endothelium-dependent vasodilatation is evident in isolated small arteries of the microcirculation despite the absence of gross atherosclerotic lesions in these vessels (Dart and Chin-Dusting, 1999; Selke, Armstrong and Harrison, 1990; Zeiher *et al.*, 1991).

Finally, the risk factors which promote atherogenesis are strongly associated with endothelial dysfunction such that the removal or modification of the risk factor (for example, lowering cholesterol, cessation of smoking, exercise, oestrogen replacement in menopausal women) improves endothelial dysfunction and may prevent the further development of the disease (Vogel, 1997).

1.3.3 Features of endothelial dysfunction

The endothelial dysfunction that results from injuries caused by one or more of the aforementioned risk factors alters the normal homeostatic properties of the endothelium thus promoting atherogenesis (Vogel, 1997). The endothelium serves a number of physiological roles as summarised previously in table 1.03. Alterations of these functions associated with 'endothelial dysfunction' include; the increased expression of adhesion molecules on the endothelial surface, the production of paracrine growth factors and chemoattractants, impaired vascular tone, the ability to oxidise LDL and to respond to oxidised lipids and lipoproteins, promotion of pro-coagulation and the modulation of plasma component levels within the vessel wall through changes in permeability.

1.3.4 Mechanisms of endothelial dysfunction

There is considerable evidence to suggest that endothelial dysfunction is associated with the loss of NO production and/or its bioavailability (Busse and Fleming, 1996; Harrison, 1997). For example, it has been demonstrated that the inhibition of NO synthesis increases the expression of genes associated with monocyte adhesion (Maxwell, Tsao and Cooke, 1998). A loss of endothelial production and/or bioavailability of NO would be expected to diminish the normally protective effects of the endothelium and predispose the vessel to atherogenesis. The mechanisms which underlie the decreased production and bioavailability of NO appear to be multifactoral and are dependent on the stage of atherosclerosis, the vascular bed and species

under study and the agonist tested (Harrison, 1997) (figure 1.07). The mechanisms of endothelial dysfunction particularly relevant to this thesis are discussed below.

Impairment of endothelial signal transduction through defects in either the membrane receptor or signalling pathways may contribute to endothelial dysfunction. For example, oxidised LDL has been shown to activate PKC (Ohgushi *et al.*, 1993) which in turn can phosphorylate the $G\alpha_i$ protein and inhibit its function (Shimokawa, 1999).

eNOS, despite being constitutively expressed, is subject to regulation. For example, cultured endothelial cells exposed to high concentrations of oxLDL have a decreased eNOS expression (Busse and Fleming, 1996; Harrison, 1997). However several factors have been shown to increase the expression of eNOS including shear stress, exposure to lysophosphatidycholine, low concentrations of oxLDL and cGMP analogues (Harrison, 1997). Indeed there is evidence to suggest that, despite a decrease in NO bioavailability, basal and stimulated NO levels are enhanced in cholesterol-fed animals (Harrison and Ohara, 1995; Minor *et al.*, 1990). These differences may be accounted for by measurements having been taken at different stages of atherogenesis.

Tetrahydropbiopterin (BH_4) is an essential cofactor for eNOS activity and is hypothesised to direct electron flow in the enzyme to L-arginine. In the absence of sufficient BH_4 Pou *et al.* reported that purified brain constitutive NOS transfers electrons to molecular oxygen, to produce the powerful oxidant O_2^- (Pou *et al.*, 1992). The addition of BH_4 to recombinant eNOS increases NO production whilst decreasing O_2^- production (Wever *et al.*, 1998). BH_4 has not been measured under pathophysiological, conditions although in hypercholesterolemic patients supplementation with BH_4 has been shown to improve endothelium-dependent vasodilation (Shimokawa, 1999; Wever *et al.*, 1998).

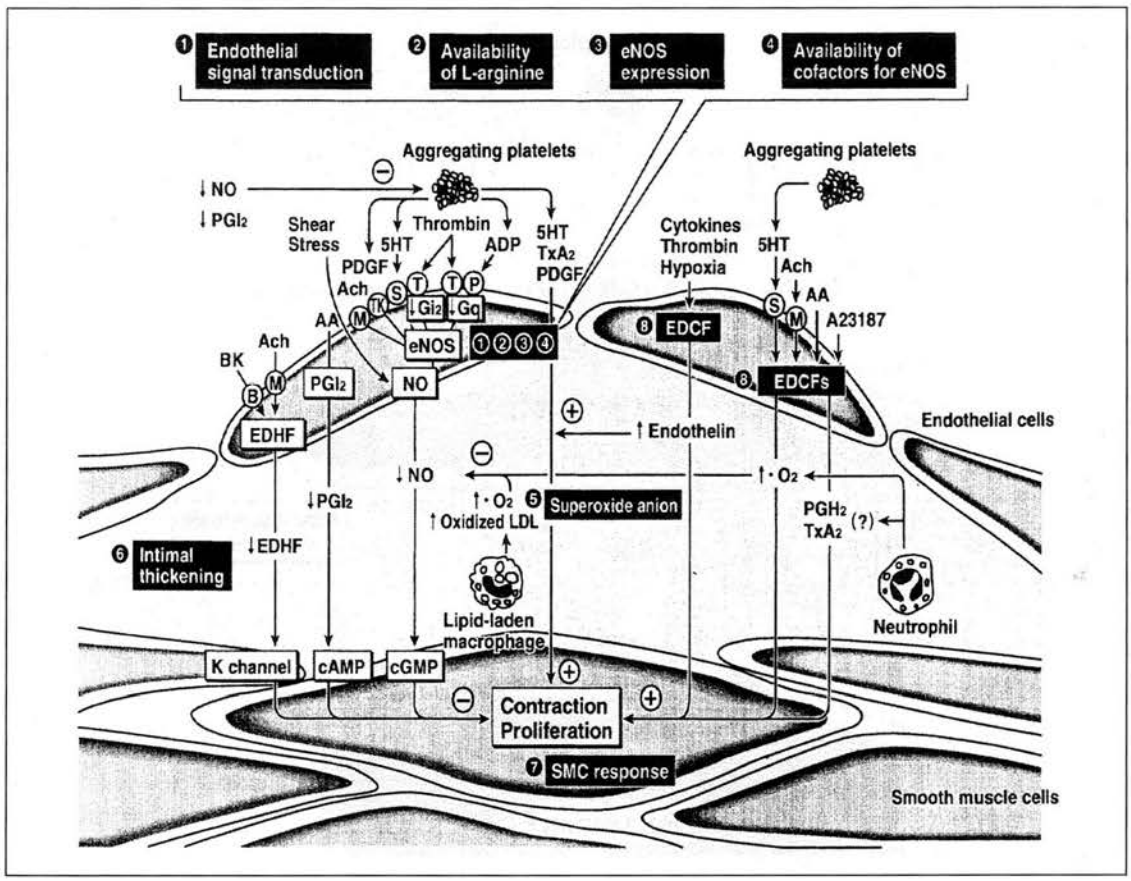


Figure 1.07. Schematic representation of the possible mechanisms that underlie endothelial dysfunction observed in hypercholesterolemia and atherosclerosis. AA, arachidonic acid; ACh, acetylcholine; ADP, adenosine diphosphate; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; EDHF, endothelium-derived hyperpolarizing factor; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; 5HT, serotonin; PGI_2, prostacyclin; M, muscarinic receptor; P_{2Y}, P_{2Y}-purinergic receptor; PDGF, platelet-derived growth factor; PGH_2, prostaglandin H_2; S, $5HT_{1B}$-serotonergic receptor; T, thrombin receptor; TK, receptor for PDGF coupled to tyrosine kinase, TXA_2, thromboxane A_2. From Shimokawa (Shimokawa, 1999).

The concomitant release of endothelium-derived contracting factors such as O_2^-, TXA_2 , angiotensin II, ET-1, PGH_2 may account for the changes in vascular tone. With the removal of the negative feedback constraints of NO, it is suggested ET-1 production is increased which may be of potential importance in atherosclerosis. Evidence consistent with this is the finding of enhanced plasma levels of ET-1 in patients with advanced atherosclerosis (Lerman *et al.*, 1991). In addition, cultured human and porcine endothelial cells exposed to oxLDL stimulated the release of ET-1 into the culture medium (Boulanger *et al.*, 1992).

The reduction in bioavailability of NO is likely to be associated with its increased inactivation. O_2^- is generated by the endothelium, smooth muscle and intimal macrophages in response to injury, ischemia, oxLDL and activated leukocytes. Excess production of O_2^- has been reported in hypercholesterolemic animals (Ohara, Peterson and Harrison, 1993) and there is also evidence that NO levels may be enhanced in atherosclerotic lesions (Harrison and Ohara, 1995; Wever *et al.*, 1998). Because NO contains an unpaired electron it is paramagnetic and reacts rapidly with O_2^- at a rate of approximately 6.7×10^{-9} Ms^{-1} (as determined by flash photolysis) to form peroxynitrite according to reaction 1 (Beckman, Wink and Crow, 1996; Beckmann *et al.*, 1994; Harrison, 1997; Kooy and Royall, 1994). This reaction is approximately three times faster than the reaction of O_2^- with superoxide dismutase and nearly thirty times faster than the reaction of NO with heme proteins.

$$NO + O_2^- \longrightarrow ONOO^- \qquad (1)$$

The presence of peroxynitrite in atherosclerotic lesions has been indirectly demonstrated through nitrotyrosine immunostaining which serves as a marker of peroxynitrite-mediated protein modification (Beckmann *et al.*, 1994). This would suggest that under conditions of oxidative stress the physiological functions of NO are inhibited by its conversion to peroxynitrite thus promoting atherogenesis.

At neutral pH the peroxynitrite radical is protonated and can form the cytotoxic peroxynitrous acid which can then spontaneously yield the hydroxyl radical and nitrite (NO_2^-) (Harrison and Ohara, 1995; Wever *et al.*, 1998). The oxidation of LDL, protein fragmentation by nitration of proteins, DNA damage, modification of iron-sulphur clusters, zinc-fingers, protein thiols and membrane lipids have all been shown to be effects of peroxynitrite. These events might well be important in the pathophysiology of atherosclerosis (Gross and Wolin, 1995; Harrison and Ohara, 1995; Wever *et al.*, 1998). Peroxynitrite, as compared to NO is a relatively weak stimulus for guanylate cyclase in vascular smooth muscle such that endothelium-dependent relaxation is impaired (Villa *et al.*, 1994).

In contrast, it is possible for peroxynitrite at low concentrations to nitrosylate sulfhydryl groups to form S-nitrosothiols which are known to promote vasodilatation, inhibit platelet aggregation and leukocyte adhesion (Maxwell, Tsao and Cooke, 1998; Stamler, Singal and Loscalzo, 1992). Whilst the formation of peroxynitrite under physiological conditions, may not be toxic, under pathological conditions where it is produced at much higher concentrations, the toxic effects of peroxynitrite may become evident.

In conclusion, the findings from human and animal studies would suggest that the inactivation of NO by O_2^- and other ROS observed under pathophysiological conditions contributes to endothelial dysfunction (Harrison, 1997). The role of ROS in atherogenesis is discussed further in section 1.3.5.

Finally, it should also be noted that the capacity of the endothelium to produce PGI_2, (whose functions include vasodilatation, the prevention of platelet aggregation, an increase in the activity of enzymes which metabolise cholesterol esters by macrophage and the prevention of smooth muscle cell proliferation) is decreased in atherosclerosis (Vane, Anggard and Botting, 1990).

1.3.5 Reactive oxygen species and the endothelium

a) Reactive oxygen species and the endothelium

The endothelial cell *in vivo* is constantly exposed to the high partial pressure of molecular oxygen in the blood as well as chemical derivatives of oxygen referred to as reactive oxygen species (ROS) (Schuppe-Koistinen *et al.*, 1994). Under normal cellular homeostasis there is a balance between the rate and magnitude of oxidant formation and the rate of oxidant elimination. The term 'oxidative stress' is used to describe the situation when the endothelial cell is exposed to excessive levels of either molecular oxygen or ROS which overwhelm the cell's antioxidant capacity.

b) Sources of reactive oxygen species

Metabolic trauma (e.g. hypercholesterolemia, diabetes mellitus, smoking, viral infection, ischemia) and physical trauma (e.g. percutaneous transluminal coronary angioplasty, hypertension) all increase vascular oxidative stress (McGorisk and Treasure, 1996). The ROS relevant to vascular biology include O_2^- , hydrogen peroxide (H_2O_2) , the hydroxyl radical (OH^-), the hydroperoxy radicals, peroxynitrite ($OONO^-$) and lipid hydroperoxides (Kojda and Harrison, 1999). There are several enzymatic sources of ROS in the mammalian cell including, mitochondrial electron transport chain, xanthine oxidase, cyclooxygenase, lipooxygenase, eNOS, heme oxygenases, peroxides, hemeproteins and NADPH oxidases.

c) Reactive oxygen species and endothelial dysfunction

There is both indirect and direct evidence that there is an excess generation of O_2^- within vessels from hypercholesterolemic subjects, that may lead to endothelial dysfunction and thus serve as an early atherosclerotic event (Mügge *et al.*, 1991; Ohara, Peterson and Harrison, 1993). In hypercholesterolemic rabbits a three-fold increase in aortic O_2^- production was measured compared to that found in aorta from normal rabbits (Ohara, Peterson and Harrison,

1993). This effect was abolished following endothelial denudation and was inhibited by oxypurinol (a non-competitive inhibitor of xanthine oxidase) inferring that the endothelium provides the major source of abnormal O_2^- production in early atherosclerosis through the activation of xanthine oxidase. In the later stages of atherosclerosis activated macrophages in the intima and smooth muscle cells are also likely to contribute to the production of vascular O_2^- as well as other ROS (Kojda and Harrison, 1999).

Damage to the endothelium by ROS, free radicals and oxidised lipids and lipoproteins has been shown to favour atherogenesis (DiCorleto and Soyombo, 1993). Oxidative stress can disrupt several physiological functions pertaining to the endothelium. These include regulation of blood flow, inhibition of leukocyte adhesion and platelet aggregation and the control of cellular growth.

The role of NO in the modulation of monocyte adhesion and migration is associated with the ability of NO to suppress the gene expression of adhesion molecules such as VCAM-1 and chemokines such as MCP-1 by modulating an oxidant sensitive transcriptional signalling pathway (Maxwell, Tsao and Cooke, 1998). It would appear that in hypercholesterolemia the loss of NO bioavailability and the increased presence of O_2^- result in the activation of the NFκB binding site present on the promoter region of the genes which regulate mechanisms such as monocyte adhesion leading to the expression of these genes (Maxwell, Tsao and Cooke, 1998; Ohara, Peterson and Harrison, 1993). Oxidative stress may also alter the expression of other genes associated with atherogenesis such as eNOS, ICAM-1, E-selectin, PDGF and FGF-2.

The O_2^- has several pro-atherogenic features including, the oxidation of LDL and lipid accumulation in the vessel wall. O_2^- can also provide a source of other ROS (H_2O_2, OH^-, singlet oxygen, hypochlorous acid and $ONOO^-$) which may participate in lipid peroxidation and thereby

have an impact on endothelial function. The altered ratio of NO/O_2^- production has been proposed to diminish the intrinsic inhibition of the transcription factor NFκB leading to an enhance expression of endothelial adhesion molecules and chemotactic factors (Busse and Fleming, 1996).

d) Oxidised LDL - a promoter of atherosclerosis

LDL molecules are large spherical particles (diameter 19-25 nm) comprising of cholesterol ester and triglycerides which form a central lipophilic core with a monolayer of amphipathic phospholipids and free cholesterol in which is embedded a large glycosylated protein, apoprotein B (apoB), which envelopes the LDL particle (figure 1.08). Several variants of apoB exist and LDL is associated with the apoB100 form of the protein. LDL also contains several lipophilic antioxidants such as α-tocopherol, γ-tocopherol, carotenoids, oxycarotenoids and ubiquinol-10. Variability in the chemical and structural composition of LDL may in part mediate the differences in susceptibility between LDL samples to oxidation (Esterbauer *et al.*, 1992). LDL particles provide the major cholesterol-carrying lipoproteins in the blood. LDL-uptake into cells is via a receptor-mediated pathway and non-specific endocytosis. Most cells contain the B/E receptor for LDL uptake but the highest concentration is found in the liver where approximately three-quarters of the LDL is removed from the blood stream (Esterbauer *et al.*, 1992).

Oxidation of LDL is thought to be crucial to the initiation of atherogenesis (Tanner *et al.*, 1991; Witzum and Steinberg, 1991). In the plasma, LDL is generally protected against oxidation by plasma antioxidants including vitamins C and E and β-carotene. However, endothelial damage may cause LDL to enter the intima where antioxidant levels are much lower and the susceptibility of LDL to oxidation may be increased. Oxidative modification of LDL has been demonstrated *in vivo*. LDL extracted from the intima includes oxLDL, characterized in terms of its physical and biological properties, as well as by Western blotting using antibodies against oxLDL (Rosenfeld, 1991; Steinberg, 1991; Ylä-Herttuala *et al.*, 1991). OxLDL and circulating

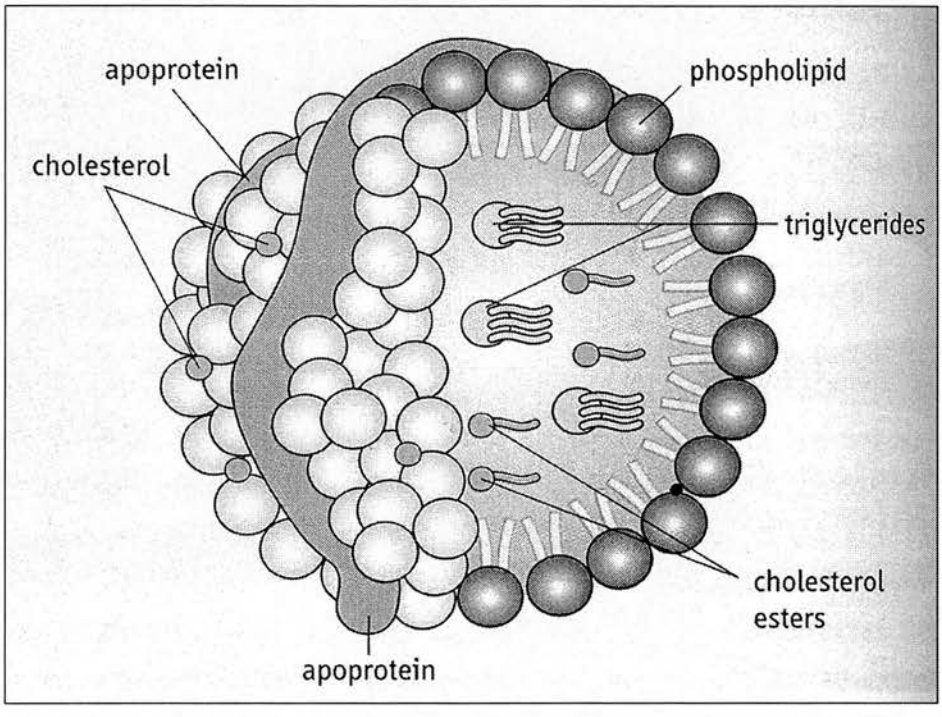


Figure 1.08. The lipoprotein particle. From Baynes and Dominiczak (Baynes and Dominiczak, 1999).

antibodies to epitopes of oxLDL are elevated in atherosclerotic patients (Esterbauer *et al.*, 1992; McGorisk and Treasure, 1996; Ylä-Herttuala *et al.*, 1989).

The mechanism by which LDL is oxidised *in vivo* is uncertain. *In vitro* studies suggest the formation of oxLDL is mediated in part by the enhanced production of ROS by each of the cells which constitute the arterial wall. The role of macrophage lipoxygenases, in particular 15-lipoxygenase, has also been implicated in LDL oxidation (Esterbauer *et al.*, 1992; Ylä-Hertulala *et al.*, 1990). Chemical and physiochemical properties of oxLDL (some of which contribute to the pro-atherogenic properties of this molecule) are summarised in table 1.04.

When LDL particles become trapped in the intima they can undergo progressive oxidation which is a prerequisite for uptake of LDL into macrophages by means of the scavenger receptors. These receptors present on the macrophage surface recognise and rapidly take up oxLDL in an uncontrolled manner (Esterbauer *et al.*, 1992). The majority of foam cells which develop in the early stages of atherogenesis develop from monocytes/macrophages which have entered the subendothelial space (Ylä-Hertulala *et al.*, 1990). The enhanced uptake of oxLDL and its degradation by macrophages leads to the increased presence of cholesteryl ester thus promoting the transition of macrophages into foam cells. The gene for the scavenger receptor has been cloned and is found in macrophages and stimulated smooth muscle cells *in vitro* and its presence *in vivo* has been demonstrated in arterial lesions (Dejager, Mietus-Synder and Pitas, 1993). Scavenger receptor activity present in endothelial cells is encoded for by a different gene. The importance of this receptor for atherogenesis has not been ascertained (Sawamura *et al.*, 1997).

Oxidised LDL affects numerous cellular functions each of which could contribute to each stage of atherosclerosis. The more established pro-atherogenic properties of oxLDL are summarised in Table 1.05, some of which are discussed in more detail below. In addition to oxLDL, minimally modified LDL (MM-LDL) may also elicit biological responses implicated in the pathogenesis of

Table 1.04. Summary of differences between oxidised LDL and native LDL. These altered properties are characteristic of LDL oxidised *in vitro* either by Cu^{++} or cells for 24 hr.

Chemical and physiochemical properties
• Complete loss of antioxidants
• Entire or near complete loss of PUFAs
• Loss of phosphatidylcholine and cholesteryl ester
• Increase in lysophosphatidylcholine and oxysterol content
• Increase in hydroxy- and hydroperoxy-PUFA content
• Increase in conjugated dienne content
• Increase in aldehydes including MDA, hexanal, HNE
• Strong fluorescence at 430 nm with excitation at 360 nm
• Partial loss of free amino groups in apoB
• Fragmentation of apoB to smaller peptides
• MDA and HNE epitopes on apoB recognised by specific antibodies
• Increased electrophoretic mobility and increased density (1.06-1.08)
• Increased tendancy to aggregate, heterogeneity in size
• Conformational rearrangement of apoB structure and phospholipid monolayer

Adapted from (Esterbauer *et al.*, 1992).

Table 1.05. Summary of the pro-atherogenic properties of oxLDL. With the exception of minimally modified LDL (MM-LDL) these properties are characteristic of LDL oxidised *in vitro* either by Cu^{++} or endothelial cells for 24 hr.

Biological properties
• Increased uptake and degradation by macrophages
• Cytotoxic to several cell types e.g. endothelial, fibroblasts and smooth muscle cells (Kosugi *et al.*, 1987; Kuzuya *et al.*, 1991; Thomas, Geiger and Girotti, 1993).
• Chemotactic for monocytes and smooth muscle cells
• Inhibition of monocyte-macrophage motility
• Inhibition of NO activation of guanylate cyclase
• Inhibition of relaxation of isolated smooth muscle strips induced by ACh, NO
• Alteration of the vasoactive compounds released by the endothelium e.g. ET-1, EDHF
• Immunogenic and able to elicit an autoantibody response
• Suppression of protein C (which increases thromboresistance) activity in cultured endothelial cells
• Suppression of the production of PDGF-mRNA and PDGF secretion by monocyte-macrophages
• Increase in macrophage glutathione concentration (approximately two-fold)
• Systemic administration into hamsters causes an immediate leukocyte adhesion to capillary endothelium
• Induction of DNA synthesis and enhancement of the proliferative response to M-CSF and GM-CSF by macrophages (Hamilton *et al.*, 1999)
• Treatment of cultured endothelial cells with MM-LDL stimulates the production of MCP-1, endothelial-leukocyte-adhesion molecules (e.g. VCAM-1 and ICAM-1), M-CSF, G-CSF and tissue factor required for coagulation (Drake *et al.*, 1991)
• MM-LDL injected into mice increases MCP-1 and CSF levels in serum and tissue
• MM-LDL inhibits mitogenesis and stimulates (at low concentrations) or inhibits PGI_2 synthesis in smooth muscle cells
• MM-LDL stimulates nuclear transcription factor (NFκB) to increase monocyte adherence

Adapted from (Esterbauer *et al.*, 1992).

atherosclerosis (Drake *et al.*, 1991). Whereas oxLDL contains high levels of thiobarbituric reactive substances (TBARS) and has major modification of apoB and is taken up by scavenger receptors, MM-LDL has lower levels of TBARS and is taken up by specific LDL receptors. oxLDL disrupts several other endothelial functions. For example, oxLDL has also been shown to stimulate platelet aggregation (Aviram, 1989), procoagulant activity (for example, by an increase in tissue thromboplastin activity on the surface of human macrophages) (Schuff-Werner *et al.*, 1989) and inhibit vasodilation induced by endothelium-derived NO (the mechanism for which has been discussed previously) (Berliner and Haberland, 1993; Chin, Azhan and Hoffman, 1992; Rosenfeld, 1991; Tanner *et al.*, 1991).

1.3.5 Antioxidants and atherosclerosis

a) Enzymatic endothelial antioxidant defence systems

The endothelial cell possesses several intracellular enzymatic antioxidant systems. These include the glutathione redox system, catalase and superoxide dismutases (figure 1.09). More recently the role of the heme-oxygenases and the thioredoxin reductase/thioredoxin system have also been implicated in antioxidant defence of the endothelial cell (Pohlman and Harlan, 2000).

Superoxide dismutases (SOD) accelerate the dismutation of $O_2^{\cdot}$ to H_2O_2 which is subsequently reduced by either catalase or glutathione peroxidase (GPX). Two forms of SOD exist in the endothelial cell: the copper/zinc-containing form is the predominant form and is expressed in the cytoplasm whilst a manganese-containing SOD is found primarily in the mitochondria.

The role of the GPX selenoenzymes in the reduction and detoxification of H_2O_2 and other ROS is discussed later in section 1.4.5a. Catalase is a ubiquitous enzyme that scavenges H_2O_2 exclusively. Whilst both GPX and catalase chemically reduce H_2O_2, catalase has a high Km for H_2O_2 and is present in much lower intracellular concentrations than GPX in most cells, with the

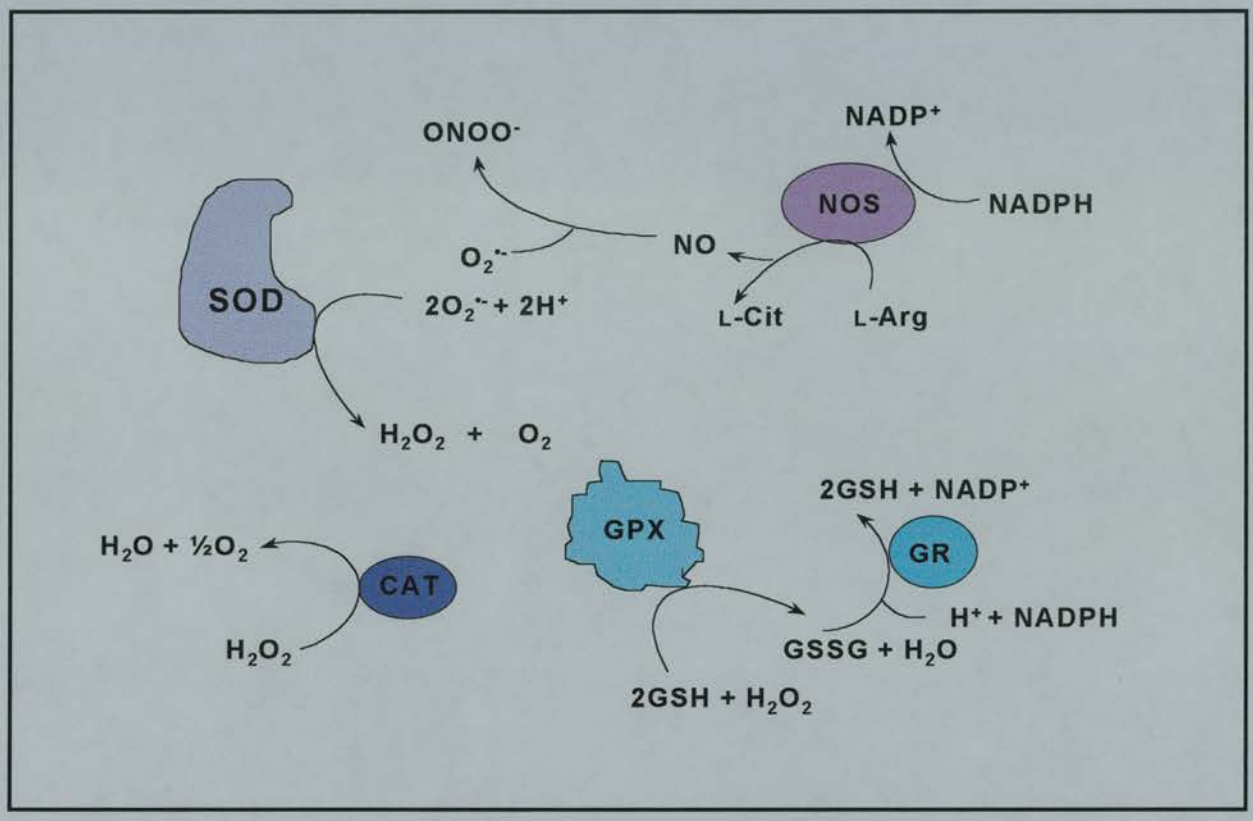


Figure 1.09. The endogenous antioxidant system of the endothelial cell. CAT, catalase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH' reduced glutathione, GSSG, glutathione disulphide; H_2O_2, hydrogen peroxide, L-Arg, L-Arginine; L-Cit, L-Citrulline; $O_2^{\bullet-}$, superoxide anion; $ONOO^-$, peroxynitrite, NO, nitric oxide, NOS, nitric oxide synthase.

exception of hepatocytes and erythrocytes (Asahi *et al.*, 1995). Impairment of the glutathione redox cycle, but not catalase, by pharmacologic manipulation increases the susceptibility of pulmonary artery endothelial cells to damage by H_2O_2 (Suttorp, Toepfer and Roka, 1986).

b) Dietary antioxidants

There are several potential strategies for the treatment or prevention of atherosclerosis. These include: lipid lowering diets, anti-thrombotic drugs, dietary supplementation with fish oil or L-arginine, angiotensin-converting enzyme inhibitors, β-blockers, apheresis and gene therapy. However much research attention has been focused on the ability of dietary antioxidant treatment to inhibit the progression of atherosclerosis. The clinical importance of an altered oxidation state in atherogenesis is attested to by the results of several case-control and cohort studies in which treatment with antioxidants significantly improved prognosis of atherosclerosis. However as yet randomised, double-blind, placebo controlled trials have not been able to assess whether there is a direct causal effect of low antioxidant status on the pathogenesis of atherosclerosis and other vascular diseases.

Laboratory data have demonstrated the beneficial effects of supplementation of SOD on endothelium-dependent vasodilatation in pig coronary arteries (Brandes *et al.*, 1997). *In vivo* studies show that antioxidants such as probucol suppress lesion formation in animal models of atherosclerosis (Witzum and Steinberg, 1991). Antioxidants have been shown to suppress LDL oxidation *ex vivo* (Ross, 1999) and in hypercholesterolemic dogs and cholesterol-fed rabbits, supplementation with vitamin E prevented the paradoxical vasoconstriction observed with endothelial dysfunction (Britten, Zeiher and Schächinger, 1999).

Preliminary clinical trials has shown that vitamin E intake is inversely correlated with the incidence of myocardial infarction and coronary heart disease whilst β-carotene appears to show no beneficial effects (Ross, 1999). Anderson *et al.* demonstrated that the combination of lipid-lowering therapy (lovastatin) and an antioxidant therapy (probucol) was more effective in

improving endothelium-dependent vasodilatation than the use of lipid lowering therapy alone (Britten, Zeiher and Schächinger, 1999).

Dietary supplementation with a combination of ascorbic acid, organic selenium, D-α-tocopheryl acetate and α-carotene was given in a randomised placebo-controlled double-blind small scale clinical trial carried out over a three month period (Nyyssonene *et al.*, 1994). The resistance of very low-density lipoprotein and LDL to oxidation was significantly increased in the subjects receiving the antioxidant supplements compared to the placebo group.

Despite these encouraging observations the evidence from case-control studies, prospective cohort studies, and primary and secondary prevention trials (recently reviewed by Gaziano (Gaziano, 1999)) attempting to link supplementation with the antioxidant vitamins C, E or β-carotene to a reduction in the risk of cardiovascular disease is equivocal. There are currently three ongoing large-scale trials underway which aim to define a role for antioxidant supplementation in the prevention of atherosclerotic disease. The Women's Health Study is studying the effect of Vitamin E and low-dose aspirin in the prevention of primary cardiovascular disease in 40,000 healthy health professionals. Secondary prevention trials such as the Women's Antioxidant Cardiovascular Study and The Heart Outcomes Prevention Evaluation Study have also been initiated. It is hoped that the results from these trials and several other small-scale trials may provide a clearer insight into the role of antioxidants in the treatment and prevention of cardiovascular disease in the near future.

1.4 SELENIUM AND SELENOPROTEINS

Dietary selenium through the expression of selenoproteins is thought to prevent damage to the endothelium caused by reactive oxygen species, thus protecting against atherogenesis.

1.4.1 Selenium - an introduction

The Swedish chemist, Jöns Jacob Berzelius discovered the element selenium in 1817 as a red deposit on the walls of a lead chamber used in the production of sulphuric acid (Reilly, 1993). It was initially regarded as an element with high toxicity and no known beneficial biological role. During the 1950s however Schwartz and Foltz demonstrated that selenium could prevent liver necrosis in vitamin-E deficient rats and suggested that it was an essential nutrient (Schwartz and Foltz, 1957). A functional role for selenium in mammals was not reported until 1973 (Rotruck *et al.*, 1973) when the element was shown to be a constituent of the antioxidant enzyme, cytosolic glutathione peroxidase (cyGPX). Reports highlighting the importance of selenium in human nutrition appeared in 1979 describing the selenium-responsive cardiomyopathy of Keshan disease in China (Group, 1979). Since then a considerable amount of further research has demonstrated that selenium is essential to human nutrition through the expression of a wide range of selenoproteins which have multiple and diverse roles.

1.4.2 The chemistry of selenium

Selenium has an atomic weight of 78.96 and is classed as a metalloid by virtue of sharing properties of both metal and non-metals (table 1.06). It lies between sulphur and tellurium in Group VI of the Periodic Table of Elements. Chemical similarities exist between selenium and sulphur; they have similar covalent radii and share the ability to use $d\pi$-$p\pi$ multiple bonding (Sunde, 1990).

Table 1.06. Some chemical and physical properties of selenium.

Properties	Values
Relative atomic mass	78.96
Atomic number	34
Atomic radius (X^2)	0.14 nm (2.02 A)
Covalent radius (X)	0.116 nm (1.17 A)
Melting point	217°C
Boiling point	684.8°C
Electronegativity	2.48
Electronic structure	$[Ar]3d^{10}4s^24p^4$
Oxidation states	-2, 0, +2, +4, +6
Stable isotopes	
mass	74 76 77 78 80 82
natural abundance (%)	0.87 9.02 7.85 23.52 49.82 9.19

From Foster and Sumar (Foster and Sumar, 1997)

Selenium exists naturally in a range of oxidation states, combining with other elements to form inorganic selenides, selenites and selenates (table 1.07). Selenium also forms organic selenoamino acids, which include selenocysteine and selenomethionine.

1.4.3 Selenium metabolism and bioavailability

a) Introduction

Despite sharing some physical and chemical properties, selenium and sulphur are not interchangeable in biological systems (Foster and Sumar, 1997). The metabolism of selenium in animals and humans, i.e. its absorption, transport, distribution, excretion, retention and transformation to an active selenide is dependent on its chemical form and the overall selenium status of the individual. Animals normally receive dietary selenium as organic selenoamino acids such as selenomethionine and selenocysteine and as methylated and non-methylated selenium, though inorganic forms, such as sodium selenite and sodium selenate, are often used in experimental diets and as supplements. Selenium is associated with tissue proteins throughout the body. Proteins that incorporate selenium through a UGA codon are referred to as selenoproteins and are metabolically active, whereas proteins that bind selenium non-specifically have been termed selenium-containing proteins.

The only metabolic pathway for selenium that has been well characterized is that based on the metabolism of most forms of selenium to selenite which is reduced to selenide. Figure 1.10 shows a hypothetical scheme of selenium metabolism (adapted from Foster and Sumar, 1997 and Sunde, 1990). The diagram shows the pathway through which most forms of selenium are metabolised and the selenium precursors for the synthesis of different forms of selenoproteins and selenium-containing proteins.

Table 1.07. Oxidation states of selenium.

Name	Formula	Oxidation state	Properties and other information
Selenate (selenium trioxide)	SeO_3	+6	m.p.120°C Stable in alkaline/oxidising conditions
Selenite	SeO_2	+4	m.p.315°C Readily oxidised to a +6 state: alkali pH Reduced to elemental selenium by SO_2, ascorbic acid; reacts with *o*-diamines Binds to iron and aluminium oxides: insoluble in soils; forms anhydride selenium oxides
Elemental selenium	Se	0	m.p.217°C, b.p.685°C Different forms include trigonal, α-monoclinic, β-monoclinic, red amphorous, black amphorous and vitreous Stable, insoluble: formed by +6 & +4 reduction
Selenide	H_2Se	-2	m.p.-66°C, b.p.-41°C a colourless flammable gas

From Foster and Sumar (Foster and Sumar, 1997).

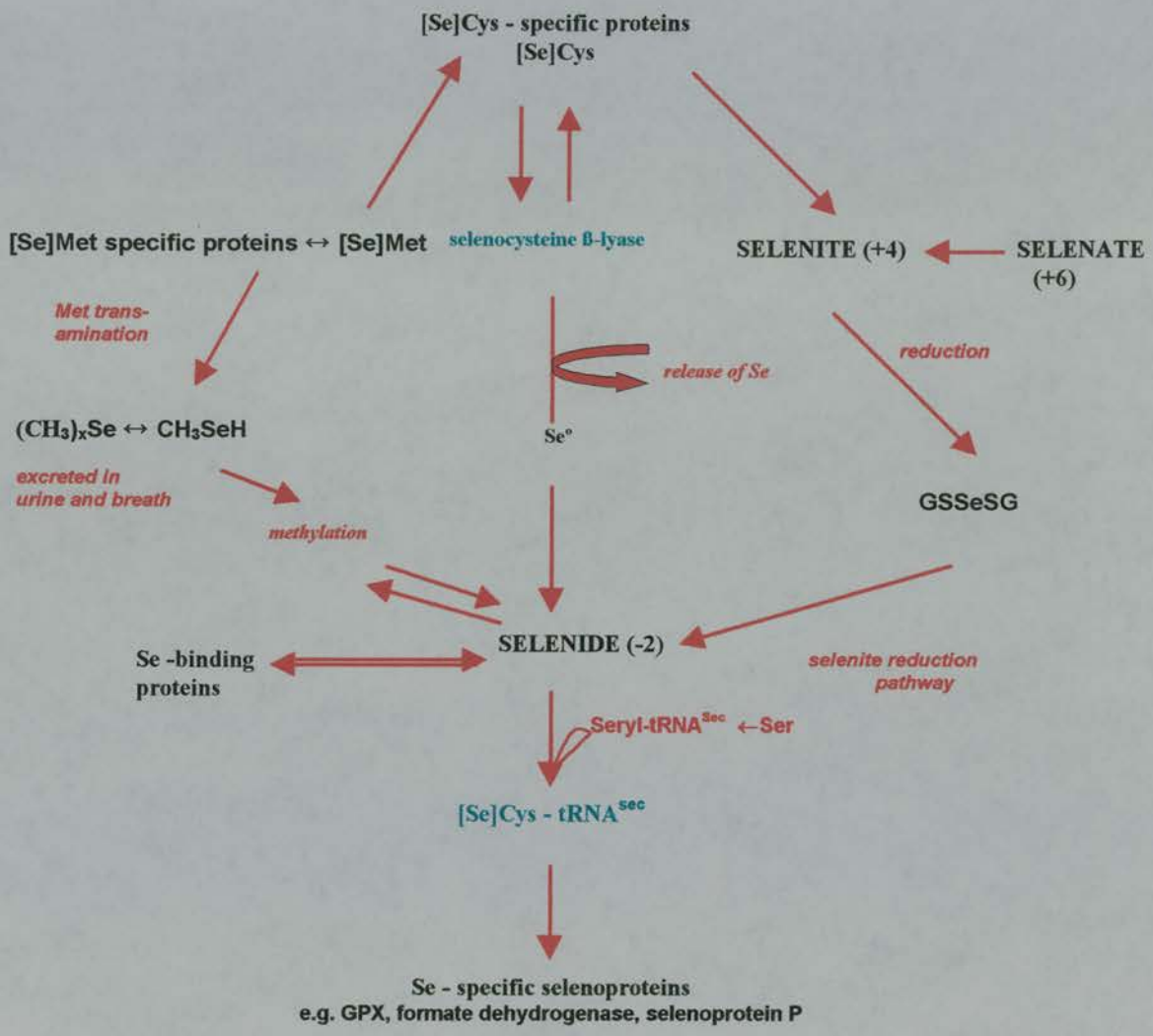


Figure 1.10. A diagram illustrating a hypothetical scheme of selenium metabolism. Adapted from Foster and Sumar (Foster and Sumar, 1997).

b) Absorption

It is generally accepted that selenium is well absorbed and under normal feeding conditions absorption is not the limiting factor to bioavailability (Mutanen, 1986). Virtually complete absorption occurs when selenium is supplied as selenomethionine (Swanson *et al.*, 1991) and other forms are generally well absorbed. Different forms of selenium are absorbed through different mechanisms, although most forms of selenium are absorbed through the duodenum (Thomson, 1998).

McConnell *et al.* demonstrated through the use of everted intestinal sacs prepared from hamster small intestine, that L-selenomethionine is absorbed using the same active transport mechanism as L-methionine (McConnell and Cho, 1965). Information on the absorption of selenocysteine is limited though it is known its absorption does not follow the same metabolic pathway as cysteine.

The details for the absorption of inorganic selenium are still unclear although the absorption of these compounds is influenced by several luminal factors. It is generally accepted that selenite is absorbed via a passive mechanism. McConnel *et al.* using the intestinal sac model showed no net exchange of selenite across the intestinal mucosa when concentrations of selenium were equal on both sides (McConnell and Cho, 1965). However further studies suggest that reduced glutathione in bile may be important in the absorption of selenite. Selenate absorption is primarily through the ileum and may share the same absorption pathway as sulphur (Daniels, 1996).

Tracer studies in humans have shown that selenium given as [^{75}Se]-selenomethionine is incorporated more efficiently into body tissues and has a longer half-life than selenium given as [^{75}Se]-selenite (Griffiths, Stewart and Robinson, 1976). There is considerable evidence to suggest that selenomethionine increases the plasma, erythrocyte and whole blood selenium more rapidly and to a greater degree than inorganic forms of selenium such as selenite and selenate (Daniels, 1996). However in individuals with low selenium status it has been shown that

organic and inorganic selenium supplementation were equally effective at raising blood glutathione peroxidase (GPX) and selenate was more effective than selenomethionine at raising platelet GPX levels (Daniels, 1996). After supplementation with inorganic selenium, blood levels decline rapidly to baseline whereas in selenomethionine supplementation, selenium levels remain elevated above baseline (Daniels, 1996). In a recent study, Brown *et al.* examined the effects of dietary organic and inorganic selenium supplementation, over a 28 day period, on GPX activities in blood cells taken from 45 men and women from a UK population (Brown *et al.*, 2000). The transient and acute changes in phospholipid GPX (PHGPX) activity observed during selenite supplementation in lymphocytes, granulocytes and platelets were largely mirrored in lymphocytes of the subjects given selenomethionine. Whereas, PHGPX activity in granulocytes and platelets from subjects given selenomethionine increased gradually over the 28 day period.

Certain interacting factors may affect the absorption of different selenium compounds. For example, in a study carried out on men, Greger and Marcus (Greger and Marcus, 1981) showed that selenium was better absorbed (72%) from a high protein diet rather than a low protein diet (44%). However supplementation of the low protein diet with cysteine and methionine so that both diets had similar total amino acid levels partly overcame the differences in absorption. However this study has been criticised, because in the high protein diet sodium selenite was used to provide all the selenium (261 µg/day) whereas in the low protein diet it only provided 150 µg/day of the selenium. Therefore these findings are difficult to interpret due to this use of different selenium compounds (Robinson and Thomson, 1983). Animal studies have shown that vitamins A and C promote absorption of selenite (Robinson and Thomson, 1983). This is an unexpected finding as ascorbic acid would be expected to reduce selenite to elemental selenium, which is probably not absorbed (Robinson and Thomson, 1983).

c) Transport

Selenium is transported in plasma bound to protein, though the proteins involved vary between species and have not been well defined in humans. Albumin seems to be the main plasma

selenium carrier in mice, whereas in humans selenium has been shown to be associated with selenoprotein P, α_2 and β-globulins and β-lipoproteins (Burk and Hill, 1994, Daniels, 1996; Robinson and Thomson, 1983).

Selenoprotein P has been identified in plasma of both rats and humans (Burk and Hill, 1994), accounting for over one half of the selenium content of mammalian plasma. Selenoprotein P has a postulated selenium transport function (Motsenbocker and Tappel, 1982), though the evidence for this is equivocal. Selenium is incorporated into selenoprotein P during protein synthesis rather than non-specifically bound, an energy consuming process that appears wasteful merely for transportation purposes. (Arthur, 1992; Hill and Burk, 1994). The half-life of selenium in selenoprotein P is not affected by the selenium status of the animal (Burk and Hill, 1993) and the only tissue which takes up selenium incorporated into selenoprotein P in selenium-deficient rats is the brain (Hill and Burk, 1994). These factors casts doubt on the role of selenoprotein P in transportation.

d) Metabolism and distribution

The metabolism of selenium is a complex process and varies according to the form ingested. Selenomethionine is readily and non-specifically incorporated in place of methionine residues into a large number of proteins especially skeletal muscle protein. The function(s) of the proteins which have a selenomethionine residue substituted for methionine appear unaltered. This non-active selenium pool makes up a large proportion of the total body selenium and does not appear to be under homeostatic control (Behne *et al.*, 1991). However, its incorporation is directly correlated with selenomethionine intake and inversely correlated to methionine intake (Burk and Hill, 1993). Selenomethionine is not directly available for utilisation in specific selenium pathways until it has been catabolized to selenide. Its metabolism to selenocysteine is via the methionine transamination and transsulphuration pathways provided adequate methionine is available (Burk, 1991; Daniels, 1996; Esaki *et al.*, 1981; Sunde, 1990). The

resulting selenocysteine does not accumulate, instead it is reduced to selenide to enable its selenium to be made available for selenoprotein synthesis. Non-methylated forms of ingested selenium are converted into methylated mono-, di- and tri-methylated selenium species in this metabolic pathway (Foster and Sumar, 1997) (Figure 1.10).

Selenocysteine does not follow the same metabolic pathway as cysteine, instead selenocysteine is rapidly converted to L-alanine by the selenocysteine-specific enzyme selenocysteine β-lyase which catalyses the β-elimination of L-selenocysteine (figure 1.10) (Esaki *et al.*, 1981). The two possible products from this mechanism are selenide, which is produced when the reaction is carried out in the presence of dithiothreitol, and elemental selenium, which is formed when the reaction is carried out anaerobically in the absence of any thiols. The action of selenocysteine β-lyase has two main purposes. It allows for the rapid elimination of the reactive molecule selenocysteine and it supplies the reduced form of selenium which is required for selenoprotein synthesis.

Inorganic sources of selenium are not stored but are metabolised according to figure 1.10. Erythrocytes rapidly take up and reduce selenite to selenodiglutathione which then undergoes a two-stage reduction to selenide in the liver and erythrocytes by glutathione reductase (Foster and Sumar, 1997).

There appear to be two distinct metabolic pools or compartments of selenium in animal tissues. The first incorporates all forms of selenium derived from inorganic selenium, including endogenously synthesised selenoproteins, the excretory trimethyl selenium and the intermediary products from the metabolism of selenite to selenide. This pool provides the metabolically active seleno-compounds. The second pool includes the proteins into which selenomethionine is non-specifically incorporated. These selenium-containing proteins have no known function other than possibly providing a selenium store. There is no evidence that the first pool contributes to the

second, but the second can contribute to the first through catabolism of selenomethionine as described above (Janghorbani *et al.*, 1990).

e) Excretion

The principle route of excretion of selenium is through the kidneys in urine as trimethyl selenium, with some loss through faeces, which comprises mainly unabsorbed selenium. Homeostatic balance of selenium in animals is primarily controlled through the regulation of its excretion through urine. Urinary concentrations of selenium are closely correlated to plasma selenium and dietary selenium within sample groups (Robinson and Thomson, 1983). Trimethyl selenium is the only metabolite identified in urine, although five other unidentified metabolites have been found.

Selenium is excreted to a small extent through dermal loss and normally only insignificant amounts are lost through exhalation, except when selenium intake is toxic when dimethyl selenium is exhaled (Diplock, 1976). This compound has a smell similar to garlic.

1.4.4 Selenoprotein synthesis

Selenium is incorporated specifically into selenoproteins as selenocysteine residues through a co-translational event directed by the UGA codon (Heider, Baron and Böck, 1992). This UGA codon was originally identified as a termination codon in the genetic code. However in selenoprotein synthesis the UGA codon signals the site of selenocysteine incorporation. This alternative interpretation of the UGA codon requires a mechanism which recognises the appropriate UGA codon and distinguishes it from the termination codon.

The mechanism for the synthesis of selenoproteins was characterised in prokaryotes using *E. coli* mutants (as reviewed in Bermano, Arthur and Hesketh, 1996; Heider, Baron and Böck, 1992). The synthesis of selenocysteine and its insertion into specific selenoproteins in

prokaryotes involves the products of four genes (*selA*, *selB*, *selC*, and *selD)* (Allan, Lacourciere and Stadtman, 1999). The products are; a selenocysteine specific tRNA species ($tRNA^{Sec}$) (*selC)* which carries the anticodon for UGA, the enzymes, selenocysteine synthase (*selA*) and selenophosphate synthetase (*selD*) that are essential for the formation of selenocysteine-$tRNA^{Sec}$ from seryl-$tRNA^{Sec}$ and the elongation factor that specifically recognises the selenocysteine-tRNA (*selB).* The major steps of this co-translational event are illustrated in figure 1.11.

The lack of a corresponding series of mutants has prevented a complete elucidation of the mechanism of selenocysteine incorporation and selenoprotein synthesis in eukaryotes. However two forms of the $tRNA^{Sec}$ have been isolated in eukayotes and both contain the UGA anticodon which is functional in *E. coli* (Kollmus, Flohè, and McCarthy, 1996; Low and Berry, 1996). Like bacterial $tRNA^{Sec}$, in eukaryotes $tRNA^{Sec}$ is esterified with serine and is subsequently converted to seryl-$tRNA^{sec}$. However, the nature of the mRNA selenocysteine insertion sequence (SECIS) elements which are responsible for the recognition of the UGA as a selenocysteine insertion codon differs between prokaryotes and eukaryotes (Kollmus, Flohè, and McCarthy, 1996). In prokaryotic selenoproteins these sequences form a stem-loop structure immediately downstream (3') from the UGA codon in the open reading frame. The movement of this sequence by more than one codon seriously compromises selenoprotein synthesis. In contrast eukaryotic SECIS elements are located in the 3'-untranslated region of the mRNA (Berry, 1991). They comprise a small number of conserved nucleotides which form a stem-loop structure (Low and Berry, 1996). Studies by Berry *et al.* have shown these SECIS elements are functionally interchangeable (Berry, 1991; Berry *et al.*, 1993).

The selenocysteine residue in most selenoproteins is necessary for the full activity of that enzyme. This has been demonstrated through the use of mutant cysteine analogues of selenoproteins whereby the selenocysteine residue of the selenoprotein has been substituted with a cysteine residue. A comparison of the enzyme activity of the selenocysteine-containing

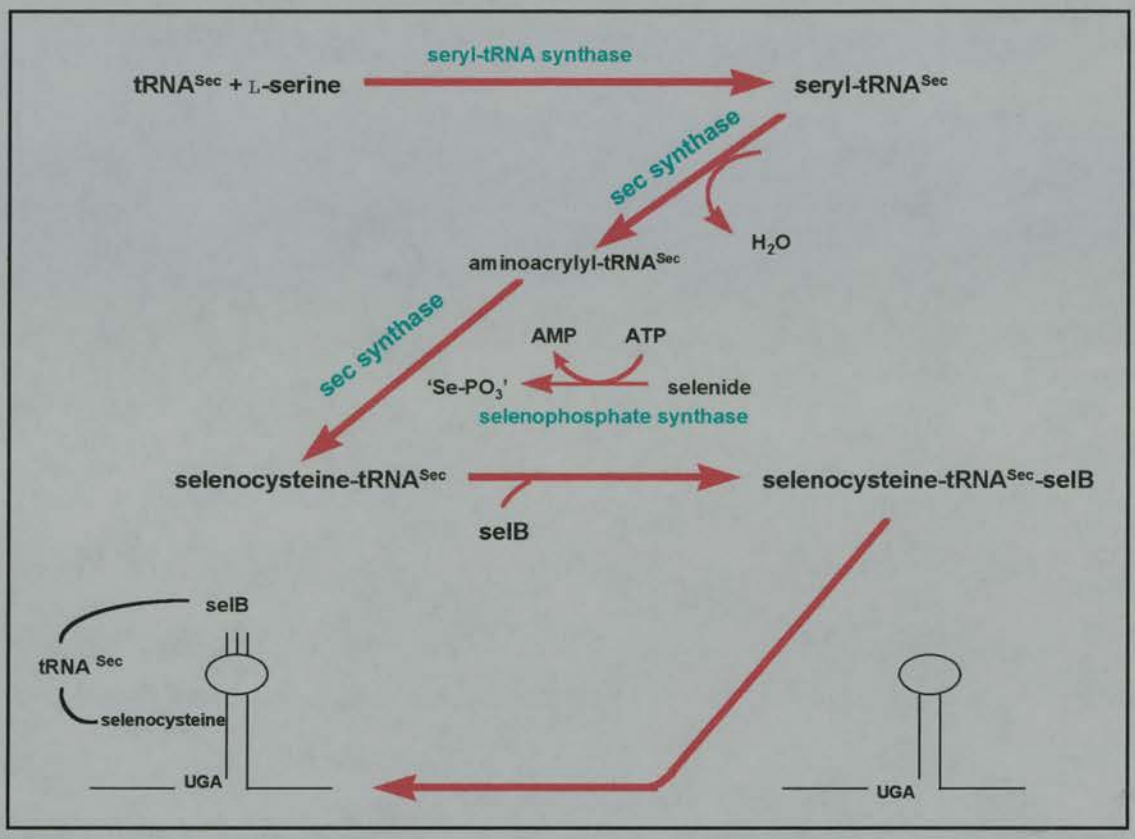


Figure 1.11 The synthesis of selenocysteine and its incorporation into selenoproteins in prokaryotes. The selenocysteine specific tRNA ($tRNA^{Sec}$) is initially esterified with L-serine catalysed by seryl-tRNA synthetase [373]. Once charged with L -serine the seryl-$tRNA^{Sec}$ can bind to selenocysteine synthase. Selenocysteine synthase is a pyridoxyl 5-phosphate enzyme which catalyses the exchange of the conversion of seryl-$tRNA^{Sec}$ to selenocysteyl-$tRNA^{Sec}$. The initial step involves a 2,3-elimination of a water molecule to form an aminoacrylyl-$tRNA^{Sec}$ intermediate. The second step involves the addition of a reactive selenium derivative across the double bond of the aminoacrylyl residue. Monoselenophosphate acts as the selenium donor in this reaction. It is produced from the selenide and ATP by the selD gene product selenophosphate synthetase. The selenocysteyl-$tRNA^{Sec}$ (sec-$tRNA^{Sec}$) is then released from the selenocysteine synthase and binds to the elongation factor selB which delivers the sec-$tRNA^{Sec}$ complex to a stem loop structure on the mRNA. In order to recognize the UGA codon as an insertion sequence for selenocysteine as opposed to a stop codon the stem loop structure has a specific sequence, positioned downstream from the UGA codon. The stem loop structure binds the *selB*-$tRNA^{Sec}$-selenocysteine complex aiding its interaction with the UGA codon and the subsequent incorporation of selenocysteine into the protein. Adapted from Leinfelder *et al.* (Leinfelder *et al.*, 1990).

type I 5' deiodinase with its cysteine-containing mutant analogue showed that the mutant enzyme was only 10-20% as active as the native selenocysteine-containing enzyme (Berry, Banu and Larsen, 1991). More recently cysteine-containing mutants have been made for TR. The NADPH-disulphide oxidoreductase activity of the purified TR mutant-enzyme was only 6-11% of that of the wild type rat liver TR (Lee *et al.*, 2000).

For many years cytoplasmic GPX (cyGPX) was the only known selenoprotein. It is now known that selenium exerts its effects through the expression of a number of different intracellular and extracellular selenoproteins (Arthur and Beckett, 1994). At least 30 selenoproteins have been identified by SDS-polyacrylamide electrophoresis (SDS-PAGE) of [^{75}Se]-labelled tissue, but only approximately 13 have been characterized by purification and cloning.

1.4.5 Mammalian selenoproteins and selenium-containing proteins

Table 1.08 lists the mammalian selenoproteins that have been characterized.

a) Glutathione peroxidases

i) Introduction

The family of glutathione peroxidases include four distinct selenoproteins. Each of these peroxidases arise from distinct gene products which are homologous with one another but structurally and phytogenetically unrelated to the selenium-containing bacterial oxidoreductases and the other mammalian selenoproteins.

Cytoplasmic glutathione peroxidase (cyGPX) was first discovered by Mills in 1957 and was shown to be important in the protection of red blood cells against hydrogen peroxide or ascorbate-induced haemoglobin oxidation (Mills, 1957; Rotruck *et al.*, 1973). Until the discovery of further selenoproteins the biological functions of selenium, including its antioxidant role, were attributed to this classic tetrameric selenoprotein cyGPX. However, in 1982 a second glutathione

Table 1.08. Mammalian selenoproteins and their postulated functions.

Selenoprotein	Postulated functions
Glutathione peroxidase (GPX)	
cytosolic GPX	intracellular antioxidant, selenium store?
phospholipid hydroperoxide GPX	intracellular antioxidant, structural role in spermatozoa
p*lasma GPX*	plasma antioxidant
gastrointestinal GPX	gastrointestinal tract antioxidant
Iodothyronine deiodinase	
type I & type II	catalyses the conversion of thyroxine (T4) to 3,5,3' triiodothyronine
type I & III	catalyses the conversion of T4 to 3,3',5' reverse triiodothyronine
Selenoprotein P	transport? Antioxidant role?
Thioredoxin reductase	
TrxR1, TrxR2 & TRß	multiple roles associated with its role as part of a dithiol-disulphide oxidoreductase system
Selenoprotein W	antioxidant role ?
Selenoprotein synthetase	catalyses the production of selenophosphate, required for selenoprotein synthesis
15 kDa selenoprotein	linked with prostate cancer?

peroxidase was identified and characterized as phospholipid hydroperoxide glutathione peroxidase (PHGPX) (Ursini *et al.*, 1982). This was followed four years later by the isolation and characterization of a glycosylated extracellular selenoprotein isolated from the plasma and aptly named plasma GPX (plGPX) (Avissar *et al.*, 1989; Takahashi and Cohen, 1986). The gastrointestinal tract yielded the fourth member of this family of selenoproteins appropriately labelled gastrointestinal GPX (giGPX) (Chu, Doroshow and Esworthy, 1993). More recently selenoproteins with structural similarities to the glutathione peoxidases, encoded by viral genomes, have been identified (Burk and Hill, 1999). For example the human immunodeficiency virus is thought to sequester selenium thus depriving infected cells (Taylor *et al.*, 1994).

As is common to most known selenoproteins, selenium is an essential requirement for the synthesis of all the glutathione peroxidases. The elimination of the selenocysteine residue or carboxymethylation of selenocysteines by iodoacetate inactivates the GPX (Ursini *et al.*, 1995).

ii) Cytoplasmic glutathione peroxidase

The structure and function of the cyGPX has been extensively studied. cyGPX comprises four identical subunits, each with a molecular weight reported to be between 19-25 kDa. Each subunit contains a glutathione binding site and an active site comprising of a single selenocysteine residue which is located at approximately the fortieth residue from the N-terminal end; the exact location depends on the tissue and species (Sunde, 1994; Zachara, 1992). Following the cloning of murine cyGPX from a mouse genomic DNA library, this enzyme has since been cloned from several tissues and species (Sunde, 1994). cyGPX is expressed in virtually all cells although its specific activity is known to vary between different species and tissues.

The antioxidant function of the glutathione peroxidases is well documented. cyGPX, as well as the other tetrameric glutathione peroxidases (plGPX and giGPX) is able to catalyse the reduction

of a variety of hydroperoxides, including hydrogen peroxide, cumene hydroperoxide, t-butyl hydroperoxide and fatty acid hydroperoxides (Flohé, 1989).

The reactions catalysed by each glutathione peroxidase (GPX) follow a similar mechanism in which the GPX catalyses the reduction of either hydrogen peroxide (H_2O_2) or an organic hydroperoxide (ROOH), using glutathione (GSH) as a reducing agent, to form water or the corresponding alcohol and glutathione disulphide (GSSG) according the equation 1 and 2. Each member of the GPX family uses a complex ter uni Ping-Pong mechanism for its actions (Ursini *et al.*, 1995; Ursini, Maiorino and Gregolin, 1985). Several different catalytic mechanisms have been postulated for these reactions. Figure 1.12 illustrates the mechanism described below. Ganther proposed that the first step involves the oxidation of the GPX active site selenol to selenenic acid by the peroxide substrate (Ganther, 1999). This is followed by the reaction of selenenic acid with the first GSH molecule to form water (or alcohol in the case of organic hydroperoxides). The final step involves the cleavage of the sulfoselenide link by a second GSH molecule, releasing oxidised glutathione and restoring GPX to its selenol form (Zachara, 1992).

$$H_2O_2 + 2GSH \xrightarrow{GPX} 2H_2O + GSSG \quad (1)$$

$$ROOH + 2GSH \xrightarrow{GPX} ROH + H_2O + GSSG \quad (2)$$

The importance of cyGPX in the physiological regulation of intracellular hydroperoxide concentrations is in doubt as selenium deficiency resulting in a loss of cyGPX activity to less than 1% of control values in the rat liver resulted in no obvious adverse clinical effects in the short term (Arthur *et al.*, 1987; Burk and Hill, 1993). In addition cyGPX knock-out mice displayed no clinical abnormalities under normal physiological conditions and in defence against hyperoxia

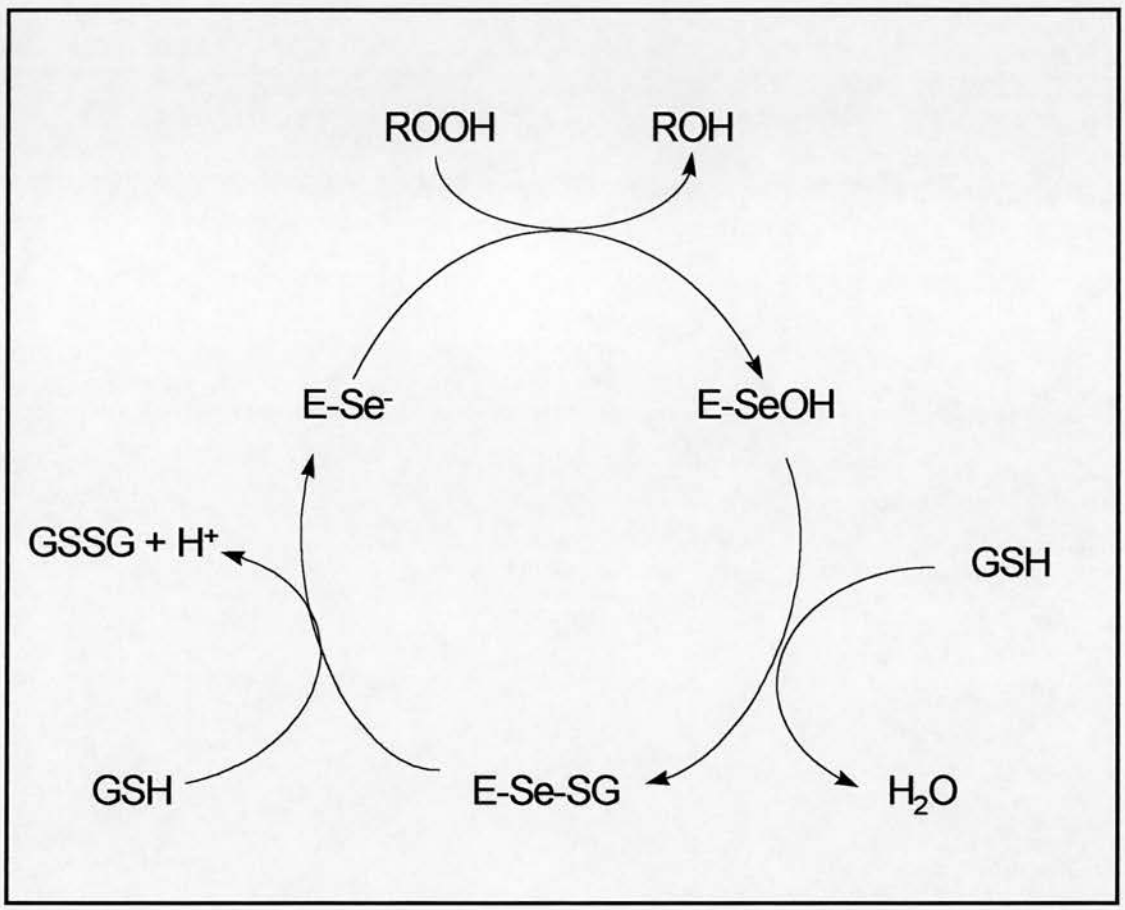


Figure 1.12. A diagram illustrating a hypothetical mechanism for the glutathione redox cycle. The first step involves the oxidation of the glutathione peroxidase active site selenol (E-Se^-) to selenenic acid (E-SeOH) by the peroxide substrate. E-SeOH then reacts with the first reduced glutathione molecule (GSH) to form water (or alcohol in the case of organic hydroperoxides). Finally the sulfoselenide (E-Se-SG) link is cleaved by a second GSH molecule releasing oxidised glutathione (GSSG) and restoring GPX to its E-Se^- form. Adapted from Zachara (Zachara, 1992).

the evidence for such a role is equivocal (Burk and Hill, 1994). The incorporation of selenium into selenoprotein P and its subsequent release are energy consuming processes which appear to be wasteful merely for transportation purposes (Arthur, 1992). The half-life of selenium in selenoprotein P is not affected by the selenium status of the animal which also questions a transportation role for selenoprotein P (Burk and Hill, 1993). Also, in the selenium-deficient rat the only tissue to accumulate selenium from selenoprotein P is the brain (Hill and Burk, 1994). Alternatively selenoprotein P may provide an important defence for endothelial cells against oxidative damage (Burk *et al.*, 1997). The association of selenoprotein P with endothelial cells has led to speculation that selenoprotein P can protect the endothelium against extracellular reactive oxygen species such as peroxynitrite and superoxide produced in cardiovascular disease (Burk *et al.*, 1997). Burk *et al.* have described an *in vivo* model which links the selenoprotein P with an antioxidant function (Burk *et al.*, 1995; Burk, Lawrence and Lane, 1980). Selenium-deficient rats administered diquat (a compound which generates superoxide in the hepatocyte) at concentrations well below the LD_{50} for selenium-sufficient rats die within hours due to liver necrosis and lipid peroxidation (Burk, Lawrence and Lane, 1980). However if selenium-deficient rats are injected with selenium 12 hr prior to diquat administration the rats survive and the degree of protection correlates with selenoprotein P levels in plasma but not with glutathione peroxidase activity (Burk *et al.*, 1995).

d) Thioredoxin reductase

i) Thioredoxin reductase - characteristics

Thioredoxin reductase (TR) is a member of the pyridine nucleotide-disulphide family of enzymes which includes lipoamide dehydrogenase, mercuric reductase, trypanothione reductase and glutathione reductase (Gasdaska *et al.*, 1999b; Lee *et al.*, 1999; Sun *et al.*, 1999). However the other members of this family lack the selenocysteine residue which characterizes TR as a selenoprotein (Tamura and Stadtman, 1996; Gladyshev, Jeang and Stadtman, 1996).

TR is a FAD-containing homodimeric selenoenzyme which, together with thioredoxin (Trx) as a substrate and NADPH as a cofactor, forms a powerful dithiol-disulphide oxidoreductase system referred to as the TR/Trx system. Each TR subunit has a molecular mass of between 54-65 kDa and contains a tightly bound FADPH which mediates the transfer of reducing equivalents from NADPH to the disulphide bond of Trx and other substrates (Holmgren, 1989). TR has a conserved Cys-Val-Asn-Val-Gly-Cys active site sequence. A single selenocysteine encoded by a UGA codon is also contained within each subunit within the sequence Cys-SeCys-Gly as the penultimate carboxyl terminal residue (Gladyshev, Jeang and Stadtman, 1996a). The function of the selenocysteine residue has not been clarified. Its presence is essential for enzyme function, as substitution of the selenocysteine residue with cysteine (Berggren *et al.*, 1997), or removal of the selenocysteine residue by peptidase treatment (Zhong *et al.*, 1998), by selective alkylation (Nordberg *et al.*, 1998) or by the removal of the selenocysteine insertion sequence (SECIS) element (Fujiwara *et al.*, 1999) abolishes TR activity. It is postulated that the selenocysteine does not form part of the catalytic site but carries reducing equivalents from the active site to the substrate (Gladyshev, Jeang and Stadtman, 1996b; Gromer *et al.*, 1998).

ii) Thioredoxin reductase - substrates

Trx is a ubiquitous low molecular weight (10-12 kDa) multifunctional protein (Holmgren, 1985; Holmgren, 1989; Holmgren and Björnstedt, 1995). It consists of a redox-active disulphide (Trx-S_2)/dithiol (Trx-$(SH)_2$ within the conserved active site sequence:- Cys-Gly-Pro-Cys-Lys (Holmgren, 1989; Holmgren and Björnstedt, 1995). The redox state and activity of Trx is regulated by TR whereby TR converts oxidised Trx back to its reduced form (Holmgren and Björnstedt, 1995).

Mammalian TR can catalyse the reduction of a variety of substrates including Trx, protein disulphide-isomerase, low molecular weight disulphides such as 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), lipoic acid as well as non-disulphides such as selenite, selenodiglutathione, vitamin K, alloxan and lipid hydroperoxides (Björnstedt *et al.*, 1995; Björnstedt, Kumar and

Holmgren, 1992; Lundström and Holmgren, 1990). The ability of TR to reduce dehydroascorbic acid to ascorbic acid has also been reported (May *et al.*, 1997).

iii) Cellular functions

The reduction of Trx (Trx-S_2) to Trx-$(SH)_2$ catalysed by TR (reaction 1) provides a powerful protein disulphide reductase (reaction 2), which has multiple roles.

$$NADPH + H^+ + Trx\text{-}S_2 \xrightleftharpoons{TR} NADP^+ + Trx\text{-}(SH)_2 \quad (1)$$

$$Trx\text{-}(SH)_2 + protein\text{-}S_2 \xrightleftharpoons{spontaneous} Trx\text{-}S_2 + protein\text{-}(SH)_2 \quad (2)$$

Interest in the TR/Trx system increased after the initial discovery that TR provides reduced Trx which serves as a hydrogen donor for ribonucleotide reductase in the initial and rate limiting step in DNA synthesis (Thelander and Reichard, 1979). The TR/ Trx system has subsequently been associated with many diverse cellular functions including the regulation of cell growth in both normal and cancer cells (possibly by increasing the sensitivity of the cell to endogenous growth factors) (Berggren *et al.*, 1996; Gallegos *et al.*, 1996) and the inhibition of apoptosis via the binding of oxidised Trx to the apoptosis signalling kinase-1 (Fujiwara *et al.*, 1999). The regeneration of proteins inactivated by oxidative stress including glyceraldehdye-3-phosphate dehydrogenase (Fernando *et al.*, 1992), phosphotyrosine phosphatase (McCarty, 1999) and nitric oxide synthase (Patel, Zhang and Block, 1996) is also thought to involve the TR/Trx system. In addition this system is believed to regulate the DNA-binding activity of transcription factors (Berggren *et al.*, 1996a; Björnstedt *et al.*, 1995; Gallegos *et al.*, 1997a), including NF-κB (Matthews *et al.*, 1992; McCarty, 1999); the glucocorticoid receptor (Makino *et al.*, 1996); TFIIIC

(Cromlish and Roeder, 1989); BZLF1 (Bannister, Cook and Kouzarides, 1991) and the modulation of activator protein 1 (fos/ Jun heterodimer) indirectly through a nuclear redox factor ref-1/HAPE (Xanthoudakis *et al.*, 1992).

The TR/Trx system can regulate the cellular redox state (Gasdaska *et al.*, 1999a) and protect against oxidative stress (Holmgren, 1985; Holmgren, 1989). For example, it can serve as a redox regulator of cellular antioxidant systems by acting as an electron donor for methionine sulfoxide reductase (Holmgren, 1985); 3-phosphoadenosine 5'-phosphosulphate reductase (Holmgren, 1985); thioredoxin-dependent peroxidase (Chae *et al.*, 1994); protein disulphide isomerase (Holmgren and Björnstedt, 1995); and vitamin K epoxide reductase (Maellaro *et al.*, 1994). Trx also facilitates the refolding of disulphide-containing protein [Lundstrom, 1990 #627].

Trx is a reactive oxygen species (ROS) scavenger and recombinant human Trx has been shown to protect against hydrogen peroxide and TNFα-induced cytoxicity (Matsuda *et al.*, 1992; Nakamura *et al.*, 1994). In a cell-free system TR has been shown to directly reduce and detoxify hydrogen peroxides, organic hydroperoxides and lipid hydroperoxides (Björnstedt *et al.*, 1995) and, serve as a electron donor to plasma glutathione peroxidase (Björnstedt *et al.*, 1994). Trx will also act as a reducing agent for type 1 iodothyronine deiodinases (Sharifi and St Germain, 1992). More recently up-regulation of the thioredoxin system has been associated with the development of cellular resistance to the chemotherapeutic agent *cis*-diamminedichloroplatinum (II) (Sasada *et al.*, 1999).

iv) Thioredoxin reductase isoforms

Recently it has been shown that several isoforms of TR exist. Studies have shown that the 57kDa TR isolated from HeLa cells and from human lung adenocarcinoma cells can be further dissociated according to their ability to bind to a heparin-agarose affinity column and their reactivity with anti-rat liver TR polyclonal antibodies (Gorlatov and Stadtman, 1999; Liu and

Stadtman, 1997). The cytosolic form of TR (classified by Gorlatov and Stadtman as TrxR1 (Gorlatov and Stadtman, 1999)) is not retained by the heparin column and shows cross-reactivity with anti-rat liver polyclonal antibodies. In contrast, the mitochondrial isoform (TrxR2) binds tightly to the heparin affinity column and shows only very weak cross-reactivity (Gorlatov and Stadtman, 1999). These two different isoforms have been cloned and sequence data suggest that they are closely related, including a conserved active site sequence (CVNVGC) and FAD-binding and NADPH-binding domains (Miranda-Vizuete *et al.*, 1999). Immunoblot analysis has confirmed that TrxR1 is expressed in the cytosol and nucleus but is also secreted (Hirota *et al.*, 1997) whilst expression of TrxR2 as a fusion protein with green fluorescent protein showed that TrxR2 is located in the mitochondria (Miranda-Vizuete *et al.*, 1999). The TrxR2 initially possesses a N-terminal extension which in the human adrenal gene product targets the enzyme from the cytoplasm to the mitochondrial membrane (Lee *et al.*, 1999; Miranda-Vizuete *et al.*, 1999). The postulated function of this alternative TR isoform is to provide a mitochondria-specific defence against ROS produced by the mitochondrial respiratory chain thus maintaining a redox balance critical for cell survival (Lee *et al.*, 1999).

Gortalov and Stadtman have recently suggested that the differing abilities of these enzymes to bind to the heparin reflect differences in conformational states determined by the redox state of the enzyme (Gorlatov and Stadtman, 1999). Gasdaska *et al.* recently reported the identification and characterization of a third human TR (TR-β), differing in molecular mass (56.5 kDa) and exhibiting a distinct pattern of tissue expression with high levels of TRβ mRNA found in the prostate, testis, liver, uterus and small intestine and only low levels in placenta, kidney, pancreas, thymus and peripheral blood leukocytes (Gasdaska *et al.*, 1999b). The cDNA sequence of TRβ is identical to that of TrxR2 from human adrenal with the exception that the former possess a Met-Ala-Ala extension at its N-terminus (Gorlatov and Stadtman, 1999). Using a polyclonal antibody made against synthetic TRβ peptide, Western blot analysis identified TRβ

in both the cytosolic and microsomal subcellular fractions of MCF-1 human breast cancer cells (Gasdaska *et al.*, 1999b).

e) Selenoprotein W

Selenoprotein W is a small intracellular selenoprotein comprising one selenocysteine residue per polypeptide chain (Burk and Hill, 1999). Four different forms of selenoprotein W have been isolated from rat muscle and a partial amino acid sequence has been determined (Allan, Lacourciere and Stadtman, 1999). The molecular masses of these isoforms range from 9.5 -10 kDa. The brain, muscle, testis and spleen contain the greatest amounts of selenoprotein W whilst the liver contains only small amounts (Burk and Hill, 1999). The catalytic activity of selenoprotein W is at present unknown although an antioxidant role has been postulated (Burk and Hill, 1999). Over-expression of selenoprotein W in cultured rat glial cells has been associated with increased protection against free-radical damage which supports a postulated antioxidant function (Sun, Gu and Whanger, 1998).

f) Selenophosphate synthetase

Selenoprotein synthetase (SPS), the Sel D gene product, is an essential component of selenoprotein synthesis, catalysing the production of selenophosphate, the selenium donor required for selenocysteine synthesis. Two homologues of human SPS have been identified. The first human SPS was cloned by Low *et al.* and is referred to as SPS1 (Low, Harney and Berry, 1995). The transfection of SPS1 cDNA into mammalian cells led to increased [^{75}Se]-labelling of type 1 iodothyronine deiodinase (Low, Harney and Berry, 1995). The following year a second SPS was identified. Designated SPS2, the gene encoding its transcription exhibits an in-frame TGA codon at the enzymes putative active site (Guimaraes *et al.*, 1996). The incorporation of [^{75}Se] into the immunopurified SPS2 protein provide further evidence that SPS2 is a selenoprotein (Guimaraes *et al.*, 1996). The requirement of SPS2 synthesis for selenium would suggest that this protein is involved in the regulation of selenoprotein synthesis *per se,*

such that selenium-deficiency would be reflected by a down-regulation of SPS2 expression with a parallel decrease in the expression of other selenoproteins. Therefore, differential expression of these SPS1 and SPS2 proteins in tissues may explain the differences between tissues in sensitivity to selenium-deficiency (discussed later in section 1.46).

g) Other selenoproteins and selenium-containing proteins

Through the use of SDS-PAGE and two-dimensional electrophoresis of [^{75}Se]-labelled animal tissue approximately 35 selenium-containing proteins or protein subunits with molecular masses ranging between 6 and 116 kDa could be distinguished (Behne *et al.*, 1988; Behne *et al.*, 1999). Naturally some of these selenium-containing proteins may represent as yet uncharacterized selenoproteins. For example a 15 kDa selenoprotein has recently been isolated and characterized in human T cells. Its function is as yet unknown but its high expression in prostate tissue has been linked with a protective role against prostate cancer (Gladyshev *et al.*, 1998).

Several selenium-containing proteins have been characterized. Fatty acid-binding proteins (FABP) constitute a family of highly conserved 14-15 kDa cytosolic proteins which are involved in the regulation of intracellular levels of long chain fatty acids (Masouyé *et al.*, 1997). Bansal *et al.* have identified a 14 kDa mouse liver selenium-binding protein as a FABP (Bansal *et al.*, 1989). Liver FABP has been implicated in the regulation of cell growth and the presence of selenium may provide a mechanism by which this occurs. To date no other members of the FABP superfamily have been identified as selenium-containing proteins. Protein disulphide isomerase (PDI) is also a selenium-containing protein, with approximate molecular weight of 58 kDa. Treatment of PDI with SDS has shown that selenium is tightly bound to PDI. However cloning and sequencing have not identified a TGA codon in the nucleotide sequence of PDI and therefore selenium is not incorporated as a selenocysteine residue (Sinha *et al.*, 1993). The role of selenium is this protein is unclear as supplementation of mouse epithelial cells with selenium

appears to have no effect on the levels of PDI (Sinha *et al.*, 1993). Also, PDI activity is not regulated by selenium in either rats or cultured cells (Arthur *et al.*, 1991; Sinha *et al.*, 1993).

1.4.6 Regulation of selenoproteins

i) Hierarchy of selenium supply

Expression of all the selenoproteins characterized to date, namely the glutathione peroxidases, thioredoxin reductases, iodothyronine deiodinases, selenoprotein P and selenoprotein W is regulated by selenium supply. Selenium deficiency results in a fall in all selenoproteins, unlike other modulators of selenoprotein expression which may be specific for particular selenoproteins. The extent to which selenium availability affects selenoprotein expression differs between tissues and between individual selenoproteins within a tissue. Several studies have been performed which demonstrate this differential regulation (Behne *et al.*, 1988; Bermano, 1995; Bermano, Arthur and Hesketh, 1996; Burk and Hill, 1993; Hill *et al.*, 1997; Weitzel, Ursini and Wendel, 1990). For example, Yang *et al.* studied the effect of changes in dietary selenium on cyGPX and plGPX activities and selenoprotein P expression, measured in rat liver and plasma (Yang, Hill and Burk, 1989). They found that as selenium was supplemented into the diet, selenoprotein P expression increased initially, followed by an increase in plGPX levels. Liver cyGPX was the last to respond to increased selenium supply. In a diet supplemented with 0.02 mg selenium/kg the selenoprotein P concentration was 48% of the control, whilst plGPX and cyGPX activities were only 12% and 1% of control values respectively.

There is a clear hierarchy in the selenium supply to different tissues (figure 1.13). Behne *et al.* were the first to show that in the rat there is a differential loss of selenium from different tissues in selenium-deficiency (Behne *et al.*, 1988). It appears that regulatory mechanisms exist which ensure that, in selenium deficiency, selenium levels are maintained in certain priority organs. Selenium is generally retained where it is most needed such as the brain, endocrine and reproductive organs thus indicating the relative importance of the element for the biological

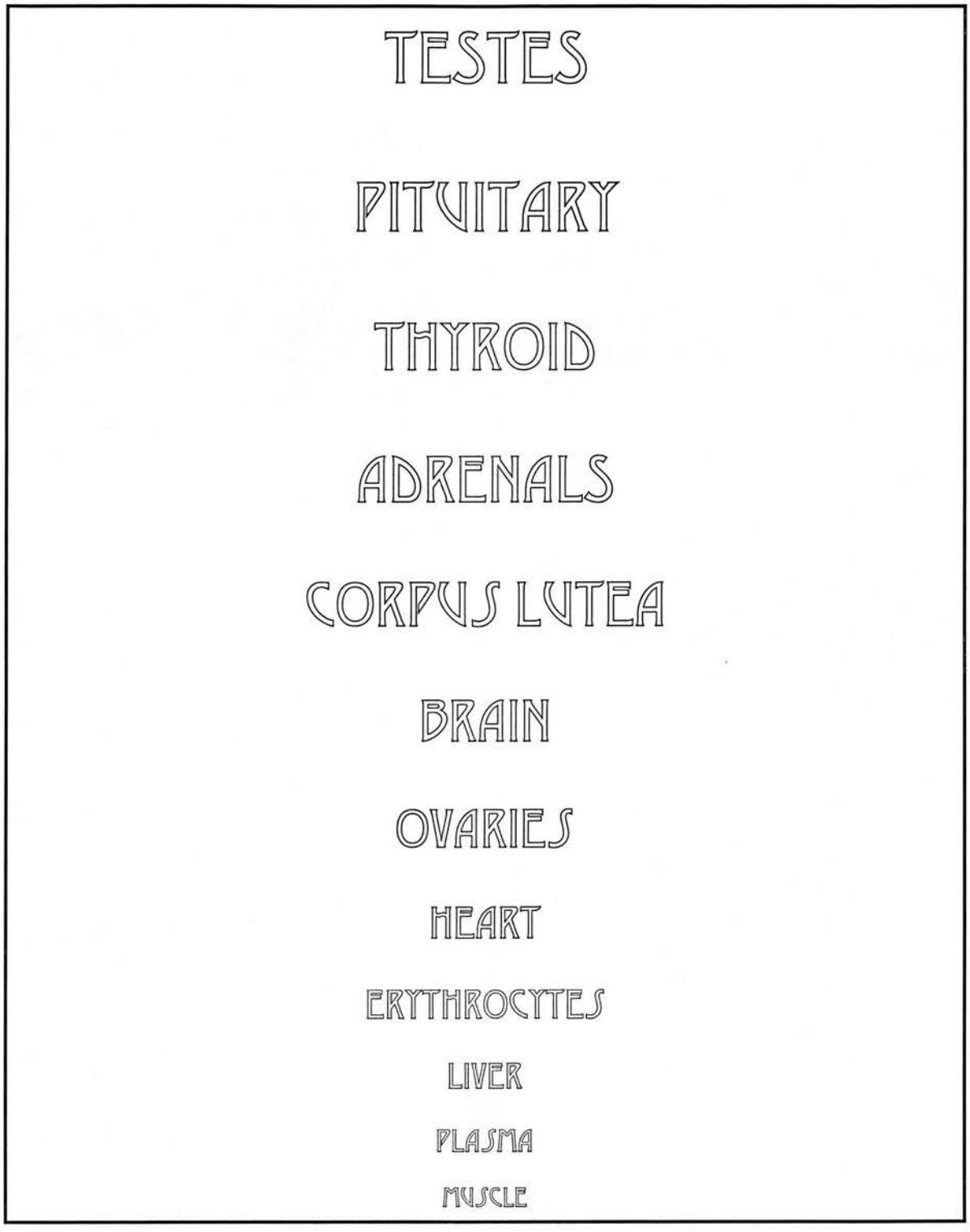


Figure 1.13. Schematically representation of the hierarchy of selenium retention by different tissues in selenium-deficient rats. Tissues at the top of the hierarchy preferentially retain selenium over those tissues at the bottom. Data taken from Behne *et al.* (Behne *et al.*, 1988).

functions of these organs. The differential regulation is not achieved by a decrease in the turnover of selenium in deficient tissues alone, re-distribution of the metabolised element and priority supply to these tissues are also involved. In selenium-deficiency, selenium supply is not only preferentially diverted to certain tissues it is also directed to specific selenoproteins within that tissue such that selenium supply to selenoproteins other than glutathione peroxidase had priority (Behne *et al.*, 1988).

Both organ-specific and selenoprotein-specific regulation are demonstrated in the study carried out by Bermano *et al.* which examined the levels of cyGPX, PHGPX and type I iodothyronine deiodinase (IDI) in the heart, liver and thyroid of rats fed a diet of varying selenium content (Bermano, 1995). In selenium-deficient rats cyGPX, PHGPX and IDI activities in the liver decreased 99%, 75% and 95% respectively. In the thyroid, cyGPX was decreased by 50%, PHGPX was unaffected and IDI was increased by 15%. Therefore with inadequate selenium intake selenium is preferentially retained by the thyroid and in this tissue the supply of selenium to PHGPX has priority over cyGPX.

Selenium status does not affect the transcription rate of genes for any of the selenoproteins studied (Burk and Hill, 1993; Wingler *et al.*, 1999). However, changes in the expression and/or the activity of selenoproteins in selenium deficiency are accompanied by changes in mRNA levels which may result from alterations in mRNA translation and/ or stability (Bermano, 1995; Bermano, Arthur and Hesketh, 1996; Gallegos *et al.*, 1997a; Saedi *et al.*, 1988; Sunde *et al.*, 1993). In addition selenium deficiency may also result in premature polypeptide chain termination owing to the recognition of the UGA codon as a normal stop codon (Burk and Hill, 1993).

ii) Regulation of thioredoxin reductase

The biosynthesis of each TR isoform, as with other selenoproteins, is dependent on selenium status (Gallegos *et al.*, 1997b; Marcocci, Flohé, and Packer, 1997). For example, *in vivo,* rats

fed a high selenium diet undergo a transitory increased expression of TR in the liver, lung and kidney (Berggren *et al.*, 1999).

The expression and activity of TR is also subject to the hierarchy of selenium supply. This was demonstrated in a recent study by Hill *et al.* in which dietary selenium deficiency in rats caused a decrease in TR activity to 4.5% of control in liver and 11% in kidney whilst TR activity in the brain was unaffected (Hill *et al.*, 1997). In cell culture studies carried out by Gallegos *et al.* both TR expression and activity were shown to be dependent on selenium availability in the media which proved to be partly due to enhanced stabilisation of mRNA by selenium (Gallegos *et al.*, 1997). Stabilisation of mRNA is not the only mechanism by which selenium status influences selenoprotein synthesis. Selenophosphate synthetase, which provides selenophosphate for selenoprotein synthesis, exists in two forms, one of which is a selenoprotein containing a UGA codon with a requirement for a source of selenium. It follows that in selenium-deficiency those tissues which preferentially retain selenium have an active selenium-dependent selenophosphate synthetase but in tissues with an inadequate supply of selenium this protein would not be active which may therefore limit selenoprotein synthesis.

The degree of change in TR activity and TR protein levels has been shown not to be directly related (Berggren *et al.*, 1999; Gallegos *et al.*, 1997). Instead, in certain rat tissues increases in TR activity were not directly correlated to increases in protein levels, instead they paralleled increases in specific activity possibly due to an increased selenocysteine incorporation (Berggren *et al.*, 1999). These particular findings are consistent with the possibility that in selenium-deficiency a truncated protein is formed in which translation is terminated at the UGA codon where selenocysteine is normally inserted (Gladyshev, Jeang and Stadtman, 1996). However without subjecting the proteins to complete amino acid analysis this is not confirmed. In contrast, it has been shown that removal of the selenocysteine residue by proteolysis, specific alkylation of the selenocysteine residue, or oxidation of the selenocysteine leads to total loss or a marked reduction in TR activity (Lee *et al.*, 2000). It has also been suggested that some

methods used to measure TR activity and mass are not entirely satisfactory (personal communication, Dr J. R. Arthur, Rowett Research Institute, Aberdeen, UK). For example, Western blot analysis of TR mass expressed in tissues identifies two immunoreactive bands close in molecular weight. The quantification of the band which represents cross-reactivity of an immunogenic plasma component in tissue may explain the lack of correlation between measurements of TR activity and TR mass. In a recent study by Berggren *et al.* selenium supplementation of rats was shown to have no effect on TR protein levels measured in kidney, liver and lung tissue as measured by Western blot analysis (Berggren *et al.*, 1999). However in a similar study carried out by Arthur *et al.* increased dietary selenium was shown to significantly increase TR protein synthesis which correlated with changes in tissue TR activity (personal communication from Dr John Arthur, Rowett Research Institute, UK).

iii) Other modulators of selenoprotein expression

Selenium status is not the only modulator of selenoprotein expression and activity. Other factors have been shown to influence selenoprotein expression at the level of the individual selenoprotein. For example copper-deficiency has been shown to down-regulate cyGPX activity and its mRNA (Sunde, 1994). Also the expression of TR has been shown to be increased by PMA, the calcium ionophore A23187 and reactive oxygen species (Howie *et al.*, 1998; Kumar and Holmgrem, 1999; Sun *et al.*, 1999). Mitchell *et al.* demonstrated that iodine-deficiency in adult rats which leads to increased H_2O_2 production increases thyroidal cyGPX activity (Mitchell *et al.*, 1996).

1.4.7 Effect of low selenium status or selenium deficiency in humans

Low dietary selenium intake has been implicated in the development of numerous health disorders in humans. These include, Kashin-Beck disease, cancer, cardiovascular disease (including Keshan disease), muscular dystrophy, malaria, alopecia areata, pregnancy hypertension syndrome, altered immune function, male infertility and even AIDS (Baum *et al.*,

1997; Foster and Sumar, 1997; Gallegos *et al.*, 1997; Levander, 1987; Ximin *et al.*, 1998). Patients on long-term parenteral nutrition without selenium-supplementation in their formulation run the risk of selenium deficiency which has been linked with myopathy (Brown *et al.*, 1986; van Rij *et al.*, 1979) and cardiomyopathy (Fleming *et al.*, 1982; Johnson *et al.*, 1981).

Kashin-Beck disease (osteoarthritis deformans endemica) is a selenium-responsive endemic osteoarthritis affecting preadolescent and adolescent children living in northern China, North Korea and eastern Siberia (Levander, 1987). The principle pathological feature of this disease is the necrotic degeneration of the chondrocytes. Joint deformation and, in extreme cases, dwarfism results from the cartilage abnormalities characteristic in the later stages of Kashin-Beck. There is some evidence that low selenium status contributes to the development of Keshin-Beck, though other etiological factors may be important, including poisoning by *Fusarium* mycotoxins and nutritional mineral imbalances (Levander, 1987). Studies have shown a dose-response relationship between urinary selenium levels and the incidence of this disease in China. Results from an uncontrolled therapeutic trial showed that 80% of children with early symptoms responded positively to treatment with sodium selenite and vitamin E (Luo, 1984. In contrast, an intervention trial carried out over a five year period to test the prophylactic effect of selenite against Kashin-Bech disease failed to demonstrate any preventative effect (Levander, 1987).

The evidence for an association between selenium status and incidence of cancer is primarily based on epidemiological evidence and animal experiments. In 1965 Shamberger and Rudolph demonstrated a significant reduction of skin cancer incidence in carcinogen-treated mice given a topical application of sodium selenite (Shamberger and Rudolf, 1965). This initiated a great number of subsequent studies, using animal models, which demonstrated the anticarcinogenic nature of selenium. Conclusions drawn from epidemiological studies are conflicting, with some showing a significant association between selenium and cancer risk (Fex, Petterson and Akesson, 1987; Salonen *et al.*, 1984; Virtamo *et al.*, 1987) and others not (Nomura *et al.*, 1987;

Ringstadt *et al.*, 1988). The most convincing evidence for the inverse relationship between selenium and certain forms of cancer comes from a randomised double blind, placebo-controlled study carried out by Clark *et al.* (Clark *et al.*, 1996). 1300 subjects (mean age at enrolment 63 yrs) from the south-eastern region of the USA either received a daily high-selenium yeast tablet containing 200 µg selenium or a placebo yeast tablet for approximately 5 years. Interim analysis of the data collected up to 1993 showed that, excluding non-melanoma skin cancers, total cancer incidence was found to be 42% lower in the selenium supplemented group compared to the placebo group with the most significant decreases in the incidence of prostate and colorectoral cancer. The total cancer death rate was found to be 52% lower in the subjects who received selenium supplementation.

1.5 SELENIUM AND CARDIOVASCULAR DISEASE

1.5.1 Selenium deficiency and cardiovascular disease

The risk of developing atherosclerosis and heart disease may be higher in people who have a low dietary selenium intake. Also, patients on parenteral nutrition without selenium supplementation have been shown to develop cardiomyopathy (Fleming *et al.*, 1982; Johnson *et al.*, 1981). The first reported link between cardiovascular disease in humans and selenium deficiency was established in the late 1970s in China (Korpela, 1993). Keshan disease, an endemic cardiomyopathy affecting mainly children and young peasant women, was shown to be regionally distributed in an area of low soil selenium content extending from north-east China down to the south-west. The acute form of the disease is characterized by the sudden onset of heart failure, whereas individuals suffering from the chronic form of the disease exhibit moderate or severe heart enlargement with varying degrees of heart failure (Ge *et al.*, 1983). The pathological features of this disease are quite distinct, differentiating this cardiomyopathy from other myocardial diseases. They include; multifocal necrosis, fibrous replacement of the myocardium and myocytolysis (Levander and Burk, 1992). The incidence of Keshan disease is dependent on age, socioeconomic status, seasonal variation and an annual shift in the epidemic foci (Foster and Sumar, 1997). Selenium deficiency appears to be the fundamental underlying condition pre-disposing individuals to the development of Keshan disease in certain endemic areas (Yang *et al.*, 1984). However supplementation only works as a prophylactic and cannot reverse cardiac failure once it occurs (Levander and Burk, 1992).

Certain features of the disease cannot be explained by selenium status alone, including the seasonal variation in the number of cases, suggesting an infectious agent may be involved. Beck and co-workers carried out animal studies in which mice given a low selenium diet were then exposed to the Coxsackievirus B3 (a family of viruses thought to be responsible for Keshan disease). These mice developed inflamed hearts within one week whereas mice fed a selenium-adequate diet were unaffected (Beck *et al.*, 1995). When the virus, recovered from the infected

selenium-deficient mice, was inoculated into selenium-adequate mice, a cardiomyopathic condition was induced. Thus it appears that in a selenium-deficient host the virus is able to mutate into a virulent cardiotoxic genotype. The isolated virus was shown to have mutated at seven sites.

The contribution of selenium deficiency to the pathogenesis of cardiovascular disease was originally suggested from epidemiological studies that correlated low selenium content of forage crops, drinking water and blood levels with regional mortality rates from cardiovascular disease (Schamberger, Willis and McCormack, 1979). Such associations are difficult to interpret because it is not possible to exclude the effects of other factors such as interactions of other nutrients, exercise, smoking, alcohol and fat intake and genetics. Whilst epidemiological studies have provided some evidence for the role of low selenium intake in the aetiology of cardiovascular heart disease and ischaemic heart disease, the results of studies within populations have often produced conflicting results.

Moore *et al.* observed an inverse correlation between low plasma selenium and the severity of atherosclerosis in 91 subjects examined by coronary angioplasty (Moore, Noiva and Wells, 1984). In a similar study the ratio of selenium to polyunsaturated fatty acid levels in serum was negatively correlated to the degree of atherosclerosis (Kok *et al.*, 1991). However, Aro *et al.* found no such correlation in a group of Finnish subjects (Aro *et al.*, 1986).

Up until 1997 the results from eight prospective studies had been published, attempting to correlate selenium status with the risk of coronary vascular disease and myocardial infarction (table 1.09) (Kok *et al.*, 1987; Miettinen *et al.*, 1983; Ringstad *et al.*, 1987; Salonen *et al.*, 1982; Salonen *et al.*, 1985; Simonetta *et al.*, 1995; Suadicani, Hein and Gyntelberg, 1992; Virtamo *et al.*, 1985). Again the results from these studies are inconsistent. From the prospective studies carried out in the 1980s only one found an inverse correlation between selenium and the risk of

Table 1.09. The association of cardiovascular disease mortality and incidence with low serum selenium in prospective case-control studies. CAD, coronary artery disease; CHD, coronary heart disease; CVD, cardiovascular disease; MI, myocardial infarction.

Study reference	Study population	Major findings
(Salonen *et al.*, 1982)	Case-control study with 283 cases, men and women aged 35-59 yrs, 7 yr follow-up	Serum selenium below 45 µg /l was associated with the increased risk of death by CHD and CVD, as well as the risk of fatal and non-fatal MI
(Miettinen *et al.*, 1983)	Case-control study of 33 middle-aged male patients with 1 or more risk factors for CHD 5-7 yr follow-up	Serum selenium (50-105 µg /l) was not associated with the development of clinical CHD
(Virtamo *et al.*, 1985)	Cohort study of 1110 male subjects aged between 55-74 yrs 5 yr follow-up	Serum selenium below 45 µg /l was negatively correlated to CVD death but not in subjects initially free of CHD No significant association between low serum selenium (< 45 µg /l) and the risk of coronary death or MI
(Salonen *et al.*, 1985)	Case-control study of 92 subjects, men and women aged 30-64 yrs 5 yr follow-up	No significant association between low serum selenium (< 45 µg /l) and the risk of death from CAD
The Tromsø Heart Study (Ringstad *et al.*, 1987)	Case-control study of 59 male subjects initially free of disease, aged 28-54 yrs, 6 yr follow-up	Low serum selenium is not associated with an excess risk of MI
(Kok *et al.*, 1987)	Case-control study of 84 subjects, men and women aged 37-87 yrs, 6-9 yr follow-up	No significant association between low serum selenium (< 105 µg /l) and the risk of subsequent death from CVD or CHD
Copenhagen Male Study (Suadicani, Hein and Gyntelberg, 1992)	Case-control study of 107 males aged 53-74 yrs, 3 yr follow-up	Individuals whose selenium level was below 1 µmol/L had an increased risk in ischemic heart disease
US Physicians Health Study (Simonetta *et al.*, 1995)	Case-control study on 251 male subjects aged between 40-84 yrs, 4-6 yr follow-up	No association between serum selenium and risk of myocardial infarction

death from cardiovascular death and myocardial infarction, two were equivocal, and no association was found in the other studies (Levander, 1987).

The results from two larger studies were published in the 1990s. In 1992 the results from the Copenhagen Male Study found that, after adjustments to take into account age, cholesterol, social class and smoking, individuals whose selenium level was below 1 µmol/L had an increased risk of coronary heart disease (Suadicani, Hein and Gyntelberg, 1992). In contrast, the study carried out by Simonetta *et al.* concluded that there was no association between serum selenium and risk of myocardial infarction (Simonetta *et al.*, 1995).

To date, therefore, the epidemiological studies linking selenium deficiency to cardiovascular disease are inconclusive. Huttunen postulated that the conflicting data from these studies can be explained by the threshold effect of selenium intake on the risk of cardiovascular disease (Huttunen, 1997). That is, in populations with low selenium status, a correlation between serum selenium and cardiovascular risk is observed, whilst populations with a high selenium intake (serum selenium levels > 45 µg/l) would show no such correlation (Korpela, 1993).

The results of a study carried out by Kardinnal *et al.* support this 'threshold hypothesis' (Kardinaal *et al.*, 1997). From the ten centres across nine European countries only one demonstrated a statistically significant association between toenail selenium levels and risk of myocardial infarction. This centre (in Germany) also has the lowest mean selenium levels.

In 1984 Finland embarked on a nationwide selenium supplementation programme. Prior to the supplementation of multimineral fertilizers with sodium selenate, dietary selenium was 40-50 µg/ day. After three years of supplementation the selenium intake plateaued at 110-120 µg/ day, remaining constant until 1990 when the selenium supplementation of fertilizers was decreased. During this time the incidences of several human pathological conditions were assessed, including the mortalities from ischemic heart disease. During the 1980s the decline in mortality

from ischemic events was almost linear and the selenium supplementation programme did not change this. Thus the decrease in mortality rates from ischemic heart disease could not be attributed to increased dietary selenium and is likely to be the result of several factors such as increased exercise, reduction in smoking, better health care and improved diet (Varo *et al.*, 1994).

1.5.2 Selenium and endothelial dysfunction

a) Introduction

Endothelial dysfunction is a primary factor in the pathogenesis of atherosclerosis as discussed previously (section 1.3). In addition to the evidence gathered from epidemiological studies discussed previously, laboratory based research has provided evidence that selenium may help prevent atherosclerotic disease.

Selenium-deficiency is associated with an increased severity of atherosclerosis in experimental animals. For example, Wójcicki *et al.* reported that the percentage of the aortic intima covered by atherosclerotic lesions in hypercholesterolemic rabbits given selenium supplementation alone decreased by 37% compared to controls (Wókcicki *et al.*, 1991). The combined effect of selenium and vitamin E supplementation diminished the area of atherosclerotic regions by approximately 48% (Wókcicki *et al.*, 1991).

The ability of certain selenoproteins to protect against atherosclerosis centres around their capacity to reduce and detoxify ROS. Initially, research focused on the glutathione peroxidases and the glutathione redox cycle and their antioxidant roles are now well established. Harlan *et al.* using pharmacologic manipulation, established a protective role against neutrophil-mediated H_2O_2 induced lysis in cultured bovine aortic and pulmonary artery endothelial cells and human umbilical vein endothelial cells (Harlan *et al.*, 1984). For example, in endothelial cells pre-treated with butathione sulfoximime, an inhibitor of glutathione synthesis, neutrophil-mediated H_2O_2

induced lysis was significantly greater compared to control cells. Using similar techniques, Ochi *et al.* confirmed the protective effects of the glutathione redox cycle in cultured bovine carotid endothelial cells, this time against the cytotoxic effects of 15(S)-hydroperoxyeicosatetraenoic acid (15-HPETE) (Ochi, Morita and Murota, 1992). They demonstrated that the stimulation of intracellular GSH synthesis in endothelial cells by treatment with L-2-oxothiazolidine-4-carboxylate decreased 15-HPETE-induced injury whilst treatment with buthionine sulfoximine, enhanced the susceptibility of endothelial cells to 15-HPETE toxicity. Also, exposure of cultured bovine endothelial cells to 15-HPETE, in addition to inducing toxicity, suppressed GPX activity. However, GPX activity was increased and 15-HPETE-induced toxicity decreased by supplementing the cells with either sodium selenite or ebselen (a synthetic organoselenium compound with cyGPX-like activity).

Selenium supplementation of bovine endothelial cells in the form of sodium selenite has also been shown to protect against oxidative damage resulting from oxLDL, cholesterol hydroperoxide and tert-butylhydroperoxide (t-BuOOH). This protection has been attributed to the antioxidant effects cyGPX and PHGPX (discussed in further detail in section five) (Thomas, Geiger and Girotti, 1993). Further evidence that cyGPX protects against oxidative damage is the observation that the susceptibility of HUVEC to oxLDL-induced injury is higher than that of the human endothelial cell line EAhy926 (Claise *et al.*, 1997). The lower antioxidant capacity of EAhy926 cells, particularly with regard to cyGPX activity, has been suggested as the cause of the increased susceptibility of EAhy926 cells to oxLDL toxicity.

In many of the previous studies concerning the role of selenium in the protection of endothelial cells from oxidative stress, the contribution of the TR/Trx system was not considered. In fact, TR may also mediate the protective effects of selenium against oxidative damage to the endothelium. For example, the TR/Trx system can maintain NOS in a reduced configuration potentially overcoming the oxidative deactivation of NOS (Patel, Zhang and Block, 1996). The

TR/Trx system has also been shown to directly reduce lipid hydroperoxides, hydrogen peroxide and organic hydroperoxides (Björnstedt *et al.*, 1995).

Peroxynitrite formation is believed to contribute to endothelial dysfunction (for further discussion see section 1.3.4). Glutathione peroxidases and possibly other selenoproteins such as selenoprotein P in human plasma have been shown to act as peroxynitrite reductases (Arteel *et al.*, 1998; Sies *et al.*, 1997). Recently it has been argued that the TR/Trx system may also contribute to peroxynitrite defence (Arteel, Briviba and Sies, 1999).

The prostaglandin-like peroxidation product of arachidonic acid, 8-isoprostane, has been implicated in atherosclerotic disease. It is used as a marker of lipid peroxidation. In agreement with the findings from Thomas's study, bovine endothelial cells cultured in selenium-deficient medium were more susceptible to damage from t-BuOOH than selenium-sufficient cells (Hara, 1998; Thomas, Geiger and Girotti, 1993). Selenium-deficient bovine endothelial cells were also found to have enhanced levels of 8-isoprostane (Hara, 1998).

Selenium supplementation has been reported to have beneficial effects on several different aspects of endothelial dysfunction. For example, selenium supplementation (4.33 $\mu mol.g^{-1}$ $bodyweight.d^{-1}$ for 3 consecutive days) enhances endothelium-dependent relaxation in response to acetylcholine in rat aortic rings (Lu, Liu and Man, 1994). The mechanism for this enhanced response may be through increased NO production mediated either directly by selenium or through the selenoprotein glutathione peroxidase. Endothelial dysfunction is also evident in HIV-positive patients (Constans *et al.*, 1998) and although pilot studies show that selenium supplementation does not improve the prognosis in these patients it does improve some markers of oxidative stress, including a decrease in serum malondialdehyde and an increase in glutathione peroxidase (Constans *et al.*, 1996). Also, in a preliminary study on a small number of HIV-infected patients, selenium supplementation with 100 µg selenite daily for 12 months

prevented the usual increase in serum concentrations of soluble thrombomodulin and von Willebrand Factor which are associated with endothelial dysfunction.

Selenium may also exert other effects in the endothelium, including the regulation of prostacyclin and platelet activating factor (PAF) (Hampel *et al.*, 1989). In selenium-deficient human umbilical vein endothelial cells, histamine-induced PGI_2 release was inhibited through the depression of cyclooxygenase activity of prostaglandin endoperoxide synthase (PGH_2 synthase) It has been proposed that this effect is due to a decrease in glutathione peroxidase activity resulting from the selenium-deficiency. This leads to the accumulation of intracellular peroxides which can in turn inhibit the cyclooxygenase activity of PGH_2 synthase (Hampel *et al.*, 1989). The effects of selenium deficiency on PGI_2 production have been reported elsewhere in cultured porcine aortic endothelial cells (Schiavon *et al.*, 1984) as well as in the aorta of selenium-deficient rats (Valentovic, Gairola and Lubaway, 1985). PGI_2 normally suppresses PAF formation. However, in selenium-deficient HUVEC with suppressed PGI_2 levels histamine-induced PAF release was stimulated (Hampel *et al.*, 1989). *In vivo* these effects may alter platelet function thus contributing to the pathogenesis of atherosclerosis.

The transmigration of leukocytes into the subendothelial space promotes a pro-inflammatory response which is a key feature of endothelial dysfunction observed in early atherogenesis (discussed previously in section 1.1.4). Recently, neutrophil adherence to bovine mammary artery endothelial cells in response to TNFα was shown to be increased in cells cultured in selenium-deficient media compared to those cultured in selenium-sufficient media (Maddox *et al.*, 1999). This effect was closely correlated to an increase in E-selectin and ICAM-1 mRNA in selenium-deficient endothelial cells. The gene sequences of both these adhesion molecules contain binding sites for NF-κB, a redox regulated transcription factor. Hydroperoxides and other ROS have been implicated as second messengers of NF-κB activation induced by cytokines such as TNF and IL-1. A mechanism by which selenoproteins such as cyGPX, PHGPX and TR may protect the endothelium from the pro-inflammatory impact of TNF and IL-1 has been

proposed (McCarty, 1999). It is hypothesised that the ability of selenoproteins to down-regulate cytokine signalling may reflect the prevention or correction of ROS-induced inhibition of phosphotyrosine phosphatase which in its active form down-regulates the cytokine signalling pathway and therefore the transcription of the pro-atherogenic adhesion molecules. Indeed, overexpression of cyGPX in T47D cells has been shown to depress TNF-induced NF-κB activation (Kretz-Remy *et al.*, 1996) whilst in ECV304 cells (a human bladder cancer cell line) interleukin-1 induced NF-κB activation was inhibited in sodium selenite supplementation (50 nM) (Brigelius-Flohè *et al.*, 1997).

Hyperhomocyst(e)inemia has been described as an independent risk factor for endothelial dysfunction and the subsequent development of atherosclerosis (Upchurch *et al.*, 1997). Homocyst(e)ine can damage the endothelium through several mechanisms including the generation of H_2O_2. Recently Upchurch and his colleagues raised the possibility that the increased H_2O_2 accumulation resulted from the ability of homocyst(e)ine to down-regulate the expression of cyGPX (Upchurch *et al.*, 1997). Several other studies have linked decreased GPX levels with an increased incidence of coronary artery disease. For example, Guidi *et al.* have shown that platelet GPX activity is impaired in patients with coronary artery disease (Guidi *et al.*, 1986).

This review does not cite every study which have taken place over the past two decades associating selenium and selenoprotein expression with endothelial dysfunction. However it does reflect the substantial evidence which supports a protective role of selenium through the expression of selenoproteins such as cyGPX, PHGPX and TR against endothelial dysfunction and atherogenesis. The ability of these selenoproteins to act as antioxidants, detoxifying lipid hydroperoxides and other ROS, appears to provide the mechanism by which these compounds exert their effects. However, the relative importance of each identified intracellular selenoprotein has yet to elucidated as does the contribution of selenium-deficiency to atherogenesis.

1.6 AIMS OF THE THESIS

The pattern of selenoprotein expression in human endothelial cells has not been previously studied. Therefore this thesis examined the selenoproteins expressed by human endothelial cells (isolated from different vascular beds) and compared the pattern of expression with that found for endothelial cells isolated from different species. The modification of selenoprotein expression through second messenger pathways and selenium supply was also studied.

Previous investigations have shown that selenium supplementation, through the modification of selenoprotein expression, confers resistance to bovine endothelial cells against the cytotoxic effect of reactive oxygen species (Ochi, Morita and Murota, 1992; Thomas, Geiger and Girotti, 1993). These studies attributed the protective effect observed to the modification of intracellular glutathione peroxidase expression. However the expression of other selenoproteins with antioxidant properties such as extracellular glutathione peroxidase, selenoprotein P and TR was not considered. Therefore the ability of sodium selenite and selenomethionine to protect against damage from oxidative stress was investigated in human umbilical vein endothelial cells, human coronary arterial endothelial cells and bovine aortic endothelial cells.

CHAPTER TWO MATERIALS AND GENERAL METHODS

2.1 CHEMICAL SUPPLIERS

The following equipment and chemicals were purchased from the commercial suppliers listed below.

Amersham International plc, Buckinghamshire, UK.

Bolton & Hunter reagent for protein iodination (18.5 MBq, 500 µCi); Iodine-125 (37 MBq, 1 mCi, specific radioactivity 16 MBq/ nmol).

Binding Sites, Birmingham, UK.

Antiserum to human cytoplasmic glutathione peroxidase.

Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK.

Glass plates (inner; 20 cm x 20 cm; outer 20 cm x 22.3 cm); low range molecular weight markers; N,N,N,'N'-tetramethyl ethylenediamine (TEMED).

Biowhittaker UK Ltd., Wokingham, Berkshire, UK.

Bovine aortic endothelial cells; endothelial growth medium bullet kit (EGM); endothelial growth medium-2 bulletkit (EGM-2); endothelial growth medium-2 supplements (i.e. ; HEPES buffered saline solution; human coronary arterial endothelial cells; trypsin/EDTA solution; trypsin neutralising solution.

Calbiochem Novobiochem, Beeston, Nottingham, UK.

Bisindolylmale I, hydrochloride (GF109203X).

Citifluor, Canterbury, UK.

Citifluor mountant.

Dako Ltd. Buckinghamshire, UK.

FITC-conjugated swine anti-rabbit immunoglobulins; normal rabbit serum; rabbit anti-human polyclonal antibody to von Willebrand factor.

European Collection of Cell Cultures, Salisbury, Wilts, UK.

HepG2 cells

Gibco, Life Technologies, Paisley, UK.

Amphotericin B solution; Dulbecco's modified Eagle's medium (DMEM) (25 mM HEPES) with 4500 mg/L/ glucose; Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 nutrient mix; Earle's balanced salt solution (EBSS); foetal bovine serum (FBS); glutamine; Hank's balanced salt solution (HBSS) (Ca^{2+}- and Mg^{2+}-free); hypoxanthine, aminopterin, thymidine (HAT); Medium 199 (M199); penicillin/streptomycin solution; plastics.

Lorne Laboratories, Twyford, Berkshire, UK.

Collagenase (type VI).

MERCK, Leicester, UK.

Acetic acid; ammonia; cyclohexane; ethanol; ethylenediaminetetraacetic acid (EDTA); glycine; hydrochloric acid; methanol; microcrystalline cellulose; orthophosphoric acid; nitric acid; perchloric acid polyethylene glycol (PEG); selenious acid.

Reactor Center, Columbia, MO, USA.

[^{75}Se] selenite (specific activity, 16 MBq/ nmol).

Scottish Antibody Production Unit, Carluke, Lanarkshire, UK.

Donkey anti-rabbit serum, normal rabbit serum.

Sigma Aldrich Company Ltd, Poole, Dorset, UK.

A23187 calcium ionophore; ammonium persulphate, aurothioglucose; bovine serum albumin powder (BSA); bradykinin; Brijj solution; Coomassie brilliant blue (R-250 and G-250); cytoplasmic glutathione peroxidase purified from human erythrocytes; dithiothreitol (DTT); lactate dehydrogenase (LDH) kit (Sigma Diagnostics); lauryl sulfate (sodium dodecylsulfate; SDS); phorbol-12-myristate 13-acetate (PMA); radiographic film Kodak X-OMAT XAR-5; sodium azide.

2.2 SOURCES OF NON-COMMERCIAL MATERIAL

2.2.1 Cell lines

The human endothelial cell line, EAhy926 was kindly donated by Professor Cora-Jean Edgell of the University of North Carolina, North Carolina, USA.

2.2.2 Antibodies

Antisera to both rat liver and human placental thioredoxin reductase (TR) were raised in rabbits to cytosolic proteins purified to homogeneity. Rat TR antisera was kindly supplied by Dr John Arthur, Rowett Research Institute, Bucksburn, Aberdeen, UK, whilst Dr Forbes Howie of this department supplied the antisera to human TR.

Antisera to rat PHGPX were raised in rabbits against PHGPX purified from rat testis. Again this antisera was kindly donated from Dr John Arthur and Mr Fergus Nicol of the Rowett Research Institute.

2.3 GENERAL METHODS

2.3.1 Introduction

The experimental methods described below are those used throughout this thesis. They include primary cell culture, maintenance of cell lines, selenoprotein determination, and selenoprotein expression and activity measurements. Where the methods diverge from those described here the modifications are explained in the relevant chapters.

2.3.2 Isolation and culture of human umbilical vein endothelial cells

Human umbilical cords (>100 mm in length) were obtained at normal deliveries or Caesarean section from non-smoking women. Immediately after delivery the cords were placed into sterile EBSS containing penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotercin B (2.5 μg/ml) at 4°C. Endothelial cells were isolated within 20 hr of delivery using a method adapted from that described previously by Jaffe *et al.* (Jaffe *et al.*, 1973). Briefly, the vein of the umbilical cord was located and cannulated with a Venflon (gauge 17/ 45 mm), which was then clamped into place. The vein was washed with 100 ml of EBSS (pre-warmed to 37°C) to remove any blood clots and the outside wiped using sterile gauze. One end of the cord was clamped shut and the opposite end infused with 0.07% collagenase in EBSS (5-15 ml). The cord was then incubated at 37°C in an atmosphere of 5% CO_2, 95% air.

After 10 min the cord was removed and massaged gently. The contents of the cord were flushed out with 30 ml of $Ca^{2+}$and Mg^{2+}- free HBSS. The resulting cell suspension was collected and centrifuged at 450 g for 10 min and the cell pellet washed once with EGM-2 containing penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotercin B (2.5 μg/ml). The cells were resuspended in 15 ml EGM-2 and plated out into one 75 cm^2 flask. This flask was then incubated at 37 °C in an atmosphere of 5% CO_2, 95% air.

After approximately 5 hr the HUVEC were washed with 2 x 10 ml EGM-2 to remove any blood, contaminant cells and cell debris. The medium was then changed and replaced with a further 15ml of EGM-2 on alternate days.

Cell reached confluence within 3-7 days. When the cells were approximately 90% confluent the HUVEC were subcultured as required. To subculture the HUVEC the overlying medium was aspirated from the flask and the flask rinsed with approximately 9 ml HBSS. The HBSS was then aspirated from the flask and replaced with 9 ml 0.025% trypsin/ 0.01% EDTA solution. The flask was then placed in the incubator at 37°C for approximately 1.5 min. The flask was then viewed under a light microscope to assess the number of cells detached and if necessary the flask was given a rap to detach any remaining cells still attached to the surface of the flask. The trypsin/ EDTA solution was then neutralised with a trypsin neutralising solution and the cell suspension transferred to a centrifuge tube. The cells were pelleted by centrifugation at 450 g for 10 min. The supernatant was then aspirated and the cells resuspended in 5 ml EGM-2. The number of cells were counted using a haemocytometer and then diluted in the appropriate amount of medium. Cells were seeded approximately 3000 cells /cm^2 into a 75 cm^2 flask.

All cells used in a given experiment were derived from a single umbilical cord unless otherwise stated.

2.3.3 Characterization of human umbilical vein endothelial cells

Cells cultured from the human umbilical vein showed the morphology characteristic of endothelial cells in culture previously described by Jaffe *et al.* (Jaffe *et al.*, 1973). Under the light microscope endothelial cells were observed as non-overlapping large polygonal cells, which after 3-7 days in culture became a confluent single monolayer of contact inhibited cells with a cobblestone appearance. Non-endothelial cell contaminants, particularly smooth muscle cells and fibroblasts were identified by light microscopy. They were distinguishable from endothelial

cells because of their morphological differences; smooth muscle cells have a characteristic 'hill and valley' morphology whilst fibroblasts are elongated cells which like smooth muscle cells form overlapping layers. HUVEC preparations which had greater than 5% contamination were discarded.

To confirm cultured HUVEC were of endothelial origin some cultures were examined for the presence of von Willebrand Factor (former designation Factor VIII-related antigen, vWF) using an indirect immuno-fluorescent detection system reported to be characteristic of endothelial cells in culture (Hoyer, De Lso Santos and Hoyer, 1973; Jaffe *et al.*, 1973).

Briefly, HUVEC were grown to approximately 70% confluence on 22 x 22 cm sterile glass coverslips in six well plates. The glass coverslips were then placed in a rack with the cell layer facing towards the front. The rack was then immersed in phosphate buffered saline (PBS) and the cells washed for 5 min. The rack was then immersed in acetone to fix the cells. After 5-10 min the cells were rinsed in fresh PBS and any excess liquid was wiped from the bottom of each coverslip.

The primary vWF antibody used was a rabbit anti-human polyclonal antibody diluted 1:100 with PBS. Prior to use the antibody was titred on human tonsil sections, which are known to be positive for vWF and then on HUVEC monolayers. A 1:100 working dilution of this antibody gave optimal staining in both human tonsil sections and HUVEC monolayers. 100 µl of the diluted antibody was then placed onto an appropriately labelled microscope slide and then each coverslip with the cells facing downwards was carefully lowered onto the microscope slide. The slides were placed into a humidity chamber and the cells were incubated at room temperature for 30 min. The coverslips are then lifted from the slides and replaced in the rack as described previously and washed twice for 5 min in PBS.

The secondary antibody used were FITC-conjugated affinity isolated swine anti-rabbit immunoglobulins (the cross reactivity of which with human immunoglobulins was low as determined by ELISA). The 30 min staining procedure was repeated with a 1:400 dilution of the secondary antibody in darkness at room temperature. After which the coverslips were washed twice for 5 min in PBS.

The coverslips were mounted onto microscope slides using a small drop of Citiflouor and examined under a Leitz Ortholux 2 microscope fitted with a Leica HBO 50W mercury vapour lamp using a Kodak Wratten No. 47B excitation filter and a Kodak Wratten No.12 barrier filter. The cells were photographed with a Leitz Vario Orthomat 2 camera using an Ektachrome 400 film.

Figure 2.01a shows positive staining with vWF which is granular in appearance and situated in the cytoplasm of the cell. Of the three HUVEC cultures stained, each tested positive for the presence of vWF. No immunofluorescent staining was observed when HACAT, a spontaneously transformed human keratinocyte cell line kindly supplied by Miss Teresa Rafferty, Department of Dermatology, University of Edinburgh, Edinburgh, UK, were incubated with vWF antigen (data not shown). EAhy926 cells display the characteristic morphology of endothelial cells as described above and have previously been shown to stain positive for vWF (Edgell, McDonald and Graham, 1983). Therefore these cells were used as a positive control for the presence of vWF. Figure 2.01b shows a positive staining for the glycoprotein vWF.

Using Southern blotting techniques the presence of significant levels of endothelial nitric oxide synthase (eNOS) mRNA were detected in HUVEC cultured in this laboratory (Jackson *et al.*, 1998). ENOS is expressed in endothelial cells in a constitutive manner bound to the endothelial membrane (Dominiczak and Bohr, 1995).

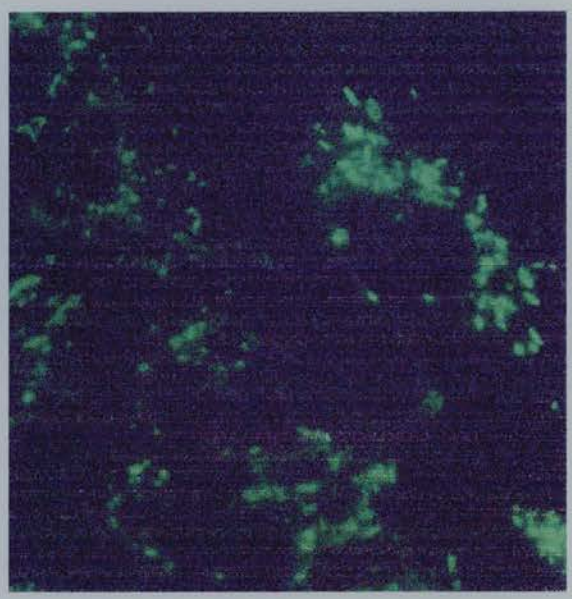


Figure 2.01a Immunofluorescence of the glycoprotein von Willebrand Factor in a non-confluent monolayer of human umbilical vein endothelial cells. x400 magnification.

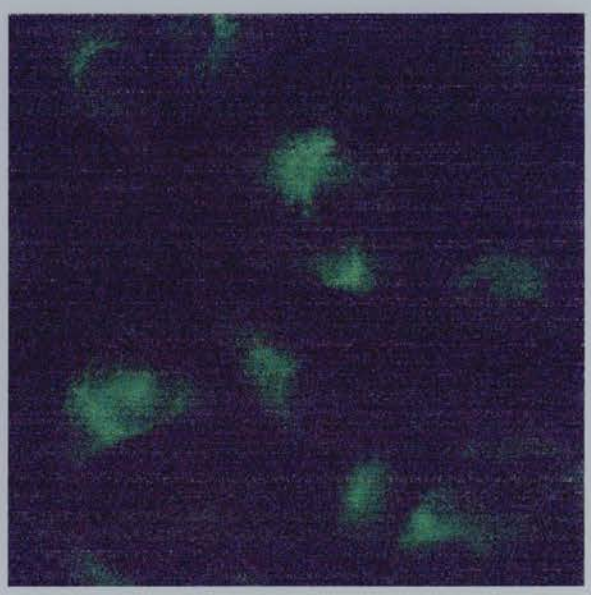


Figure 2.01b Immunofluorescence of the glycoprotein von Willebrand Factor in a non-confluent monolayer of Eahy926 cells. x400 magnification.

2.3.4 Maintenance of EAhy926 endothelial cell line

EAhy926 endothelial cells were maintained unless otherwise stated in high glucose (4.5 g/L) DMEM containing 10% FBS, 5 mM hypoxanthine , 0.02 mM aminopterin, 0.8 mM thymidine, penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml). The cells were incubated at 37°C in an atmosphere of 5% CO_2, 95% air. The cells were passaged weekly using 0.25% trypsin- 0.02% EDTA solution.

EAhy926 cells display the characteristic morphology of endothelial cells in culture as described above and have previously been shown to stain positive for vWF (Edgell, McDonald and Graham, 1983). The presence of the glycoprotein vWF was confirmed in this laboratory (figure 2.01b).

2.3.5 Maintenance of human coronary arterial endothelial cells

Human coronary arterial endothelial cells (HCAEC) were maintained in endothelial growth medium-2 bulletkit (EGM-2) containing penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (2.5µg/ml). The cells were incubated at 37 °C in an atmosphere of 5% CO_2, 95% air. The cells were passaged every 7-9 days as described in section 2.3.2.

HCAEC were purchased from Biowhittaker UK Ltd. The certificate of analysis supplied with these cells stated the HCAEC tested positive for the presence of vWF and acetylated LDL (an alternative method for the specific identification of endothelial cells in culture) (Voyta *et al.*, 1984). HCAEC also displayed the characteristic morphology of endothelial cells as described above and cultures where non-endothelial contaminants were observed were discarded.

2.3.6 Isolation and culture of human umbilical artery endothelial cells

Human umbilical artery endothelial cells (HUAEC) were isolated and cultured by methods similar to those described for HUVEC in section 2.3.2. The exception being that the umbilical artery was cannulated which required a smaller Venflon (gauge 20 / 32 mm). Also due to the smaller surface area of the umbilical artery, fewer cells were isolated and therefore they were resuspended in 10 ml EGM-2 and then plated out into one T25 flask.

Cells were maintained in endothelial growth medium-2 bulletkit (EGM-2) containing penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (2.5µg/ml). The cells were incubated at 37 °C in an atmosphere of 5% CO_2, 95% air.

When the cells were approximately 90% confluent the HUAEC were subcultured in T75 flasks as described in section 3.3.2.

HUAEC were confirmed as being of endothelial origin by virtue of the characteristic morphology displayed as described above. Cultures that showed greater than 5% contamination from non-endothelial cell types were discarded.

2.3.7 Isolation and culture of porcine aortic endothelial cells

Segments of porcine aorta were obtained within 5-10 min of slaughter from pigs aged under two years old. Upon receipt the aorta was immediately placed into sterile EBSS containing penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml) at 4°C. Endothelial cells were isolated within 2-3 hr of dissection using a method adapted from that previously described by Slater and Sloan (Slater and Sloan, 1975). Any fat or connective tissue was removed from the dissected aorta. Segments of about 5-10 cm were cut and any minor vessels were ligated using strong cotton. The segments were washed with approximately 25 ml EBSS (pre-warmed to

37°C). One end of the aorta was then clamped shut and the opposite end infused with 0.1% collagenase in EBSS (approximately 10 ml). This end was then clamped shut and the cord was incubated at 37°C in an atmosphere of 5% CO_2, 95% air.

After 15 min the segment was removed and gently massaged. The contents of the aortic segment were then collected into a sterile universal container. The vessel was then cut along its longitudinal axis and the luminal surface gently scraped gently with a sterile stainless steel scalpel blade angled at approximately 60° to the intimal surface.

The blade was then washed with EBSS and the wash was added to the cell suspension previously collected. The sample was then centrifuged at 450 g for 10 min and the resulting cell pellet was washed with M199 containing 20% FBS, penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotercin B (2.5 µg/ml). The cells were resuspended into a 25 cm^2 flask and incubated at 37°C in an atmosphere of 5% CO_2, 95% air. The growth medium was changed and replaced after 24 hr and then on alternate days.

Cells reached confluence within 5-9 days. When the cells were approximately 90% confluent the porcine aortic endothelial cells (PAEC) were subcultured as required.

To subculture the PAEC the overlying medium was aspirated from the flask and the flask rinsed with HBSS. The HBSS was aspirated from the flask and replaced with 2 ml 0.05% trypsin/ 0.02% EDTA in Puck's modified saline, which was agitated gently over the cells. The flask was incubated at 37°C in an atmosphere of 5% CO_2, 95% air for approximately 1.5 min. The flask was then viewed under a light microscope to assess the number of cells detached and if necessary the flask was given a rap to detach any remaining cells still attached to the surface of the flask. The trypsin/ EDTA solution was then neutralised with growth medium and the cell suspension transferred to a centrifuge tube. The cells were pelleted by centrifugation at 450 g for

10 min. The supernatant was then aspirated and the cells resuspended in 5 ml growth medium. The number of cells were counted using a haemocytometer and then diluted in the appropriate amount of medium. Cells were seeded at approximately 9000 cells /cm^2 into a 75 cm^2 flask.

PAEC were not tested for the presence of vWF, however cultures of these cells displayed the characteristic morphology of endothelial cells without the presence of non-endothelial contaminants.

2.3.8 Maintenance of bovine aortic endothelial cells

Bovine aortic endothelial cells (BAEC) were maintained in endothelial growth medium bulletkit (EGM) containing penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (2.5µg/ml). The cells were incubated at 37 °C in an atmosphere of 5% CO_2, 95% air.

The cells were passaged every 7-9 days as described in section 2.3.2.

BAEC were purchased from Biowhittaker UK Ltd. The certificate of analysis supplied with this product stated that the BAEC tested positive for acetylated LDL. Also in culture BAEC displayed the characteristic morphology of endothelial cells as described above and cultures where non-endothelial contaminants were observed were discarded.

2.3.9 Isolation and culture of human thyrocytes

Human thyrocytes were isolated as previously described using thyroid tissue which was surplus to routine histopathological examination obtained from patients with Grave's disease (Beech *et al.*, 1993; Rapoport, 1975). These cells were cultured and maintained in DMEM/ 10% CPSR-1 (FCS, treated to remove endotoxins and immunoglobulins). This procedure was carried out by Dr Forbes Howie of this department.

2.3.10 Maintenance of HepG2 cells

HepG2 cells are a human foetal liver-derived cell line which were maintained in DMEM/ Ham's F-12 nutrient mix containing L-glutamine and 15mM Hepes to which 5% (v/v) foetal bovine serum was added. The cells were cultured in 75cm^2 culture flasks and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air.

2.3.11 Measurement of selenium content of culture medium

The method used to measure the selenium content of the different culture media used in this study was based on the formation of a piazselenol between selenium and 2,3-diaminonaphthalene followed by extraction and fluorometry.

This assay was carried out by Mr Fergus Nicol of the Rowett Research Institute, Aberdeen and has been previously described by Olsen *et al.* (Olsen, Palmer and Carey, 1975). 10 ml of each medium sample was boiled down to 1 ml before being digested overnight in 2 ml concentrated nitric acid in a 75ml glass boiling tube. The following day samples were heated slowly up to boiling point and boiled until the loss of brown fumes (approximately 5 min). 2 ml concentrated perchloric acid was then added dropwise and the sample was boiled until the appearance of white fumes. After 30 min boiling, hydrochloric acid was added dropwise to drive off excess nitric oxide and to convert selenium as selenate to selenite.

When cooled, 5 ml hydroxylamine/ EDTA solution (25 g hydroxylamine and 9.24 g EDTA per litre distilled water) was added to each sample. Five drops of a cresol red solution (0.05 g cresol red in 250 ml distilled water containing 1 ml of 40% (v/v) ammonia. A 40% ammonia solution (v/v in distilled water) was added to each sample until the sample turned green, then 10% HCl (v/v) was added until the solution turned orange. The pH range of the sample at this stage was

between 1.5 and 2.5 suitable for the formation of the diaminonaphthaline-selenium complex. The samples were then diluted to 50 ml with distilled water.

Diaminonaphthalene solution (5 ml) was added to each sample and incubated for 30 min at 50°C in a covered water bath. After cooling the samples to room temperature, 6 ml cyclohexane was added to each sample. Each tube was covered with a glass stopper and shaken vigorously for 20 sec to extract the diaminonapthalene/ selenium complex. The sample was left for 10 min to allow the diaminonapthalene layer to separate. 2.5 ml of the top layer was removed and fluorescence was measured. The samples were read form a standard curve, prepared from selenious acid supplied at 1 mg/ml. The standards were as follows:- 0 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml. A dried blood standard was used as a control.

2.3.12 Determination of protein concentration

The protein concentration of cell lysates and extracellular proteins was determined using the Bradford dye-binding method (Bradford, 1976) adapted for the Cobas Fara centrifugal analyser (Roche Diagnositcs, Welwyn Garden City, UK).

The Bradford reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol. To this solution is added 100 ml of 85% (w/v) phosphoric acid and stirred for 30 min. The resulting solution is diluted with distilled H_2O to a final volume of 1000 ml, filtered through Whatman, grade 1 filter paper and stored at room temperature in a closed bottle.

Bradford reagent (256 µl) was added to each cuvette and incubated for 100 sec at 37°C. The initial absorbance reading (595 nm), was taken at 95 sec. Sample (25 µl) and H_20 diluent (50 µl) were then added to the cuvettes and incubated at 37°C for 180 sec when a final absorbance reading (595 nm) was taken.

Bovine serum albumin (BSA) standards were used at a concentration range of between 0-100 mg/L. Unless otherwise stated BSA standards were dissolved in distilled H_20. The change in absorbance reading resulting from the addition of the standard was used to construct a standard curve. Samples with unknown protein concentrations were then interpolated from this curve. Samples with a protein concentration greater than 100 mg/L were diluted in distilled H_20 until they fell within the range of the standard curve (0-100 mg/L).

2.3.13 Sodium-dodecyl sulphate-polyacrylamide gel electrophoresis

The [^{75}Se]-selenoproteins in cell lysates and culture medium was separated by sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were prepared for electrophoresis by diluting each sample to a common protein concentration with 60 mM Tris buffer, pH 7.8 at room temperature, containing 1 mM EDTA and 1 mM dithiothreitol. The diluted samples were then further diluted 2:1 (sample: "boiling mix") with "boiling mix" (SDS 35 mM, glycerol 1.4 mM, 2-mercaptoethanol 0.3 mM and bromophenol blue 15 mM) and heat-treated at 90°C for 10 min.

Electrophoresis was carried out at room temperature using a Protean II electrophoresis system (Bio-Rad Laboratories Ltd, Watford, Herts, UK). The discontinuous gel (dimensions 0.1 cm x 16.5 cm x 18 cm) is cast as two separate gels sandwiched between two glass plates set in a casting stand. The lower 12% resolving gel (14 cm long) was made up of 17 ml distilled H_2O; 32 ml acrylamide solution (30% acrylamide, 0.8% bis-acrylamide); 30 ml 1M Tris/ HCl, pH 8.85; 0.8 ml 10% SDS; 0.15 ml N,N,N',N'-tetramethylethylene diamine (TEMED); 0.15 ml 0.1% ammonium persulphate. The upper stacking gel (approximately 4 cm long) consisted of 32 ml distilled H_2O; 7.2 ml acrylamide; 20 ml 0.375M Tris/ HCl, pH 6.8; 0.6 ml 10% SDS; 0.2 ml TEMED; 0.2 ml 0.1% ammonium persulphate. The gel sandwiches were removed from the casting stand once set and transferred to the buffer chamber. The upper and lower buffer chambers were filled with SDS-electrophoresis buffer (0.3% (w/v) Tris; 1.44% glycine; 0.1%

SDS). Low range molecular weight markers (14.4 kDa – 97.4 kDa) and samples were loaded into the wells. The gel was run at 200 volts (V), 35 milliamperes (mA), 50 Watts through the stacking gel until the bromophenol blue tracking dye entered the resolving gel when the voltage and current was increased to 300 V and 50 mA respectively. The power supply was disconnected once the tracking dye had reached the bottom of the resolving gel.

The gels were stained in 0.2% (w/v) Coomassie Brilliant Blue R in a methanol: acetic acid: distilled H_2O solution (50: 7: 50 ratio) for 30 min and then destained in two changes of a methanol: acetic acid: distilled H_2O solution (5: 7: 88 ratio) overnight.

The gels were then dried under vacuum sandwiched between two layers of pre-soaked cellophane support using a Bio-Rad gel drier (model 583) set at 80°C for 2 hr.

2.3.14 Autoradiography of SDS-PAGE gels

[^{75}Se]-labelled selenoproteins were visualised by autoradiography using Kodak X-OMAT XAR-5 film. The dried gels were placed in close contact with the film within an exposure cassette at -70°C for between 12 hr and 4 days. The staff of the Department of Radiology, The Royal Infirmary of Edinburgh kindly developed the films.

The molecular weights of the standard protein molecular weight markers were plotted against the distance travelled and a curve fitted using Fig P (produced by Fig P Software Incorporation, Durham, NC, USA), which was used to determine the molecular weights of unknown selenoproteins.

2.3.15 Phosphoimage analysis

Two types of phosphoimage analysis were used in this study.

To quantify the expression of the major [^{75}Se]-selenoproteins in HUVEC as a percentage of the total intracellular [^{75}Se]-selenoprotein labelling, Coomassie Blue gels were scanned using a Bio-Rad model GS-525 Molecular Imager System to create a digitised image. The radioactivity in each band was quantified by means of radioactive band densitometry using the Bio-Rad Molecular Imager Analyst/ PC image-analysis software. The data was presented as a percentage of the total intracellular [^{75}Se]-labelled proteins expressed.

Quantification of TR expression from Western blots and changes in the [^{75}Se]-labelling of the major selenoproteins after PMA and A23187 treatment was carried out by Mr Fergus Nicol of the Rowett Research Institute, Bucksburn, Aberdeen, UK. Autoradiographs were scanned and the digitised image analysed by means of radioactive band densitometry, using an Epson$_{GT}$ - 9500 scanner with Phoretix software.

2.3.16 Western blot analysis

Thioredoxin reductase (TR) and phospholipid hydroperoxide glutathione peroxidase (PHGPX) were identified in HUVEC using Western blot analysis. The identification of TR and PHGPX was carried out by Mr Fergus Nicol of the Rowett Research Institute, Bucksburn, Aberdeen, UK.

For the Western blot analysis of TR, proteins resolved by SDS-PAGE (section 2.3.13) were transferred to Immobilon P membranes which were then blocked using a 10% (v/v) horse serum with 0.025 M Tris buffer/0.5 M sodium chloride (pH 7.5) and 0.05% Tween. The membranes were then probed with affinity-purified anti-rat TR antibody at a final dilution of 1:500. Enhanced

chemiluminescence was used as a detection method to visualise the immunoreactive proteins as described previously by McLeod *et al* (McLeod *et al.*, 1997).

Previous studies performed by Dr Forbes Howie of this department with the antiserum to TR and the purified 58 kDa selenoprotein from [^{75}Se]-labelled HepG2 confirmed that the antiserum reacts only with TR and not another selenoprotein with a similar molecular mass (Howie *et al.*, 1998).

PHGPX expression was analysed by Western blotting using a protocol based on that described for TR. The PHGPX antibody was raised against rat testes PHGPX and was used at a final dilution of 1:500.

2.3.17 Radioimmunoassay of thioredoxin reductase

Thioredoxin reductase (TR) was measured using an 'in-house' radioimmunoassay developed in this laboratory by Dr. Forbes Howie (unpublished).

a) Purification of thioredoxin reductase

TR was purified from normal 40 week full-term human placentas obtained within two hours of delivery. The method used for purification was based on that of Holmgren and Björnstedt (Holmgren and Björnstedt, 1995). This procedure was kindly carried out by Dr Forbes Howie.

b) Details of antibodies

The primary antibody used was a kind gift from Dr Forbes Howie of this department. It was raised by subcutaneous injection of purified human placental TR emulsified with an equal volume of Freund's complete adjuvant into New Zealand White rabbits. Six weeks after the primary immunisation each rabbit was given a booster injection of the protein-containing

emulsion. After two further weeks, the animals were anaesthetised and the blood recovered by cardiac puncture. The blood was centrifuged at 3000 g for 30 min, and the serum removed and stored at -70°C at a dilution of 1:100 in 0.05 M phosphate buffer, pH 7.4, containing 0.1% BSA and 0.02% sodium azide. To determine the titre of primary antibody required for the radioimmunoassay an antibody dilution curve was performed. The antibody dilution used was that which gave approximately 60% of the maximum total binding. This was determined for each batch of tracer used. The primary antibody titre used was in the region of 1:30000 (approximately 15% binding).

The secondary antibody reagent was made by adding 25 ml of donkey anti-rabbit serum to 1.5 ml normal rabbit serum and mixing overnight at room temperature to precipitate out the immunoglobulins. After centrifugation (230 g for 5 min) the supernatant was discarded and the precipitate washed four times using 0.05 M phosphate buffer, pH 7.4, containing 0.1% BSA and 0.02% sodium azide. After the final wash the supernatant was removed and the pellet was resuspended in the same 0.05 M phosphate buffer as described above, to give a final volume of 100 ml

c) Preparation of the assay diluent

The assay diluent used for both the antibody and the tracer was a 0.05 M phosphate buffer, pH 7.4, containing 0.1% BSA and 0.02% sodium azide. Prior to use in the assay, dithiolthreitol (DTT) was added to the buffer at a final concentration of 10 mM.

d) Preparation of ^{125}I-TR tracer, standards, controls and samples

Purified human placental TR was iodinated by Dr Forbes Howie using the Bolton-Hunter iodination procedure (Bolton and Hunter, 1973). The ^{125}I-TR trace was immediately diluted 1:2 with FBS and stored at -20°C for up to three months. For use in the radioimmunoassay the ^{125}I-

TR trace was diluted with assay diluent containing DTT such that approximately 10000 cpm was added to each tube (mass approximately 50-100 pg TR/ tube).

Standards were made up by diluting a stock solution of purified placental human TR (1 mg TR/L) with FBS to give the following concentrations: 0.5, 1, 2, 5, 10, 25, 50µg TR/ L. Two controls were made by diluting a stock solution of human placental cytosol with FBS to values of approximately 5 and 25 µg TR/ L. Both standards and controls were dispensed into aliquots which were frozen at -70°C.

Cell pellets were stored at -70°C immediately after harvesting. Prior to being assayed samples were removed from the freezer and kept on ice at 4°C until required. Each sample was diluted with radioimmunoassay buffer (without the added DTT) between 1:50 and 1:200.

e) TR radioimmunoassay

Antibodies, trace, standards, controls and samples were all brought to room temperature prior to setting up the assay. All samples, standards and controls were performed in duplicate. The radioimmunoassay was performed as follows: 100 µl of standard, control or sample was added with 100 µl ^{125}I-TR tracer. Primary antibody (100 µl) was then added to each tube with the exception of the total counts tubes. The tubes were then mixed by vortexing for 30 sec and then incubated overnight at 4°C.

Following the over night incubation, 100 µl of the secondary antibody (at room temperature) was added to each tube with the exception of the total count tubes. The tubes were vortexed and incubated for a further hour at room temperature with shaking. 1.5 ml of wash solution (0.05% Brij solution containing approximately 200 mg microcrystalline cellulose) was added to each tube (with the exception of the total count tubes) and the tubes were centrifuged for 30 min at 1800 g (4°C). The supernatant was decanted, the precipitate was washed with a further 1.5 ml wash

solution, centrifuged and decanted as previously described. The resultant precipitate was counted in the LKB 1261 Multigamma gamma counter and the data processing performed using the LKB 1224-RIACalc RIA evaluation program.

Figure 2.02 shows a typical standard curve for the TR radioimmunoassay.

Controls were run as 9 replicates within a single assay to determine the intra-assay precision data. To determine the inter-assay precision controls were included at the beginning and the end of each assay for 12 consecutive assays.

f) Intra-assay precision data

The intra-assay precision data calculated from one assay (in which 9 duplicates of each pool (5 and 25 µg TR/ L) were read from a single standard curve, is shown in table 2.01.

Table 2.01. Intra-assay variation for two pools run in one assay for TR. (Coefficient of variation is referred to as CV)

pools	n	mean	SD	CV (%)
5 µg TR/ L	9	5.062	0.733	14.473
25 µg TR/ L	9	24.391	0.963	3.949

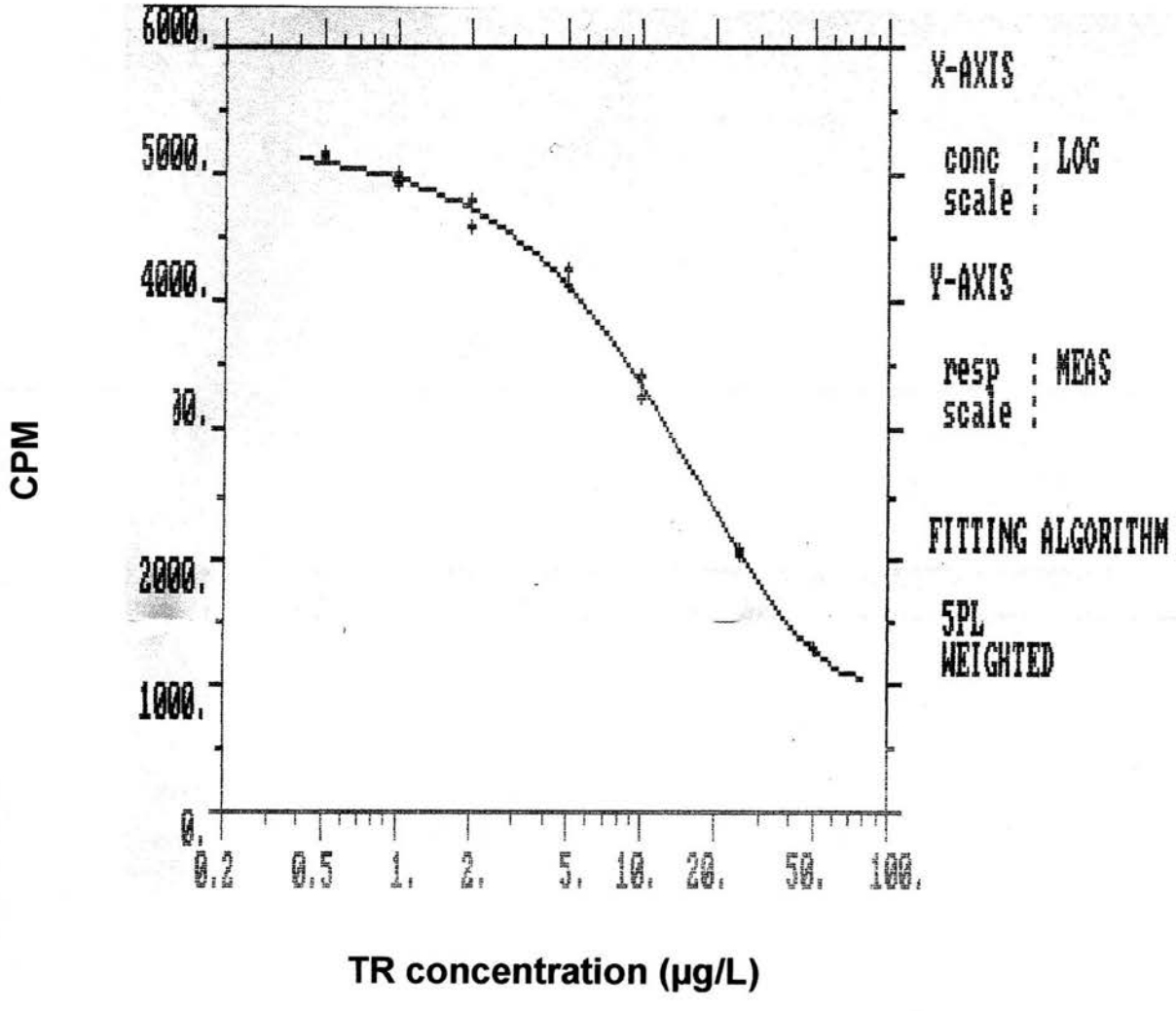


Figure 2.02. A typical standard curve for the thioredoxin reductase radioimmunoassay. The standard curve is a scan from an original printout from the LKB1224-RIA CalcRIA evaluation program.

g) Inter-assay precision data

The inter-assay variation was determined by running the controls in each assay for 12 consecutive assays. The results are shown in table 2.02 .

Table 2.02. Inter-assay variation for two pools run in twelve consecutive assays for TR. (Coefficient of variation is referred to as CV).

pools	n	mean	SD	CV (%)
5 µg TR/ L	12	5.569	0.446	8.009
25 µg TR/ L	12	25.151	2.811	11.176

h) Detection limit of the TR radioimmunoassay

To determine the functional sensitivity of the assay a precision profile was constructed, calculated from 9 consecutive assays (figure 2.03). The minimal detection limit of the TR assay (functional sensitivity) was calculated as 1.50 µg TR /L which represented the TR concentration which had a CV of 22.5% (McConway *et al.*, 1989). The working range of this assay was 3.50 - 50.0 µg TR/ L and was defined as the concentration range which had a CV of less than 10%.

2.3.18 Thioredoxin reductase activity assay

TR activity was measured in cell homogenates using a method adapted from that previously described by Hill *et al.* (Hill, McCollum and Burk, 1997). Assays were performed by Miss Michelle Lewin of this department.

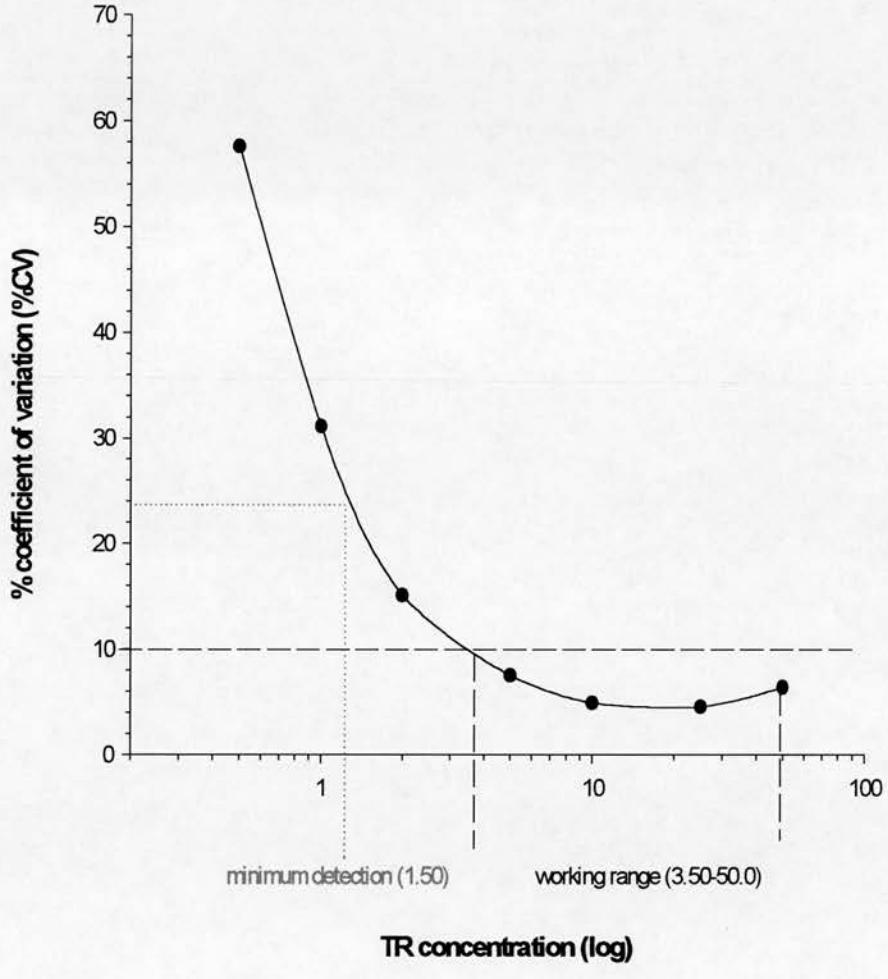


Figure 2.03. Plot showing the mean % coefficient of variation (CV) (mean ± SD, n=9) for each standard. The minimum detection limit of the assay is the concentration of TR (μg/L) with a CV of 22.5%. The working range of the assay represents the concentration range which had a CV of less than 10%.

The TR activity assay follows the NADPH-dependent-reduction of the substrate 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to 5'-thionitrobenzoic acid (TNB) according to reaction (1).

$$\mathrm{DTNB + NADPH + H^+ \xrightarrow{TR} 2TNB + NADP^+} \qquad (1)$$

TR activity was determined by following the rate of DTNB reduction to TNB measured as a increase in absorbance at 412 nm using a Cobas Fara Centrifugal Analyser (Roche Diagnostics, Welwyn Garden City, UK). To correct for the reduction of DTNB by enzymes other than TR and by endogenous thiols such as glutathione, the assay was performed in the presence of aurothioglucose (720 nM) which inhibits TR activity (Hill, McCollum and Burk, 1997). The aurothioglucose at a concentration of 720 nM, used in our assay, has been optimised to selectively inhibit TR activity but does not significantly decrease the activity of any of cyGPX and PHGPX (unpublished data, personal communication from Miss Michelle Lewin of this department).

a) Preparation of reagents

The assay buffer consisted of 2.194 g potassium phosphate, 0.47 g potassium chloride, 0.47 g EDTA and 0.025 g BSA made up to 100 ml with distilled water to give final concentrations of 0.1 M, 50 mM, 10 mM and 0.2 mg/ml respectively. The pH of the buffer was adjusted to pH 7.0 using orthophosphoric acid and NADPH was added to the mixture to give a final concentration of 0.48 mM. This buffer was stored at 4°C until required.

The reaction mixtures were made prior to the beginning of the assay. To make reagent A, 6 mg NADPH was added to 25 ml of the assay buffer to give a final NADPH concentration of 0.48 mM. This solution was stored in the dark until use. To make reagent B 0.66 g DTNB was made up to 25 ml in absolute ethanol.

b) Preparation of samples

Cell pellets were stored at -70°C immediately after harvesting. Prior to the assay samples were removed from the freezer and kept on ice at 4°C until required.

c) Thioredoxin reductase activity assay

Samples (180 µl) were aliquoted into Cobas cups in the presence of either aurothioglucose at a final concentration of 720 nM or assay buffer to give a final volume of 200 µl.

Prior to each assay run a fresh reaction mixture was made which consisted of 4.6 ml reagent A and 400 µl reagent B.

To start the reaction 190 µl reaction mixture was added to 30 µl sample in a final volume of 250 µl (made up with distilled water). The increase in absorbance at a wavelength of 412 nm (37°C) was followed through thirty 10 sec intervals with the first reading taken at 0.5 sec. A blank containing assay buffer in place of sample was run at the beginning and the end of each assay.

One unit of TR activity was defined as that which reduces 1 µmole of DTNB per minute at 37°C and pH 7.0. The molar extinction coefficient for TNB is 13600 M^{-1} cm^{-1}. The conversion factor used to convert absorption units into units of activity for this assay when a sample volume of 30 µl was used is 612.7.

Both the blank activity and the activity measured in the aurothioglucose-inhibited sample was subtracted from the activity measured in the respective sample. These activity measurements were then corrected for protein to give TR activity measured in mU/ mg protein.

2.3.19 Cytosolic glutathione peroxidase assay

Cell pellets were stored at -70°C immediately after harvesting. Samples were then transported on dry ice to the Rowett Research Institute, Aberdeen, UK where cytosolic glutathione peroxidase (cyGPX) activities were measured by Miss Karen Pickering of the Rowett Research Institute using a method previously described by Beckett *et al.* (Beckett *et al.*, 1990). The measurement of cyGPX employs an indirect, coupled method. Briefly, the oxidised glutathione produced during the cyGPX enzyme activity (figure 1.12, section one) is reduced by NADPH and glutathione reductase. cyGPX activity is thus determined by following the rate at which NADPH is oxidised to $NADP^+$ measured as a change in absorbance at 340 nm in the presence of the substrate hydrogen peroxide using a Unicam UV/Vis spectrometer (UV4). The spectrometer was linked to a computer installed with Vision software for the calculation of cyGPX activity.

a) Preparation of the samples and standards for the cyGPX activity assay

Cell pellets were resuspended in 0.2 ml 0.125 M phosphate buffer containing 1 mM EDTA and 0.1% Triton X-100.

b) Preparation of the reaction mix

A reaction mix was made up of the following: 5 mg $NADPH_2$, 46 mg reduced glutathione, 3 ml distilled water, 24 ml PBS (pH 7.6), 1 ml sodium azide (0.1125 M), 20 U glutathione reductase and 0.1% peroxide free Triton X-100. The reaction mix was placed in a water bath at 37°C.

To measure cyGPX activity 990 µl reaction mix was added to 10 µl sample in a 1 ml glass cuvette. The blank rate was followed for 5 cycles at 340 nm (30 seconds per cycle). To start the reaction 20 µl of the substrate solution hydrogen peroxide (0.0022 M) was added and the subsequent reaction rate was followed for a further five cycles (30 seconds per cycle) at 340 nm.

A unit of cyGPX is defined as that which oxidises 1 µmole of NADPH per min. The molar extinction coefficient of NADPH is 6220 M^{-1} cm^{-1}. The blank rate was subtracted from the rate obtained after substrate addition. The conversion factor for the assay when a 20 µl sample was used was 8.0385.

2.3.20 Phospholipid glutathione peroxidase assay

Cell pellets for measurement of phospholipid hydroperoxide glutathione peroxidase (PHGPX) activities were stored and transported as described for cyGPX activity measurement (section 2.3.19). All measurements were carried out by Miss Karen Pickering of the Rowett Research Institute.

The same protocol was used for the measurement of PHGPX activity as described in section 2.3.19 with the exception that an alternative substrate of phosphatidyl choline hydroperoxide was used and purified PHGPX was used as a control to quantify this substrate. The conversion factor for the calculation of the results was the same as that used in the cyGPX activity assay which was 8.0385.

a) *Preparation of substrate (phosphatidyl choline hydroperoxide) for the PHGPX activity assay*

The substrate phosphatidyl choline hydroperoxide was prepared by dissolving 10 mg phosphatidyl choline in 4 ml of 3% deoxycholate. This mixture was diluted with 21 ml 0.2M sodium borate, pH 9.0 to which 100 µl of lipoxidase was added. The solution was placed in a water bath at 37°C for 1 hr with oxygen passed through the mixture every 15 min.

After incubation the mixture was passed through a Sep pak C18 cartridge, that had been previously washed with methanol and equilibrated with distilled water. Distilled water (100 ml) was added to the column and the phosphatidyl choline hydroperoxide was eluted with 2 ml methanol.

The substrate could be stored frozen for up to 2 months (-40°C).

2.3.21 Cell viability measurements (lactate dehydrogenase activity assay)

The viability of cells was measured by the cellular retention of lactate dehydrogenase (LDH). Intracellular and extracellular LDH activity was determined by following the rate at which NADH is converted to NAD^+ measured as a decrease in absorbance at 340 nm in the presence of pyruvate using a Sigma Diagnostics LDH kit adapted for the Cobas Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK). The rate of decrease in absorbance at 340 nm measured at 37°C is directly proportional to LDH activity in the sample. The percentage LDH activity retained was calculated as follows; intracellular LDH activity/ (extracellular LDH activity + intracellular LDH activity) x 100.

a) Preparation of reagents

LDH reagent A contains NADH (0.194 mM) in phosphate buffer, pH7.5 (54 mM). LDH reagent B is a solution of pyruvate (16.2 mM) to which non-reactive stabilisers and filler have been added. Both reagents were reconstituted by the addition of deionized water as indicated on the vial. After addition of water, the vial was sealed and then gently inverted several times.

b) Sample preparation

All cells for cell viability studies were plated into 12 well plates at a density of 10000 cells/cm^2. Immediately after treatment with tert-butylhydroperoxide the medium (1 ml) was removed from the cells into labelled eppendorf tubes. Cells were then washed with 2 x 1 ml phosphate

minutes the cell lysates were collected into labelled eppendorf tube and the wells were washed in a further 0.5 ml PBS and the contents added to the respective eppendorf tube. Cell debris which may interfere with the assay was removed by micro-centrifugation of all samples at 11500g.

c) LDH activity assay

Samples (200 µl) were transferred into Cobas cups for LDH activity analysis. The change in absorbance at a wavelength of 340 nm was measured in 25 µl of sample in a final volume of 290 µl (200 µl reagent A and 25 µl reagent B). The reaction mixture was maintained at 37°C and the LDH activity expressed as units per litre (U/L) was determined by kinetic analysis.

LDH activity was also measured in culture media which had not been in contact with cells as a measure of background absorbance which was subsequently subtracted from each extracellular LDH activity. The LDH activity in both the medium and cell lysates was then calculated as described above.

2.3.22 Statistical analysis

The statistical analyses in this thesis were carried out using the computer statistics package GraphPad InStat version 3 for Windows 95.

To test whether the means from two groups differed significantly the unpaired 't' test was used. This test assumes that the standard deviations of the two populations do not differ significantly. In cases where there was found to be a significant difference the Welch correction was used. One-way analysis of variance (ANOVA) was used when differences where suspected between the means of a number of groups of data. In the cases where the difference was significant ($p>0.05$) a Tukey-Kramer multiple comparison was used which compares all columns of data.

CHAPTER THREE SELENOPROTEIN EXPRESSION IN ENDOTHELIAL CELLS

3.1 INTRODUCTION

3.1.1 Antioxidant enzymes in plasma and endothelial cells

Selenium is found in all human tissues at an average concentration of 0.2 µg per gram in association with tissue protein, either loosely bound or as selenium analogues of sulphur amino acids forming selenoproteins (Reilly, 1993). Selenium is thought to exert its effects through the expression of both intracellular and extracellular selenoproteins (Arthur and Beckett, 1994). Since the identification of cytoplasmic glutathione peroxidase (cyGPX) in 1973, approximately 30 different selenoproteins in mammals have been identified by SDS-PAGE of [^{75}Se]-labelled tissue. Of these 30 selenoproteins, only about 13 have been characterized using purification and sequencing of the protein and/ or cloning and sequencing of the cDNAs. The biochemical roles of these 13 selenoproteins have been elucidated in all but three of the selenoproteins identified; however the precise characterisation and detailed mechanisms of action of some have still to be determined.

The intracellular selenoproteins identified to date consist of cyGPX, phospholipid hydroperoxide glutathione peroxidase (PHGPX), gastrointestinal glutathione peroxide, three different forms of both thioredoxin reductase (TR) and iodothyronine 5'-deiodinase (IDI), selenoprotein W and the 58, 56 and 14 kDa selenium-binding proteins.

Labelling with [^{75}Se]-selenite provides a reliable, precise and sensitive method for assessing the expression of selenoproteins which require insertion of selenocysteine residues at specific sites. However, since equilibration of exogenous [^{75}Se]-selenite with the endogenous pool of selenium and selenoproteins can take within excess of 27 hr (Beech *et al.*, 1994), labelling experiments to

detect specific selenoproteins are usually carried out over at least a 24-36 hr period. The use of [^{75}Se]-labelling to study the effects of selenium on selenoenzyme induction is potentially problematic. Isotope dilution with added selenium would be likely to obscure any real changes in selenoenzyme expression.

The [^{75}Se]-labelling of endothelial cells has not been previously reported. However the presence of specific selenoproteins in endothelial cells has been demonstrated. Thomas *et al.*, (Thomas, Geiger and Girotti, 1993), and Claise *et al.*, (Claise *et al.*, 1997) have measured cyGPX and PHGPX activity in cultured bovine and human endothelial cells respectively, whilst cyGPX mRNA has been detected using Northern blot analysis in human umbilical vein endothelial cells (HUVEC) (Jornot and Junod, 1995; Ricetti *et al.*, 1994).

Human plasma contains several antioxidant enzymes including superoxide dismutase, catalase and the extracellular selenoproteins, extracellular glutathione peroxidase (EGPX) (Takahashi *et al.*, 1987) and selenoprotein P (Arteel *et al.*, 1998; Burk and Hill, 1994; Mostert, Lombeck and Abel, 1998). Whilst, endothelial cells are reported to be the main source of extracellular superoxide dismutase (Marklund, 1984), Avissar *et al.* were unable to detect EGPX in the media of HUVEC using immunoprecipitation. They concluded that either endothelial cells do not synthesise and secrete EGPX or the levels were below the detection limit of their immunopurification method (Avissar *et al.*, 1989). Selenoprotein P was the second extracellular selenoprotein to be identified and comprises of approximately 65% of the plasma selenium in rats (Burk and Hill, 1994). Immunohistochemical techniques have shown that selenoprotein P is associated with endothelial cells in brain, capillary endothelial cells of the renal glomeruli and the endothelial cells of the liver (Burk *et al.*, 1997). However the synthesis of selenoprotein P by HUVEC or other endothelial cells has not been previously reported.

Selenium status can modify selenoprotein expression. In studies where rats received selenium-deficient diets substantial decreases in hepatic IDI and cyGPX activities and mRNA levels, compared to selenium-adequate controls, were observed (Bermano, 1995; Mitchell *et al.*, 1996). In cell culture studies, selenium supplementation of approximately 174 nM and 520 nM significantly increased cyGPX activity in HUVEC (Ricetti *et al.*, 1994). The selenium concentration of different culture media can vary significantly and this factor should be considered when comparing selenoprotein expression and activities of cells cultured using different media.

3.1.2 Model systems of endothelial cell function

The use of cell culture has allowed the study of homogeneous cell populations without being complicated by the confounding factors of other tissues. Large vessel endothelial cell culture is a well-established model for the study of the endothelium. The human umbilical vein is often the chosen vessel for the study of human endothelial function as it has several advantages as a source of endothelial cells. It is a non-branching vessel, with a large intimal surface area, making it technically very easy to isolate cells. However there are many variables which can affect the viability of the isolated cells, including foetal stress, maternal anaesthesia and smoking. The use of HUVEC is also complicated by the genetic variability between preparations, limited population doublings, difficulty in culture and the requirement for specialised growth factors.

An alternative to the use of HUVEC is to use human transformed cell lines such as EAhy926. EAhy926 is an endothelial cell line established by hybridising primary HUVEC with A549 human lung tumor cells (Edgell, McDonald and Graham, 1983). Cells in primary culture have a limited replication potential and tend to senesce in culture (Ager *et al.*, 1982), whilst EAhy926 cells retain many of the differentiated functions common to primary endothelial cells beyond 100 passages. These functions include; the expression of von Willebrand Factor (Edgell, McDonaldand Graham, 1983), prostacyclin formation (Suggs *et al.*, 1986) and expression of

endothelin-1 (Saijonmaa *et al.*, 1991). The selenoprotein profile of EAhy926 cells has not been previously determined, which is essential in order to establish whether this more convenient cell line would provide a suitable model for future studies of selenoprotein expression in endothelial cells.

Endothelial cells isolated from a number of different species have been used as model systems to investigate human pathologies. For example bovine aortic endothelial cells (BAEC) were used in the earliest studies due to their ease of isolation and subcultivation. Porcine aortic endothelial cells (PAEC) were initially isolated and characterised by Slater and Sloan (Slater and Sloan, 1975) and were thought to provide a suitable alternative to HUVEC based on similarities of the porcine cardiovascular system with that in man. In addition porcine aorta is subject to atheroma formation and should provide a useful model for the study of atherosclerosis (Rosenthal and Gotleib, 1990). However variation between endothelial cells isolated from different species has been acknowledged; for example PAEC unlike HUVEC and BAEC do not have Factor VIII-related antigen immunoreactivity (Rosenthal and Gotleib, 1990).

As well as species differences in the properties of endothelial cells it has also been shown that endothelial cells isolated from different sites in the vasculature exhibit different properties. Arterial and venous endothelial cells show differences in the production of angiotensin-converting enzyme (Johnson, 1980) and their response to cytokine stimulation (Hauser, Johnson and Madri, 1993). These observations have led to the suggestion that HUVEC, despite being used by a number of researchers in the field of vascular disease [Milner, 1990; Jornot, 1997; Zhao, 1998] may not be the most suitable model in the study of human cardiovascular disease. Indeed a more suitable model to study vascular disease may be endothelial cells isolated from arterial vascular beds as opposed to venous. Thus, human coronary arterial endothelial cells may provide a good model as the coronary artery in particular is one of the main vascular beds affected by the formation of atherosclerotic lesions.

Furthermore if the efficacious effect of selenium on endothelial cell function is to be studied it is essential that a model system is chosen which reflects the selenoprotein expression and function of endothelial cells which line vessels prone to developing vascular disease.

The experiments reported here aim to:

- determine the labelling conditions required to study selenoprotein expression in endothelial cells using [^{75}Se]-selenite
- examine the expression of extracellular and intracellular selenoproteins in [^{75}Se]-labelled HUVEC
- compare the selenoprotein profile of HUVEC with that of the endothelial cell line, EAhy926
- compare the selenoprotein profile of HUVEC with those of endothelial cells isolated from human umbilical artery and human coronary artery
- compare the pattern of selenoprotein expression observed in HUVEC with that of endothelial cells isolated from alternative species
- to arrive at an appropriate model to investigate the role of selenium on endothelial cells.

3.2 METHODS

3.2.1 Time-course of [^{75}Se]-labelling in HUVEC

HUVEC were isolated and maintained as described in section 2.3.2. Cells were grown to confluence and the culture medium was changed and replaced with fresh medium. [^{75}Se]-selenite (0.02 MBq/ml) was added to the culture medium of duplicate T75 flasks at time 0, 12, 24, 48, 72 and 96 hr. After 96 hr the medium was removed from all the flasks and the cells were washed three times with EBSS (4°C). The cells were harvested into 20 ml of EBSS by scraping and centrifuged at 2000 g for 10 min at 4°C. The supernatant was aspirated off and the cell pellet resuspended in 200 µl of 60 mM Tris buffer, pH 7.8, containing 1 mM EDTA and 1 mM dithiothreitol (Tris buffer). The cells were then lysed by sonication on ice using the Soniprep 150.

Protein concentrations were measured using the Bradford assay (section 2.3.12) and the samples were diluted to a common protein concentration with Tris buffer. The cell lysates were prepared for separation by SDS-PAGE (section 2.3.13) and the [^{75}Se]-labelled proteins present in 25 µg of protein were separated by SDS-PAGE (section 2.3.13). The resulting gel was dried and the [^{75}Se]-labelled selenoproteins were visualised by autoradiography using Kodak X-OMAT XAR-5 film (section 2.3.14).

3.2.2 The intracellular and extracellular selenoproteins expressed by HUVEC

In experiments where only the intracellular selenoproteins were monitored the following protocol was used. HUVEC were isolated and maintained as described in section 2.3.2. Cells were grown to confluence and the culture medium was changed and replaced with fresh medium. [^{75}Se]-selenite (0.02MBq/ml) was added to a T75 flask of HUVEC and incubated for 48 hr to allow a steady state of labelling to be achieved (as ascertained in the experiment described in section 3.2.1). After a 48 hr labelling period the cells were harvested and lysed in Tris buffer as described above in section 3.2.1.

3.2.1). After a 48 hr labelling period the cells were harvested and lysed in Tris buffer as described above in section 3.2.1.

To monitor both the intracellular and extracellular selenoprotein expression, duplicate T75 flasks containing confluent cultures of HUVEC were incubated with [^{75}Se]-selenite (0.02 MBq/ml) for 48 hr. After 30 hr the EGM-2 culture medium was removed and the cells washed. Cells were then incubated in protein-free culture medium EBM-2 containing [^{75}Se]-selenite (0.02 MBq/ml). After a further 18 hr this culture medium was removed, centrifuged at 2000 g for 20 min and then dialysed over two days to remove any unbound [^{75}Se]-selenite. Dialysis was against 2 litres of Tris buffer at 4°C (which was changed four times over the two day period). The dialysed solution was then concentrated by placing the dialysis sack into a saturated solution of poly(ethylene glycol) prepared in dialysis buffer. Meanwhile the cells were harvested and lysed in Tris buffer (section 3.2.1).

Protein concentrations of both the HUVEC lysate and the dialysed medium were measured using the Bradford assay (section 2.3.12). The samples were prepared for separation by SDS-PAGE (section 2.3.13) and the ^{75}Se-labelled proteins present in 25 μg of protein in the HUVEC and in 12.5 μg protein in the culture medium were separated by SDS-PAGE (section 2.3.13). The resulting gel was dried and the [^{75}Se]-labelled selenoproteins were visualised by autoradiography using Kodak X-OMAT XAR-5 film (section 2.3.14).

Some of the gels were scanned using a Bio-Rad model GS-525 Molecular Imager System to create a digitilized image (section 2.3.15). The radioactivity in each band was quantified using the Bio-Rad Molecular Analyst/ PC image-analysis software. This method was used to assess the relative amounts of each selenoprotein in a given selenoprotein profile.

3.2.3 *Identification of intracellular selenoproteins expressed by HUVEC*

Thioredoxin reductase (TR), cytosolic glutathione peroxidase (cyGPX) and phospholipid hydroperoxide glutathione peroxidase (PHGPX) were identified in HUVEC using Western blot analysis. The methodology used is described in section 2.3.16 and was carried out by Mr Fergus Nicol of the Rowett Research Institute, Bucksburn, Aberdeen, UK and Dr. Forbes Howie of the Department of Clinical Biochemistry, The University of Edinburgh, Edinburgh, UK.

3.2.4 *The effect of passage number on [^{75}Se]-labelling in HUVEC*

The intracellular [^{75}Se]-selenoprotein profiles of eight passages of a single preparation of HUVEC isolated from a single umbilical vein were compared. HUVEC were isolated and maintained as described in section 2.3.2. The HUVEC from the primary isolate (passage zero) were grown to confluence and passaged into at least three T75 flasks (passage one). At confluence, two of the T75 flasks were labelled with [^{75}Se]-selenite (0.02MBq/ml) for 48 hr, whilst the third was sub-cultured to provide passage two HUVEC. This procedure was continued until cells had reached passage eight, at which point distinct morphological changes were observed, such as significant cell enlargement and a partial loss of the characteristic cobblestone appearance.

After a 48 hr labelling period HUVEC of each passage were harvested and stored at -21°C until HUVEC had been collected for each passage number.

Samples were thawed, lysed and prepared for separation on an SDS-PAGE as described in section 3.2.1. The [^{75}Se]-labelled selenoproteins present in 25 µg of protein of each sample of HUVEC were then separated on a single SDS-PAGE gel (section 2.3.13) to allow a direct comparison between HUVEC of a number of passages. The resulting gel was dried and the [^{75}Se]-labelled selenoproteins were visualised by autoradiography using Kodak X-OMAT XAR-5 film (section 2.3.14).

3.2.5 The [^{75}Se]-labelling of endothelial cells isolated from different vasculature

The intracellular selenoprotein profile of the cell line EAhy926 cells, human coronary artery endothelial cells (HCAEC), human umbilical artery endothelial cells (HUAEC) and bovine aortic endothelial cells (BAEC) were each compared to that of HUVEC. Each cell type were isolated and/or maintained as previously described (sections 2.3.4, 2.3.5, 2.3.6, 2.3.8 and 2.3.2). Duplicate flasks of both HUVEC and the comparative endothelial cell type were seeded and maintained in their respective growth media. At confluence the cells were labelled with [^{75}Se]-selenite (0.02MBq/ml). After 48 hr incubation the cells were harvested as described in section 3.2.1.

Cell lysates were then prepared for and the [^{75}Se]-labelled proteins separated by SDS-PAGE (section 3.2.1). The resulting gel was dried and the [^{75}Se]-labelled selenoproteins were visualised by autoradiography using Kodak X-OMAT XAR-5 film (section 2.3.14). The [^{75}Se]-labelled selenoproteins of the HUVEC and the comparative endothelial cell type were separated on a single SDS-PAGE gel to allow a direct comparison between the two cell types.

The intracellular selenoprotein profile of porcine aortic endothelial cells (PAEC) was established using the same protocol as described above in this section except that HUVEC were not labelled in parallel with PAEC and therefore their intracellular selenoprotein profiles were not directly compared on a single SDS-PAGE gel. (PAEC were isolated and maintained as described in section 2.3.7).

3.2.6 Intracellular selenoprotein expression and activity of endothelial cells isolated from different vasculature

HUVEC, EAhy926 cells, HCAEC and BAEC were isolated and/or maintained as previously described (see sections 2.3.2, 2.3.4, 2.3.5 and 2.3.6).

For measurement of TR mass and activity, cyGPX activity and PHGPX activity (sections 2.3.17, 2.3.18, 2.3.19 and 2.3.20), triplicate T75 flasks of each cell types were grown and maintained as previously described (3.2.2, 2.3.4, 2.3.5 and 2.3.8). At confluence the cells were harvested, lysed in 0.125 M potassium phosphate buffer, pH 7.4, and sonicated on ice (section 3.2.1). The cell lysates were subsequently frozen at -70°C until assayed. All the samples for each measurement were analysed in the same assay to avoid any between assay variation. All the TR activity assays described in this chapter were carried out by Miss Michelle Lewin of the Department of Clinical Biochemistry, The University of Edinburgh, Edinburgh, UK whilst Miss Karen Pickard and Mr Fergus Nicol both of the Rowett Research Institute, Bucksburn, Aberdeen, UK carried out the GPX activity assays.

TR, cyGPX and PHGPX activity were not measured in HCAEC, whilst TR mass was not measured in BAEC due to a shortage of cells.

3.2.7 Comparison of [^{75}Se]-labelling of cell types isolated from different human tissues

Cells originating from different human tissues (HUVEC, human thyrocytes and a human foetal liver-derived cell line, HepG2) were isolated and maintained as described in sections 2.3.2, 2.3.9 and 2.3.10. HUVEC, human thyrocytes and hepG2 cells were grown to confluence in T75 flasks and then each was labelled for 48 hr with [^{75}Se]-selenite (0.02 MBq/ml). Cells were then harvested, lysed in Tris buffer and separated by SDS-PAGE (section 3.2.1). The resulting gels were dried and the [^{75}Se] labelled selenoproteins visualised by autoradiography using Kodak X-OMAT XAR-5 film. The autoradiographs obtained for each of the different cell types were then compared with each other.

3.2.8 Measurement of the selenium content of various culture mediums

The selenium content of the following culture media was measured using the method described in section 2.3.11: EGM-2, EBM-2, DMEM containing 10% FBS and 1% HAT, M199 containing 20% FCS and EGM.

3.2.9 The effect of increasing doses of sodium selenite on [^{75}Se]-labelling of HUVEC

T75 flasks of HUVEC were isolated and maintained as described in section 2.3.2. Cells were grown to confluence at which point the culture medium was changed and replaced with fresh medium containing one of the following concentrations of sodium selenite; 0 nM, 0.1 nM, 1.0 nM, 10 nM and 100 nM. [^{75}Se]-selenite (0.02 MBq/ml, 1.25 nM selenite) was added to each flask. After 48 hr the cells were harvested and lysed in Tris buffer and the intracellular selenoproteins separated by SDS-PAGE as described in section 3.2.1.

3.2.10 Statistical analysis

An unpaired 't' test was used to test the significant difference between the levels of selenoprotein expression or enzyme activity in the different endothelial cell types. The Students 't' test assumes that the standard deviations (SD) are equal. In cases where there was found to be a significant difference in the SD the 't' test was used with a Welch correction.

3.3 RESULTS

3.3.1 [^{75}Se]-labelling time-course of HUVEC

Figure 3.01 shows an autoradiograph of an SDS-PAGE gel demonstrating the changes in [^{75}Se]-labelling with increasing time. Selenoproteins were faintly labelled after a 12 hr exposure to [^{75}Se]-selenite. The intensity of labelling of all selenoproteins increased up until 48 hr at which time a steady state of labelling was achieved and no further increase in labelling was observed.

3.3.2 The intracellular and extracellular selenoproteins expressed by HUVEC

Figure 3.02 shows an autoradiograph of an SDS-PAGE gel of the intracellular [^{75}Se]-labelled selenoproteins in HUVEC. Four major [^{75}Se]-labelled selenoproteins were observed. A single selenoprotein with a molecular mass of 58.1± 1.0 kDa (mean ± SEM, n=5) was the dominant [^{75}Se]-labelled band accounting for 42.9±1.4% (mean ± SEM, n=3) of the total intracellular [^{75}Se]-selenoproteins. Two other selenoproteins that showed pronounced [^{75}Se]-labelling had calculated molecular masses of 21.7±0.5 kDa (mean ± SEM, n=5) and 24.4±0.5 kDa (mean ± SEM, n=5) and accounted for 14.6±1.0% (mean ± SEM, n=3) and 15.1±2.8% (mean ± SEM, n=3) respectively of the total intracellular [^{75}Se]-labelling. The fourth prominently [^{75}Se]-labelled selenoprotein ran slightly above the 14.4 kDa molecular weight marker and had a molecular mass of approximately 15 kDa and accounted for 14.7±5.4% (mean ± SEM, n=3) of the total intracellular [^{75}Se]-selenoproteins. In addition to these four major bands a number of minor labelled selenoproteins were observed, including a band which ran slightly above the 58 kDa [^{75}Se]-selenoprotein and a band with an approximate molecular mass of 72 kDa. No distinct variations in the pattern of intracellular [^{75}Se]-labelled selenoproteins between different preparations of HUVEC were observed (figure 3.03). The variation in the intensities of autoradiographic bands between preparations can be accounted for by differing exposures of the SDS-PAGE gels to the autoradiographic film.

No significant [^{75}Se]-labelling was detected in the culture medium of HUVEC (figure 3.02).

3.3.3 Identification of intracellular selenoproteins expressed by HUVEC

Using antiserum to rat thioredoxin reductase (TR) and human TR, the 58 kDa [^{75}Se]-labelled band in HUVEC was identified by Western blotting as TR (refer to lane 4, figure 3.20).

The 22 kDa [^{75}Se]-labelled band was identified as phospholipid hydroperoxide glutathione peroxidase (PHGPX) using antisera to rat PHGPX by Western blot analysis (figure 3.04). The molecular weight of crocodile and human PHGPX was higher than that of rat PHGPX.

Using antiserum to human cyGPX it was not possible to visualise an immunoreactive band in HUVEC (data not shown). The purified cyGPX separated by SDS-PAGE revealed several bands but only one band had an electrophoretic mobility of 24 kDa. This 24 kDa protein (observed on the Coomassie Blue gel) was superimposable on the 24 kDa [^{75}Se]-labelled protein band observed on the SDS-PAGE gel autoradiograph (figure 3.05).

The 58 kDa, 22 kDa and 24 kDa bands observed on an autoradiograph of an SDS-PAGE gel showing the [^{75}Se]-selenoproteins expressed by HUVEC are thought to represent TR, PHGPX and cyGPX respectively. Therefore, these bands, whether expressed by HUVEC or other endothelial cells, are referred to as the selenoproteins they are believed to represent.

3.3.4 The effect of passage number on [^{75}Se]-labelling in HUVEC

Figure 3.06 demonstrates that the pattern of intracellular [^{75}Se]-labelled selenoproteins expressed by HUVEC of eight passages from a single preparation did not show any distinct alteration with increasing passage.

3.3.5 The intracellular selenoproteins expressed by the human endothelial cell line EAhy926

Figure 3.07 shows that the overall pattern of selenoprotein expression in EAhy926 cells resembles that observed in HUVEC with some significant differences in the levels of expression of a few selenoproteins. TR is dominantly labelled and to a similar extent in both HUVEC and EAhy926 cells. Figure 3.08 compares the TR mass and activity of both cell types and confirms that there is no significant difference in the TR expression in these cell types.

The [^{75}Se]-labelled selenoprotein with an approximate molecular weight of 64 kDa in HUVEC is not apparent in EAhy926 cells whilst an unidentified selenoprotein with an estimated molecular mass of approximately 18 kDa is more prominently labelled in EAhy926 cells as compared to HUVEC.

A lower expression of cyGPX and PHGPX can be observed in figure 3.07 in EAhy926 cells compared to HUVEC. Figure 3.09 confirms this observation showing that cyGPX activity in EAhy926 cells (0.086 ±0.001 U/mg protein, mean ± SD, n=3) is 27% of that in HUVEC (0.023 ±0.001 U/mg protein, mean ± SD, n=3, $p<0.01$). The PHGPX activity in the EAhy926 cells tended towards being lower than that observed in HUVEC, although this difference was not shown to be significant (figure 3.10).

3.3.6 The selenoproteins expressed by human coronary artery endothelial cells

Figure 3.11 shows that the pattern of [^{75}Se]-labelled selenoproteins in HCAEC was very similar to that observed in HUVEC. The autoradiograph shows little difference in the degree of labelling and hence the expression of some of these selenoproteins. TR is more dominantly labelled in HCAEC compared to HUVEC, whilst the selenoprotein with an approximate molecular mass of 22 kDa is labelled to a slightly lesser extent in HCAEC compared to HUVEC.

Figure 3.12 compares the TR mass between HUVEC and HCAEC. The expression of TR is 1.89-fold higher in HCAEC compared to HUVEC.

3.3.7 The pattern of selenoprotein expression in human umbilical artery endothelial cells

Figure 3.13 demonstrates that venous endothelial cells and arterial endothelial cells isolated from the same umbilical cord differ very slightly. In HUVEC one extra selenoprotein with a molecular mass of approximately 27 kDa is labelled which is not observed in HUAEC.

TR expression in HUAEC appears to be greater than in the HUVEC.

3.3.8 The pattern of selenoprotein expression in porcine aortic endothelial cells

Figure 3.14 shows the [^{75}Se]-labelled selenoproteins in a preparation of PAEC. Compared to HUVEC, PAEC show quite distinct differences in the pattern of [^{75}Se]-labelled selenoproteins. TR is no longer the most prominently labelled band. Instead a band with an approximate molecular weight of 15 kDa predominates.

3.3.9 The selenoproteins expressed by bovine aortic endothelial cells

Figure 3.15 shows that the pattern of [^{75}Se]-labelled selenoproteins varies considerably between HUVEC and BAEC. The [^{75}Se]-labelling of most selenoproteins was significantly lower in BAEC, with the exception of PHGPX which was labelled more prominantly in BAEC. The [^{75}Se]-labelled band with an approximate molecular weight of 15 kDa migrated at a slightly higher molecular weight in BAEC than that observed in HUVEC, but the selenoprotein was labelled to a similar degree.

TR activity measured in BAEC was approximately 6% of that measured in HUVEC (figure 3.16). TR mass was not measured in BAEC as the antiserum to human TR did not cross-react with bovine TR. There was no significant difference in the cyGPX and PHGPX activities between HUVEC and BAEC (figures 3.17 and 3.18).

3.3.10 Comparison of [^{75}Se]-labelling of cell types isolated from different human tissues

Distinct differences in the ratios of the intracellular [^{75}Se]-labelled selenoproteins were observed between cells isolated from different human tissues (figure 3.19). In the human foetal liver-derived cell line, HepG2, the band with an approximate molecular mass of 58 kDa is observed as a doublet rather than the single band found in both HUVEC and human thyrocytes. The same [^{75}Se]-labelled 58 kDa band was much more prominent in the HUVEC compared to HepG2 cells and human thyrocytes. (It should be noted that these cell types were labelled and separated on different SDS-PAGE gels, therefore some of the differences may be due to variability in the labelling and processing conditions).

The [^{75}Se]-labelled 58 kDa band observed in both HUVEC and HepG2 cells was identified as TR by Western blot analysis as previously described in section 2.3.16 (figure 3.20). Quantification of Western blots showed HUVEC expressed high concentrations of TR under basal conditions (4.36 ±0.63 µg/ mg, mean ± S.E.M., n=3). The concentration of TR expressed by HepG2 cells was approximately one tenth of that expressed in HUVEC. The Western blot was insufficiently sensitive to detect TR in human thyrocytes grown under basal conditions (figure 3.20).

3.3.11 Comparison of selenium content of various culture media

Figure 3.21 shows the measured selenium concentrations of the culture media used for the growth and maintenance of some of the endothelial cells described in this chapter. The results

show that there is no significant difference between the selenium content of the culture medium used to grow the human endothelial cell line EAhy926 i.e. DMEM containing 10% FBS and 1% HAT and the culture medium used for HUVEC, HCAEC and HUAEC i.e. EGM-2. The selenium content of the culture medium used to maintain BAEC has a significantly higher selenium concentration than the other two culture media.

The culture medium used to maintain human thyrocytes contained a selenium concentration of 5.4 nM as measured by Beech *et al.* in a previous study (Beech *et al.*, 1995).

The main source of selenium in EGM and EGM-2 media is selenious acid which can be metabolised by the cultured cells for incorporation into selenoproteins. In contrast, the selenium contained in most culture media is incorporated into serum proteins within the FBS which is not easily utilised by cultured cells (Brigelius-Flohé *et al.*, 1996). Therefore the selenium content of the EAhy926 culture media is unlikely to provide a bioavailable source of selenium. Thus the absolute levels of selenium measured in this study do not necessarily correlate to the ability of the selenium to increase selenoprotein expression.

The selenium concentration was not measured in the complete media used to maintain HepG2 cells or PAEC. The source of selenium in both types of media is from FBS as both DMEM/ Ham's F-12 nutrient mix containing L-glutamine and 15mM Hepes and M199 contain only negligible levels of selenium (data not shown).

3.3.12 The effect of increasing doses of sodium selenite on [^{75}Se]-labelling of HUVEC

Figure 3.22 shows an autoradiograph of an SDS-PAGE demonstrating the effects of increasing concentrations of sodium selenite on the [^{75}Se]-labelling of HUVEC. An observable decrease in

labelling was observed between 10 and 100 nM sodium selenite. Concentrations of sodium selenite above 1000 nM were toxic to HUVEC.

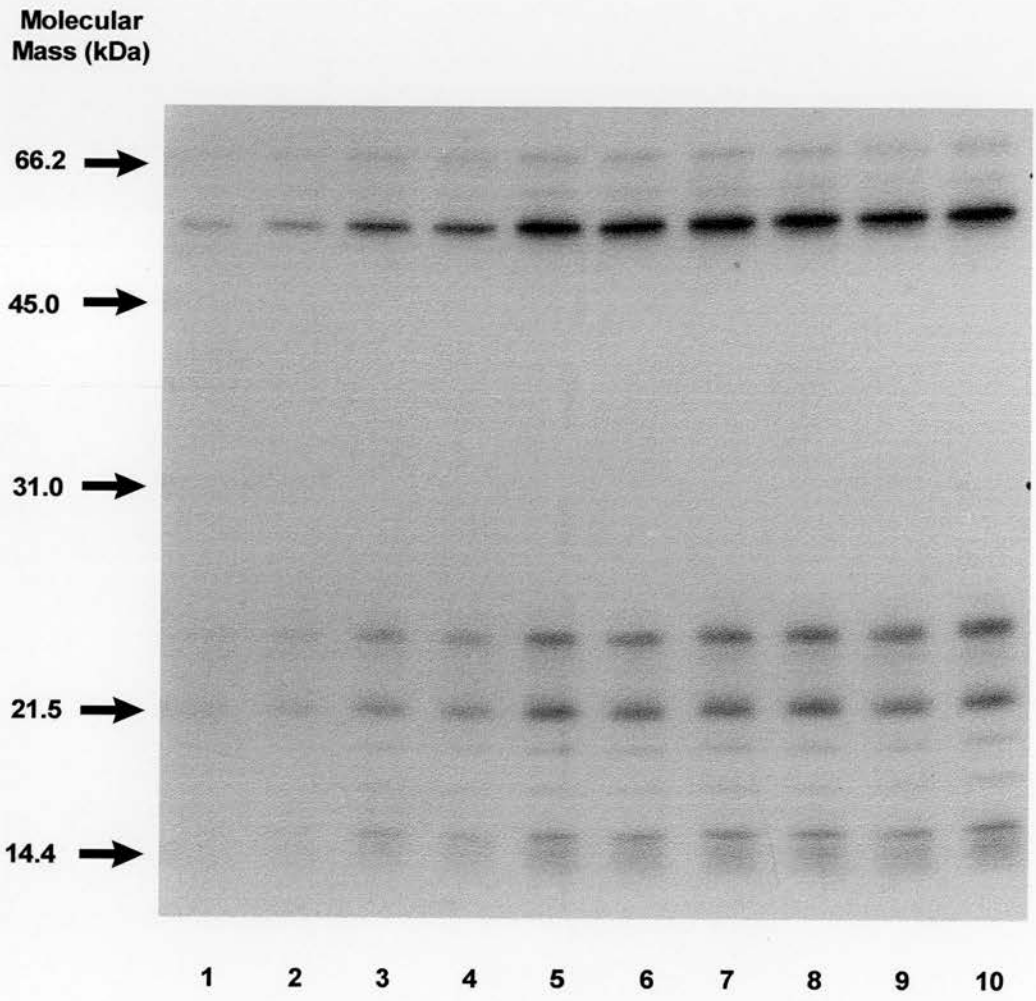


Figure 3.01. Autoradiograph of an SDS-PAGE gel of human umbilical vein endothelial cells (HUVEC) labelled with [^{75}Se]-selenite (0.02 MBq/ml) for stated lengths of time. Duplicate flasks of HUVEC were labelled for each time point. Lanes 1 and 2, 12 hr; lanes 3 and 4, 24 hr; lanes 5 and 6, 48 hr; lanes 7 and 8, 72 hr; lanes 9 and 10, 96 hr. Each lane was loaded with 25μg of protein.

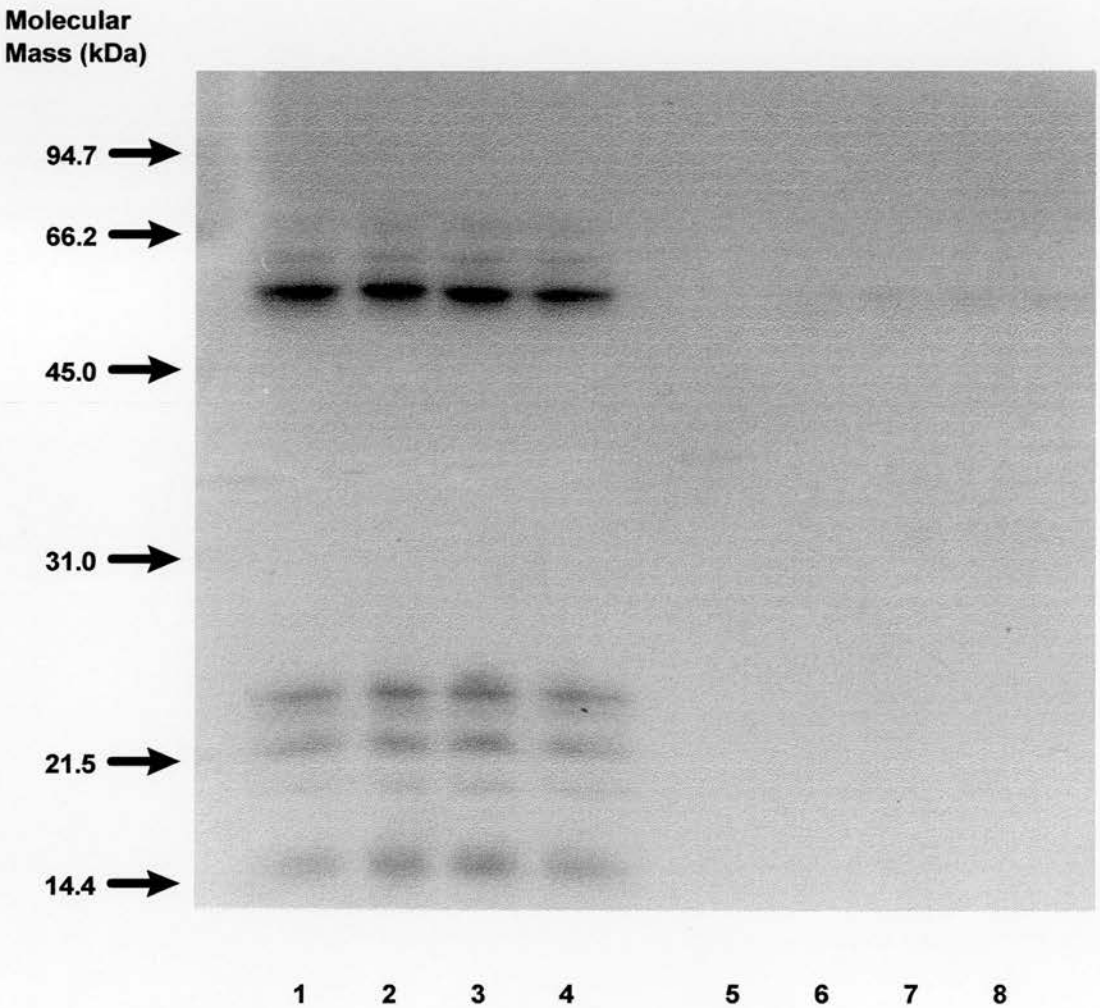


Figure 3.02. Autoradiograph of an SDS-PAGE gel of human umbilical vein endothelial cell (HUVEC) intracellular and extracellular selenoproteins labelled with [^{75}Se]-selenite (0.02 MBq/ ml) for 48 hr. Quadruple flasks of HUVEC from a single isolation were used. Lanes 1-4, intracellular selenoproteins (each lane was loaded with 25 µg protein); lanes 5-8, extracellular selenoproteins (each lane was loaded with 12.5 µg protein).

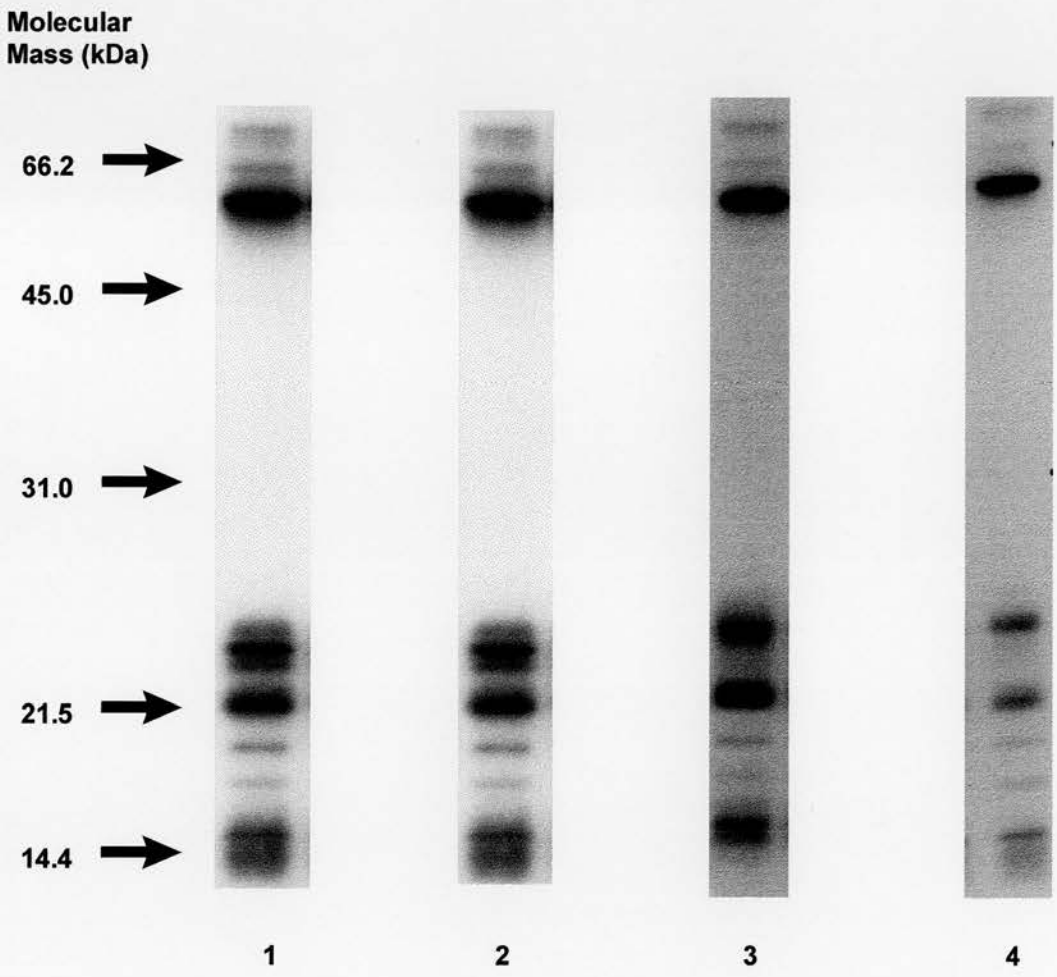


Figure 3.03. Autoradiographs of four SDS-PAGE gels showing the intracellular selenoproteins from four different preparations of human umbilical vein endothelial cells (HUVEC) labelled with [^{75}Se]-selenite (0.02 MBq/ ml) for 48 hr. Lane 1, HUVEC prep 7; lane 2, HUVEC prep 13; lane 3, HUVEC prep 29 and lane 4, HUVEC prep 31. Each lane was loaded with 25 µg of protein.

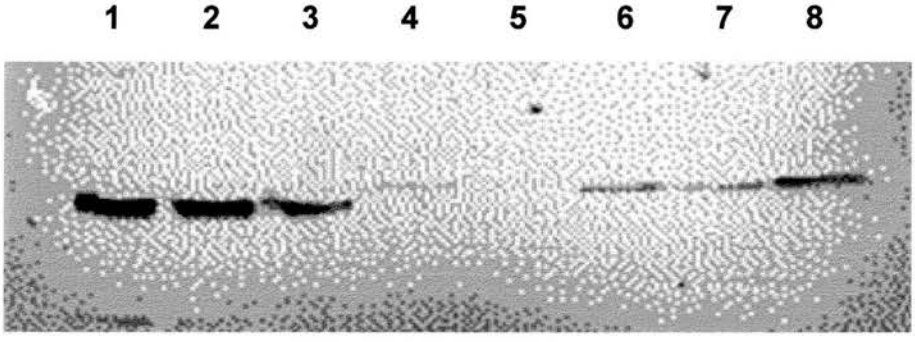


Figure 3.04. Western blot of human umbilical vein endothelial cells (HUVEC) and crocodile phospholipid hydroperoxide glutathione peroxidase (PHGPX) using antiserum to rat PHGPX. Lane 1, purified rat PHGPX standard (28 ng); lane 2, purified rat PHGPX standard (14 ng); lane 3, purified rat PHGPX standard (7 ng); lane 4, HUVEC grown in EGM-2 culture medium (50 µg protein loaded); lane 5, HUVEC grown in M199 culture medium (50 µg) protein loaded); lanes 6, 7 and 8, semi pure crocodile PHGPX.

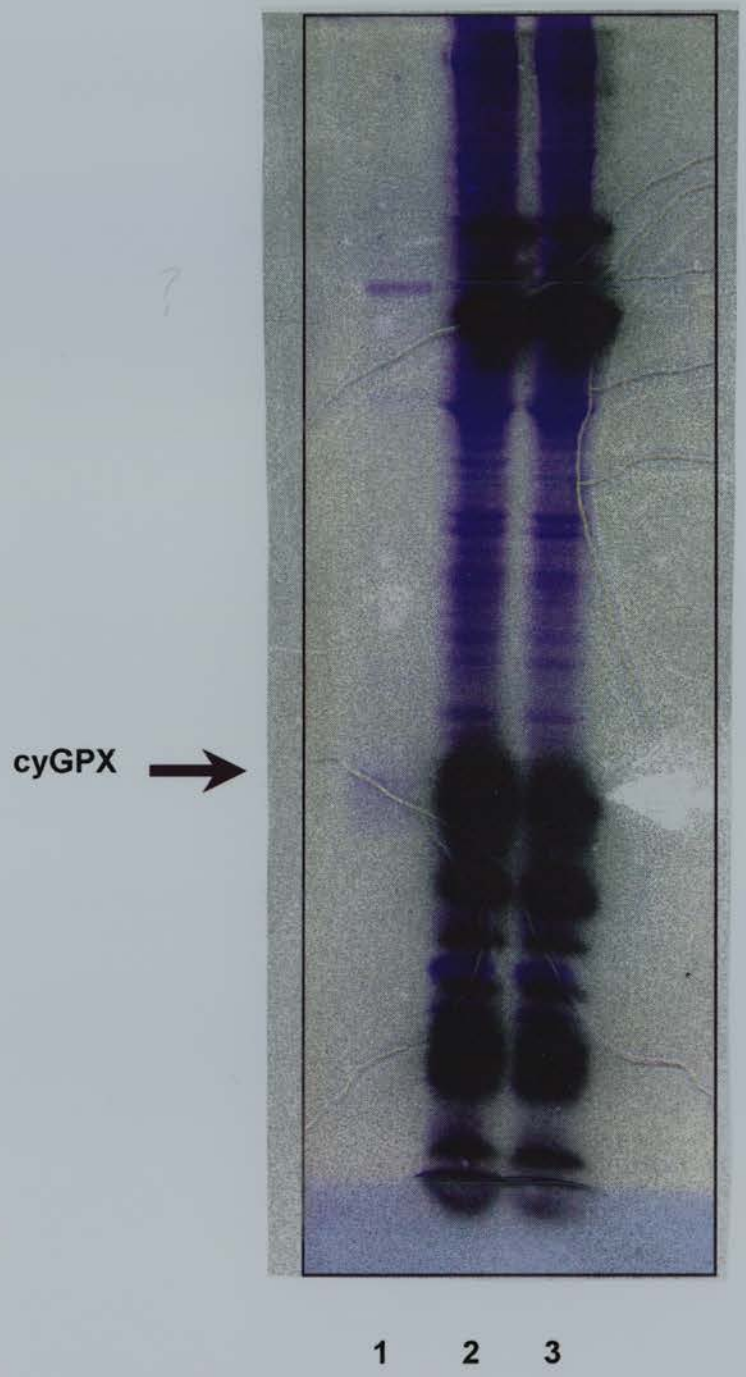


Figure 3.05. Autoradiograph of an SDS-PAGE gel of human umbilical vein endothelial cell (HUVEC) intracellular selenoproteins labelled with [^{75}Se]-selenite (0.02MBq/ml superimposed onto a Coomassie Blue gel of purified human cytoplasmic glutathione peroxidase (cyGPX). Lane 1, purified human cyGPX (10 µg); lanes 2 and 3 (Coomassie Blue gel), intracellular proteins expressed by HUVEC (25 µg); lanes 2 and 3 (autoradiograph), intracellular selenoproteins expressed by HUVEC (25 µg). **cyGPX, cytoplasmic glutathione peroxidase.**

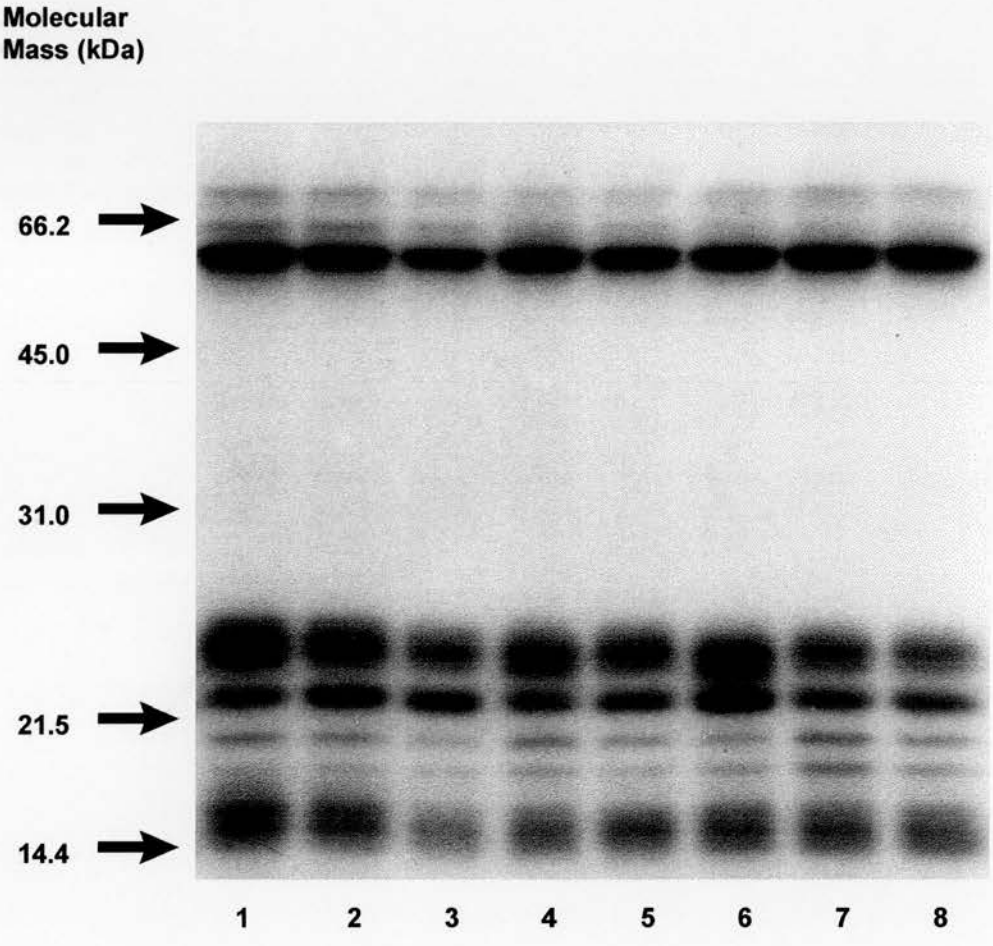


Figure 3.06. Autoradiograph of an SDS-PAGE gel of a single isolation of human umbilical vein endothelial cells (HUVEC) at different passages each labelled with [^{75}Se]-selenite (0.02 MBq/ ml) for 48 hr. Lane 1, passage 1; lane 2, passage 2; lane 3, passage 3; lane 4, passage 4; lane 5, passage 5; lane 6, passage 6; lane 7, passage 7 and lane 8, passage 8. Each lane was loaded with 25 µg of protein.

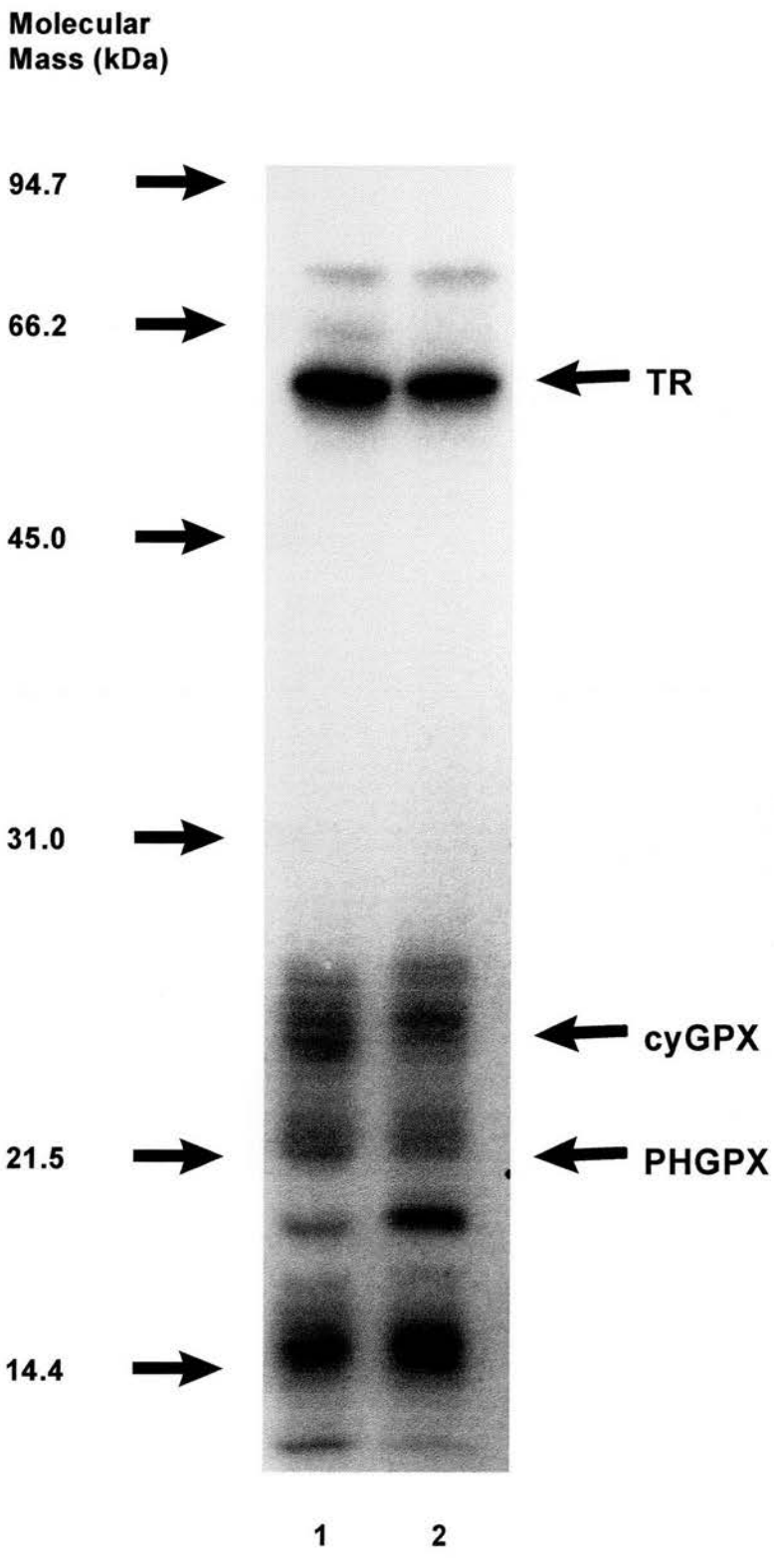


Figure 3.07. Autoradiograph of an SDS-PAGE gel of the intracellular selenoproteins of human umbilical vein endothelial cells (HUVEC) and EAhy926 cells labelled with [^{75}Se]-selenite (0.02 MBq/ ml) for 48 hr. Lane 1, HUVEC; lane 2, EAhy926 cells. Both lanes were loaded with 25 µg protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

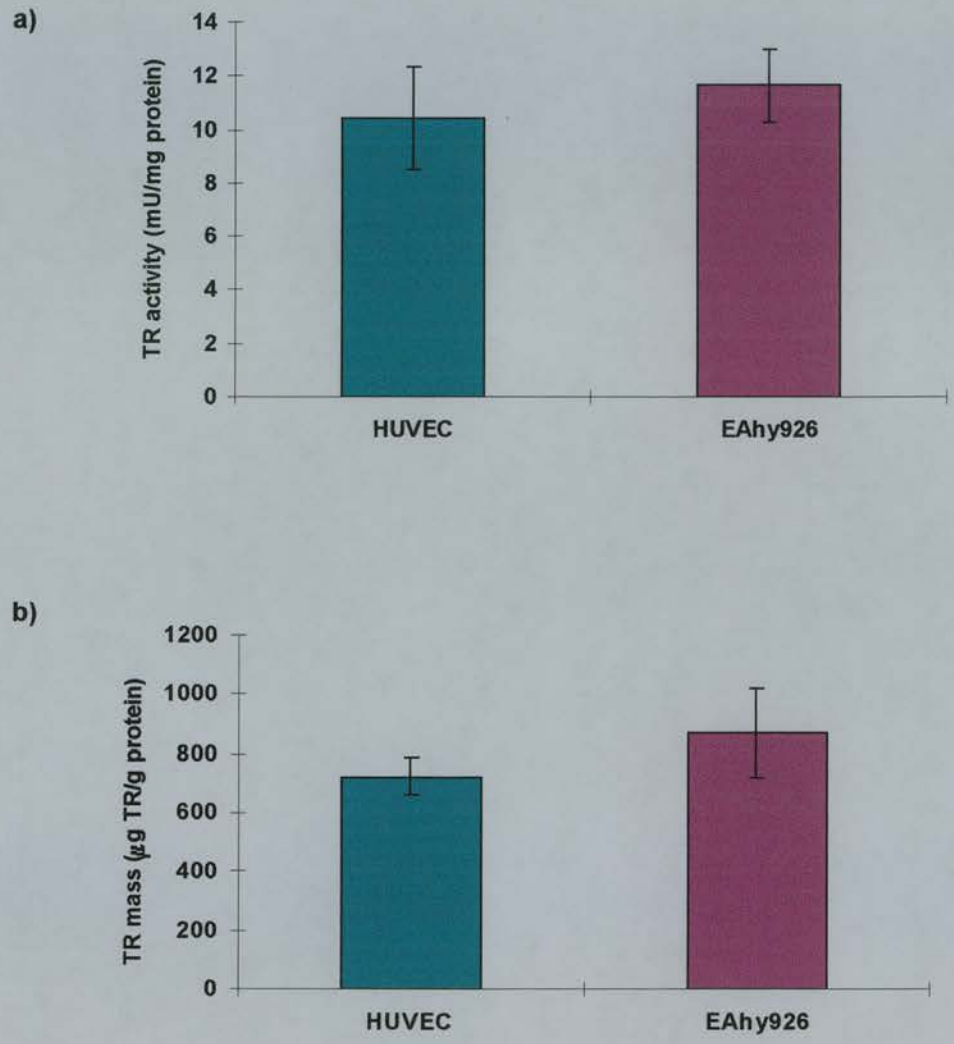


Figure 3.08 Thioredoxin reductase (TR) activity (a) and mass (b) comparison in human umbilical vein endothelial cells (HUVEC) cultured in EGM-2 compared with EAhy926 cells cultured in DMEM + 10% FBS + 1% HAT. Results shown are those of the mean of 4 flasks ± SD. No significant differences were calculated.

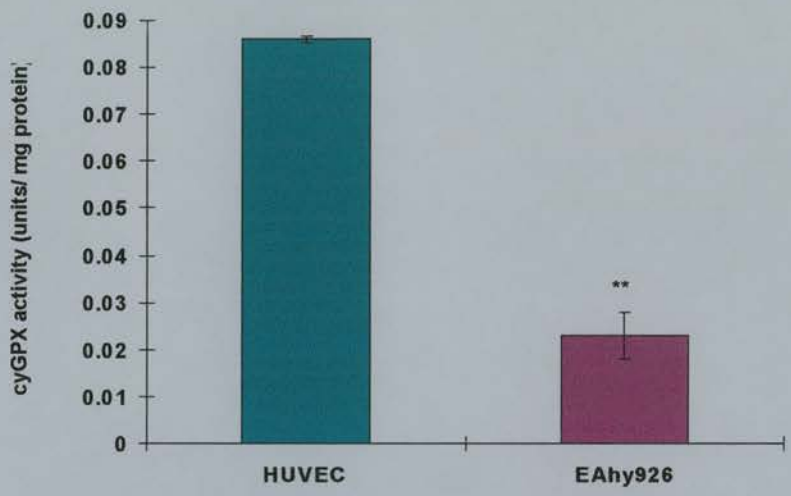


Figure 3.09. Cytoplasmic glutathione peroxidase (cyGPX) activity comparison between human umbilical vein endothelial cells (HUVEC) cultured in EGM-2 and EAhy926 cells cultured in DMEM + 10% FBS + 1% HAT. Results shown are those of the mean of 3 flasks ± SD. $p<0.001^{**}$.

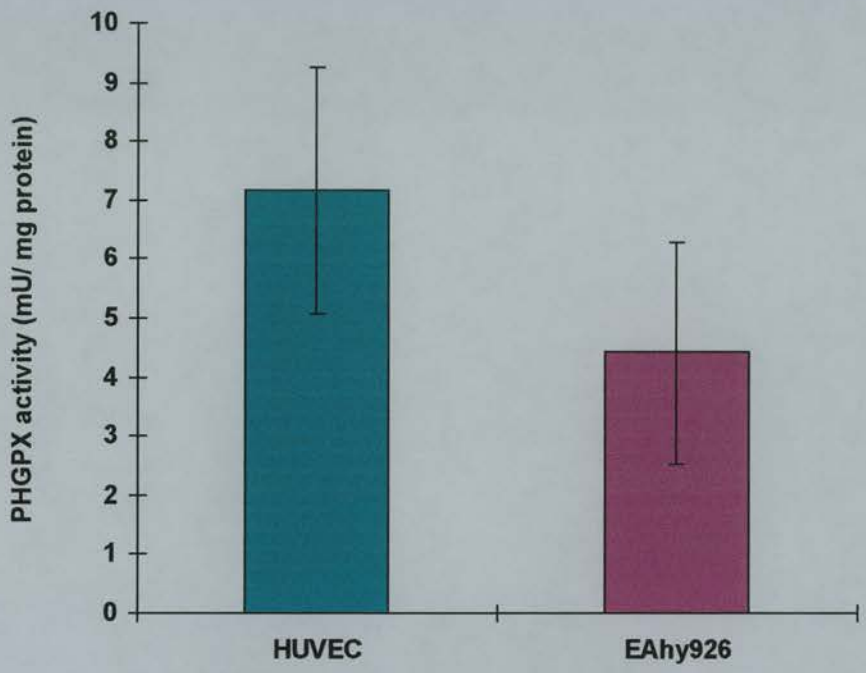


Figure 3.10. Phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity comparison between human umbilical vein endothelial cells (HUVEC) cultured in EGM-2 and EAhy926 cells cultured in DMEM + 10% FBS +1% HAT. Results shown are those of the mean of 3 flasks ± SD. No significant difference in PHGPX activity between the different cell types was found.

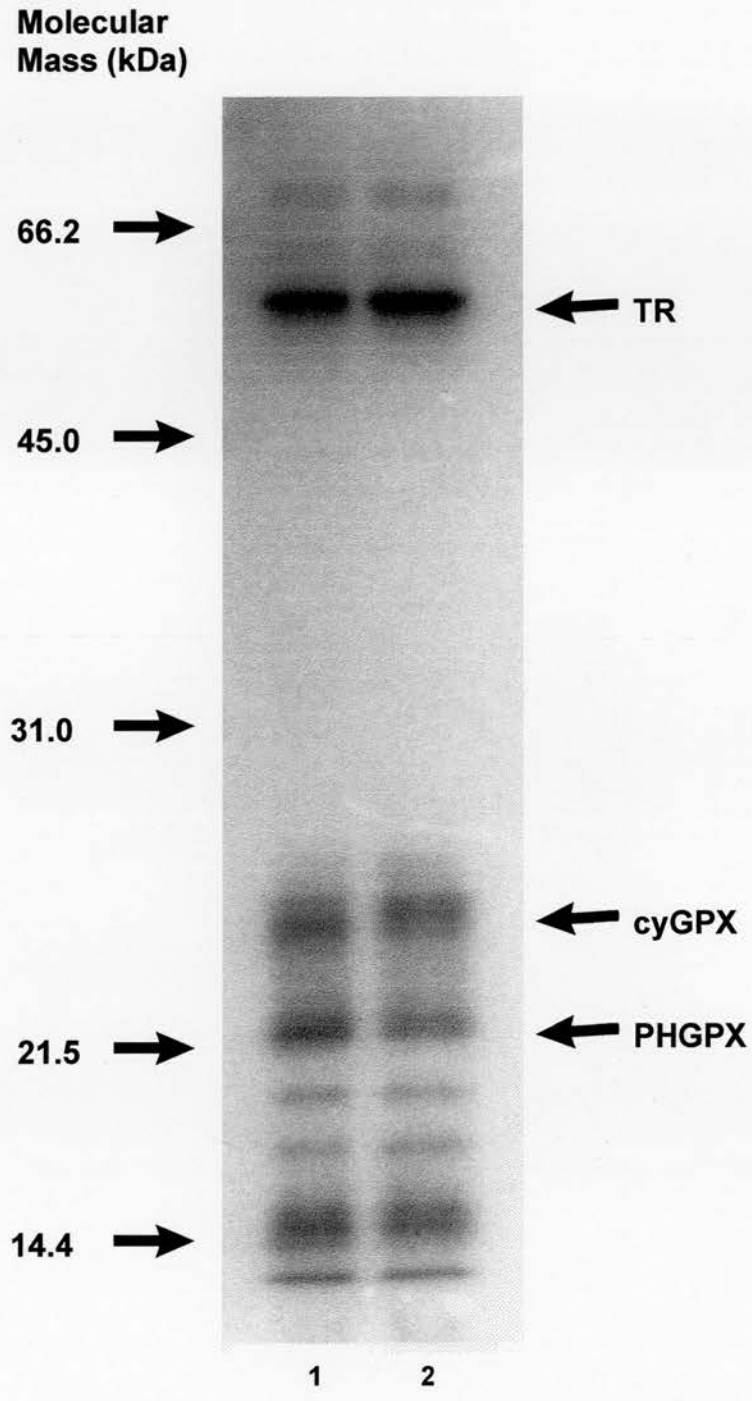


Figure 3.11. Autoradiograph of an SDS-PAGE gel of the intracellular selenoproteins of human umbilical vein endothelial cells (HUVEC) and human coronary artery endothelial cells (HCAEC) labelled with [^{75}Se]-selenite (0.02 MBq/ ml) for 48 hr. Lane 1, HUVEC; lane 2, HCAEC. Both lanes were loaded with 25 µg protein.

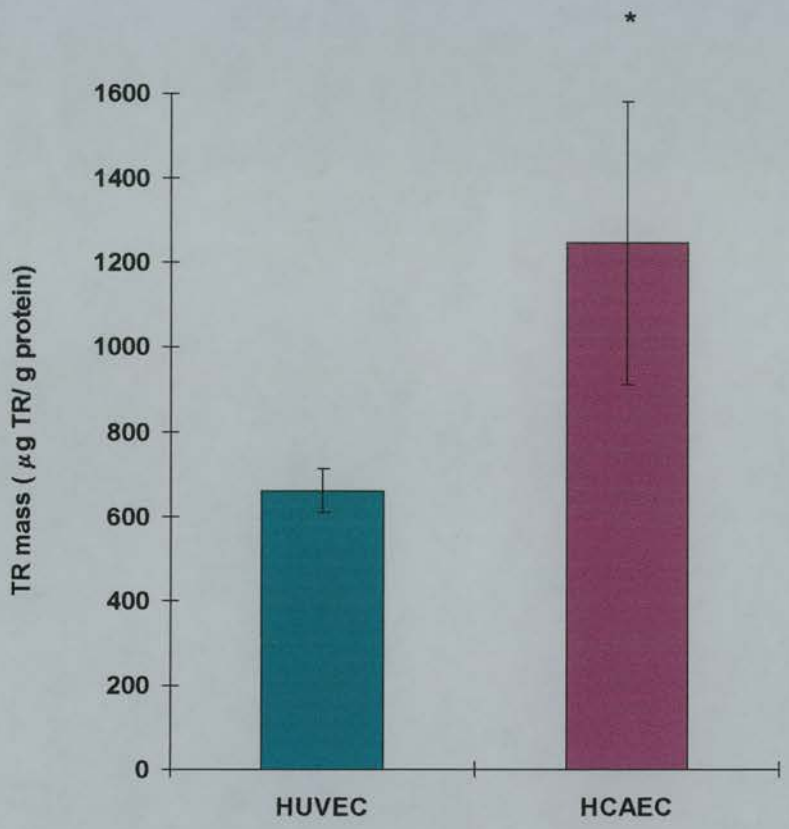


Figure 3.12. Thioredoxin reductase (TR) comparison between human umbilical vein endothelial cells (HUVEC) and human coronary artery endothelial cells (HCAEC) both grown in EGM-2 culture medium. Results shown are the mean of four flasks ± SD. $p < 0.05^{*}$.

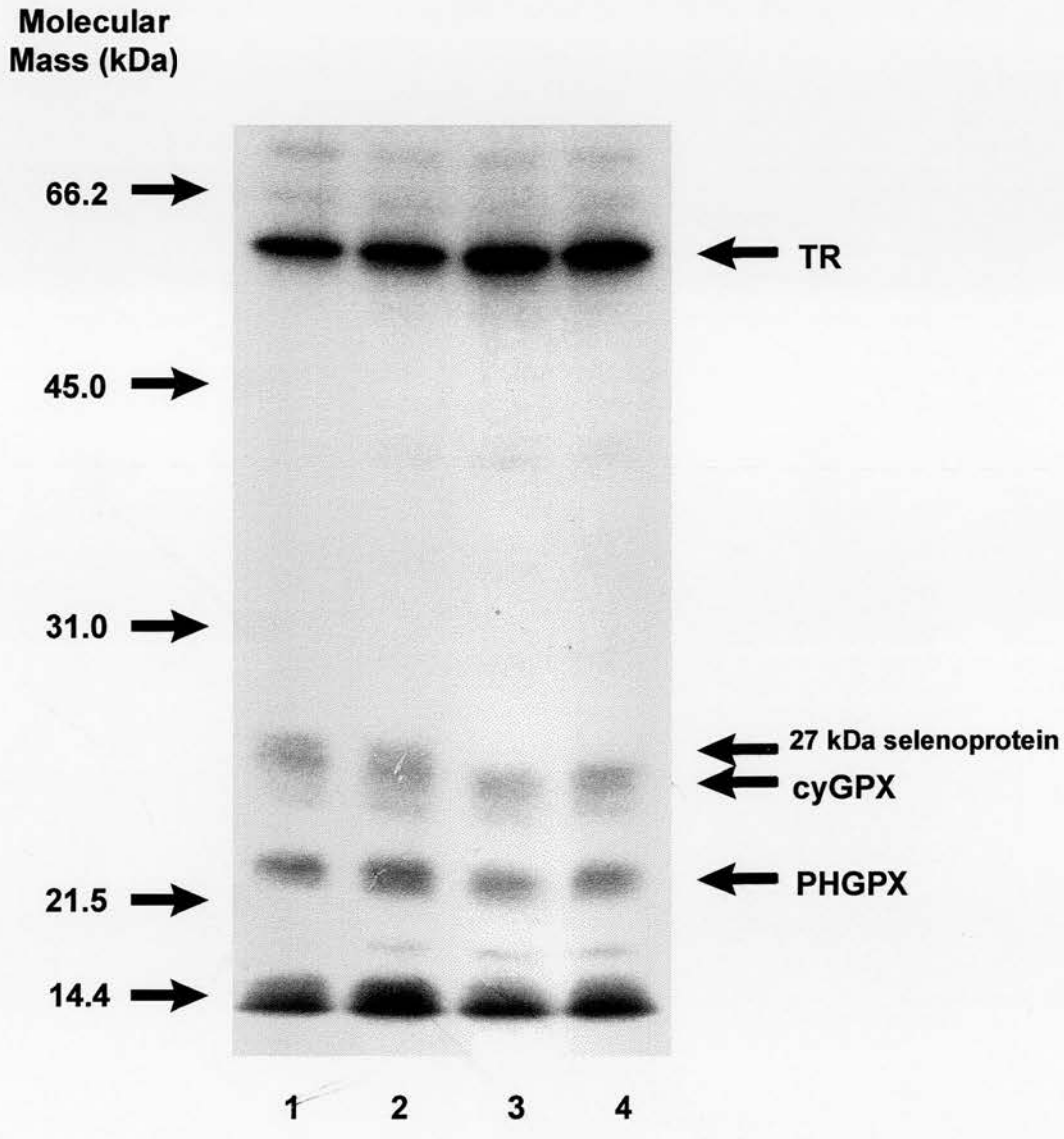


Figure 3.13. Autoradiogrpah of an SDS-PAGE gel of the intracellular selenoproteins of human umbilical vein endothelial cells (HUVEC) and human umbilical artery endothelial cells (HUAEC) labelled with [^{75}Se]-selenite (0.02 MBq/ml) for 48 hr. Both HUVEC and HUAEC were isolated from a single umbilical cord and samples were taken from duplicate flasks. Lanes 1 and 2, HUVEC; lanes 3 and 4, HUAEC. Each lane were loaded with 25 µg protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

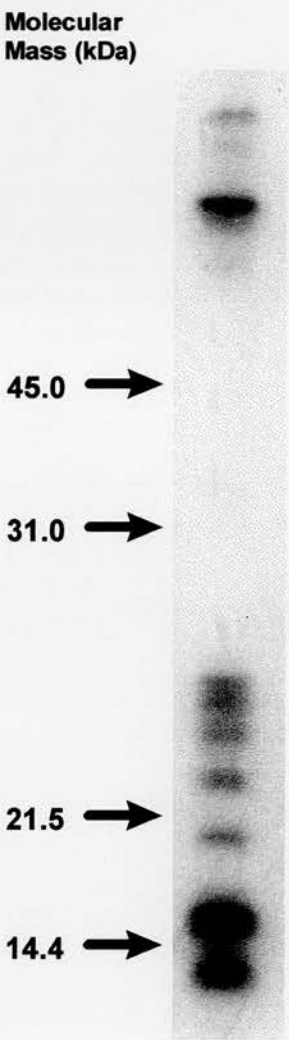


Figure 3.14. Autoradiograph of an SDS-PAGE gel of porcine aortic endothelial cells labelled with [^{75}Se]-selenite (0.02 MBq/ ml) for 48 hr. The lane was loaded with 25 µg of protein.

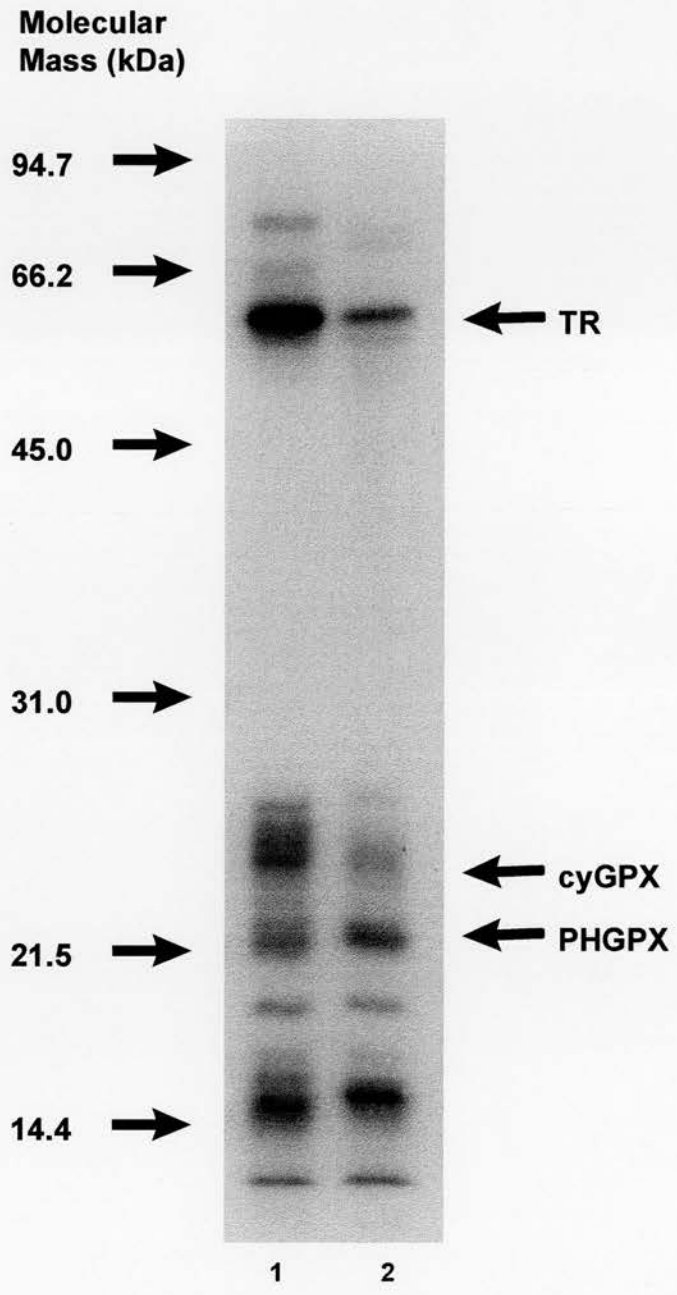


Figure 3.15. Autoradiograph of an SDS-PAGE gel of the intracellular selenoproteins of human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC) labelled with [^{75}Se] selenite (0.02 MBq/ ml) for 48 hr. Lane 1, HUVEC; lane 2, BAEC. Both lanes were loaded with 25 µg protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

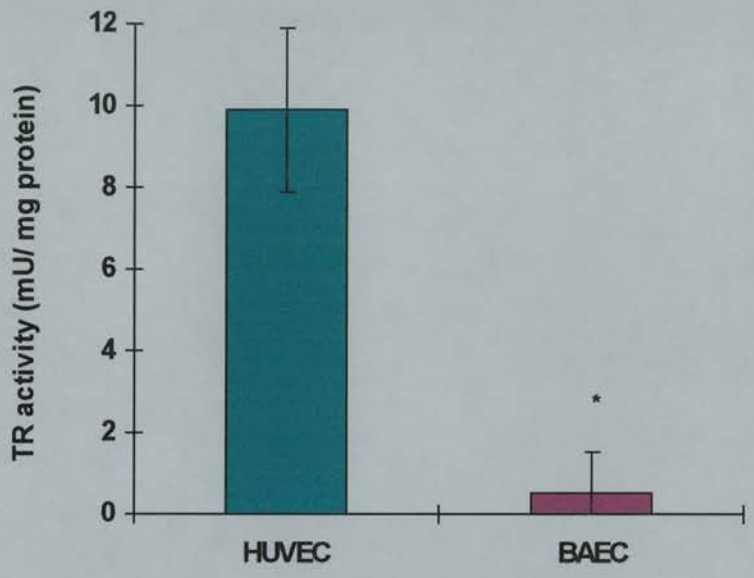


Figure 3.16. Thioredoxin reductase (TR) activity comparison between human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC). Results shown are those of the mean of 3 flasks ± SD. $p<0.01$*.

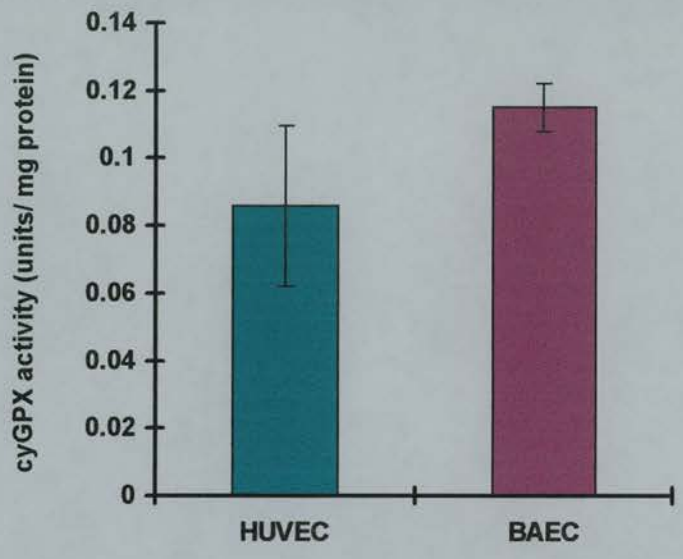


Figure 3.17. Cytoplasmic glutathione peroxidase (cyGPX) activity comparison between human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC). Results shown are those of the mean of three flasks ± SD. No significant difference in cyGPX activity between cell types was found.

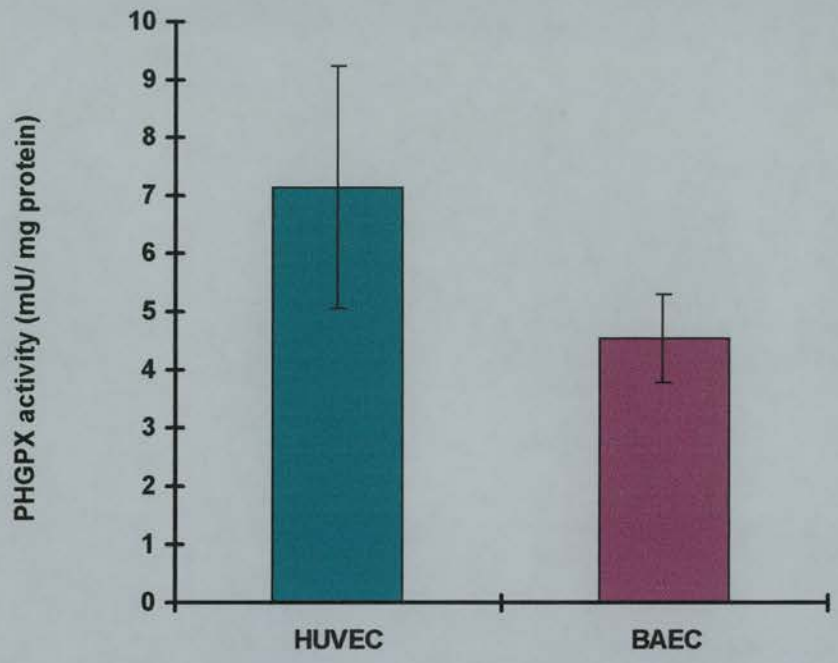


Figure 3.18. Phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity comparison between human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC). Results shown are those of the mean of three flasks ± SD. No significant difference in PHGPX activity between the different cell types was found.

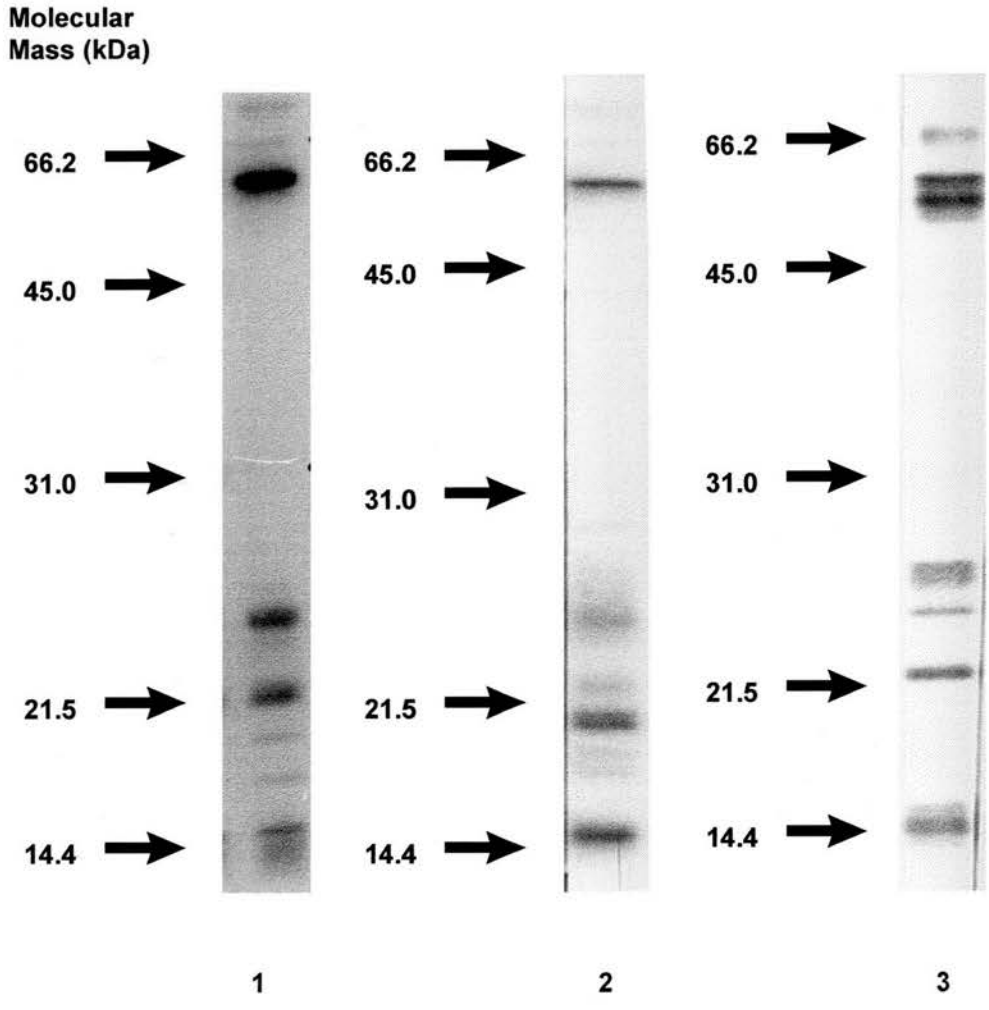


Figure 3.19. Autoradiograph of an SDS-PAGE gel of the intracellular selenoproteins of different human cells isolated from three types of human tissue labelled with [^{75}Se]-selenite (0.02 MBq/ml) for 48 hr. Lane 1, human umbilical vein endothelial cells; lane 2, human thyrocytes; lane 3, HepG2 cells. Each lane was loaded with 25 µg of protein.

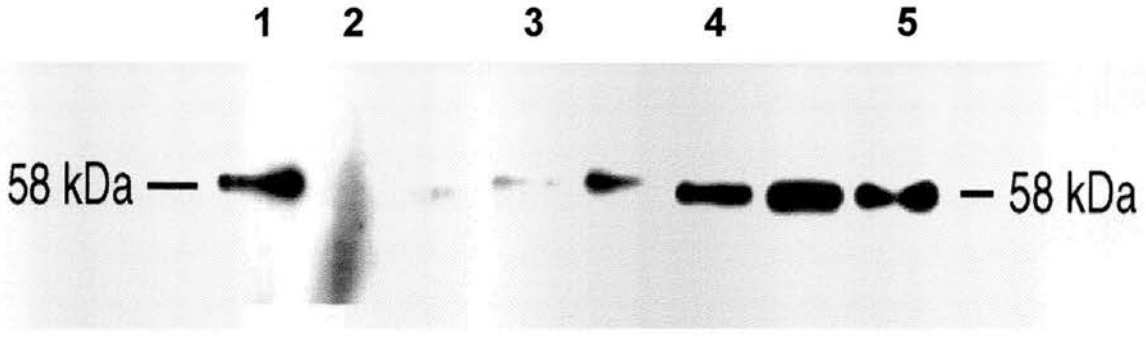


Figure 3.20. Western blot of human thyrocytes, HepG2 cells and human umbilical vein endothelial cells (HUVEC) using antiserum to thioredoxin reductase. Lane 1, purified rat thioredoxin reductase standard (5 ng); lane 2, untreated human thyrocytes (20 µg protein loaded); lane 3, untreated HepG2 cells (20 µg protein loaded); lane 4, untreated HUVEC (20 µg protein loaded); lane 5, purified human thioredoxin reductase standard (5 ng).

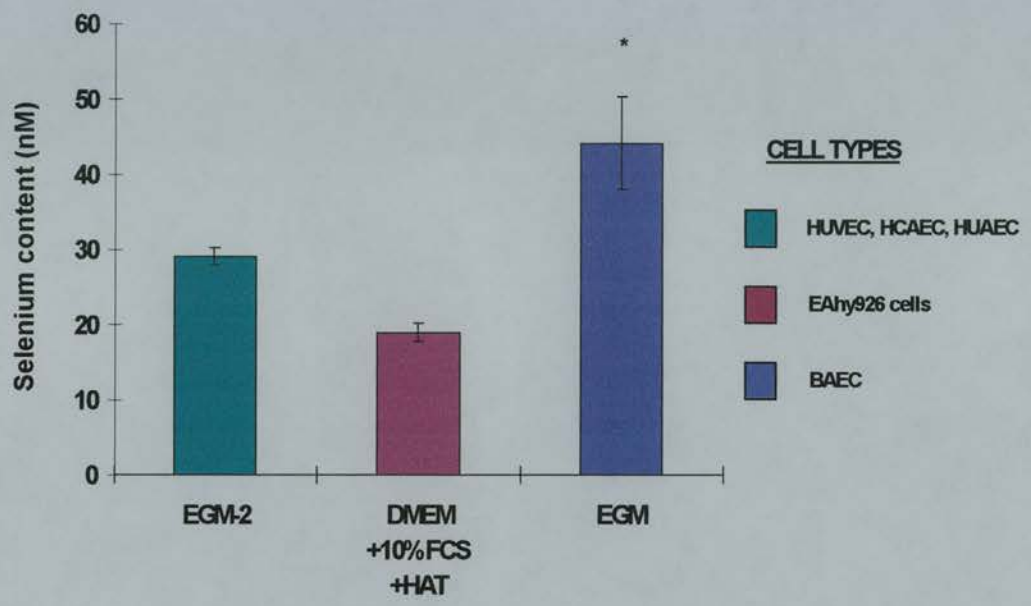


Figure 3.21. Comparison of selenium contents of various culture media used to grow and maintain some of the endothelial cells described in this chapter. The results shown are the means of triplicate measurements ± SD. $p<0.01$* from EGM-2. DMEM, Dulbecco's modified Eagle medium; EGM, endothelial growth medium; EGM-2, endothelial growth medium-2; FCS, foetal calf serum. The cell types associated with each particular culture medium are listed in the key.

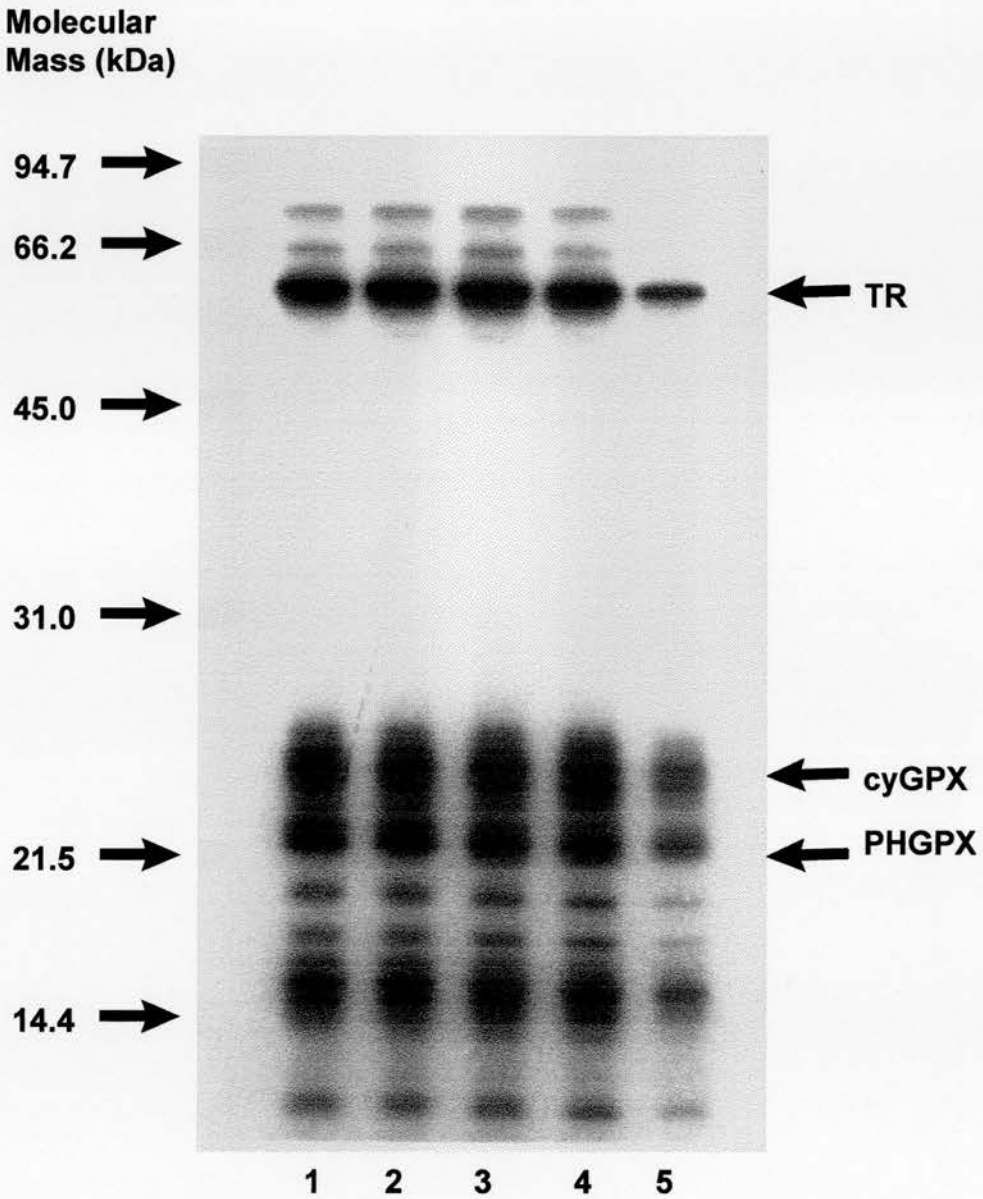


Figure 3.22. Autoradiograph of an SDS-PAGE gel of human umbilical vein endothelial cells (HUVEC) labelled with [^{75}Se]-selenite (0.02MBq/ml) in the presence of increasing doses of sodium selenite for 48 hr. Lane 1, control; lane 2, 0.1 nM sodium selenite; lane 3, 1.0 nM sodium selenite; lane 4, 10 nM sodium selenite: lane 5, 100 nM sodium selenite. Each lane was loaded with 25 µg of protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

3.4 DISCUSSION

Labelling with [^{75}Se]-selenite *in vivo* provides a precise and sensitive method to assess selenoprotein expression in tissues and cells. [^{75}Se] is either specifically directed to and incorporated into selenocysteine residues which are incorporated into selenoproteins such as in glutathione peroxidase (GPX), or it covalently binds to proteins at undefined sites as is the case with fatty-acid binding protein and protein disulphide isomerase. The time taken for the incorporation of [^{75}Se] to reach equilibrium with the endogenous pool of selenium can vary between different cell types. For example, in human thyrocytes [^{75}Se]-labelling reaches a steady state in excess of 27 hr, determined by measuring [^{75}Se]-counts at specific time points, (Beech *et al.*, 1994), whereas in HUVEC our data show that [^{75}Se] incorporation, determined by [^{75}Se]-selenoprotein labelling, reached a steady state after 48 hr. This difference can probably be ascribed to variability in selenoprotein turnover in different tissues or possibly to different rates of [^{75}Se]-selenite uptake between cells from different tissue types.

Through [^{75}Se]-labelling of intracellular selenoproteins, we have shown that human umbilical vein endothelial cells (HUVEC) show dominant expression of a selenoprotein with a molecular mass of 58 kDa. Western blot analysis using an antiserum to thioredoxin reductase (TR), characterised previously by Howie *et al.* (Howie *et al.*, 1998) identified this 58 kDa selenoprotein as TR. The presence of TR in porcine pulmonary arterial endothelial cells has been previously reported (Zhang *et al.*, 1998). However, until recently the identification of TR in endothelial cells derived from human tissue had not been described (Anema *et al.*, 1999).

Two other prominent [^{75}Se]-selenoproteins expressed by HUVEC, though labelled to a lesser extent than TR, had molecular masses of 22 kDa and 24 kDa. These two selenoproteins were tentatively identified as being PHGPX and cyGPX, respectively. Western blot analysis of HUVEC using antiserum raised to rat liver phospholipid hydroperoxide glutathione peroxidase (PHGPX) produced a single band with a slightly higher molecular mass than the rat standard used.

(PHGPX purified from rat testis was used as a standard because no human PHGPX standard was available). The single immunoreactive band in HUVEC had the same electrophoretic mobility as purified crocodile PHGPX. It is possible that the difference in electrophoretic mobility between human, crocodile and rat PHGPX may reflect a species difference in the molecular mass of PHGPX. Indeed small variations in the molecular mass and electrophoretic mobility of iodothyronine deiodinase (IDI) have been previously reported (Schoenmakers, Pigmans and Visser, 1992). HUVEC also had basal PHGPX activity. Although, the possibility that the 22 kDa [^{75}Se]-labelled selenoprotein does not represent PHGPX cannot be excluded.

It was not possible to visualise an immunoreactive band in HUVEC using human antiserum to cytoplasmic glutathione peroxidase (cyGPX). Using a commercial preparation of purified human cyGPX a protein band with a molecular weight of approximately 24 kDa was observed using Coomassie Blue staining on an SDS-PAGE gel. This band was superimposable with the 24 kDa [^{75}Se]-labelled band on the autoradiograph suggesting that this 24 kDa band was cyGPX. The Western blot was insufficiently sensitive to detect cyGPX in HUVEC lysates directly and confirmation of the identity of this 24 kDa [^{75}Se]-selenoprotein would be desirable. Other groups have shown that HUVEC express cyGPX. For example Ricetti *et al.* and Jornot and Jornod detected cyGPX mRNA through Northern blot analysis (Ricetti *et al.*, 1994; Jornot and Junod, 1997), whilst others like ourselves have demonstrated cyGPX activity in endothelial cells (Crosby, Wahle and Duthie, 1996; Ricetti *et al.*, 1994; Thomas, Geiger and Girotti, 1993). The molecular mass of cyGPX has been reported to be in the range of 19-26 kDa (Gladyshev *et al.*, 1999; Jornot and Junod, 1995; Sunde, 1994), a range which includes the 24 kDa selenoprotein band labelled in HUVEC. This evidence suggests that the 24 kDa [^{75}Se]-selenoprotein labelled in HUVEC is cyGPX although the possibility that this band may represent an alternative selenoprotein cannot not be excluded at this stage.

Many of the [^{75}Se]-selenoproteins labelled in HUVEC were unidentified. These include a [^{75}Se]-labelled protein with a molecular mass of approximately 15 kDa which could be either the uncharacterised 15 kDa selenoprotein described by Gladyshev *et al* (Gladyshev *et al.*, 1998) or epidermal fatty acid-binding protein, which has been identified previously in cultured HUVEC using immunohistochemistry (Masouyé *et al.*, 1997). Indeed, Masouyé *et al.* have demonstrated the presence of E-FABP in HUVEC (Masouyé *et al.*, 1997). Nevertheless it should be noted that immunohistochemistry carried out on the endothelium of the umbilical vein from which the endothelial cells were isolated was negative for the presence of E-FABP (Masouyé *et al.*, 1997). This implies that the expression E-FABP may be induced by cell culture and therefore this *in vitro* model may not reflect the *in vivo* E-FABP content.

In overexposed autoragiographs (figure 3.22) it was possible to visualise additional bands compared to the less exposed autoradiographs (figure 3.01). These minor bands may result from proteolysis or may be selenoprotein products of post-translational modification. However it is possible that these minor bands, only visible on overexposed autoradiographs, may be of biological importance.

The cyGPX/glutathione reductase system has been implicated in hydrogen peroxide metabolism thus forming part of the multicomponent enzymatic antioxidant defence system expressed in cultured endothelial cells which works in conjunction with SOD and catalase to maintain the redox potential within endothelial cells (Michiels, Toussaint and Remacle, 1990; Vercellotti *et al.*, 1988). PHGPX can reduce and detoxify the membrane-associated lipid hydroperoxides such as the cholesterol hydroperoxides which are inaccessible to cyGPX (Thomas *et al.*, 1990). The TR/Trx system has the ability to scavenge reactive oxygen species (Björnstedt *et al.*, 1995) indicating that it may contribute to the conventional antioxidant enzyme systems already described.

confirm the observations made by Avissar *et al* that HUVEC do not synthesise and secrete EGPX (Avissar *et al.*, 1989).

HUVEC was the chosen model in this study and was compared to the endothelial cell line, EAhy926 as well as endothelial cells isolated from different vascular beds and species. However, HUVEC can show genetic variability between preparations and cells in primary culture have a limited replication potential, tending to senesce in culture (Ager *et al.*, 1982). Consequently some enzyme activities can vary with the number of passages (Oberley *et al.*, 1995). We observed no differences in the [^{75}Se]-selenoprotein labelling of HUVEC throughout eight passages of the same preparation cultured using identical growth conditions. These findings suggest that the expression of selenoproteins is not significantly altered over eight passages. Throughout this thesis HUVEC were used at passage numbers below nine and most often using cells between passage one and four.

The intracellular [^{75}Se]-selenoprotein profiles from four different HUVEC preparations were also compared and showed no significant differences in the ratios of the various selenoproteins expressed. Thus it would appear that similar patterns of selenoprotein expression occur between most HUVEC preparations and that this profile does not change throughout eight passages.

The use of cell lines in studies of this nature is often the preferred alternative to primary cell culture, which is often complicated by genetic variability between preparations, limited population doublings and requirements for specialised growth factors. However it is possible for cell lines to lose the differentiated functions characteristic of their primary cell type which may make the cell line unsuitable as a model. For this reason, the [^{75}Se]-selenoprotein profile of the endothelial cell line, EAhy926 was compared to that of HUVEC. Differences in the intensities of labelling of some of the [^{75}Se]-selenoproteins were observed between the two cell types.

The [^{75}Se]-labelling of TR, TR mass and activity were not significantly different in HUVEC compared to EAhy926 cells. However, the 24 kDa [^{75}Se]-labelled band was less intensely labelled in EAhy926 cells. We have provided evidence to suggest that this 24 kDa selenoprotein is cyGPX and this is supported by the finding that cyGPX activity was shown also to be significantly lower in EAhy926 cells. This observation confirms the findings of Claise *et al.* who reported lower cyGPX activity in EAhy926 cell homogenates as compared to HUVEC (Claise *et al.*, 1997). The intensity of [^{75}Se]-labelling of PHGPX was slightly lower in EAhy926 cells compared to HUVEC. The measurement of PHGPX activity did not fully confirm this observation as the difference in PHGPX activity measured in HUVEC and EAhy926 cells was not significant. This disparity between the [^{75}Se]-labelling results and the activity assay may be accounted for by the poor sensitivity of the PHGPX assay.

HUVEC and EAhy926 cells were grown in culture media with a similar selenium content, although the level of bioavailable selenium is likely to be different between the two media. Therefore the possibility exists that the differences in selenoprotein expression and activity between HUVEC and EAhy926 cells, are the consequence of this variability in bioavailable selenium and may not reflect true differences between the two cell types.

Endothelial cells show functional differences according to the vascular bed from which they are derived. Arterial endothelial cells differ from venous endothelial cells in a number of respects, such as the production of angiotensin-converting enzyme, ability to form prostacyclin (Johnson, 1980) and their cell adhesion molecule response to cytokine stimulation (Hauser, Johnson and Madri, 1993). The selenoproteins expressed by endothelial cells isolated from different vascular beds were therefore compared. HUVEC and human coronary artery endothelial cells (HCAEC) were grown in the same culture medium and the cells compared were at the same passage. The [^{75}Se]-selenoprotein profiles of both cell types were very similar. The only observable differences were that in HCAEC the [^{75}Se]-labelled TR was more prominent, whilst the PHGPX was labelled

to a lesser extent. A comparison of the TR mass between the two cell types confirmed that TR expression was significantly higher in the HCAEC. These differences are more likely to reflect true differences between endothelial cells isolated from different vascular beds because as far as possible the growth and labelling conditions were identical. However it should be noted that this comparison was only carried out using one preparation of both cell types and would need to be repeated using several different preparations before drawing a final conclusion as to the extent of these differences.

Differences between the [^{75}Se]-labelled selenoprotein profiles of endothelial cells isolated from the human umbilical vein and human umbilical artery of the same umbilical cord were observed. The extra [^{75}Se]-selenoprotein in HUVEC not labelled in HUAEC, with an approximate molecular weight of 27 kDa, was unidentified but has an electrophoretic mobility consistent with it being IDI. TR mass or activity was not measured in HUAEC, though there was an increased [^{75}Se]-labelling of TR in these cells compared to HUVEC. Arterial and venous endothelial cells *in vivo* would be subject to different oxygen tensions and therefore different redox states. The expression of some selenoproteins has been shown to be regulated by oxygen tension (Berggren *et al.*, 1996b; Jornot and Junod, 1997; Das, Guo and White, 1999). The possibility that an *in vitro* cell culture model in which cells are exposed to the same oxygen tensions underestimates variations in selenoprotein expression, cannot be excluded.

The differences in [^{75}Se]-labelling of selenoproteins between HUVEC and HUAEC could reflect true differences in basal expression reflecting functional variations as both cell types were isolated from the same umbilical cord, grown in the same culture medium and were exposed to the same oxygen tension post-isolation.

Marked differences in the pattern of selenoprotein expression between human endothelial cells and endothelial cells isolated from bovine and porcine vascular beds were observed. In HUVEC

and other human endothelial cells studied, TR was the dominantly [^{75}Se]-labelled band. Although a [^{75}Se]-labelled band with a molecular mass of 58 kDa thought to be TR was observed in porcine aortic endothelial cells (PAEC), the predominant selenoprotein had an approximate molecular mass of 15 kDa. This 15 kDa [^{75}Se]-selenoprotein was also found in HUVEC but to a lesser extent. The identity of the 15kDa selenoprotein was not established, although its electrophoretic mobility is consistent with it being either the uncharacterised 15 kDa selenoprotein described by Gladyshev *et al.* (Gladyshev *et al.*, 1998) or the selenium-containing protein, epidermal fatty acid-binding protein (E-FABP) (Masouyé *et al.*, 1997).

The interpretation of the differential selenoprotein expression between HUVEC and PAEC is limited because the two cell types were grown in different culture media and were not directly compared on a single SDS-PAGE. The HUVEC were grown in EGM-2, which has a selenium concentration of approximately 29 nM, whilst PAEC were maintained in M199 supplemented with 20% FBS. The selenium content of the latter was not measured although M199 was shown to contain only trace levels of selenium and, when supplemented with 20% FBS, probably contains within the region of 25 nM selenium in total. Though the bioavailablility of this selenium to PAEC in culture is thought to be limited.

The effect of selenium-supplementation on [^{75}Se]-selenoprotein labelling was studied in HUVEC (figure 3.22). It was shown that a significant dilution of the [^{75}Se]-label was observed between 10 and 100 nM sodium selenite but in HUVEC the relative band intensities of [^{75}Se]-selenoproteins remained constant. It is possible that the lower intensity of labelling of the [^{75}Se]-selenoproteins could be the result of an isotope dilution effect although this is only likely if there is a parallel decrease in the intensity of [^{75}Se]-labelling of all the selenoproteins. The band which runs at approximately 15 kDa was more intensely labelled in PAEC than in HUVEC suggesting that its expression is truly greater in PAEC. These are only preliminary findings as only one preparation of PAEC was studied.

The pattern of [^{75}Se]-selenoprotein expression in bovine aortic endothelial cells (BAEC) differed considerably from that observed in HUVEC. The [^{75}Se]-labelling of the majority of selenoproteins was significantly lower in BAEC compared to HUVEC. One exception was a band with an approximate molecular mass of 22kDa, which was more intensely labelled in BAEC. Another exception was a [^{75}Se]-selenoprotein with an approximate molecular mass of 15kDa which in both BAEC and HUVEC was [^{75}Se]-labelled to a similar intensity. It is unknown whether this band represents the same selenoprotein or a different one.

The selenoprotein profiles of HUVEC and BAEC are not strictly comparable because each cell type required a different culture medium for optimal growth. The EGM culture medium (used to maintain the BAEC) had a selenium content approximately 20 nM higher than the EGM-2 (used to maintain HUVEC). Therefore it is possible that the lower intensity of labelling of the [^{75}Se]-selenoproteins in BAEC could be the result of an isotope dilution effect. However it is unlikely to provide a complete explanation, since PHGPX was more intensely labelled in BAEC than HUVEC and a 15 kDa selenoprotein was labelled to a similar intensity in both HUVEC and BAEC. The cyGPX activity in BAEC and HUVEC was compared and showed no significant difference. This suggests that the less intensely labelled 24 kDa band (cyGPX) is the result of an isotope dilution effect. The TR activity of HUVEC and BAEC was also compared and the results confirmed the [^{75}Se]-labelling findings, i.e. that TR activity is lower in BAEC even under conditions of higher selenium concentration which has been shown to significantly increase TR activity in BAEC (see chapter 5). The SDS-PAGE gel shows that PHGPX is expressed to a greater extent in BAEC than HUVEC. However this was not confirmed as PHGPX activity measured in HUVEC was not significantly different from that measured in BAEC.

The comparison of the GPX activities between HUVEC and BAEC showed that both PHGPX activity and cyGPX activity were not significantly different between the two cell types. The apparent disparity between the results from the SDS-PAGE and the activity assays may be

because there is a difference in turnover number between the same enzyme in different species. This effect has been reported in the selenium-containing enzyme IDI where the rate of substrate turnover per mole of protein is variable between different species (Vissar *et al.*, 1988) (Foster, Thoday and Beckett, 2000). Alternatively, the possibility that this disparity between the [^{75}Se]-labelling results and the activity results may be accounted for by the poor sensitivity of the activity assays used, cannot be excluded.

The [^{75}Se]-selenoprotein profiles of HUVEC exhibit a distinct pattern of selenoprotein expression compared to human thyrocytes and the human foetal liver-derived cell line, HepG2. In HUVEC a 58 kDa [^{75}Se]-selenoprotein, identified as TR, was the dominant selenoprotein expressed. In HepG2 cells the expression of TR was one tenth of that found in HUVEC, whilst in human thyrocytes, TR expression was too low to be detected by Western blotting; [^{75}Se]-selenoprotein labelling suggests that its expression is half of that seen in HepG2 cells. These different patterns of selenoprotein expression are presumed to underlie the requirement for different selenoproteins to contribute to the specific functions of the various cell types.

In conclusion, this study has confirmed the presence of cyGPX and PHGPX in HUVEC; however TR was found to be the dominant intracellular selenoprotein expressed by HUVEC in culture. No extracellular selenoproteins were synthesised by HUVEC. Distinct differences were observed in selenoprotein expression and activity in cells originating from various human tissues and endothelial cells isolated from different species. However these differences were less pronounced when comparing endothelial cells from different human vascular beds and the human endothelial cell line, EAhy926. These differences cannot be wholly explained by differences in selenium content and selenium bioavailability of the various culture media which were used.

The most appropriate cell culture model for the study of selenoprotein expression in atherosclerotic disease in man would be HCAEC. However the tissue from which these cells are isolated was not readily available for this study. Therefore a number of different cell culture models were investigated. Our studies have shown that endothelial cells isolated from bovine and porcine aortae may not provide a suitable alternative to HCAEC. In contrast, a distinct similarity in selenoprotein characteristics between HCAEC and HUVEC was observed. Therefore HUVEC can be regarded as a suitable alternative to HCAEC and are used as the cell culture model throughout this study.

CHAPTER FOUR REGULATION OF THIOREDOXIN REDUCTASE AND GLUTATHIONE PEROXIDASE EXPRESSION THROUGH DIFFERENT SECOND MESSENGER PATHWAYS

4.1 INTRODUCTION

The diverse homeostatic functions of the endothelium are modulated by various endogenous and exogenous biochemical and mechanical stimuli which act through a number of second messenger pathways. The signalling pathways of the endothelium include adenylate cyclase (AC), guanylate cyclase (GC) and phospholipase C (PLC) (see section 1.2.3).

The expression of some selenoenzymes has been shown to be regulated through second-messenger systems. Howie *et al.* reported that in the human foetal liver cell line, HepG2 and human thyrocytes, thioredoxin reductase (TR) expression was significantly increased by the addition of the calcium ionophore A23187 (1 μM) and PMA (1 μM) (Howie *et al.*, 1998). In contrast, extracellular glutathione peroxidase (EGPX) secretion from human thyrocytes was inhibited by A23187 (1 μM), whilst PMA (1 μM) had little or no effect (Howie *et al.*, 1995). Activation of the calcium-phosphoinositol pathway has been shown to down-regulate the expression of type-1 iodothyronine deiodinase in human thyrocytes, whilst the activation of the cyclic AMP (cAMP) pathway stimulates the expression of this selenoenzyme (Beech *et al.*, 1995). These observations suggest that activation of second messenger pathways may be important in the regulation of selenoprotein expression.

A number of different compounds have been used to stimulate second messenger pathways directly. PMA is a phorbol ester which activates protein kinase C (PKC) *in vitro* and *in vivo* at nM concentrations (Anema *et al.*, 1999; Hirata *et al.*, 1995; Keaney *et al.*, 1996; Wheeler-Jones, Sayed and Persaud, 1995). The calcium ionophore A23187 has been extensively used in

studies for increasing intracellular calcium (Corder *et al.*, 1993; Howie *et al.*, 1998; Kumar and Holmgren, 1999). Bradykinin (BK), histamine, adenosine triphosphate (ATP) and several other ligands have been shown to elevate intracellular calcium levels in cultured endothelial cells up to high nanomolar concentrations (Schmidt, Mayer and Kukovetz, 1989; Warren, 1990). The AC/cAMP pathway has been studied using 8-bromoadenosine 3' 5'-cyclic monophosphate which is a cell-permeable cAMP analogue (Beech *et al.*, 1995; Boyer and Thiery, 1993; Howie *et al.*, 1995) whilst, forskolin, directly activates AC which results in increased cAMP levels (Crutchley *et al.*, 1993).

Howie *et al.* observed a significant induction of TR expression after incubation of [^{75}Se]-selenite pre-labelled (24 hr) human thyrocytes with A23187 and PMA for 6 hr (Howie *et al.*, 1998), with a maximal induction of the enzyme occurring after 24 hr of treatment. Although TR expression was not altered after a 2 hr incubation with PMA and A23187 it is possible that short-term exposure to these agents could produce rapid effects on the signalling pathways in the cell, which elicit subsequent changes in selenoprotein expression that may only be observable after several hours.

The modification of selenoprotein expression through the activation of second messenger pathways has not been studied extensively in cultured large vessel endothelial cells. However Jornot and Jornod previously reported that treatment of human umbilical vein endothelial cells (HUVEC) with the phorbol ester phorbol 12, 13-dibutyrate induced a 2-fold increase in cytoplasmic glutathione peroxidase (cyGPX) mRNA levels which occurred 24-48 hr after treatment (Jornot and Junod, 1997).

Differences and similarities in the basal expression of intracellular selenoproteins between endothelial cells isolated from different vascular beds and different species were described in the previous chapter. Since the signalling pathways which modify selenoprotein expression may

also be dependent on species and the vascular bed used for the endothelial cell isolation, these issues are addressed in this chapter.

This study aims to:

- examine the effects of the phorbol ester PMA and the calcium ionophore A23187 on selenoprotein expression and activity in HUVEC
- examine the effects of alternative agents which stimulate the Ca^{2+}-phosphoinositol signalling pathway on selenoprotein expression in HUVEC
- examine the effect of activation of the adenylate cyclase pathway on selenoprotein expression in HUVEC
- compare the effects of PMA on selenoprotein expression in human coronary arterial endothelial cells and bovine aortic endothelial cells to those observed in HUVEC.

4.2 METHODS

4.2.1 Introduction

For all the experiments described in this section human umbilical vein endothelial cells (HUVEC) were isolated and maintained as described in section 2.3.2. Both PMA and A23187 were dissolved in dimethylsulphoxide (DMSO). Unless otherwise stated, when PMA and/or A23187 were used, the effects of DMSO were controlled for by treating the cells with 0.05% DMSO.

4.2.2 Changes in selenoprotein expression in response to protein kinase C activation in HUVEC

a) *The effect of PMA on [^{75}Se]-selenoprotein expression in HUVEC for different times.*

The concentration of PMA (0.5 μM) used for this experiment was chosen as it was the optimal dose of PMA to induce TR expression in human thyrocytes (personal communication with Dr. Forbes Howie, of this department).

The effects of PMA on the second messenger pathways in HUVEC may be rapid but the changes in selenoprotein expression may only be observable after several hours. The following experiments were designed to i) determine the effect of continual exposure of HUVEC to PMA on selenoprotein expression and ii) the influence of shorter 'pulsed' exposure times to PMA on selenoprotein expression observed after a 48 hr 'lag-period'.

i) The effect of continued exposure of PMA on selenoprotein expression in HUVEC (figure 4.01)

Confluent cultures of HUVEC were pre-labelled to steady state for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. After 48 hr the culture medium was replaced with a further 15 ml of medium containing 0.02 MBq/ml [^{75}Se]-selenite to ensure that the selenium supply was not exhausted. The effect of PMA (0.5 μM) on the selenoprotein profile was studied by the inclusion of this compound in the

culture medium for 1 min, 12 hr, 24 hr, 48 hr, 72 hr or 96 hr in the continuing presence of 0.02 MBq/ml [^{75}Se]-selenite. The additions were timed to ensure that the cells for each data point were harvested immediately after the defined incubation period with PMA but after the same overall length of culture time, ensuring that all cells were cultured with [^{75}Se]-selenite for a total culture time of 144 hr which included the 48 hr pre-incubation period.

After incubation the cells were harvested into 20 ml EBSS by scraping and centrifuged at 2000 g for 10 min at 4°C. The resulting cell pellet was resuspended in 200 µl 60 mM Tris buffer, pH 7.4 (4°C), containing 1 mM EDTA and 1 mM dithiothreitol (Tris buffer). The cells were lysed by sonication on ice for 30 sec using the Soniprep 150.

Protein concentrations were measured using the Bradford assay (section 2.3.12) and the samples were diluted to a common protein concentration with Tris buffer. The cell lysates were prepared for separation by SDS-PAGE (section 2.3.13) and the [^{75}Se]-labelled proteins present in 25 µg of protein were separated by SDS-PAGE (section 2.3.13). The resulting gel was dried and the [^{75}Se]-labelled selenoproteins visualised by autoradiography using Kodak X-OMAT XAR-5 film (section 2.3.14).

The SDS-gels were scanned using an Epson$_{GT}$-9500 to create a digitilized image (section 2.3.15). The radioactivity in each band was quantified using the Phoretix software. The same system was used to quantify TR expression in the Western blots.

ii) The effect of 'pulsed' exposure to PMA on selenoprotein expression in HUVEC (figure 4.02)

Confluent cultures of HUVEC were pre-labelled to a steady state for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. The addition of PMA (0.5 µM) was made to these pre-labelled cells for 1 min, 10 min, 1 hr or 12 hr in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. Each addition was made at the same time and after each time of exposure the culture medium was removed from

the flask and the cells were washed to remove any residual PMA. Fresh culture medium containing 0.02 MBq/ml [^{75}Se]-selenite but without PMA, was added and the cells were incubated for a further period such that the total length of incubation from when PMA was initially added was 48 hr. This total incubation time of 48 hr was chosen because the time course experiment (described in 'i' above) showed that at 48 hr in the continual presence of PMA maximal changes in the expression of [^{75}Se]-selenoproteins occurred. HUVEC were harvested and the intracellular [^{75}Se]-selenoproteins separated by SDS-PAGE, visualized by autoradiography and analysed using densitometry as described in section 4.2.2ai).

b) ***The effect of PMA on thioredoxin reductase and glutathione peroxidase expression in HUVEC***

Triplicate T75 flasks of confluent cultures of HUVEC were incubated in the presence of PMA (0.5 µM) for 48 hr. The cells were harvested as described in section 4.2.2a and lysed by sonication in 0.125 M potassium phosphate buffer, pH 7.4, containing 1mM EDTA for 30 sec using the Soniprep 150 (4°C).

The HUVEC lysates were then assayed for thioredoxin reductase (TR) mass and activity, cytoplasmic glutathione peroxidase (cyGPX) activity and phospholipid hydroperoxide glutathione peroxidase activity (PHGPX) activity as described in sections 2.3.17, 2.3.18, 2.3.19 and 2.3.20 respectively.

c) Effects of GF109203X and PMA on the expression of [^{75}Se]-selenoproteins in HUVEC

Confluent cultures of HUVEC were pre-labelled to a steady state with 0.02 MBq/ml [^{75}Se]-selenite for 48 hr prior to investigating the effect of the specific PKC inhibitor GF109203X in the presence and absence of PMA on [^{75}Se]-selenoprotein expression in HUVEC (figure 4.03). After the 48 hr pre-labelling period, the culture medium was removed and replaced with fresh medium containing 0.02 MBq/ml [^{75}Se]-selenite. GF109203X at 3 different doses (5 µM, 1 µM, 0.5 µM)

was added to the culture medium 1 hr prior to any PMA additions, (previous studies in HUVEC have shown PKC activity is inhibited under these conditions (Villard *et al.*, 1998)). After the addition of PMA (0.5 μM), the HUVEC were incubated for a further 48 hr before the cells were harvested and [^{75}Se]-labelled selenoproteins separated by SDS-PAGE as described previously (section 4.2.2a). The [^{75}Se]-selenoproteins were visualized by autoradiography as described in section 2.3.14.

d) The effects of prolonged exposure and cumulative doses of PMA on [^{75}Se]-selenoprotein expression in HUVEC

Confluent cultures of HUVEC were pre-labelled to a steady state with 0.02 MBq/ml [^{75}Se]-selenite for 48 hr prior to investigating the effects of cumulative doses of PMA on [^{75}Se]-selenoprotein expression (figure 4.04). The effect of PMA on the [^{75}Se]-labelled selenoproteins was studied by the inclusion of PMA (0.5 μM) in the culture medium for 1 min, 48 hr and 96 hr in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. The additions were timed so that the cells for each data point were harvested at 96 hr after the initial pre-labelling period. For the 96 hr time point two flasks were incubated with PMA (0.5 μM). In one of these flasks after 48 hr in the presence of PMA a further addition of PMA was made to give a final concentration of 1 μM PMA.

HUVEC were harvested as described above and the intracellular [^{75}Se]-selenoproteins were separated by SDS-PAGE and visualized by autoradiography (section 4.2.2a).

4.2.3 The effects of PMA on the selenoprotein expression in human coronary arterial endothelial cells

Human coronary arterial endothelial cells (HCAEC) were maintained as described in section 2.3.5. Confluent cultures of HCAEC were pre-labelled to steady state for 48 hr in the presence of 0.02 MBq/ml [^{75}Se]-selenite. The cells were then incubated with PMA (0.5 μM) for 48 hr.

HCAEC were harvested as described above and the intracellular [^{75}Se]-selenoproteins were separated by SDS-PAGE and visualized by autoradiography (sections 2.3.13 and 2.3.14).

For measurement of TR mass and cyGPX and PHGPX activity, triplicate T75 flasks of confluent cultures HCAEC were incubated in the presence of PMA (0.5 µM) for 48 hr and cell lysates prepared as described for HUVEC in section 4.2.2b.

The HCAEC lysates were then assayed for TR mass, cyGPX activity and PHGPX activity as described in sections 2.3.17, 2.3.19 and 2.3.20. TR activity was not measured in HCAEC.

4.2.4 The effects of PMA on the selenoprotein expression in bovine aortic endothelial cells

The effect of PMA (0.5 µM) on the [^{75}Se]-selenoprotein expression in bovine aortic endothelial cells (BAEC) was established using the same protocol as described above for HCAEC in section 4.2.3.

For measurement of TR activity triplicate T75 flasks of confluent cultures of BAEC were incubated in the presence of PMA (0.5 µM) for 48 hr and cell lysates prepared and assayed for TR activity as described for HUVEC in section 4.2.2b.

The BAEC cell lysates were not assayed for TR mass because there was a lack of cross-reactivity between the anti-human TR antibody with bovine TR in the radioimmunoassay. cyGPX and PHGPX activities were also not measured in BAEC.

4.2.5 Changes in selenoprotein expression in response to the calcium ionophore A23187 in HUVEC

a) Dose-response effects of A23187 on the [^{75}Se]-selenoprotein expression in HUVEC

Confluent cultures of HUVEC were pre-labelled to a steady state with 0.02 MBq/ml [^{75}Se]-selenite for 48 hr (as described in section 4.2.2) prior to investigating the effects of A23187 on the [^{75}Se]-labelling of intracellular selenoproteins in HUVEC. A23187 was then added to HUVEC to achieve final concentrations of 0.5 nM, 5 nM, 50 nM and 0.5 µM in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. After 24 hr HUVEC were harvested and the intracellular [^{75}Se]-selenoproteins separated by SDS-PAGE and visualized by autoradiography as described in section 4.2.2a.

b) The effect of A23187 on [^{75}Se]selenoprotein expression in HUVEC for different times

The concentration of A23187 (0.5 µM) used for this experiment was selected on the basis of the experiment described in section 4.2.4a, which had shown optimal changes in [^{75}Se]-labelling of selenoproteins at this concentration.

As with PMA the effects of A23187 (0.5 µM) on the second messenger pathways in HUVEC may be rapid, whilst the changes in selenoprotein expression may only be observable after several hours. The time course experiments used to study the effects of PMA were therefore repeated using A23187 as described section 4.2.2a. In the first series of experiments continuous exposure of A23187 was studied (figure 4.05), whilst in the second series of experiments the influence of short 'pulsed' exposure times of HUVEC to A23187 on the subsequent maximal changes observed in the expression of selenoproteins was studied (figure 4.06). The incubation time chosen to study the maximal changes in selenoprotein expression in response to A23187 i.e. the second series of experiments, was 35 hr not 48 hr. This was chosen as it was not always possible to use time points in excess of 35 hr as A23187 occasionally caused cell detachment if time periods longer than this were used.

4.2.6 The effect of PMA and A23187 added alone or in combination on the expression of selenoproteins

The effects of PMA (0.5 µM) and A23187 (0.5 µM), added individually or in combination on the expression of [^{75}Se]-selenoproteins were investigated by the inclusion of the compounds in the culture medium of HUVEC for 35 hr in the continuous presence of 0.02 MBq/ml [^{75}Se]-selenite. Prior to treatment with PMA and/or A23187 HUVEC were pre-labelled with 0.02 MBq/ml [^{75}Se]-selenite for 48 hr. HUVEC were harvested and the intracellular [^{75}Se]-selenoproteins were separated by SDS-PAGE and visualized by autoradiography as described in section 4.2.2a. The TR expression in HUVEC incubated with PMA and/or A23187 was quantified by Western blot analysis using the method described in section 2.3.16.

4.2.7 The effects of acetylcholine, adenosine triphosphate and bradykinin on the [^{75}Se]-selenoprotein expression in HUVEC

The effects of acetylcholine (1 mM) and adenosine triphosphate (1 mM) on the [^{75}Se]-selenoprotein expression was studied by the inclusion of these compounds (made up in EBSS) individually in confluent cultures of HUVEC in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. The cells had been pre-labelled to a steady state for 48 hr with 0.02 MBq/ ml [^{75}Se]-selenite prior to the addition of the compounds to be tested. After 48 hr HUVEC were harvested and the intracellular [^{75}Se]-selenoproteins were separated by SDS-PAGE and visualized by autoradiography as previously described in section 4.2.2a.

The effect of bradykinin (1 µM) was studied by the inclusion of this compound into the culture medium of pre-labelled HUVEC for 6 hr, 12 hr, 24 hr and 48 hr in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. The additions were timed to ensure that all the cells for each data point were harvested immediately after the defined incubation period but so that cells were cultured

for the same period of time. HUVEC were harvested as described above and the intracellular [^{75}Se]-selenoproteins were separated by SDS-PAGE and visualized by autoradiography.

4.2.8 The effect of forskolin on the [^{75}Se]-selenoprotein expression in HUVEC

HUVEC were pre-labelled to a steady state for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. The effect of forskolin on the [^{75}Se]-selenoprotein expression was studied by the inclusion of forskolin (0.1 μM) in the culture medium in the continued presence of 0.02 MBq/ml [^{75}Se]- selenite. This concentration of forskolin was chosen as it induced steroidogenic responses in bovine adrenal cells (personal communication from Mrs Moira Nicol of this department). After 48 hr HUVEC were harvested and the intracellular [^{75}Se]-selenoproteins were separated by SDS-PAGE and visualized by autoradiography as previously described in sections 4.2.2a.

To control for any possible effects of ethanol, in which the forskolin was dissolved, HUVEC were treated with 0.1% ethanol.

4.2.9 Statistical analysis

An unpaired 't' test was used to test for significance between the levels of selenoprotein expression or activity in treated cells compared to control cells. The Students 't' test assumes that the standard deviations (SD) are equal. In cases where there was found to be a significant difference in the SD the 't' test was used with a Welch correction.

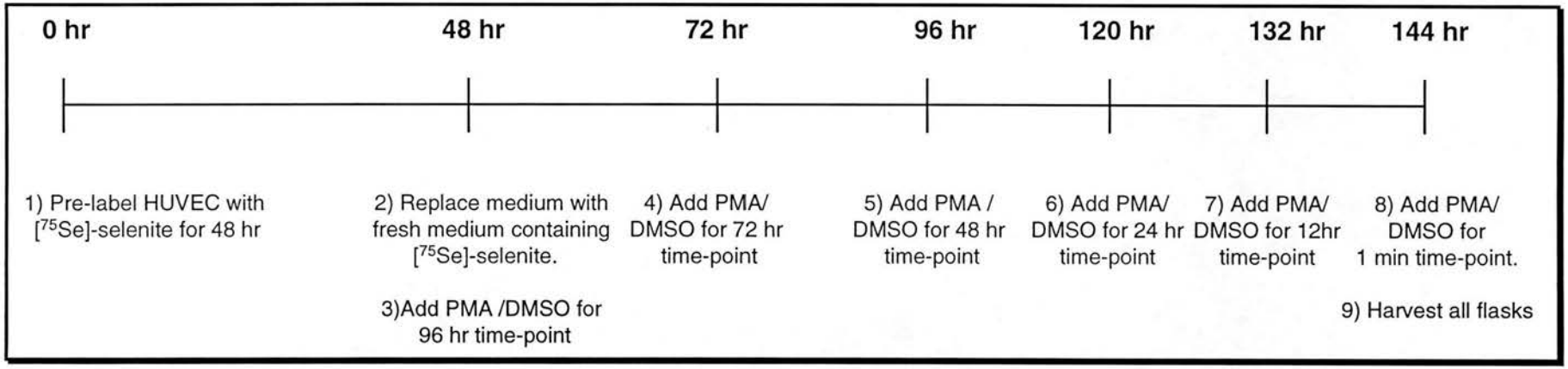


Figure 4.01. Schematic representation to illustrate the key steps in the procedure used to determine the effect of continual exposure of PMA on selenoprotein expression in HUVEC. 1) Confluent cultures of HUVEC were pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. 2) At 48 hr the culture medium from all flasks was replaced with a further 15 ml of medium containing 0.02 MBq/ml [^{75}Se]-selenite. 3) At 48 hr 0.5 µM PMA or 0.05% DMSO was added to a flask of HUVEC for the 96 hr time-point. 4) At 72 hr 0.5 µM PMA or 0.05% DMSO was added to a flask of HUVEC for the 72 hr time-point. 5) At 96 hr 0.5 µM PMA or 0.05% DMSO was added to a flask of HUVEC for the 48 hr time-point. 6) At 120 hr 0.5 µM PMA or 0.05% DMSO was added to a flask of HUVEC for the 24 hr time-point. 6) At 132 hr 0.5 µM PMA or 0.05% DMSO was added to a flask of HUVEC for the 12 hr time-point. 7) At 144 hr 0.5 µM PMA or 0.05% DMSO was added to a flask of HUVEC for the 1 minute time-point. 8) At 144 hr and 1 minute all the flasks of HUVEC were harvested beginning with the 1 minute time-point.

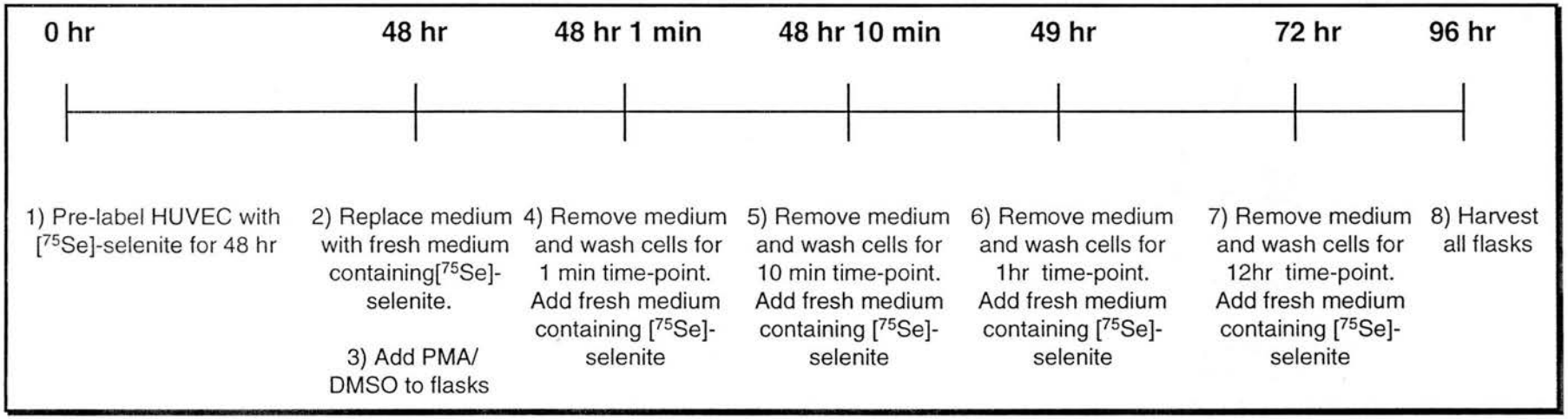


Figure 4.02. Schematic representation to illustrate the key steps in the procedure used to determine the effect of 'pulsed' exposure to PMA on selenoprotein expression in HUVEC. 1) Confluent cultures of HUVEC were pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. 2) At 48 hr the culture medium from all flasks was replaced with a further 15 ml of medium containing 0.02 MBq/ml [^{75}Se]-selenite. 3) At 48 hr 0.5 µM PMA or 0.05% DMSO was added all flasks of HUVEC. 4) At 48 hr and 1 minute the culture medium was removed from the 1 minute time-point flask and the HUVEC washed. Fresh medium containing 0.02 MBq/ml [^{75}Se]-selenite was added to this flask. 5) At 48 hr and 10 minutes the culture medium was removed from the 10 minute time-point flask and the HUVEC washed. Fresh medium containing 0.02 MBq/ml [^{75}Se]-selenite was added to this flask. 6) At 49 hr the culture medium was removed from the 1 hr time-point flask and the HUVEC washed. Fresh medium containing 0.02 MBq/ml [^{75}Se]-selenite was added to this flask. 7) At 72 hr the culture medium was removed from the 12 hr time-point flask and the HUVEC washed. Fresh medium containing 0.02 MBq/ml [^{75}Se]-selenite was added to this flask. 8) The HUVEC from all flasks were harvested.

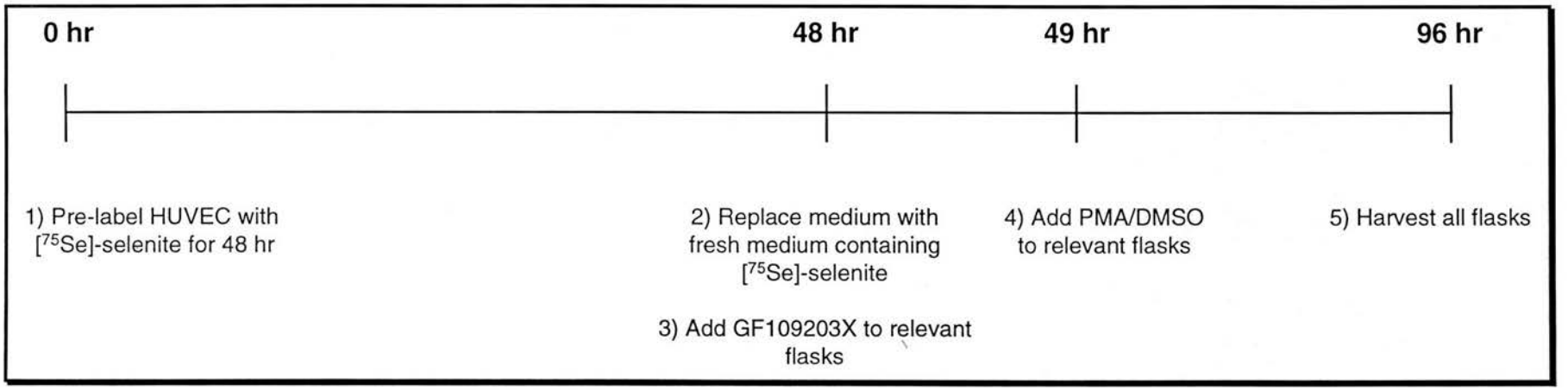


Figure 4.03. Schematic representation to illustrate the key steps in the procedure used to determine the effects of GF109203X and PMA on the expression of selenoproteins in HUVEC. 1) Confluent cultures of HUVEC were pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. 2) At 48 hr the culture medium from all flasks was replaced with a further 15 ml of medium containing 0.02 MBq/ml [^{75}Se]-selenite. 3) At 48 hr different concentrations of GF109203X (5 μM, 1 μM, 0.5 μM) were added to the relevant flasks of HUVEC. 4) At 49 hr 0.5 μM PMA or 0.05% DMSO was added to the relevant flasks of HUVEC. 5) The HUVEC from all flasks were harvested.

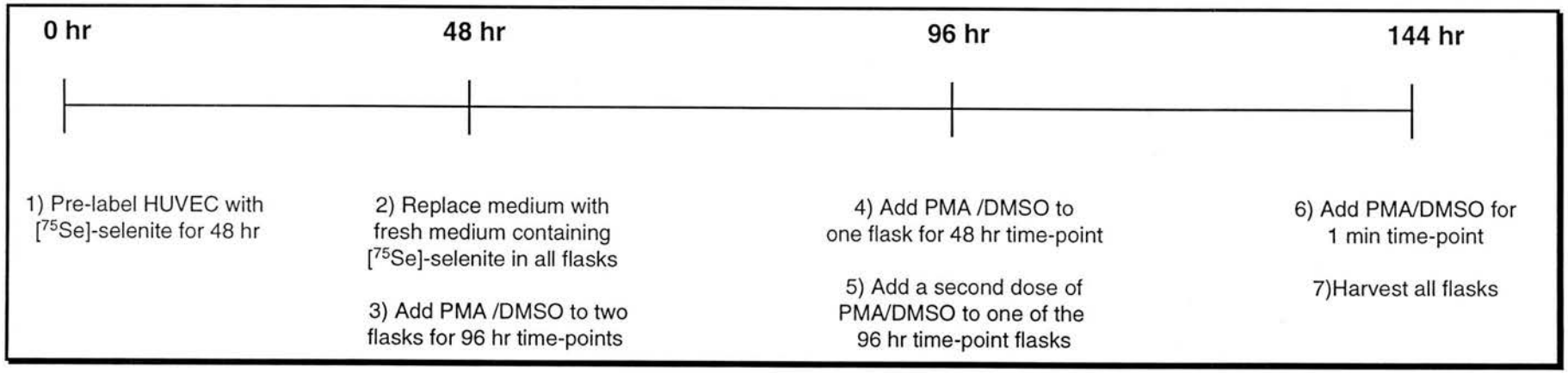


Figure 4.04. Schematic representation to illustrate the key steps in the procedure used to determine the effects of prolonged exposure and cumulative doses of PMA on the expression of selenoproteins. 1) Confluent cultures of HUVEC were pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. 2) At 48 hr the culture medium from all flasks was replaced with a further 15 ml of medium containing 0.02 MBq/ml [^{75}Se]-selenite. 3) At 48 hr 0.5 µM PMA or 0.05% DMSO was added to two flasks of HUVEC for the 96 hr time-points. 4) At 96 hr 0.5 µM PMA or 0.05% DMSO was added to a flask of HUVEC for the 48 hr time-point. 5) At 48 hr a second dose of 0.5 µM PMA or 0.05% DMSO was added to one of the 96 hr time-point flasks of HUVEC. 6) At 144 hr 0.5 µM PMA or 0.05% DMSO was added to a flask of HUVEC for the 1 minute time-point. 7) At 144 hr and 1 minute all the flasks of HUVEC were harvested beginning with the 1 minute time-point.

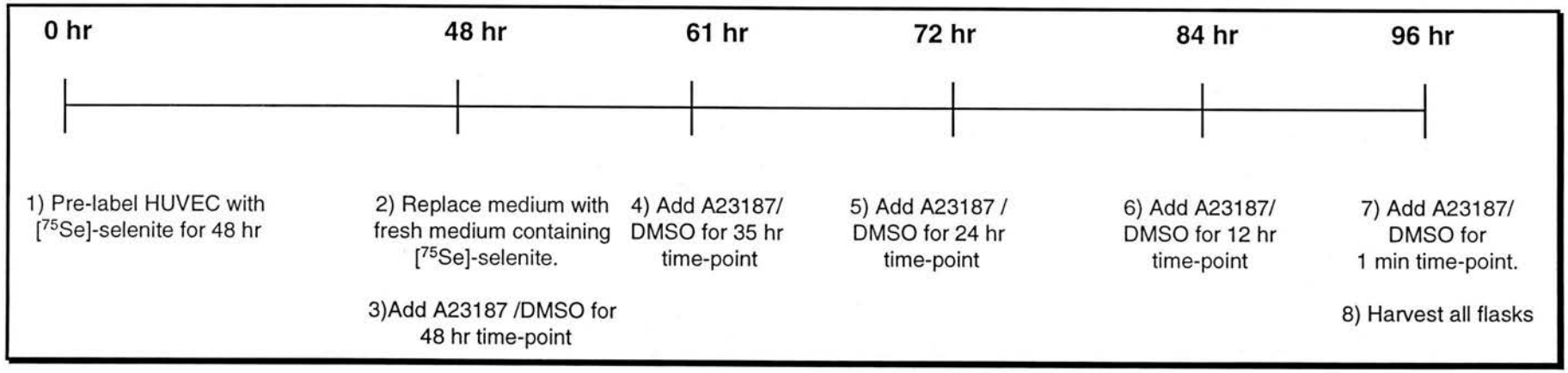


Figure 4.05. Schematic representation to illustrate the key steps in the procedure used to determine the effect of continual exposure of A23187 on selenoprotein expression in HUVEC. 1) Confluent cultures of HUVEC were pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. 2) At 48 hr the culture medium from all flasks was replaced with a further 15 ml of medium containing 0.02 MBq/ml [^{75}Se]-selenite. 3) At 48 hr 0.5 μM A23187 or 0.05% DMSO was added to a flask of HUVEC for the 48 hr time-point. 4) At 61 hr 0.5 μM A23187 or 0.05% DMSO was added to a flask of HUVEC for the 35 hr time-point. 5) At 72 hr 0.5 μM A23187 or 0.05% DMSO was added to a flask of HUVEC for the 24 hr time-point. 6) At 84 hr 0.5 μM A23187 or 0.05% DMSO was added to a flask of HUVEC for the 12 hr time-point. 7) At 96 hr 0.5 μM A23187 or 0.05% DMSO was added to a flask of HUVEC for the 1 minute time-point. 8) At 96 hr and 1 minute all the flasks of HUVEC were harvested beginning with the 1 minute time-point.

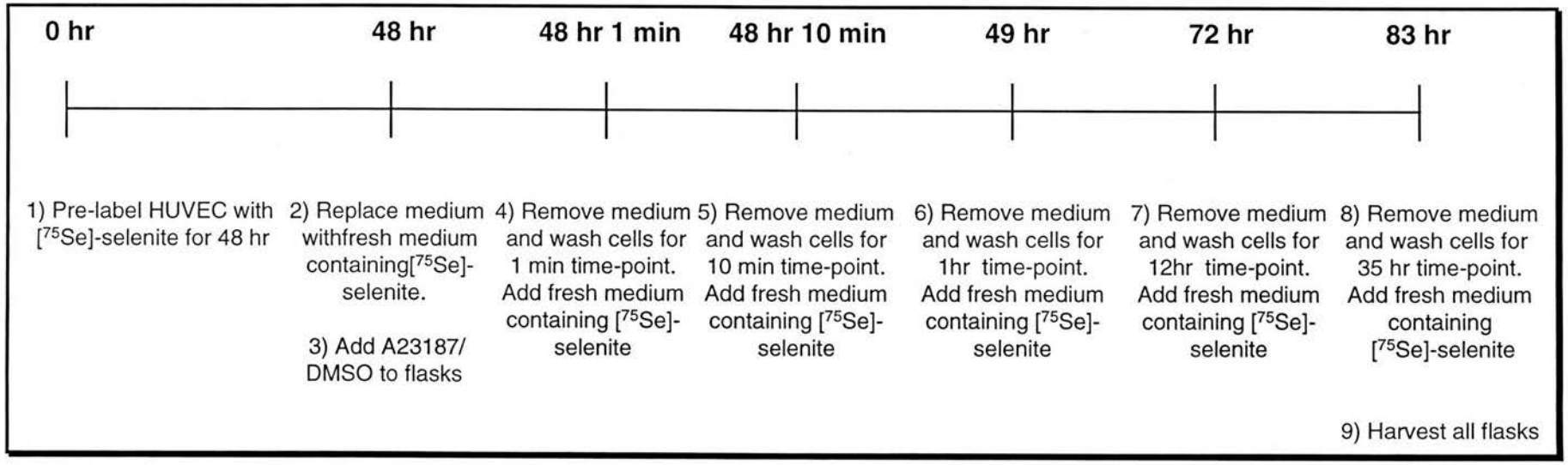


Figure 4.06. Schematic representation to illustrate the key steps in the procedure used to determine the effect of 'pulsed' exposure to A23187 on selenoprotein expression in HUVEC. 1) Confluent cultures of HUVEC were pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. 2) At 48 hr the culture medium from all flasks was replaced with a further 15 ml of medium containing 0.02 MBq/ml [^{75}Se]-selenite. 3) At 48 hr 0.5 μM A23187 or 0.05% DMSO was added all flasks of HUVEC. 4) At 48 hr and 1 minute the culture medium was removed from the 1 minute time-point flask and the HUVEC washed. Fresh medium containing 0.02 MBq/ml [^{75}Se]-selenite was added to this flask. 5) At 48 hr and 10 minutes the culture medium was removed from the 10 minute time-point flask and the HUVEC washed. Fresh medium containing 0.02 MBq/ml [^{75}Se]-selenite was added to this flask. 6) At 49 hr the culture medium was removed from the 1 hr time-point flask and the HUVEC washed. Fresh medium containing 0.02 MBq/ml [^{75}Se]-selenite was added to this flask. 7) At 72 hr the culture medium was removed from the 12 hr time-point flask and the HUVEC washed. Fresh medium containing 0.02 MBq/ml [^{75}Se]-selenite was added to this flask. 8) At 83 hr the culture medium was removed from the 35 hr time-point flask and the HUVEC washed. Fresh medium containing 0.02 MBq/ml [^{75}Se]-selenite was added to this flask. 9) The HUVEC from all flasks were harvested.

4.3 RESULTS

4.3.1 Introduction

The 22 kDa, 24 kDa and 58 kDa bands observed on an autoradiograph of an SDS-PAGE gel showing the [^{75}Se]-selenoproteins expressed by HUVEC have been identified in section three as PHGPX, cyGPX and TR respectively. Therefore these bands whether expressed by HUVEC or other endothelial cells are referred to as these selenoproteins in the text.

4.3.2 Changes in selenoprotein expression in response to protein kinase C activation in HUVEC

a) *The effect of PMA on [^{75}Se]-selenoprotein expression in HUVEC for different times*

The effect of PMA on the expression of intracellular selenoproteins was time-dependent. The continued presence of PMA (0.5 μM) markedly decreased the expression of TR and PHGPX. In contrast, the expression of cyGPX was increased in the presence of PMA. The change in the expression of the TR following exposure to PMA was first apparent after 12 hr, with the lowest level of expression observed after 48 hr exposure (figure 4.07). At 72 hr and 96 hr of exposure, the expression of TR started to rise but the level did not return to the level observed in the control DMSO-treated HUVEC. The changes in expression of both the PHGPX and cyGPX were observed at 12 hr exposure and at each subsequent time point thereafter the expression of PHGPX continued to decrease, whilst the expression of cyGPX continued to rise.

The effects of the shorter 'pulsed' exposures to PMA (0.5 μM) are shown in figure 4.08. In HUVEC transiently exposed to PMA for 1 min to 12 hr, a decrease in the expression of both TR and PHGPX was measured after a 48 hr lag period following the initial exposure to PMA for 1 min. In contrast, the increased expression of cyGPX was observed after a 10 min exposure to PMA measured after a 48 hr lag period.

b) ***The effect of PMA on thioredoxin reductase and glutathione peroxidase expression in HUVEC***

Changes in TR mass and activity, cyGPX and PHGPX activities in HUVEC were measured after a 48 hr incubation with PMA (0.5 μM) (figures 4.09-4.12).

The TR mass and activity decreased significantly ($p<0.01$) in the presence of PMA, whilst no significant change in either the activity of cyGPX or PHGPX was measured despite the trend of increased cyGPX activity observed in the presence of PMA.

c) Effects of GF109203X and PMA on the expression of [^{75}Se]-selenoproteins in HUVEC

Treatment of pre-labelled HUVEC with PMA (0.5 μM) for 48 hr resulted in a decrease in expression of both TR and PHGPX, whilst the expression of cyGPX was increased (figure 4.13). Pre-incubation of the HUVEC with the protein kinase C inhibitor GF109203X 1 hr prior to the addition of PMA (0.5 μM) partially reversed these changes in selenoprotein expression in a dose dependent manner. The addition of the highest dose of GF109203X (5.0 μM) with PMA (0.5 μM) allowed only a very small net change in selenoprotein expression whilst the lowest dose of GF109203X (0.5 μM) was very much less effective at attenuating the selenoprotein response to PMA. No change in selenoprotein expression was observed when HUVEC were incubated with GF109203X alone at all the concentrations tested.

d) The effects of prolonged exposure and cumulative doses of PMA on [^{75}Se]-selenoprotein expression in HUVEC

The effect of PMA on the expression of TR, PHGPX and cyGPX after 48 hr continuous exposure to PMA was consistent with the changes described in section a) above. At 48 hr the expression of both TR and PHGPX was decreased whilst the expression of cyGPX was increased compared to that observed in control cells which were not treated with PMA (figure 4.14).

However between 48 hr and 96 hr the expression of both TR and PHGPX decreased further, whilst the expression of cyGPX continued to increase. The changes in selenoprotein expression described above were maximal in HUVEC to which two doses of PMA had been added, one of which was added 48 hr after the first.

4.3.3 The effects of PMA on the selenoprotein expression in human coronary arterial endothelial cells

Whilst the basal expression of some of the major selenoproteins measured in human coronary artery endothelial cells (HCAEC) and HUVEC differed (as discussed in section 3.3.6), the changes in selenoprotein expression observed in both cell types followed the same general trend when incubated for 48 hr with PMA (0.5 μM). The expression of TR and PHGPX decreased whilst the expression of cyGPX increased as visualised by autoradiography (figure 4.15).

Figure 4.16 shows that in both HUVEC and HCAEC which had been treated with PMA (0.5 μM) for 48 hr, TR expression is significantly decreased ($p<0.01$). In HUVEC, PMA reduced the mass of TR to approximately 35% of control cells and in HCAEC to approximately 42% of control cells.

The cyGPX activity in both HUVEC and HCAEC treated with PMA (0.5 μM) were not statistically different from the activities measured in untreated cells, though there was a trend towards a general increase which was more obvious in HCAEC (figure 4.17).

In HUVEC treated with PMA (0.5 μM) for 48 hr no significant effect on PHGPX activity was observed (figure 4.18). In contrast in PMA-treated HCAEC the PHGPX activity was approximately 37% of control values, significantly lower than HCAEC treated with DMSO ($p<0.01$).

4.3.4 The effects of PMA on the selenoprotein expression in bovine aortic endothelial cells

Figure 4.19 shows that in HUVEC treated with PMA (0.5 μM) for 48 hr both TR and PHGPX expression decreased whilst the expression of cyGPX increased. However, in PMA-treated bovine aortic endothelial cells (BAEC) no noticeable changes in the selenoprotein expression as visualized by autoradiography were observed. The labelling of the BAEC selenoproteins was very low in this particular experiment (in contrast to figure 3.15), which could not be explained by different labelling conditions or by protein loading errors.

Figure 4.20 shows that the TR activity of HUVEC incubated with 0.5 μM PMA for 48 hr was significantly lower than that measured in control HUVEC. In contrast, in BAEC treated in an identical fashion there was no difference in TR activity.

4.3.5 Changes in selenoprotein expression in response to the calcium ionophore A23187 in HUVEC

a) Dose-response effects of A23187 on the [^{75}Se]-selenoprotein expression in HUVEC

The effects of the calcium ionophore A23187 on the expression of [^{75}Se]-selenoproteins was dose dependent (figure 4.21). The continued presence of 0.5 μM A23187 for 24 hr significantly increased the expression of TR. Other changes in the unidentified selenoproteins included a small up-regulation of a 64 kDa selenoprotein whilst the expression of a 72 kDa selenoprotein was significantly attenuated. At the lower doses of A23187 tested, no noticeable change in selenoprotein expression was observed as visualized by autoradiography.

b) The effect of A23187 on [^{75}Se]-selenoprotein expression in HUVEC for different times

As with PMA, the effects of A23187 on selenoprotein expression were time-dependent (figure 4.22). The continued presence of A23187 (0.5 µM) in the culture medium resulted in an induction of TR first observed at 12 hr which was augmented after 24 and 35 hr exposure (figure 4.22). The response was maximal at 48 hr. In contrast, the expression of a 64 kDa selenoprotein was increased after 24 hr and was maximal at 48 hr. A23187 appeared to increase the expression of cyGPX whilst the expression of PHGPX was unchanged. A clear increase in the expression of the selenoprotein with an approximate molecular mass of 15 kDa was observed in the presence of A23187.

In some experiments where HUVEC were exposed to A23187 for 38 hr and longer, cells started to detach from the monolayer, suggesting possible toxic effects of the ionophore. Therefore in future experiments a total incubation time of 35 hr was chosen, as this allowed maximal changes in selenoprotein expression to occur without any observable effect on cell attachment.

The effect of the shorter pulse times of exposure to A23187 is shown in figure 4.23. HUVEC transiently exposed to A23187 (0.5 µM) for 1 hr showed a slight induction of TR expression. This observed induction became more obvious with longer exposure times such that a clear increase in the 58 kDa selenoprotein was observed at 12 hr, with maximal induction after 35 hr of exposure.

4.3.6 The effect of PMA and A23187 added alone or in combination on the expression of selenoproteins

Treatment of HUVEC with PMA (0.5 µM) alone significantly down-regulated the expression of TR and PHGPX, whilst the expression of cyGPX was up-regulated as visualized by autoradiography (figure 4.24). The addition of the calcium ionophore A23187 significantly

increased the expression of TR and to a slightly lesser extent the expression of an unidentified 64 kDa selenoprotein. The expression of a 72 kDa selenoprotein appeared to be completely suppressed in the presence of A23187. PMA and A23187 added in combination produced an overall net increase in the expression of TR and a 64 kDa selenoprotein 35 hr later but the expression of both was lower than that seen when A23187 was added alone. The addition of PMA and A23187 however, had an additive effect on the down-regulation of PHGPX, reducing its expression significantly more in the presence of both compounds compared to the level of expression when PMA and A23187 were added alone. The expression of the 72 kDa selenoprotein in the presence of both compounds was attenuated to the same degree as when A23187 was added alone.

Quantification of the 58kDa [^{75}Se]-labelled band (TR) using the Molecular Imager System showed that treatment with PMA decreased the intensity of the 58 kDa band by 31.36±2.46% of basal levels (mean ± SEM, n=3), whereas treatment with A23187 increased the intensity of the same band by 1.69±0.40-fold over basal levels (mean ± SEM, n=3). PMA and A23187 added in combination produced an overall increase in the intensity of the 58 kDa band by 1.40±0.26-fold (mean ± SEM, n=3) over basal levels. Poor resolution of the [^{75}Se]-selenoproteins meant that it was not possible to quantitate the 22 kDa and 24 kDa [^{75}Se]-labelled bands (PHGPX and cyGPX respectively) in this manner.

The effects of PMA and A23187 treatment on TR were confirmed by Western blotting using antiserum to rat TR (figure 4.25). Western blotting showed that PMA significantly ($p< 0.05$, ANOVA and test of least significant difference) decreased the expression of TR from 4.36±0.63 µg/mg of protein (n=3), expressed under basal conditions, to 2.97 µg/mg of protein (n=3), a decrease of 29.6±9.7%. In contrast treatment with A23187 significantly ($p<0.01$) increased TR expression to 8.38±0.69 µg/mg of protein (n=3), a 1.96±0.13-fold increase over basal levels. When HUVEC were treated with PMA and A23187 in combination there was a net increase in

TR expression to 6.58±0.42 µg/mg of protein (n=3), a 1.57 ± 0.24-fold increase over basal levels.

4.3.7 The effects of acetylcholine, adenosine triphosphate and bradykinin on the [^{75}Se]-selenoprotein expression in HUVEC

Treatment of HUVEC with acetylcholine (1 mM) slightly decreased the expression of all the [^{75}Se]-labelled selenoproteins whilst adenosine triphosphate (1 mM) had no observable effects on the expression of any [^{75}Se]-labelled selenoproteins (figure 4.26).

No changes in the expression of [^{75}Se]-labelled selenoproteins were observed in HUVEC treated with bradykinin (1 µM) for any incubation time studied (figure 4.27).

4.3.8 The effect of forskolin on the [^{75}Se]-selenoprotein expression in HUVEC

Treatment of HUVEC with forskolin (0.1 µM) for 1 min and 48 hr had no observable effect on the expression of [^{75}Se]-selenoproteins relative to cells treated with ethanol (control) as visualized by autoradiography (figure 4.28).

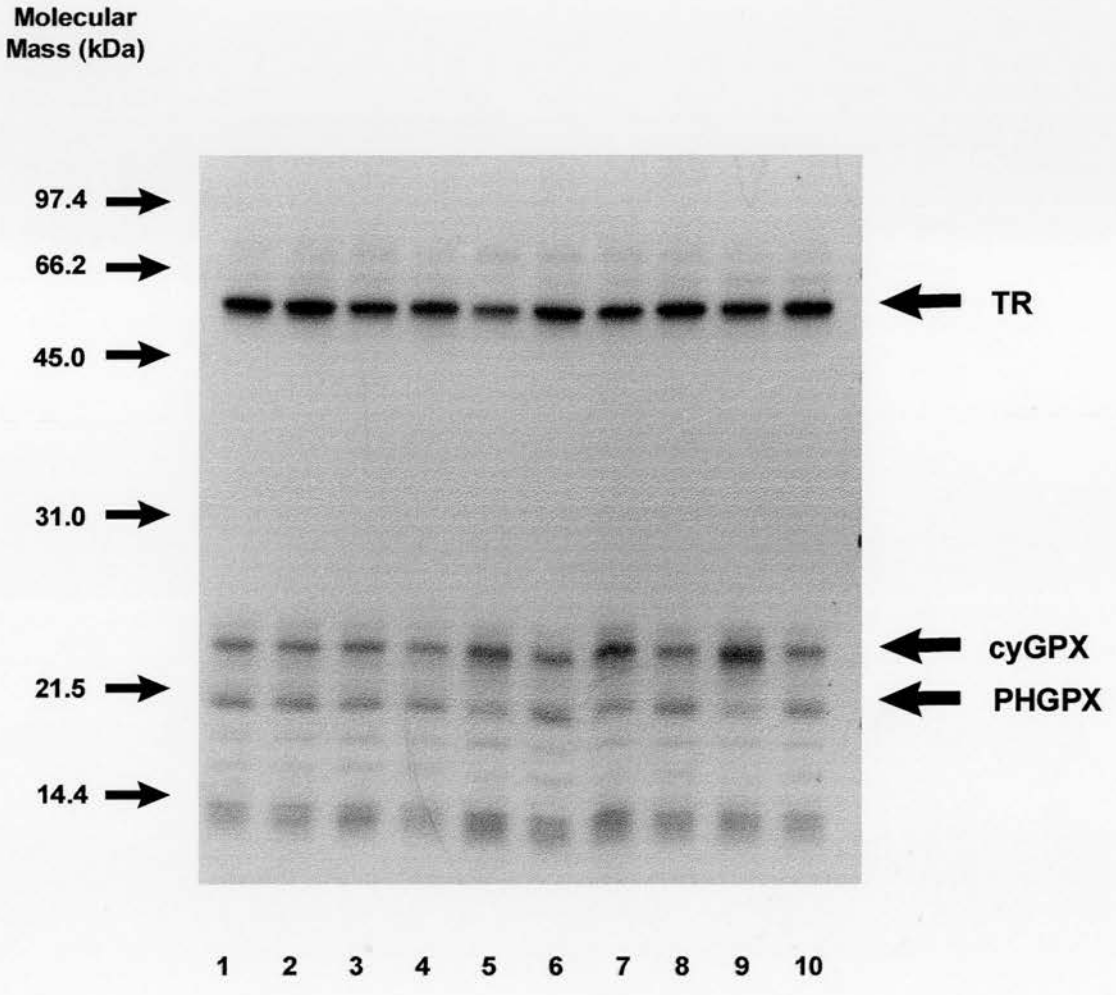


Figure 4.07. Autoradiograph of an SDS-PAGE gel showing the expression of [^{75}Se]-selenoproteins in HUVEC incubated in the continued presence of PMA (0.5 µM) for various times. Confluent cultures of HUVEC were pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. The effect of PMA on the expression of [^{75}Se]-selenoproteins was studied by including either PMA (0.5 µM) or DMSO (0.05%) in the culture medium for 1 min, 12 hr, 48 hr, 72 hr or 96 hr in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. Lane 1, PMA, 1 min; lane 2, DMSO, 1 min; lane 3, PMA, 12 hr; lane 4, DMSO, 12 hr; lane 5, PMA, 48 hr; lane 6, DMSO, 48 hr; lane 7, PMA 72 hr; lane 8, DMSO, 72 hr; lane 9, PMA, 96 hr; lane 10, DMSO, 96 hr. Each lane was loaded with 25 µg of protein. TR, Thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

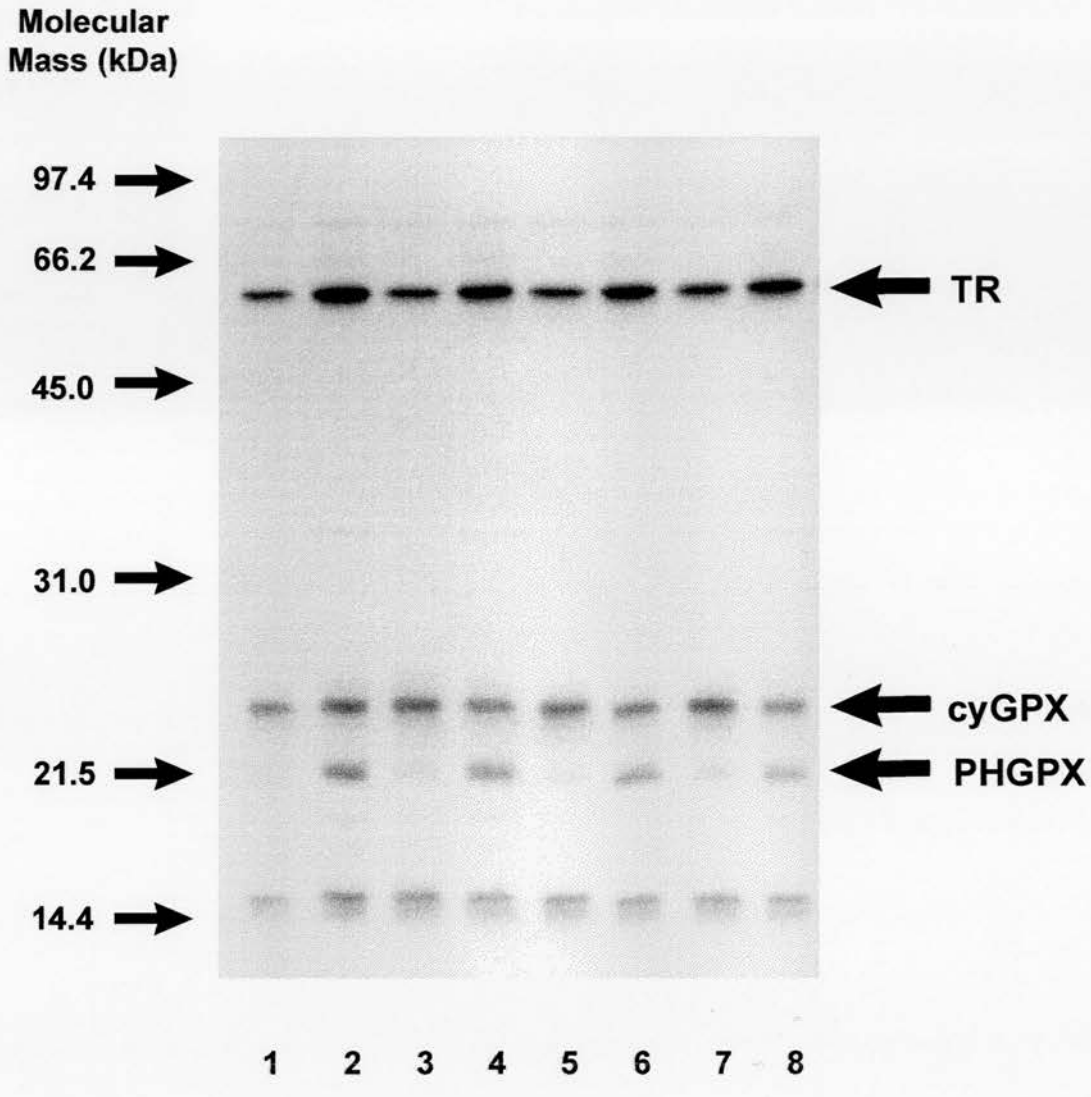


Figure 4.08. Autoradiograph of an SDS-PAGE gel showing the expression of [^{75}Se]-selenoproteins in HUVEC incubated with PMA (0.5 µM) for various times. Confluent cultures of HUVEC pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite were used. The effect of PMA on the expression of [^{75}Se]-selenoproteins was studied by including either PMA (0.5 µM) or DMSO (0.05%) in the culture medium for 1 min, 10 min, 1 hr or 12 hr in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. After each time-point the medium was removed, the cells were washed and fresh culture medium containing 0.02 MBq/ml [^{75}Se]-selenite without PMA or DMSO added. The cells were incubated for a total incubation time of 48 hr. Lane 1, PMA, 1 min; lane 2, DMSO, 1 min; lane 3, PMA, 10 min; lane 4, DMSO, 10 min; lane 5, PMA, 1 hr; lane 6, DMSO, 1 hr; lane 7, PMA, 12 hr; lane 8, DMSO, 12 hr. Each lane was loaded with 25 µg of protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

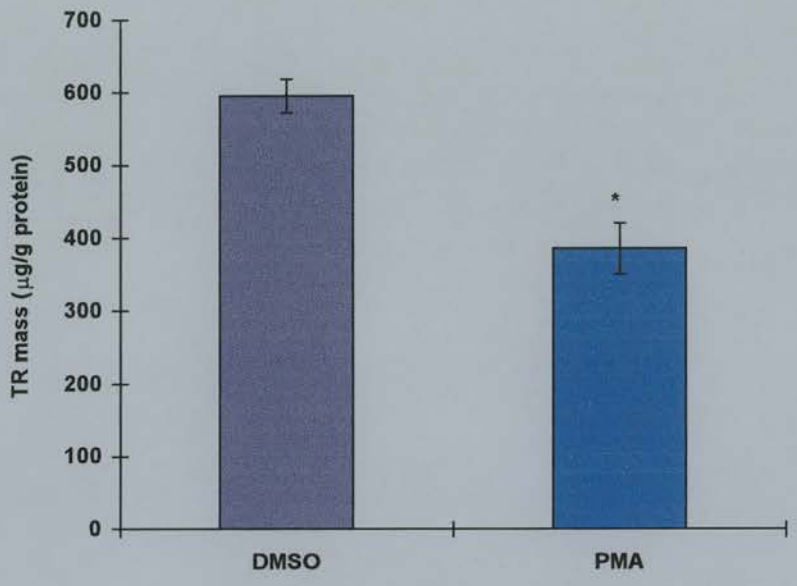


Figure 4.09. Thioredoxin reductase (TR) mass in HUVEC treated with either DMSO (0.05%) or PMA (0.5 µM) for 48 hr. Results shown are those of the mean of 3 flasks ± SD. $p<0.01$.

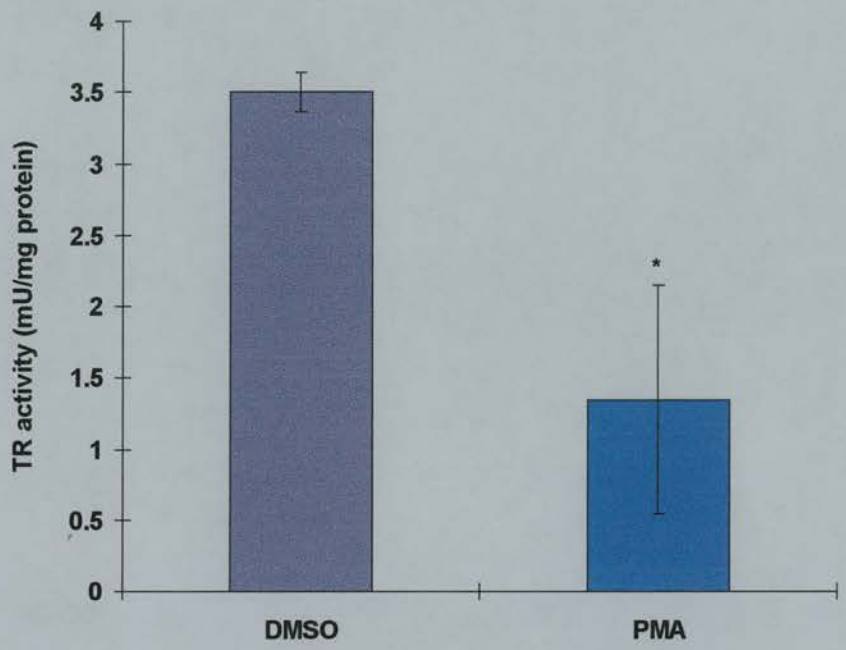


Figure 4.10. Thioredoxin reductase (TR) activity in HUVEC treated with either DMSO (0.05%) or PMA (0.5 µM) for 48 hr. Results shown are those of the mean of 3 flasks ± SD. $p<0.05$.

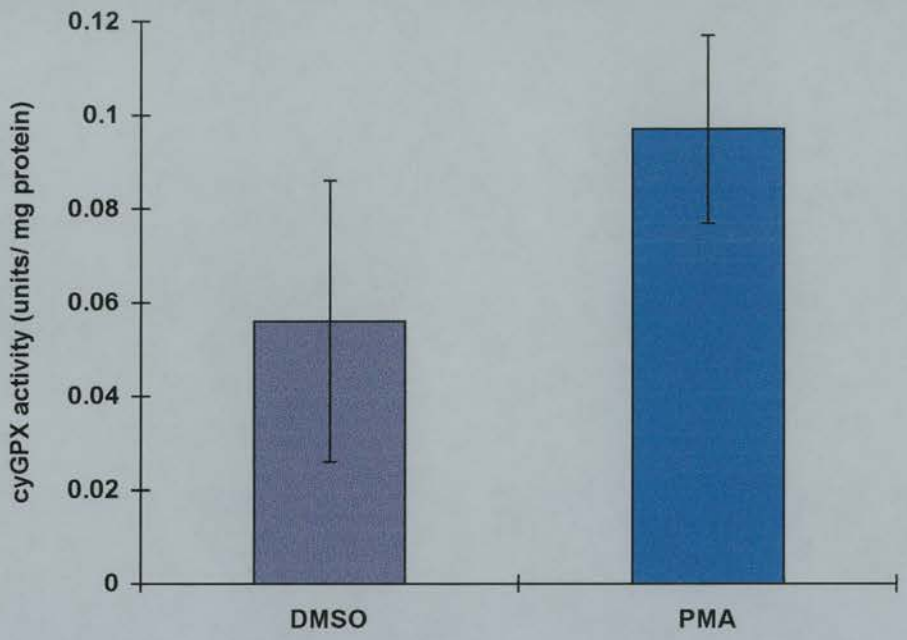


Figure 4.11. Cytoplasmic glutathione peroxidase (cyGPX) activity in HUVEC treated with either DMSO (0.05%) or PMA (0.5 µM) for 48 hr. Results shown are those of the mean of 3 flasks ± SD. No significant difference in cyGPX activity between HUVEC treated with DMSO and those treated with PMA was shown.

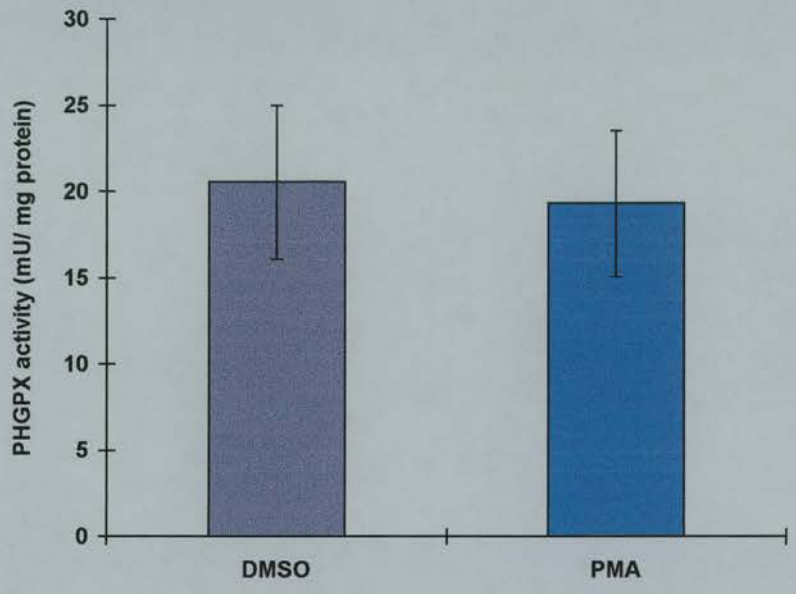


Figure 4.12. Phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity in HUVEC treated with either DMSO (0.05%) or PMA (0.5 µM) for 48 hr. Results shown are those of the mean of 3 flasks ± SD. No significant difference in PHGPX activity between HUVEC treated with DMSO and those treated with PMA was shown.

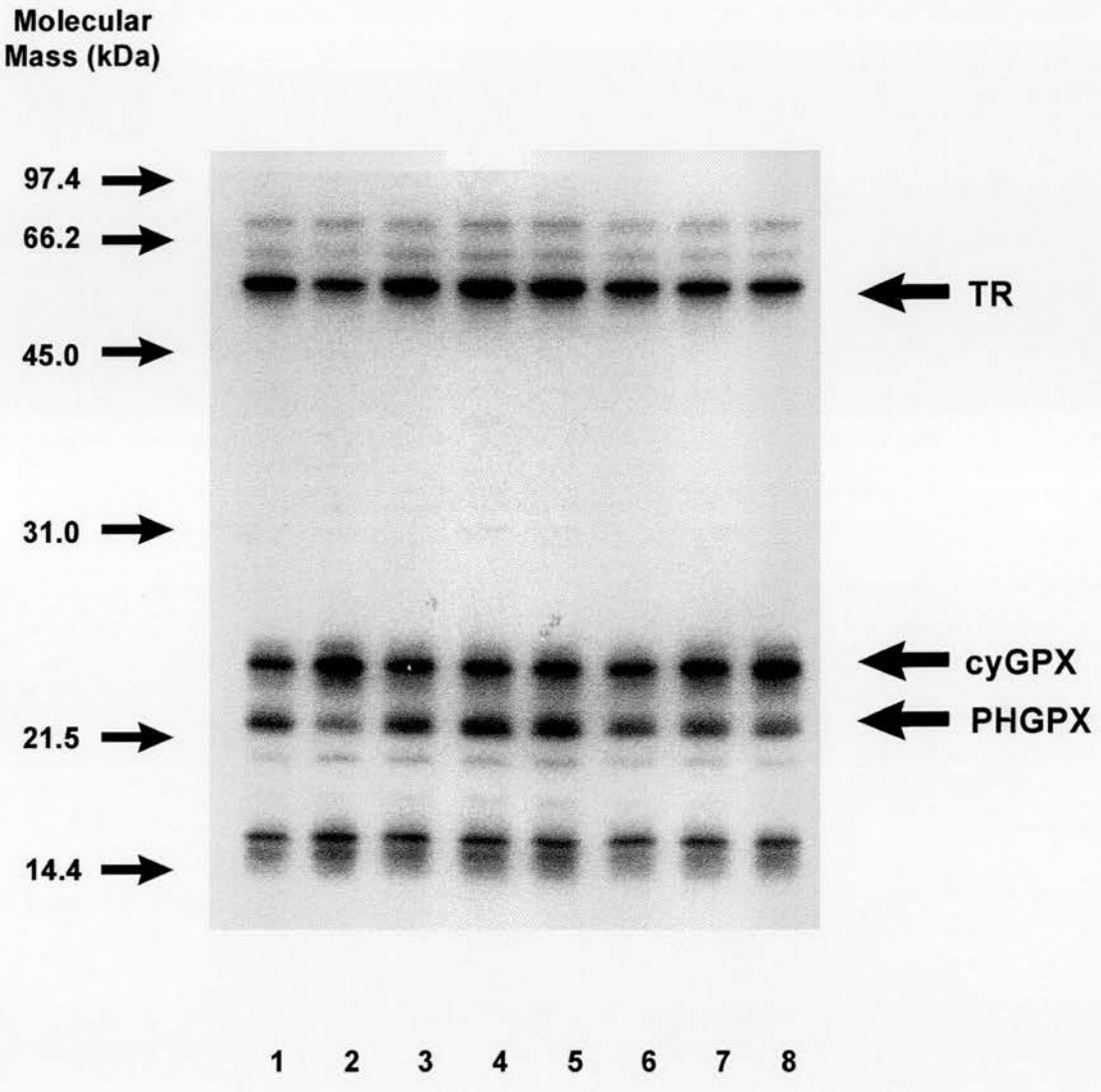


Figure 4.13 Autoradiograph of an SDS-PAGE gel showing the expression of [^{75}Se]-selenoproteins in HUVEC incubated with the PKC inhibitor GF109203X at various doses and/or PMA (0.5 µM). Confluent cultures of HUVEC pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite were used. The effect of GF109203X and PMA on the expression of [^{75}Se]-selenoproteins was studied by the inclusion of different concentrations of GF109203X and PMA (0.5 µM) in the culture medium either alone or in combination. GF109203X was added 1 hr prior to the 48 hr PMA incubation in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. Lane 1, DMSO; lane 2, PMA alone (0.5 µM); lane 3, GF109203X alone (5.0 µM); lane 4, GF109203X alone (1.0 µM); lane 5, GF109203X alone (0.5 µM); lane 6, GF109203X (5.0 µM) and PMA (0.5 µM); lane 7, GF109203X (1.0 µM) and PMA (0.5 µM); lane 8, GF109203X (1.0 µM) and PMA (0.5 µM). Each lane was loaded with 25 µg of protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

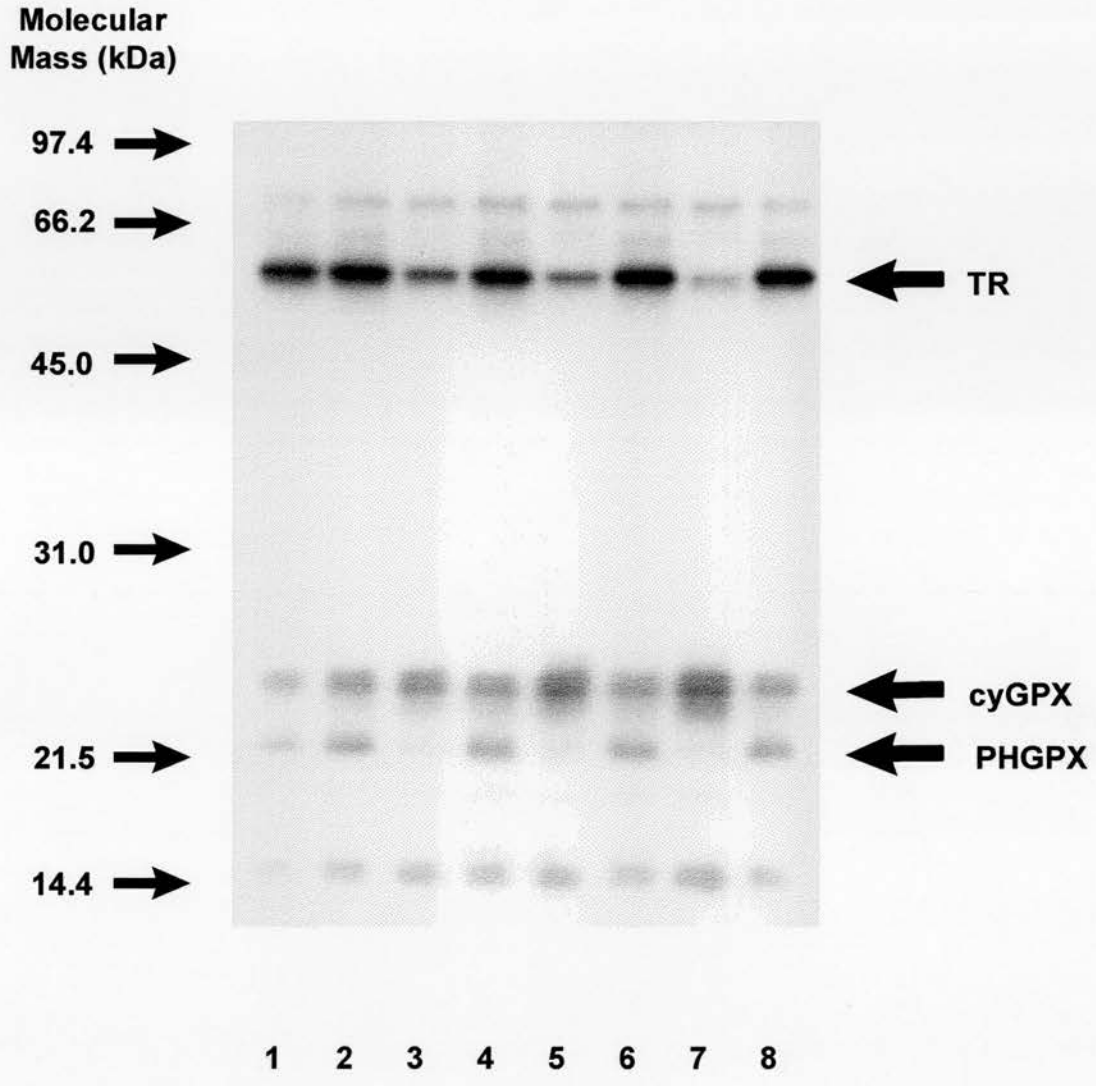


Figure 4.14 Autoradiograph of an SDS-PAGE gel showing the expression of [^{75}Se]-selenoproteins in HUVEC incubated in the continued presence of PMA (0.5 μM) for various times with single and cumulative doses. Confluent cultures of HUVEC pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite were used. The effect of PMA on the expression of [^{75}Se]-selenoproteins was studied by including either PMA (0.5 μM) or DMSO (0.05%) in the culture medium for 1 min, 48 hr, and 96 hr in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. The effect a cumulative dose of PMA on the expression of [^{75}Se]-selenoproteins was studied by adding a further dose of PMA (final concentration 1 μM) to HUVEC previously incubated with PMA for 48 hr, followed by a further 48 hr incubation. Lane 1, PMA (0.5 μM), 1 min; lane 2, DMSO (0.05%), 1 min; lane 3, PMA (0.5 μM), 48 hr; lane 4, DMSO (0.05%), 48 hr; lane 5, PMA (0.5 μM), 96 hr, lane 6, DMSO (0.05%), 96 hr; lane 7, PMA (10^{-6}M), 96 hr; lane 8, DMSO (0.10%), 96 hr. Each lane was loaded with 25 μg of protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

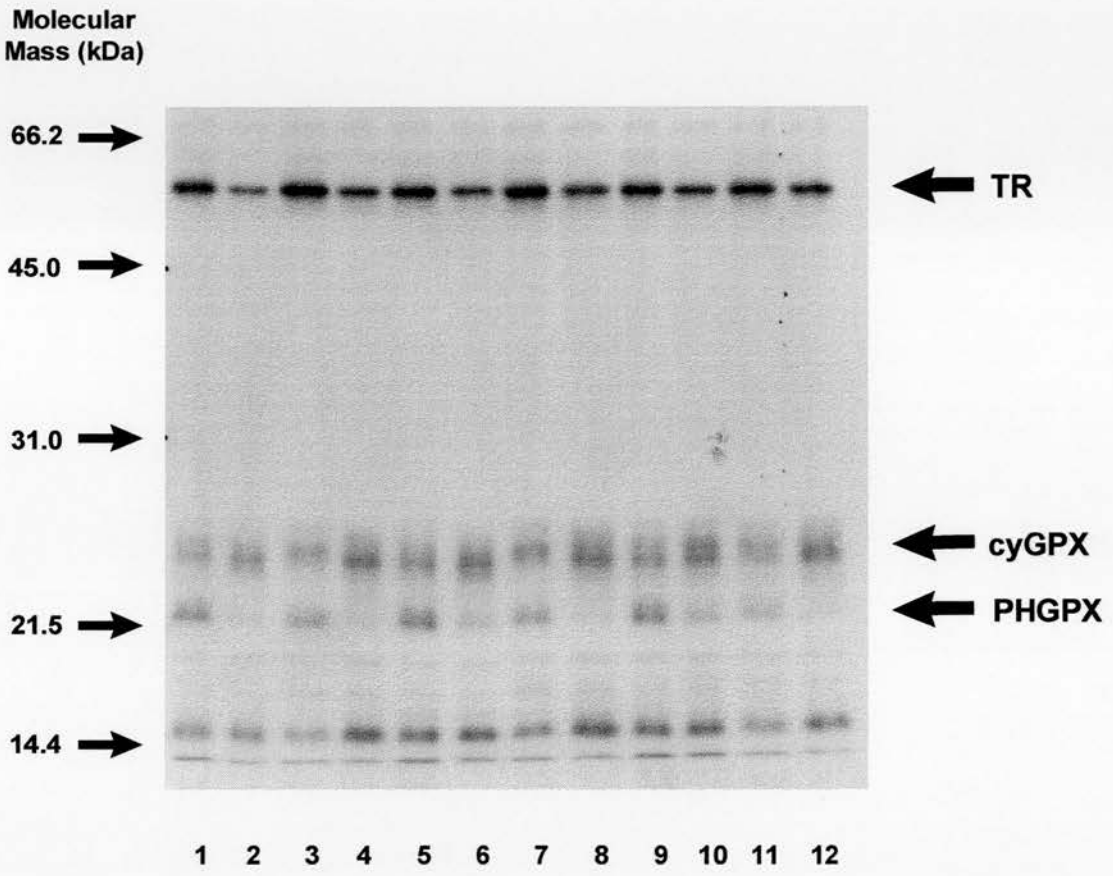


Figure 4.15 Autoradiograph of an SDS-PAGE gel showing the expression of [^{75}Se]-selenoproteins in both human umbilical vein endothelial cells (HUVEC) and human coronary artery endothelial cells (HCAEC) incubated in the continued presence of PMA (0.5 µM) for 48 hr. Confluent cultures of HUVEC and HCAEC were pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. The effect of PMA on the expression of [^{75}Se]-selenoproteins was studied by including either PMA (0.5 µM) or DMSO (0.05%) in the culture medium of both cell types for 48 hr in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. Lane 1, HUVEC, DMSO; lane 2, HUVEC, PMA; lane 3, HCAEC, DMSO; lane 4, HCAEC, PMA; lane 5, HUVEC, DMSO; lane 6, HUVEC, PMA; lane 7, HCAEC, DMSO; lane 8, HCAEC, PMA, lane 9, HUVEC, DMSO, lane 10, HUVEC, PMA; lane 11, HCAEC, DMSO; lane 12, HCAEC, PMA. The samples loaded are those from triplicate flasks of both cell types for each treatment. Each lane was loaded with 25 µg of protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

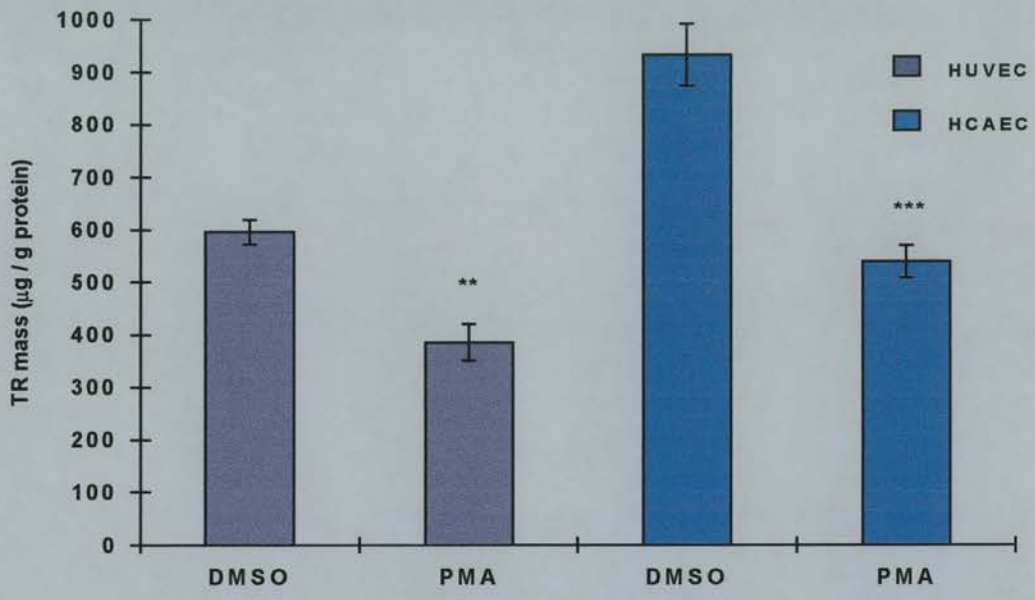


Figure 4.16. Thioredoxin reductase (TR) mass in HUVEC and HCAEC treated with either DMSO (0.05%) or PMA (0.5 µM) for 48 hr. Results shown are those of the mean of 3 flasks ± SD. Results which are significantly different from DMSO-treated cells are denoted as follows:- $p<0.001^{***}$, $p<0.01^{**}$.

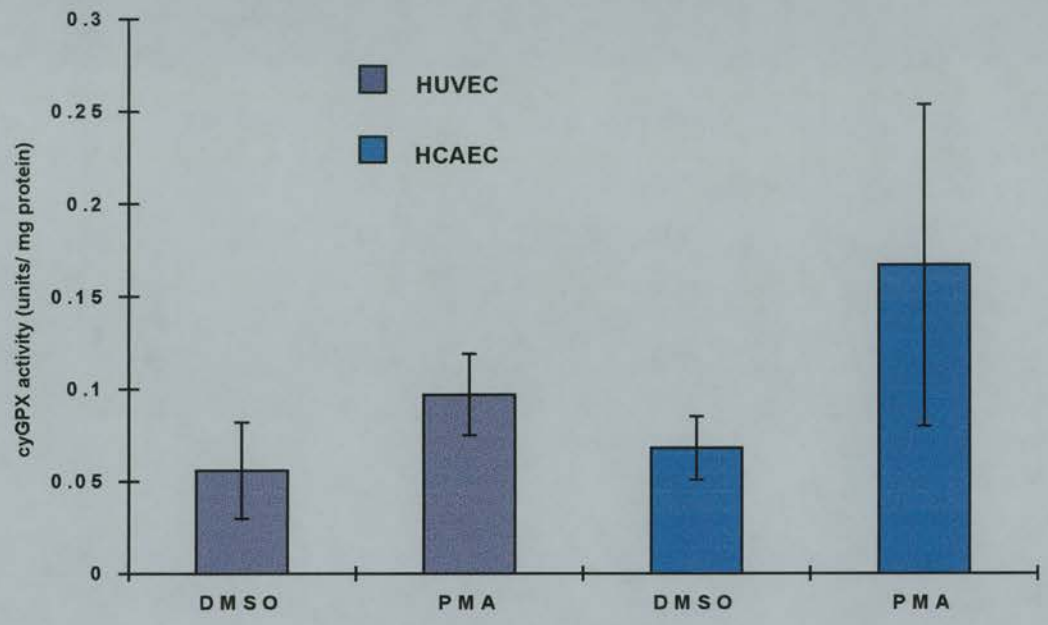


Figure 4.17. Cytoplasmic glutathione peroxidase (cyGPX) activity in HUVEC and HCAEC treated with either DMSO (0.05%) or PMA (0.5 µM) for 48 hr. Results shown are those of the mean of 2 or 3 flasks ± SD. No significant difference in cyGPX activity between DMSO- and PMA-treated HUVEC or HCAEC was shown.

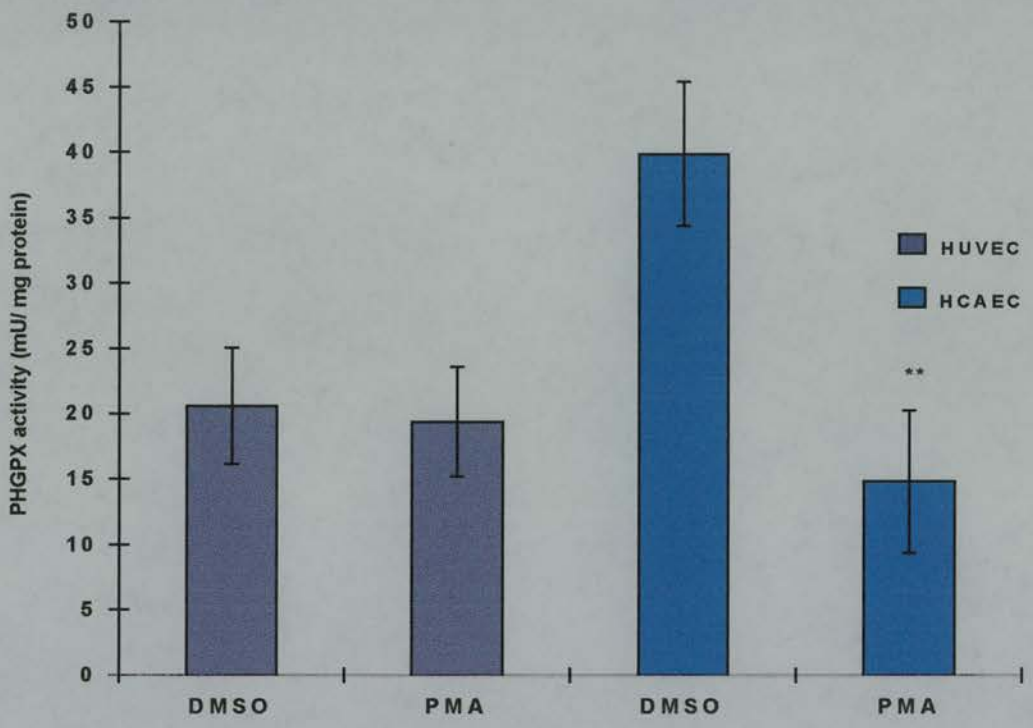


Figure 4.18. Phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity in human umbilical vein endothelial cells (HUVEC) and human coronary arterial endothelial cells (HCAEC) treated with either DMSO (0.05%) or PMA (0.5 µM) for 48 hr. Results shown are those of the mean of 3 flasks ± SD. Results which are significantly different from DMSO-treated cells are denoted as follows:- $p<0.01^{**}$.

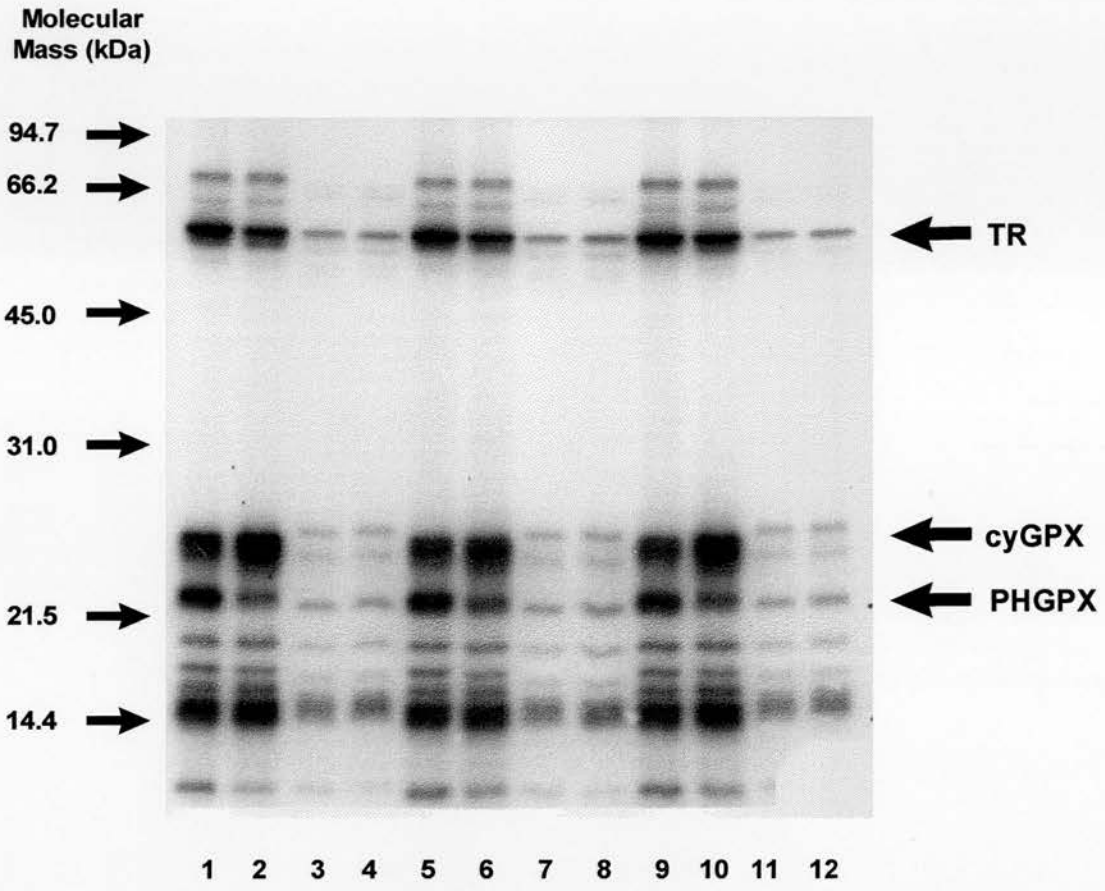


Figure 4.19. Autoradiograph of an SDS-PAGE gel showing the expression of [^{75}Se]-selenoproteins in both human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC) incubated in the continued presence of PMA (0.5 µM) for 48 hr. Confluent cultures of HUVEC and BAEC were pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. The effect of PMA on the expression of [^{75}Se]-selenoproteins was studied by including either PMA (0.5 µM) or DMSO (0.05%) in the culture medium of both cell types for 48 hr in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. Lane 1, HUVEC, DMSO; lane 2, HUVEC, PMA; lane 3, BAEC, DMSO; lane 4, BAEC, PMA; lane 5, HUVEC, DMSO; lane 6, HUVEC, PMA; lane 7, BAEC, DMSO; lane 8, BAEC, PMA, lane 9, HUVEC, DMSO, lane 10, HUVEC, PMA; lane 11, BAEC, DMSO; lane 12, BAEC, PMA. The samples loaded are those from triplicate flasks of both cell types for each treatment. Each lane was loaded with 25 µg of protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

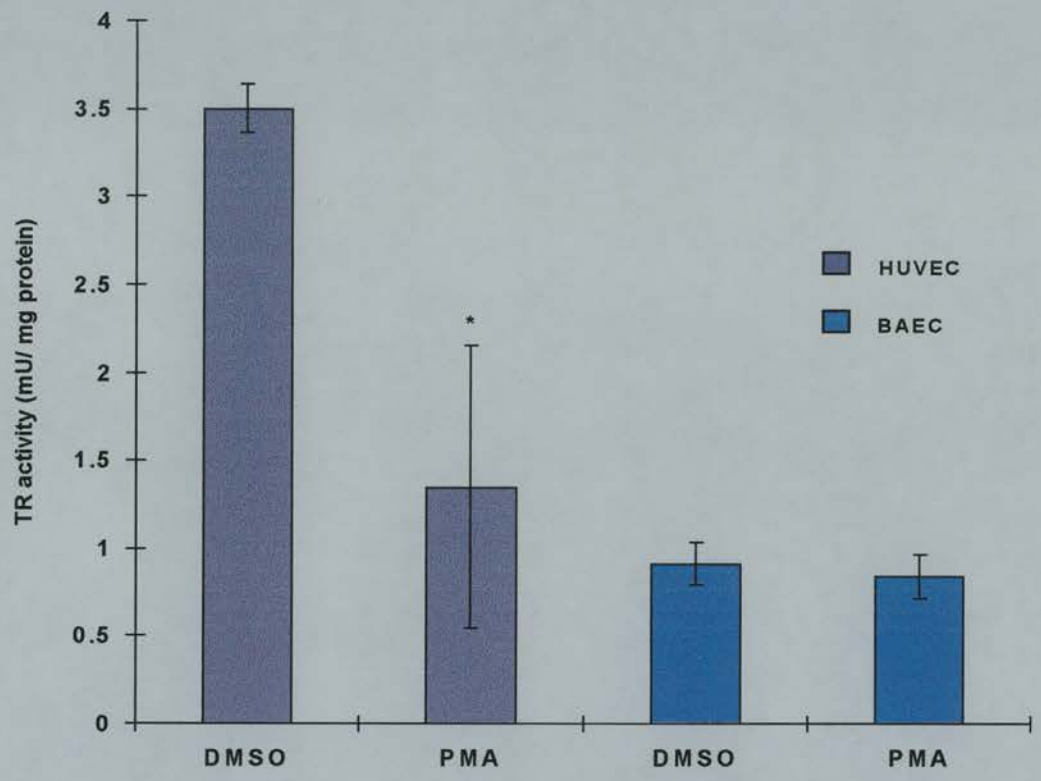


Figure 4.20. Thioredoxin reductase activity in human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC) treated with either DMSO (0.05%) or PMA (0.5 µM) for 48 hr. Results shown are those of the mean of 3 flasks ± SD. Results which are significantly different from DMSO-treated HUVEC or BAEC are denoted as follows:- $p<0.05^{*}$.

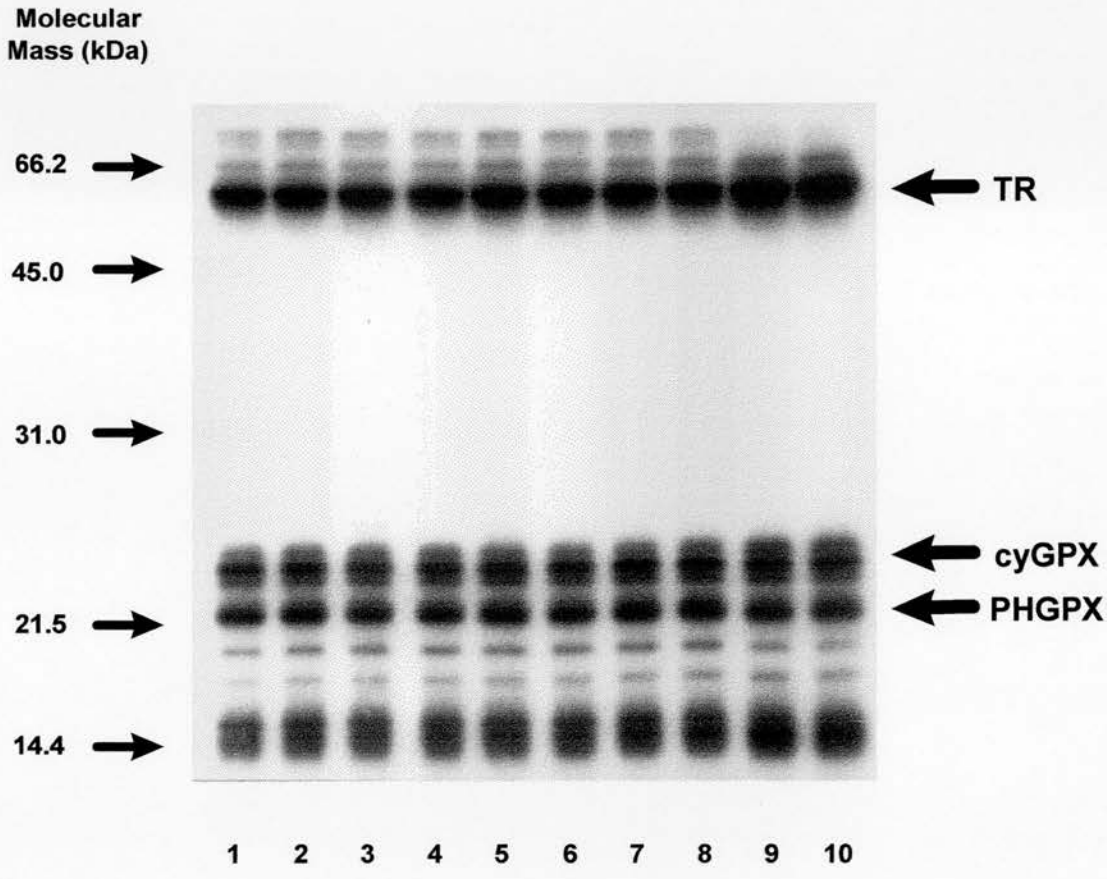


Figure 4.21. Autoradiograph of an SDS-PAGE gel showing the expression of [^{75}Se]-selenoproteins in both human umbilical vein endothelial cells (HUVEC) in the continued presence of various doses of A23187 for 24 hr. Confluent cultures of HUVEC were pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. The effect of A23187 on the expression of [^{75}Se]-selenoproteins was studied by including different concentrations of A23187 in the culture medium for 24 hr in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. Lane 1, No additions; lane 2, DMSO (0.05%), lanes 3 and 4, A23187 (0.5 nM); lanes 5 and 6, A23187 (5 nM); lanes 7 and 8 (50 nM); lanes 9 and 10, A23187 (0.5 µM) Each lane was loaded with 25 µg of protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

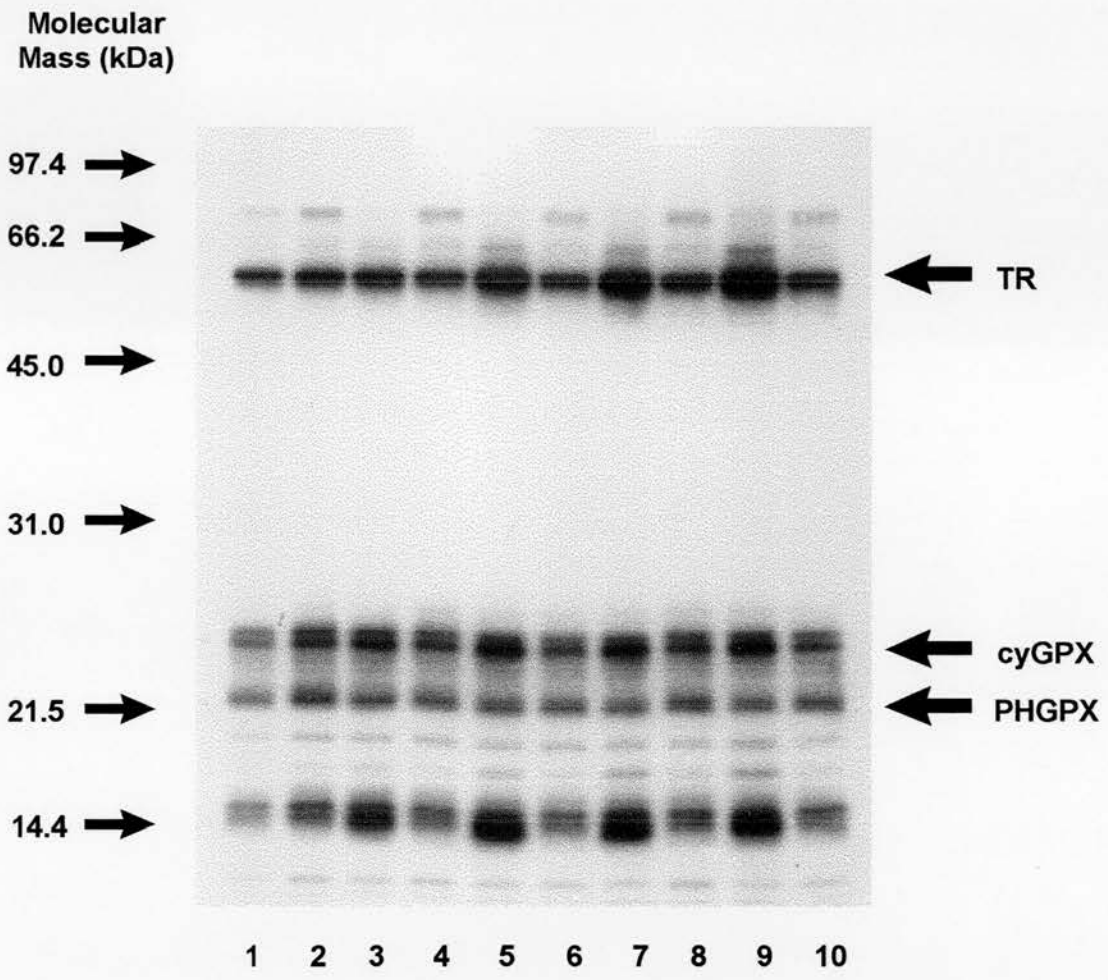


Figure 4.22. Autoradiograph of an SDS-PAGE gel showing the expression of [^{75}Se]-selenoproteins in human umbilical vein endothelial cells (HUVEC) incubated in the presence of A23187 (0.5 µM) for various times. Confluent cultures of HUVEC were pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. The effect of A23187 on the expression of [^{75}Se]-selenoproteins was studied by including A23187 (0.5 µM) in the culture medium for 1 min, 12 hr, 24 hr 35 hr or 48 hr in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. Lane 1, A23187, 1 min; lane 2, DMSO, 1 min; lane 3, A23187, 12 hr; lane 4, DMSO, 12 hr; lane 5, A23187, 24 hr; lane 6, DMSO, 24 hr; lane 7, A23187, 35 hr; lane 8, DMSO, 35 hr; lane 9, A23187, 48 hr; lane 10, DMSO, 48 hr. Each lane was loaded with 25 µg of protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid glutathione peroxidase.

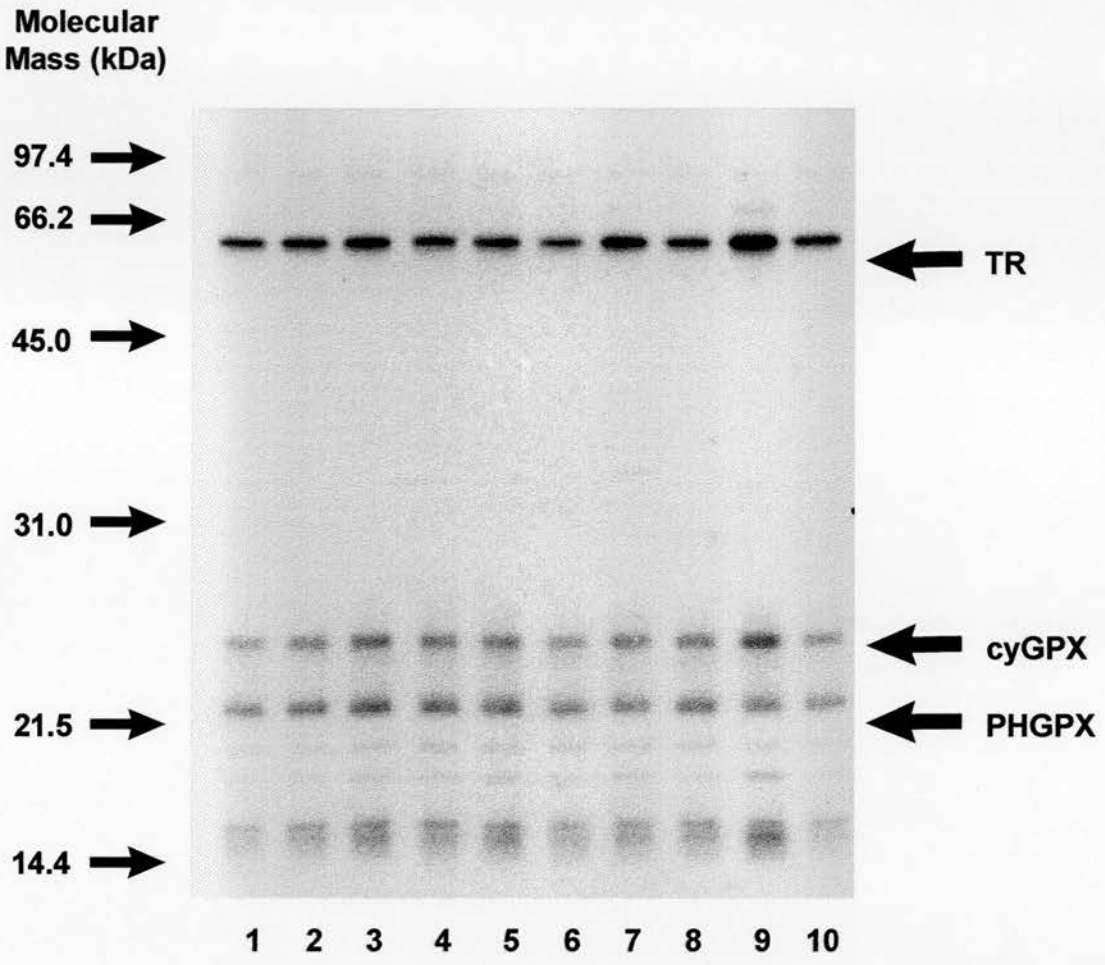


Figure 4.23. Autoradiograph of an SDS-PAGE gel showing the expression of [^{75}Se]-selenoproteins in human umbilical vein endothelial cells (HUVEC) incubated in the continued presence of A23187 (0.5 μM) for various times. Confluent cultures of HUVEC were pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenoproteins was studied by including either A23187 (0.5 μM) or DMSO (0.05%) in the culture medium for 1 min, 10 min, 1 hr, 12 hr or 48 hr in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. After each time point the medium was removed and the cells washed and fresh medium containing 0.02 MBq/ml [^{75}Se]-selenite without A23187 or DMSO added. The cells were incubated for a total time of 48 hr. Lane 1, A23187, 1 min; lane 2, DMSO, 1 min, lane 3, A23187, 10 min; lane 4, DMSO, 10 min; lane 5, A23187, 1 hr; lane 6, DMSO, 1 hr; lane 7, A23187, 12 hr; lane 8, DMSO, 12 hr; lane 9, A23187, 35 hr, lane 10, DMSO, 35 hr. Each lane was loaded with 25 μg of protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

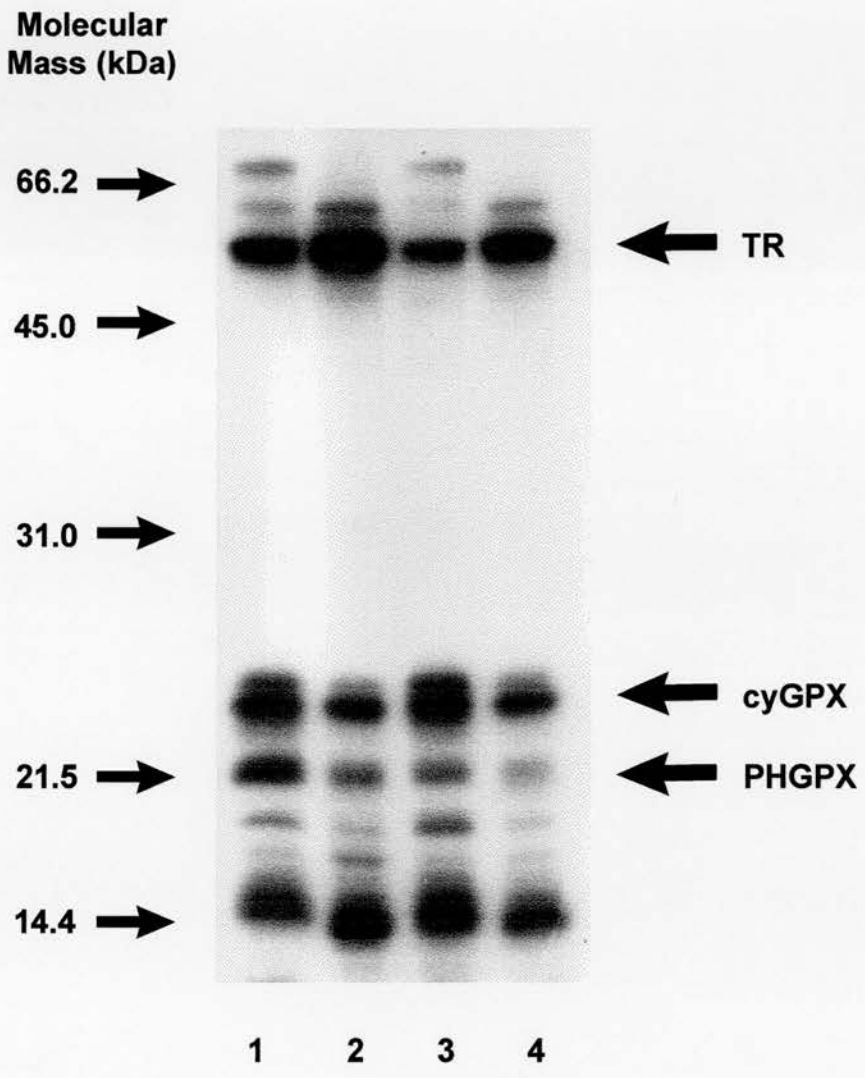


Figure 4.24. Autoradiograph of an SDS-PAGE gel showing the expression of [^{75}Se]-selenoproteins in human umbilical vein endothelial cells (HUVEC) labelled with [^{75}Se]-selenite (0.02 MBq/ml) for 35 hr in the presence and absence of various agents. Lane 1, no additions; lane 2, A23187 (0.5 μM); lane 3, PMA (0.5 μM); lane 4, A23187 (0.5 μM) and PMA (0.5 μM). Each lane was loaded with 25 μg of protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

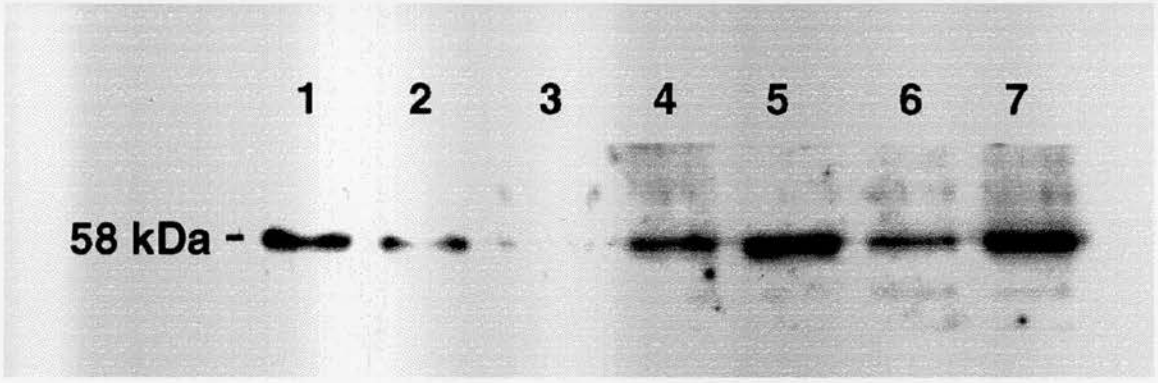


Figure 4.25. Western blot of human umbilical vein endothelial cells (HUVEC) using antiserum to thioredoxin reductase (TR). HUVEC were incubated in the presence and absence of various agents, as specified, prior to the Western blotting of the cell lysates. Lanes 1-3, purified human TR standard (4, 2, and 1ng respectively); lane 4, HUVEC - no additions; lane 5, HUVEC - A23187 (0.5 µM); lane 6, HUVEC - PMA (0.5 µM); lane 7, HUVEC - A23187 (0.5 µM) and PMA (0.5 µM).

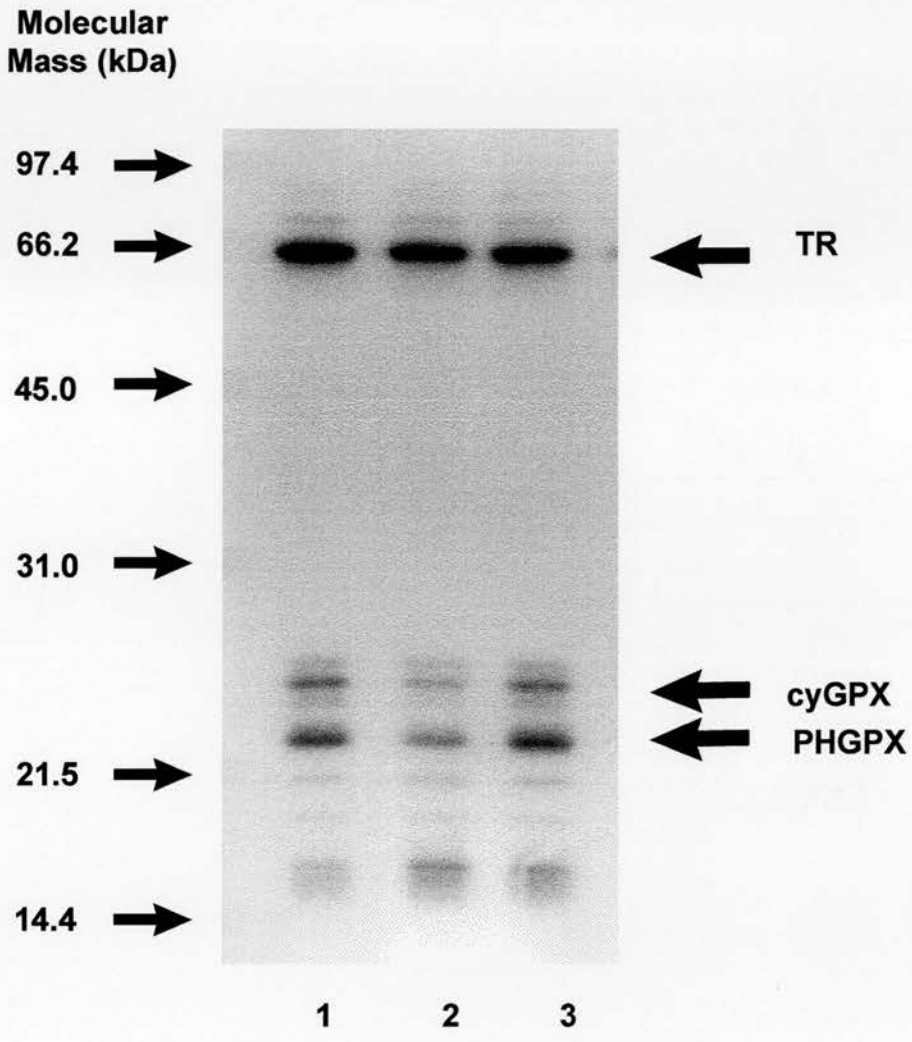


Figure 4.26. Autoradiograph of an SDS-PAGE gel showing the expression of [^{75}Se]-selenoproteins in human umbilical vein endothelial cells (HUVEC) in the continued presence of various agents. Confluent cultures of HUVEC were pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. The effects of acetylcholine (1 mM) and adenosine triphosphate (1 mM) on the expression of [^{75}Se]-selenoproteins was studied by including each of these agents in the culture medium for 48 hr in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. Lane 1, no additions; lane 2, acetylcholine; lane 3, adenosine triphosphate.. Each lane was loaded with 25 µg of protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

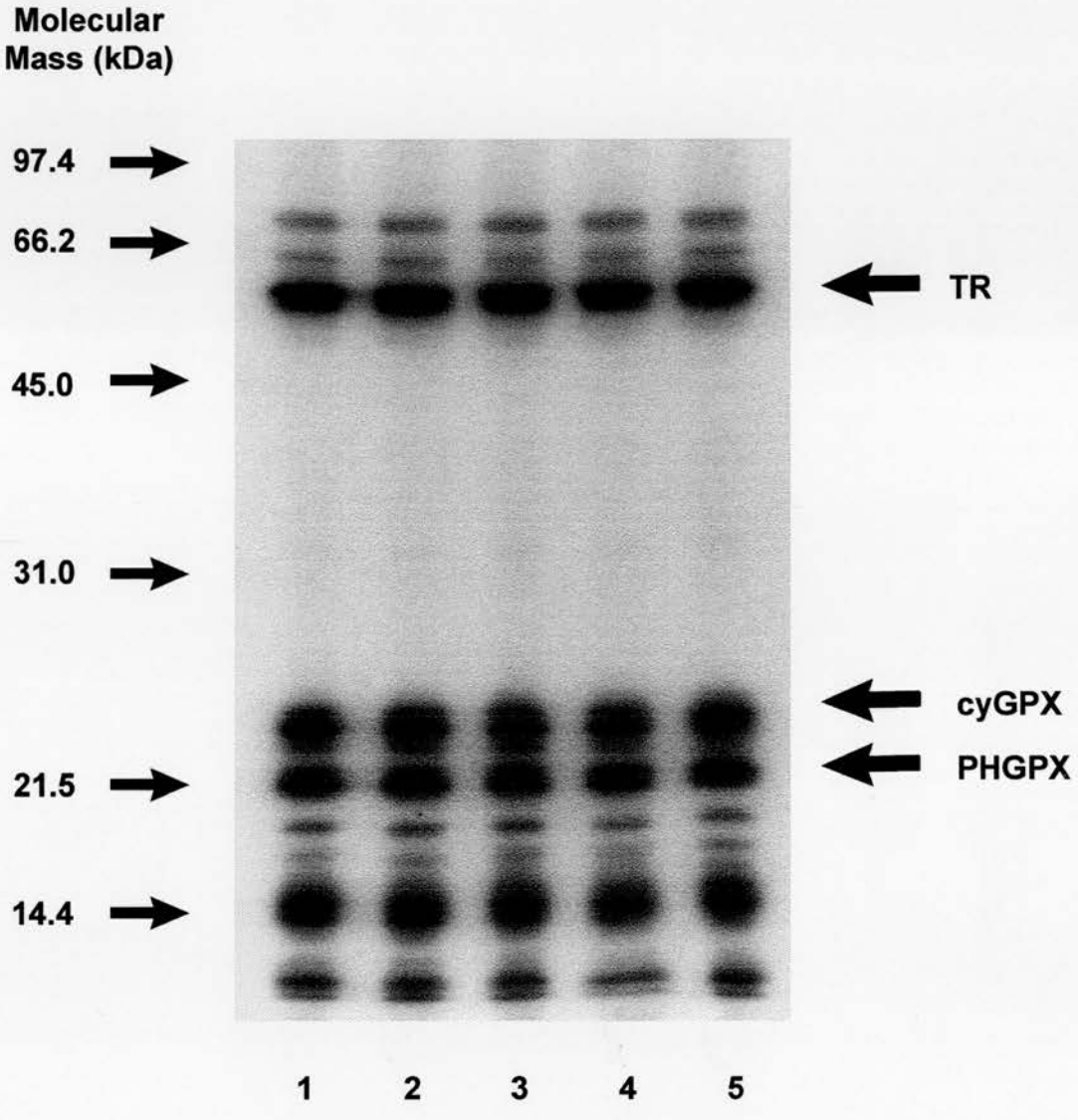


Figure 4.27. Autoradiograph of an SDS-PAGE gel showing the expression of [^{75}Se]-selenoproteins in human umbilical vein endothelial cells (HUVEC) incubated in the continued presence of bradykinin (1 μM) for various times. Confluent cultures of HUVEC were pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. The effects of bradykinin (1 μM) on the expression of [^{75}Se]-selenoproteins was studied by including bradykinin in the culture medium for 6 hr, 12 hr, 24 hr and 48 hr in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. Lane 1, no additions; lane 2, 6 hr; lane 3, 12 hr; lane 4, 24 hr; lane 5, 48 hr. Each lane was loaded with 25 μg of protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

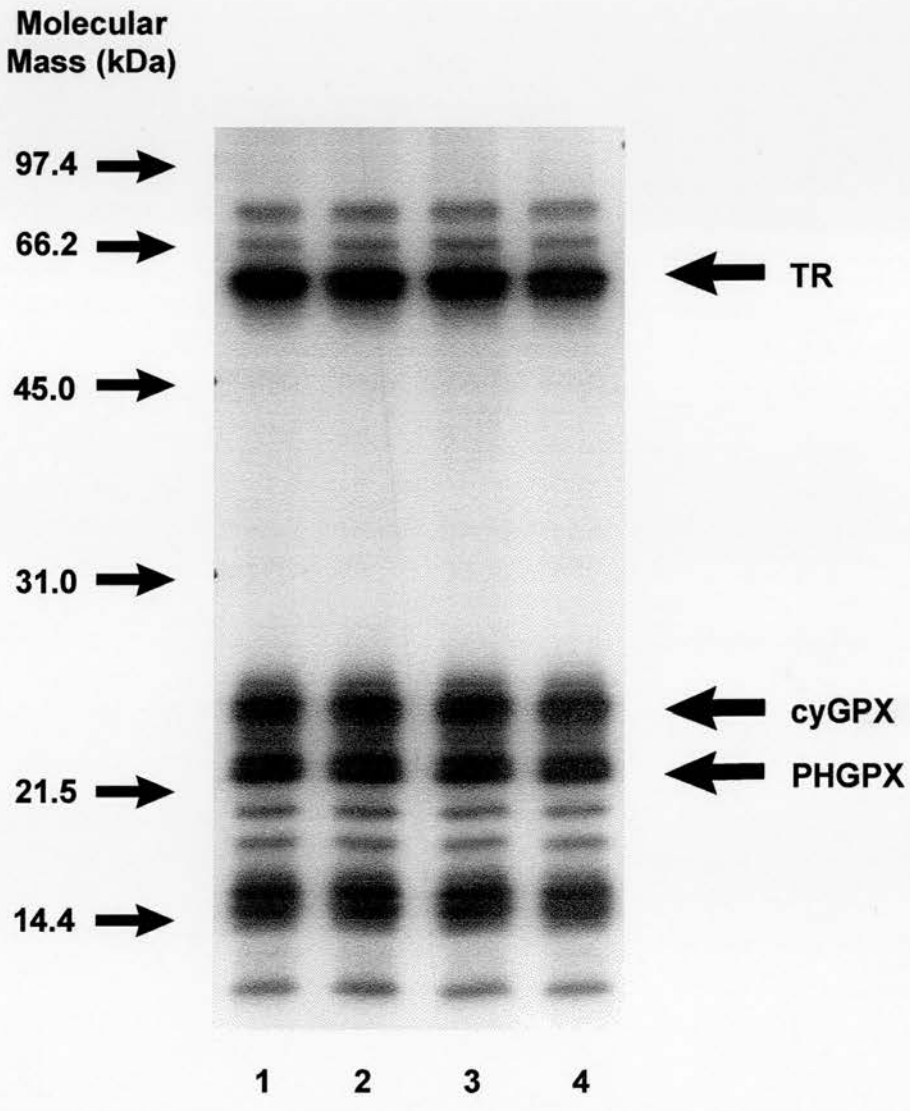


Figure 4.28. Autoradiograph of an SDS-PAGE gel showing the expression of [^{75}Se]-selenoproteins in human umbilical vein endothelial cells (HUVEC) in the continued presence of forskolin for 48 hr. Confluent cultures of HUVEC were pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. The effect of forskolin on the expression of [^{75}Se]-selenoproteins was studied by including forskolin (0.1 μM) in the culture medium for 48 hr in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. Lane 1, forskolin, 1 minute; lane 2, ethanol (0.1%), 1 minute; lane 3, forskolin, 48 hr; lane 4, ethanol (0.1%), 48 hr. Each lane was loaded with 25 μg of protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

4.4 DISCUSSION

The treatment of HUVEC with the phorbol ester PMA and/or the calcium ionophore A23187 has clear effects on intracellular selenoprotein expression. In HUVEC exposed to PMA for times as short as 1 minute, a down-regulation of TR and PHGPX was observed. In contrast, an up-regulation of cyGPX was found after 10 min exposure to PMA (as visualized by autoradiography). These changes in selenoprotein expression were only apparent after a 48 hr lag period following these brief exposure times to PMA. The degree of change in the expression of these major selenoproteins was similar to that observed in HUVEC which had been continually exposed to PMA for 48 hr. These results strongly support an effect of PMA on selenoprotein expression through the activation of PKC rather than its desensitization which has been previously reported after prolonged PMA exposure (Emori *et al.*, 1991; Santell, Bartfield and Levin, 1992). It is possible that desensitization of PKC was not observed in HUVEC exposed to PMA for times in excess of 48 hr because the initial levels of PMA were depleted. However, our results showed that this was unlikely as the double dose of PMA had an additive effect on the changes in selenoprotein expression observed after 96 hr (figure 4.14).

The data presented using the PKC inhibitor GF109203X provided further evidence that the change in selenoprotein expression induced by PMA was through the activation of PKC.

Furthermore the use of this inhibitor strongly suggests that the effects of PMA on selenoprotein expression did not result from a down-regulation of PKC since GF109203X added alone had no observable effect on selenoprotein expression. However, in cells pre-incubated with GF109203X, the effects of PMA on selenoprotein expression were attenuated.

Overall the results from the [^{75}Se]-selenoprotein labelling experiments and the Western blot analysis provide good evidence that the expression of TR, PHGPX and cyGPX is modulated through the activation of PKC by the phorbol ester PMA. Changes in TR expression in response

to phorbol esters have also been reported in human thyrocytes and HepG2 cells but in contrast to HUVEC PMA led to an increase in TR expression in these cells (Howie *et al.*, 1998). Recently Kumar and Holmgren demonstrated that a topical application of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (8 nM) significantly induces TR activity in skin (Kumar and Holmgren, 1999). The down-regulation of TR expression in response to PMA in HUVEC thus contrasts with TR up-regulation reported in these other cell and tissue types. It is possible that the same protein (in this case TR) may be differentially regulated by PKC perhaps through the expression of different isoforms of PKC (Hug and Sarre, 1993). The responses of PHGPX and cyGPX expression to PMA have not been reported elsewhere although treatment of HUVEC with phorbol ester has been shown to increase cyGPX mRNA levels (Jornot and Junod, 1997).

The decreases in TR mass and activity in HUVEC in response to a 48 hr treatment with PMA confirmed the findings from the [^{75}Se]-selenoprotein labelling experiments. However the down-regulation of [^{75}Se]-labelled PHGPX and the up-regulation of [^{75}Se]-labelled cyGPX observed in HUVEC treated with PMA were not confirmed by the activity assays for PHGPX and cyGPX. Although there was a trend towards increased cyGPX activity in the presence of PMA such changes did not reach statistical significance and no change in PHGPX activity was apparent. The reason for this discrepancy may be due to the fact that the GPX activities being measured were very low and therefore may be at the detection limit of the assay. Thus at such levels it may be difficult to detect the modest changes in activity which occur in response to PMA treatment. Indeed in HCAEC, which express higher levels of PHGPX, a significant effect of PMA on PHGPX activity could be observed. Though, it is also a possibility that the selenoprotein bands with molecular masses of 21 kDa and 24 kDa identified in this study as being PHGPX and cyGPX respectively were not homogenous and that other selenoproteins with identical molecular masses were being affected by PMA. Two-dimensional gels would be required to clarify this point.

Despite some differences in the basal expression of selenoproteins in HCAEC and HUVEC both cell types respond to a 48 hr incubation with PMA in a similar manner. PMA-treatment resulted in the down-regulation of both PHGPX and TR, whilst the expression of cyGPX was increased as visualized by autoradiography. The HCAEC were also assayed for TR mass, PHGPX and cyGPX activity. PMA was shown to down-regulate TR mass to a similar degree in both HUVEC and HCAEC. In HCAEC the change is cyGPX activity in response to PMA treatment was not significant, despite a trend for higher cyGPX activity in cells treated with PMA. The disparity between the results from the [^{75}Se]-labelling experiment and the selenoenzyme activities is likely to be due to a sensitivity problem in the activity assay discussed previously.

We have shown that in HUVEC and HCAEC, both TR and PHGPX are down-regulated through PKC activation, whilst the expression of cyGPX is increased in response to PMA. The up-regulation of cyGPX may not result from the direct activation of PKC but rather as a consequence of the increased oxidative stress resulting from the down-regulation of the antioxidant enzymes TR and PHGPX. Activation of cyGPX expression may thus occur in order to maintain the antioxidant capacity of the cell. Indeed when hydrogen peroxide production is stimulated in the rat thyroid during iodine deficiency, there is an associated increase in cyGPX activity possibly to protect against the increased oxidative stress (Mitchell *et al.*, 1996). The pulsed time course data is in agreement with this hypothesis as the induction of cyGPX as visualized by autoradiography appears to occur after the down-regulation of TR and PHGPX expression. There is evidence that exposure of endothelial cells to oxidants causes a time and dose-dependent stimulation of the phospholipase C signal transduction pathway (Shasby, Yorek and Shasby, 1988). In inflammatory cells activation of PKC has been associated with a burst of superoxide production (Harrison and Ohara, 1995). More recently the activation of PKC through oxidized low-density lipoprotein has been implicated in vascular disease (Ohgushi *et al.*, 1993). We have shown that activation of the PKC pathway attenuates the expression of TR and PHGPX. Since these selenoproteins may have an antioxidant function within the endothelial cell,

down-regulation of TR and PHGPX may diminish the cells antioxidant capacity and render it more susceptible to oxidative damage and to the development of atheroma.

The selenoprotein expression of BAEC did not appear to be regulated by PMA as visualized by autoradiography. Also, treatment of BAEC with PMA did not modify TR activity. These results suggest that in BAEC, any possible modification of selenoprotein expression must occur through a signal transduction mechanism other than the phosphoinositol pathway. Differences in signal transduction pathways have been previously reported. For example, bradykinin, a potent stimulus for IP_3 formation and Ca^{2+} mobilization in bovine aortic and cerebral microvascular endothelium is only partially effective in HUVEC (Garcia and Natarajan, 1992).

The addition of the calcium ionophore A23187 increased the expression of TR 1.96 ± 0.13-fold over basal levels in HUVEC. A similar response of TR to A23187 was reported in human thyrocytes and HepG2 cells (Howie *et al.*, 1998). A23187 has a number of actions, in addition to increasing the intracellular concentration of Ca^{2+}. It may act as an uncoupler of oxidative phosphorylation and as an inhibitor of mitochondrial ATPase activity (Reed and Lardy, 1972). The action of A23187 on Ca^{2+} influx is a rapid response. For example, the A23187-induced cGMP response in cultured endothelial cells reaches a plateau within 3 minutes (Schmidt, Mayer and Kukovetz, 1989). However, in our studies the increase of TR expression in response to A23187 was only observed when A23187 was present continuously in the culture medium for periods in excess of 12 hr. This raises the possibility that the regulation of TR expression by A23187 is a result of the toxic effect of A23187 rather than a calcium-signalling effect. In agreement with this Dr Forbes Howie has found that the calcium ionophore ionomycin has no effect on TR expression in human or sheep thyrocytes (personal communication).

Studies involving the Ca^{2+} regulation of TR expression are not in agreement. The claim by Schallreuter and colleagues that purified human TR is regulated by Ca^{2+} at physiological levels

has been refuted (Oblong and Powis, 1993). Oblong and Powis suggested that the ambiguity concerning the Ca^{2+} regulation of TR results from Schallreuter *et al.* using only a partially purified TR preparation which may contain a less stable isoform of TR or another reductase (Oblong and Powis, 1993). Howie *et al.* have claimed that the up-regulation of TR in cultured human thyrocytes was a calcium signalling effect and postulated that TR may be important in detoxifying the high concentrations of peroxides produced during thyroid hormone synthesis (Howie *et al.*, 1998). Therefore the possibility exists that increased TR levels are a consequence of increased reactive oxygen species in thyrocytes and other cell types rather than a straightforward Ca^{2+} response. In agreement with this hypothesis the addition of A23187 to FRTL-5 rat thyroid cell line and to human thyroid slices produced a marked stimulation of hydrogen peroxide production (Björkman and Ekholm, 1992; Corvilain *et al.*, 1994). Indeed, our data also suggests that calcium signalling does not regulate TR expression, but that the up-regulation of TR is likely to be a consequence of toxicity, possibly an oxidative stress resulting from the presence of A23187.

The TR/Trx system provides a powerful dithiol/disulphide oxidoreductase system which has been shown to reduce and detoxify lipid hydroperoxides, hydrogen peroxides, and organic hydroperoxides (Björnstedt *et al.*, 1995). The accumulation of such compounds in tissues exerts deleterious effects. For example the hydroperoxide (15S)-hydroperoxy-(5Z), (8Z), 11 (Z), 13 (E)-eicosatetraenoic acid ([15S]-HPETE) oxidizes low-density lipoprotein to a form which under some conditions renders the particle more toxic to the endothelium, with implications for the development and progression of atherosclerosis (Björnstedt *et al.*, 1995).

In HUVEC, the A23187-induced increase in TR expression may therefore act as a detoxification mechanism induced by the increased presence of reactive oxygen species which result from the presence of A23187. The addition of tert-butylhydroperoxide (an oxidative agent) to HUVEC in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite for 20 hr resulted in an increased

labelling of the 58 kDa selenoprotein (TR) (data shown in figure 5.02, section five). In addition A23187 treatment of BAEC has been shown to induce oxidant production as measured by luminol-enhanced chemiluminescence (Kooy and Royall, 1994). These findings also support the interpretation that the up-regulation of TR in response to A23187 may be a beneficial response to oxidative stress rather than a specific induction occurring through calcium signalling.

The effect of A23187 on cyGPX and PHGPX was variable in our studies. In one experiment (figure 4.24) PHGPX appeared to be down-regulated after A23187 treatment, but in a further two experiments (figures 4.21 and 4.22) no clear change was observed. The reasons for this difference are unknown, but may be accounted for by the age, selenium status, oxidant status or the ethnic group of the individual from whom the cells were isolated.

In the unlikely event that A23187 was exerting its effects on TR expression in HUVEC through a calcium response, it would appear that the calcium signalling branch antagonises the phosphoinositol signalling branch of the calcium-phosphoinositol signalling pathway. There are other examples in the endothelium where this is the case. For example, constitutive endothelial nitric oxide synthase activity in endothelial cells is stimulated by A23187, but the subsequent A23187-induced nitric oxide release is inhibited by PKC activation using the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (Hirata *et al.*, 1995). There are also examples in endothelial cells where the two branches of the calcium-phosphoinositol signalling pathway act synergistically. Matsubara and Ziff have shown that in HUVEC, simultaneous stimulation with PMA and A23187 produces a large increase in superoxide anion release at submaximal concentrations, which, when added separately, only cause a minimal response. (Matsubara and Ziff, 1986).

Bradykinin (BK) and adenosine triphosphate (ATP) activate the calcium-phosphoinositol signalling pathway by increasing intracellular Ca^{2+} in endothelial cells (Buchan and Martin, 1991;

Busse, Trogisch and Bassenge, 1985; Colden-Stanfield *et al.*, 1987; Natarajan, 1995). Our data shows that the treatment of cultured HUVEC with BK and ATP induced no change in [^{75}Se]-labelling of any of the selenoproteins. These compounds, through an increase in intracellular calcium, may not affect selenoprotein expression, thereby supporting a non-specific effect of A23187. There are a number of alternative possible explanations for this lack of response. Firstly, it is possible that the BK and ATP receptors are lost during isolation and subculture of these cells or secondly, the receptors may be down-regulated as a result of the growth factors present in the culture medium. These possibilities were not investigated further in any more detail. However, no intracellular cGMP response to BK could be measured in the HUVEC used in this study (data not shown).

The inability of acetylcholine (ACh) to promote changes in the [^{75}Se]-labelling of selenoproteins is likely to be the effect of the loss of muscarininc receptors during cell culture. The literature reports that freshly isolated endothelial cells respond to ACh (Fransen, Katnik and Adams, 1998; Hartmann, Saeed and Bing, 1987) whilst there are a number of reports showing that cultured endothelial cells do not respond to ACh (Cocks *et al.*, 1985; Gryglewski, Moncada and Palmer, 1986; Schmidt, Mayer and Kukovetz, 1989).

Forskolin (a diterpine compound that activates adenylate cyclase) had no effect on the [^{75}Se]-selenoprotein expression in HUVEC. The activation of adenylate cyclase by forskolin and subsequent increase in cAMP levels has been previously reported in endothelial cells (Crutchley *et al.*, 1993; Ferro *et al.*, 1999). These findings suggest therefore that selenoprotein expression in HUVEC is not regulated by the adenylate cyclase signal transduction pathway, in contrast to thyrocytes where this signalling pathway is extremely important.

In conclusion, in HUVEC and HCAEC the expression of TR, PHGPX and cyGPX is regulated by the activation of PKC. Since oxidised LDL has been shown to activate PKC the changes in

selenoprotein expression which we observed in this study may reflect those seen *in vivo* in endothelial cells exposed to reactive oxygen species. In particular TR and PHGPX are potentially important antioxidant enzymes in the vascular endothelium and their down-regulation could result in an increased susceptibility to oxidative damage and thus the development of atheroma. Again the findings of this chapter demonstrate that HUVEC but not BAEC provide a good model for HCAEC as both HUVEC and HCAEC show similar responses in selenoprotein expression to PMA whilst in BAEC treatment with PMA induces no such changes.

CHAPTER FIVE THE ROLE OF SELENIUM IN THE PREVENTION OF OXIDATIVE DAMAGE

5.1 INTRODUCTION

Aerobic cells are constantly exposed to oxidative stresses from reactive oxygen species (ROS) such as superoxide, hydroxyl radicals, hydrogen peroxide and singlet oxygen. At a cellular level ROS are responsible for a number of different disruptive processes, including: lipid peroxidation, protein cross-linking and degradation, cleavage of DNA, polymerization of polysaccharides and ultimately cell mortality (Hennig and Chow, 1988; Kirkland, 1991; Pohlman and Harlan, 2000; Schuppe-Koistinen *et al.*, 1994; Thomas, Geiger and Girotti, 1993). Oxidative damage to the endothelium is one of the principle mechanisms in the pathogenesis of atherosclerosis (Gimbrone, 1995; McGorisk and Treasure, 1996; Ross, 1993). Cytoprotection against oxidative damage is accomplished by diverse enzymatic and non-enzymatic antioxidant systems. In the endothelial cell these systems principally consist of antioxidant vitamins, superoxide dismutase, catalase and selenoproteins such as the glutathione peroxidases and possibly thioredoxin reductase (TR).

Low plasma selenium levels have been associated with an increased risk of cardiovascular disease including coronary atherosclerosis (Kok *et al.*, 1991; Salonen *et al.*, 1982; Suadicani, Hein and Gyntelberg, 1992). Studies *in vitro* have demonstrated that selenium supplementation confers resistance on bovine endothelial cells to oxidative damage (Ochi, Morita and Murota, 1992; Thomas, Geiger and Girotti, 1993). Ochi *et al.* demonstrated that sodium selenite and ebselen (a synthetic glutathione peroxidase mimic) dramatically decreased the toxicity of the hydroperoxide (15*S*)-hydroperoxy-(5*Z*), (8*Z*), 11(*Z*), 13(*E*)-eicosatetraenoic acid ([15*S*]-HPETE) (which is formed *in vivo* by oxygenation of arachidonic acid by arachidonic acid 15-lipoxygenase) (Björnstedt *et al.*, 1995) in a dose-dependent manner (10-100nM) (Ochi, Morita and Murota, 1992). These findings and further investigations manipulating the glutathione redox cycle

suggested that (15S)-HPETE-induced cytoxicity could be attributed to a decreased glutathione peroxidase (GPX) activity. In agreement with Ochi *et al.,* Thomas *et al.* using bovine endothelial cells, proposed that increased cytoxicity in selenium-deficient endothelial cells to both tert-butylhydroperoxide (t-BuOOH) and photogenerated oxidized low density lipoprotein (oxLDL) resulted from a decrease in both cytoplasmic glutathione peroxidase (cyGPX) and phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity (Thomas, Geiger and Girotti, 1993). However, the expression of other selenoproteins with antioxidant properties such as extracellular glutathione peroxidase, selenoprotein P and TR was not considered in either of these studies.

cyGPX catalyzes the reduction of a variety of hydroperoxides, including hydrogen peroxide, cumene hydroperoxide, t-BuOOH, and fatty acid hydroperoxides (Flohé, 1989; Rotruck *et al.*, 1973). In contrast, PHGPX has been shown to catalyze the reduction of fatty acid hydroperoxides and cholesterol hydroperoxides (Ursini, Maiorino and Gregolin, 1985). The modification of the expression and activity of these selenoproteins in cultured cells through selenium supplementation has been widely reported (Ricetti *et al.*, 1994; Takahashi, Newburger and Cohen, 1986; Thomas, Geiger and Girotti, 1993). It is therefore possible that decreased expression and activity of these selenoproteins in selenium-deficiency may increase the susceptibility of the endothelium to oxidative damage.

Knockout mice which are deficient in cyGPX have been developed as a model to study the role of cyGPX in normal physiology and the pathogenesis of a number of diseases (Ho *et al.*, 1997). Using this model Ho *et al.* showed that mice deficient in cyGPX developed normally and showed no increased sensitivity to pulmonary hyperoxia (Ho *et al.*, 1997). In contrast, oxidative stress induced by 30 mg/kg of paraquat, which is approximately LD_{20} for mice, killed all the cyGPX knock-out mice, whereas control mice showed no signs of toxicity at this dose (de Haan *et al.*, 1998). The possible protective role of cyGPX in the pathogenesis of atherosclerosis has not

been ruled out and the use of this model in future studies could help define which selenoproteins contribute to the protective effect of selenium against oxidative damage in endothelial cells.

Data presented in this thesis have shown that TR is the dominantly expressed selenoprotein in both HUVEC and HCAEC. Despite this, an antioxidant function for TR in the endothelium has not been reported. TR can reduce and detoxify lipid hydroperoxides and organic hydroperoxides (Björnstedt *et al.*, 1995). For example, the hydroperoxide (15*S*)-HPETE oxidizes low density lipoprotein to a form that under some conditions renders it more toxic to the endothelium, with implications for the pathogenesis of atherogenesis (Björnstedt *et al.*, 1995). TR can detoxify (15*S*)-HPETE and other lipid hydroperoxides (Björnstedt *et al.*, 1995).

An important mediator of oxidative damage to the endothelium *in vivo* is oxLDL (Nielson, 1999; Steinberg, 1991; Witzum and Steinberg, 1991). OxLDL is not a single homogenous entity, instead it consists of many oxidation products that form from both the peroxidation and fragmentation of the lipid components of LDL, and the modification and oxidation of the apoprotein B (Rosenfeld, 1991; Witzum and Steinberg, 1991). Studies using oxLDL are confounded by the considerable variation in the products formed between different preparations. It appears that the products formed are dependent on several factors including; the cell type used to initiate oxidation, the metal ion concentration in the medium as well as the composition of the medium, the incubation conditions and the inherent susceptibility of the native LDL to be oxidised in the peroxidation conditions used for different studies (Esterbauer and Jürgens, 1993; Kuzuya *et al.*, 1991; Witzum and Steinberg, 1991). Therefore the toxic composition of any one batch of oxLDL made using LDL isolated from different sources, even when using constant conditions, is known to vary, which can make interpretation of results difficult. t-BuOOH has been previously used as a membrane permeable oxidative stress inducing reagent (Elliot, Doan and Henschke, 1995; Schupe, Moldéus and Cotgreave, 1992; Thomas, Geiger and Girotti, 1993). This is the agent adopted for the studies in this chapter. Although it could be argued that

t-BuOOH is not physiological agent, unlike oxLDL it is not subject to between-batch variation to the same extent.

Organic selenium such as selenomethionine and selenocysteine provide the main source of selenium ingested in the normal human diet. However inorganic forms of selenium such as sodium selenite are often used in experimental diets and as supplements (Daniels, 1996). In man, tracer studies have shown that selenomethionine is incorporated more efficiently into body tissues and has a longer half-life than selenite (Griffiths, Stewart and Robinson, 1976). The properties of selenomethionine result from the ability of selenomethionine to be substituted into proteins at methionine residues (Behne *et al.*, 1991). It is possible that sodium selenite is a more bioactive form of the trace element selenium. Studies comparing the efficacy of sodium selenite and selenomethionine in the prevention of oxidative damage to endothelial cells have not been previously performed.

Differences in the expression of intracellular selenoproteins occur between endothelial cells isolated from different vascular beds. Also, there are clear species differences in selenoprotein expression in the endothelium (chapter three). It is therefore possible that the antioxidant systems of endothelial cells may vary between cells isolated from different vascular beds and different species.

This study aims to:

- determine the effects of t-BuOOH on [^{75}Se]-selenoprotein expression in HUVEC
- examine the ability of sodium selenite supplementation to protect HUVEC, HCAEC and BAEC from cytotoxicity resulting from t-BuOOH treatment
- associate any observed protective effects with changes in the expression and activity of TR and the activity of cyGPX and PHGPX

- assess the direct effect of sodium selenite in the protection of HUVEC against oxidative damage resulting from t-BuOOH treatment
- to compare the potency of inorganic sodium selenite with organic selenomethionine on the protection of HUVEC against oxidative damage resulting from t-BuOOH treatment.

5.2 MATERIALS AND METHODS

5.2.1 Introduction

To study the ability of selenium to protect endothelial cells against oxidative damage from tert-butylhydroperoxide (t-BuOOH), HUVEC, HCAEC and BAEC were isolated, cultured and maintained as described in sections 2.3.2, 2.3.5 and 2.3.8, respectively. Prior to each experiment the cells were sub-cultured and seeded according to a specific protocol using M199 medium to which were added the supplements used in EGM-2 (i.e. FBS (2%), hydrocortisone (0.04%), ascorbic acid (0.1%), long R insulin-like growth factor-1 (0.1%), heparin (0.1%), human fibroblast growth factor (0.4%), human recombinant vascular endothelial growth factor (0.1%), human recombinant epidermal growth factor (0.1%) and gentamicin sulphate/ amphotericin B (0.1%)). The selenium content of the M199 medium containing these supplements was determined by acid digestion followed by fluorimetric analysis, as described in section 2.3.11. M199 medium containing all the above listed supplements had a selenium concentration of 4.73 nM, and was classified as effectively selenium-deficient medium.

Selenium-sufficient medium refers to a culture medium with a selenium concentration of approximately 48 nM. It comprises of a basal medium, with added selenium, and contains the same supplements as the selenium-deficient medium described above.

5.2.2 The effect of t-BuOOH on the expression of [^{75}Se]-selenoproteins in HUVEC

For these experiments HUVEC derived from the same primary cell isolation were seeded into both 12 well plates (seeding density 10000 cell/cm^2) and T75 flasks (seeding density 3000 cells/cm^2) and maintained in selenium-sufficient medium (48 nM).

a) The effect of different concentrations of t-BuOOH on cell viability

To determine a sub-toxic concentration of t-BuOOH, triplicate wells of confluent wells of HUVEC, which had been cultured in selenium-sufficient medium, were incubated with defined concentrations of t-BuOOH (0, 50, 60, 70, 80, 90, 100, 500 µM) for 48 hr. After this incubation period, both the culture medium and cells were harvested and analysed for LDH activity as described in section 2.3.21.

b) The effect of a sub-toxic dose of t-BuOOH on the expression of [^{75}Se]-selenoproteins

Confluent cultures of HUVEC in T75 flasks, which had been cultured in selenium-sufficient medium, were pre-labelled to a steady state with 0.02 MBq/ml [^{75}Se]-selenite for 48 hr (as described in section 3.2.2) prior to investigating the effects of a sub-toxic dose of t-BuOOH (as determined from the experiment described in section 'a' above). The effect of t-BuOOH on the [^{75}Se]-labelled selenoproteins was studied by the inclusion of t-BuOOH (90 µM) in the culture medium for 48 hr in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite.

After 48 hr, HUVEC were harvested into 20 ml EBSS by scraping and centrifuged at 2000 g for 10 min at 4°C. The resulting cell pellet was resuspended in 200µl 60 mM Tris buffer, pH 7.8, containing 1 mM EDTA and 1 mM dithiothreitol (Tris buffer). The cells were lysed by sonication on ice using the Soniprep 150.

Protein concentrations were measured using the Bradford assay (section 2.3.12). The samples were then diluted to a common protein concentration with Tris buffer and prepared for separation by SDS-PAGE as described in section 2.3.13.

The [^{75}Se]-labelled selenoproteins present in 25 µg protein were separated by SDS-PAGE (section 2.3.13). The resulting gel was dried and the [^{75}Se]-labelled selenoproteins visualized by autoradiography using Kodak X-OMAT XAR-5 film (section 2.3.14).

5.2.3 The effect of different doses of t-BuOOH on LDH activity in HUVEC cultured in selenium-deficient medium

HUVEC were sub-cultured and seeded into 12 well plates with selenium-deficient medium. The effect of different doses of t-BuOOH (0, 75, 150, 300 μM) on HUVEC viability was determined using triplicate wells of confluent HUVEC. After 20 hr incubation in the presence of t-BuOOH both the medium and cells were harvested and analysed for LDH activity (section 2.3.21).

5.2.4 The ability of sodium selenite to protect against oxidative damage from t-BuOOH in HUVEC

To investigate the possible protective effect of sodium selenite against oxidative damage, HUVEC were sub-cultured into 12 well plates at a density of approximately 10000 cells/cm^2 using selenium-deficient medium to which different concentrations of sodium selenite had been added. After three days the culture medium was removed and replaced with fresh medium containing the same respective concentration of sodium selenite.

Due to clear variability in the susceptibility of different preparations of HUVEC to be killed by t-BuOOH it was necessary to determine a concentration of t-BuOOH which would be significantly toxic to a particular preparation of HUVEC. This was done before each main experiment and required an additional 12 well plate of HUVEC seeded in selenium-deficient medium. Concentrations of t-BuOOH ranging from 0-300 μM were added to the cells and after 20 hr exposure to t-BuOOH the HUVEC were observed under the light microscope. The concentrations of t-BuOOH chosen for the subsequent experiment spanned the range of concentrations which were found to cause significant cell death as observed under the light microscope.

The remaining HUVEC when fully confluent (between 5 to 6 days) were used to monitor the effects of sodium selenite at preventing cell death in the presence of two or three concentrations of t-BuOOH. After 20 hr of exposure to t-BuOOH both the medium and cells were harvested and analysed for LDH activity as described in section 2.3.21.

5.2.5 Assessment of the direct effect of sodium selenite in the protection of HUVEC against oxidative damage from t-BuOOH

To determine whether sodium selenite can exert a direct antioxidant effect against t-BuOOH in HUVEC, rather than through modification of selenoprotein expression, the following procedure was adopted. Briefly, HUVEC were cultured in selenium-deficient medium until confluent and then supplemented with different concentrations of sodium selenite (0, 5, 10, 40, 160 nM) simultaneously with the addition of t-BuOOH (100 μM). In contrast, HUVEC, were sub-cultured in selenium-deficient medium, were supplemented with different concentrations of sodium selenite (0, 5, 10, 40, 160 nM) prior to treatment with t-BuOOH.

HUVEC were sub-cultured into 12 well plates at a density of approximately 10000 cells/cm^2 in selenium-deficient medium. When the cells were fully confluent (between 5 to 6 days) the culture medium was removed and replaced with fresh medium containing sodium selenite (0, 5, 10, 40, 160 nM) to which t-BuOOH (100 μM) was added. Control cells received no t-BuOOH or sodium selenite supplementation. After 20 hr both the medium and cells were harvested and analysed for LDH activity as described in section 2.3.21.

In parallel, HUVEC derived from the same primary cell preparation were sub-cultured at a density of approximately 10000 cells/cm^2 in selenium-deficient medium containing defined concentrations of sodium selenite (0, 5, 10, 40, 160 nM). When the cells were fully confluent (between 5 to 6 days) the culture medium was removed and replaced with fresh medium

containing sodium selenite (0, 5, 10, 40, 160 nM) to which t-BuOOH (100 µM) was added. Again control cells received no t-BuOOH or sodium selenite supplementation.

After 20 hr treatment with t-BuOOH the medium and cells from the experiments were harvested and analysed for LDH activity as described in section 2.2.21.

The above procedure was carried out on two different preparations of HUVEC.

5.2.6 The ability of sodium selenite to protect against oxidative damage from t-BuOOH in HCAEC

The procedures used to study the protective effect of sodium selenite against oxidative damage from t-BuOOH in HCAEC were as described for HUVEC (section 5.2.4).

Unfortunately, due to a limited supply of HCAEC, it was not possible to repeat these experiments on different preparations of cells. However the experiment was repeated on the same preparation of cells but at a different passage to show that the effects observed were reproducible.

5.2.7 The ability of sodium selenite to protect against oxidative damage from t-BuOOH in BAEC

The procedures used to study the protective effect of sodium selenite against oxidative damage from t-BuOOH in BAEC were as described previously for HUVEC (section 5.2.4).

Again due to a limited supply of BAEC this experiment was repeated on the same preparation of cells at a later passage.

5.2.8 The effect of sodium selenite supplementation on intracellular selenoprotein expression and activity in different vascular endothelial cells

Measurements of TR mass and activity, cyGPX and PHGPX activity, were run in parallel with the cell viability determinations. HUVEC, HCAEC and BAEC were sub-cultured as described above (sections 5.2.4, 5.2.6 and 5.2.7) and a proportion of the cells were seeded at a density of approximately 3000 cells/cm^2 into T75 flasks. The cells were cultured and maintained in selenium-deficient medium containing sodium selenite concentrations which corresponded to those used for each of the cell viability experiments. The cultured medium was removed and replaced with fresh medium containing sodium selenite on every other day. Upon reaching full confluence (approximately 9 to 12 days) the cells were harvested, lysed in 0.125 M potassium phosphate buffer, pH 7.4, and sonicated on ice. The HUVEC lysates were then subsequently frozen at -70°C until assayed. TR mass and activity, cyGPX and PHGPX activity were determined as described in sections 2.3.17, 2.3.18, 2.3.19 and 2.3.20, respectively.

It was not possible to determine TR expression in BAEC due to the lack of cross-reactivity of the antibody to human TR with the bovine protein. The cyGPX and PHGPX activity were not measured in all experiments due to a shortage of cells.

5.2.9 A comparison of the protective effect of sodium selenite and selenomethionine against oxidative damage from t-BuOOH in HUVEC

HUVEC were sub-cultured into 12 well plates at a density of approximately 10000 cells/cm^2 in selenium-deficient medium to which different concentrations of either sodium selenite or selenomethionine had been added (0, 10, 40, 160, 320, 640 nM). On the third day after sub-culture the medium was removed and replaced with fresh medium containing the same respective dose of sodium selenite or selenomethionine. Upon reaching confluence (between 5 to 6 days) the culture medium was removed and replaced with fresh medium which contained

either sodium selenite or selenomethionine to which different concentrations of t-BuOOH had been added (0, 100, 200 μM). After 20 hr incubation with t-BuOOH both the medium and cells were harvested and analysed for LDH activity as described in section 2.3.21.

5.2.10 Statistical analysis

One-way analysis of variance (ANOVA) was used to test for significant differences in % LDH activity retained in response to t-BuOOH between cells cultured in selenium-sufficient media and cells cultured in selenium-deficient media. In the cases where the difference was significant ($p<0.05$) a Tukey-Kramer multiple comparisons post test was used to test for significant differences in % LDH retained in response to t-BuOOH for sodium selenite concentrations.

These statistical tests were also used to investigate significant differences between levels of selenoprotein expression and activity in cells cultured in different concentrations of sodium selenite.

5.3 RESULTS

5.3.1 The effect of t-BuOOH on the expression of [^{75}Se]-selenoproteins in HUVEC

a) The effect of different concentrations of t-BuOOH on cell viability

The changes in %LDH activity retained by HUVEC incubated for 48 hr with different concentrations of t-BuOOH (0, 50, 60, 70, 80, 90, 100 and 500 μM) are shown in figure 5.01. Concentrations of 90 μM t-BuOOH and above significantly decreased the %LDH activity retained by HUVEC ($p<0.001$). The concentration of t-BuOOH chosen to study the effects of this compound on the expression of intracellular [^{75}Se]-selenoproteins was 90 μM. This concentration produced a small, albeit significant, toxic effect.

b) The effect of a sub-toxic dose of t-BuOOH on the expression of [^{75}Se]-selenoproteins

Treatment of HUVEC with 90 μM t-BuOOH for 20 hr significantly up-regulated the expression of the 58kDa selenoprotein identified as TR (figure 5.02). No other changes in expression of intracellular [^{75}Se]-labelled selenoproteins were observed.

5.3.2 The effect of different doses of t-BuOOH on LDH activity in HUVEC cultured in selenium-deficient medium

The cell viability of HUVEC, cultured in selenium-deficient medium, was decreased in a dose-dependent manner in response to treatment with t-BuOOH (figure 5.03). The cytotoxic effect was initially observed at 75 μM. Concentrations of 150 μM and 300 μM t-BuOOH decreased the % LDH activity retained to its lowest levels of 11.25 ± 1.05% and 18.87 ±0.70% (mean ± SD, n=3), respectively.

5.3.3 The ability of sodium selenite to protect against oxidative damage from t-BuOOH in HUVEC

The protective effect of sodium selenite against oxidative damage resulting from t-BuOOH exposure was established in four different preparations of HUVEC.

a) Protection by selenite added at concentrations up to 1000 nM

Figure 5.04 shows that HUVEC cultured in the presence of all the concentrations of sodium selenite tested (40, 200, 1000 nM) were significantly less sensitive to the cytotoxic effects of t-BuOOH (100 μM) compared to HUVEC cultured in selenium-deficient medium. The maximal protective effect of sodium selenite was observed at 200 nM sodium selenite though 40 nM sodium selenite conferred almost full protection against 100 μM t-BuOOH ($p<0.001$).

TR activity and mass and cyGPX and PHGPX activity were determined in the same preparation of HUVEC as the cell viability experiment shown in figure 5.04. Figures 5.05 a and b show that both TR activity and mass were significantly increased in HUVEC cultured in the presence of 40 nM sodium selenite (by 2.2-fold and 3.0-fold, respectively) compared to HUVEC cultured in selenium-deficient medium ($p<0.05$). The TR activity was maximal in HUVEC cultured in 40 nM sodium selenite although with all concentrations of sodium selenite tested the TR activity was higher than basal activity. At 1000 nM sodium selenite, TR expression was maximally increased by 2.9-fold over basal. Figure 5.05 c shows that cyGPX activity was significantly increased in HUVEC grown in increasing concentrations of sodium selenite ($p<0.01$). The maximal increase in cyGPX activity was a 5.4-fold ($n=3$) response to 1000 nM sodium selenite. Figure 5.05 d shows that PHGPX activity is increased in a dose-dependent manner to sodium selenite, with the increase significant at 200 nM and 1000 nM sodium selenite ($p< 0.05$).

b) Protection by selenite added at concentrations between 0-160 nM

In the following experiment HUVEC were cultured in a more limited range of sodium selenite concentrations (10, 40, 160 nM) than used in the previous experiment in order to more accurately define a level of sodium selenite at which protection against t-BuOOH could be observed. These concentrations of sodium selenite were also more physiological than those used in the previous experiment. Different concentrations of t-BuOOH (100, 200, 300 μM) were also used, again in an attempt to more closely define the protective effect of sodium selenite.

Figure 5.06 shows that HUVEC cultured in 10 nM sodium selenite appear to be slightly less sensitive to the cytotoxic effects of 100 μM t-BuOOH compared to those cells cultured in selenium-deficient medium. This protective effect was only significant and maximal in HUVEC cultured in 40 nM and 160 nM sodium selenite. In HUVEC exposed to 200 μM and 300 μM t-BuOOH no protection was afforded by sodium selenite at any concentration tested (10, 40, 160 nM). It was observed, however, that in HUVEC exposed to these higher concentrations of t-BuOOH (200 and 300 μM) the % LDH activity retained by the cell significantly decreased with increasing sodium selenite concentrations (200 μM, $p< 0.01$; 300 μM, $p<0.05$).

TR activity and mass and cyGPX and PHGPX activity were determined in these HUVEC cultured in different concentrations of sodium selenite (0, 10, 40, 160 nM). TR activity and mass and cyGPX activity for all concentrations of sodium selenite were higher than levels measured in selenium-deficient cells and this effect was maximal at 40 nM sodium selenite in all cases, with n-fold increases of 2.5, 1.5, and 5.3, respectively (n=3; figures 5.07 a, b and c.). PHGPX activity was also increased significantly in HUVEC cultured in 10 nM and 160 nM sodium selenite ($p<0.05$). The increase was maximal in HUVEC grown in the presence of 10 nM sodium selenite which expressed a 3.7-fold increase in PHGPX activity over cells cultured in selenium-deficient medium (n=3).

Figure 5.08 shows the protective effect of different doses of sodium selenite (0, 1, 5, 10, 40 nM) against cytoxicity from t-BuOOH (0, 100, 200 μM) in a third HUVEC preparation. A significant protective effect against 100 μM t-BuOOH was observed in HUVEC cultured in 5 nM sodium selenite compared to selenium-deficient cells ($p<0.001$). The sensitivity to 100 μM t-BuOOH was decreased further at 10 nM and protection was maximal at 40 nM sodium selenite resulting in 93.4% LDH activity retained. Again, sodium selenite did not exhibit a protective effect against 200 μM t-BuOOH. However, as observed previously, the sensitivity, as measured by % LDH retained, to this concentration of t-BuOOH was significantly augmented at all concentrations of sodium selenite ($p<0.05$). In addition, in HUVEC exposed to 100 μM t-BuOOH at 1 nM sodium selenite (a concentration at which no protection was observed) the % LDH retention was significantly attenuated ($p< 0.001$).

The TR activity and mass was measured in the third experiment and both increased in response to sodium selenite in a dose-dependent manner. As observed previously the increase was maximal in HUVEC grown in the presence of 40 nM sodium selenite which expressed a 3.9-fold and 2.0-fold increase in TR activity and TR mass respectively, over cells which were cultured in selenium-deficient medium (n=3; figure 5.09).

Figure 5.10 shows the protective effect of different doses of sodium selenite (0, 1, 5, 10, 40, 160 nM) against cytoxicity from different concentrations of t-BuOOH (0, 100, 200 μM) in the fourth HUVEC preparation. A near maximal, highly significant protective effect was observed at 10 nM sodium selenite against 100 μM t-BuOOH ($p<0.001$). The decreased sensitivity to 100 μM t-BuOOH was maintained in HUVEC cultured in 40 nM and 160 nM sodium selenite. Again it was observed that sodium selenite did not exhibit a protective effect against 200 μM t-BuOOH at any concentration, and as previously recorded, the sensitivity to this dose of t-BuOOH was significantly increased at all concentrations of sodium selenite ($p<0.001$) in a dose-dependent manner. HUVEC cultured in 5 nM sodium selenite and then exposed to 100 μM t-BuOOH (a

sodium selenite concentration at which no protection was observed) the % LDH retention was significantly attenuated ($p< 0.001$).

Both the TR activity and mass were measured in the fourth HUVEC preparation and both increased in response to sodium selenite in a dose-dependent manner. On this occasion the increase in TR activity was maximal in HUVEC grown in the presence of 160 nM sodium selenite which expressed a 6.4-fold increase in TR activity over cells cultured in selenium-deficient medium. TR mass was maximal in HUVEC cultured in 40 nM sodium selenite which expressed a 3.7-fold increase (n=3; figure 5.11) compared to selenium-deficient medium.

5.3.4 Assessment of the direct effect of sodium selenite in the protection of HUVEC against oxidative damage from t-BuOOH

Figure 5.12 shows the effects of sodium selenite on the % LDH retention in HUVEC. The selenite was added either prior to or at the same time as t-BuOOH (100 µM) treatment. In HUVEC to which different concentrations of sodium selenite (0, 5, 10, 40, 160 nM) were added at the same time as t-BuOOH treatment no protection was afforded to the HUVEC. In contrast, HUVEC which had been pre-cultured in sodium selenite were significantly protected against 100 µM t-BuOOH at 40 nM and 160 nM sodium selenite concentrations ($p<0.001$).

These effects were paralleled in a further experiment which used the same protocol but with HUVEC from a different preparation (data not shown).

5.3.5 The ability of sodium selenite to protect against oxidative damage from t-BuOOH in HCAEC

The protective effect of sodium selenite against cytotoxicity from t-BuOOH observed in HUVEC was also evident in HCAEC. Figure 5.13 clearly demonstrates that the sensitivity of HCAEC to

100 μM t-BuOOH is significantly decreased in cells cultured in concentrations of sodium selenite of 10 nM and above ($p<0.001$). The protective effect of sodium selenite was sub-maximal at 10 nM sodium selenite. At 40 nM and 160 nM sodium selenite, the decreased sensitivity to 100 μM t-BuOOH was maximal with % LDH activity retained measured at 94.7 ± 0.4% and 95.1 ± 0.8% respectively (mean ± SD, n=3). HCAEC treated with 150 μM and 200 μM t-BuOOH responded in the same manner as observed in HUVEC, showing a significant reduction in % LDH retention in cells cultured in increasing concentrations of sodium selenite (150 μM, $p< 0.05$; 200 μM, $p<0.001$).

TR activity and mass and cyGPX and PHGPX activity were determined in these HCAEC cultured in different concentrations of sodium selenite (0, 1, 5, 10, 40, 160 nM) (figure 5.14). TR activity and mass significantly increased in response to sodium selenite concentrations of 5 nM sodium selenite and above compared to HCAEC cultured in selenium-deficient medium ($p<0.001$). The increases in TR activity and mass were maximal in HUVEC grown in 160 nM sodium selenite which produced a 4.1-fold and 1.9-fold increase, respectively (n=3). CyGPX activity also increased dose-dependently with sodium selenite. The augmented activity was significant at 10 nM sodium selenite ($p<0.05$) and maximal at 160 nM which increased activity 4.0-fold over levels measured in cells cultured in selenium-deficient medium. In contrast PHGPX activity was shown not to be significantly different from basal levels in HCAEC cultured in sodium selenite at all concentrations tested. However there was a trend towards a small increase in at 40 nM and 160 nM sodium selenite.

The experiment was repeated in the same preparation of HCAEC of a later passage with a similar protective effect against 100 μM t-BuOOH observed (figure 5.15). However, the effect was close to maximal at 5 nM sodium selenite resulting in a 80.9 ±3.5% retention of % LDH activity (mean ± SD, n=3). Again, the HCAEC treated with 200 μM t-BuOOH showed a

significantly attenuated % LDH retention in response to sodium selenite concentrations of 5 nM and above ($p< 0.01$).

TR activity and mass and cyGPX and PHGPX activity were measured in these HCAEC cultured in different concentrations of sodium selenite (0, 1, 5, 10, 40 nM) (figure 5.16). The TR activity and mass increased in an almost identical dose-dependent manner to sodium selenite as observed in the previous HCAEC. In contrast, the cyGPX activity in these cells showed no significant changes with added sodium selenite, although there was a trend towards increased activity in response to sodium selenite concentrations of 5 nM and above. PHGPX activity increased in a dose-dependent manner to increasing concentrations of sodium selenite with a significant response to 10 nM and 40 nM sodium selenite ($p<0.05$).

Thus the only selenoprotein which changed in activity at 5nM sodium selenite, a dose which gave full protection against 100 µM t-BuOOH was TR.

5.3.6 The ability of sodium selenite to protect against oxidative damage from t-BuOOH in BAEC

Figure 5.17 shows the protective effect of different concentrations of sodium selenite (0, 1, 5, 10, 40, 160 nM) against cytoxicity from different concentrations of t-BuOOH (100 and 200 µM) in cultured BAEC. At concentrations of 10 nM sodium selenite and below no protective effect against t-BuOOH (100 µM) was found, though cells cultured in 40 nM and 160 nM sodium selenite showed a moderate level of protection with 46.9 ± 26.9% and 35.8± 5.4 % LDH retention, respectively (mean ± SD, n=3). No significant differences were observed in cell damage for BAEC cultured in sodium selenite at any concentration and then treated with 200 µM t-BuOOH. Thus full protection was not afforded by any concentration of sodium selenite tested.

This experiment was repeated on the same preparation of BAEC of a later passage with similar results (figure 5.18).

TR activity was measured in response to increasing concentrations of sodium selenite in the two different passages of BAEC which in the cytoxicity experiments had shown a similar protective profile against 100 μM t-BuOOH. Figure 5.19 shows the changes in TR activity in BAEC parallel the % LDH retention data represented in figure 5.17. BAEC cultured in 40 nM and 160 nM sodium selenite significantly increased the activity of TR ($p<0.05$) from 0 mU/mg protein to 0.594 ± 0.121 mU/mg protein and 0.484 ± 0.174 mU/mg protein, respectively. In contrast in the subsequent experiment for which the cytoxicity data was illustrated in figure 5.18, TR activity was significantly augmented in BAEC cultured in sodium selenite concentrations of 5 nM and above (figure 5.20; $p<0.001$). The maximal increase in TR activity was measured in BAEC cultured in 40 nM sodium selenite whereby TR activity was 1.1-fold higher than measured in cells cultured in the absence of sodium selenite.

At each concentration of sodium selenite TR activity was significantly higher in HUVEC compared to BAEC.

5.3.7 A comparison of the protective effect of sodium selenite and selenomethionine against oxidative damage from t-BuOOH in HUVEC

Selenomethionine was less potent at protecting HUVEC against damage from t-BuOOH (100 μM) then sodium selenite. Figure 5.21 shows a comparison of the protective effects of various concentrations of sodium selenite and selenomethionine (0, 10, 40, 160, 320, 640 nM) against oxidative damage from t-BuOOH (100 and 200 μM).

HUVEC cultured in the presence of all the concentrations of sodium selenite tested (10, 40, 160, 320, 640 nM) were significantly less sensitive to the cytotoxic effects of 100 μM t-BuOOH

compared to HUVEC cultured in selenium-deficient medium (figure 5.21). Also, at all concentrations of sodium selenite tested the protective effect was near maximal with LDH activity retention at approximately 90.0%. As observed in previous experiments in section 5.3.3, sodium selenite did not exhibit a protective effect against 200 μM t-BuOOH, though the sensitivity to this dose of t-BuOOH was significantly augmented at all concentrations of sodium selenite tested ($p<0.05$).

In contrast in the same preparation of HUVEC, cultured in the presence of selenomethionine (10, 40, 160, 320, 640 nM), no indication of a protective effect against damage from either dose of t-BuOOH (100 and 200 μM) was observed. At each concentration of either sodium selenite or selenomethionine tested a significant difference between the % LDH response to 100 μM t-BuOOH was found ($p<0.001$) between selenite and selenomethionine-treated cells .

The second experiment comparing the protective effects of sodium selenite with selenomethionine was carried out using exactly the same procedure on a different preparation of HUVEC but using a different stock of selenomethionine. This experiment again demonstrated that selenomethionine was less potent at protecting HUVEC against damage from t-BuOOH (100 μM) than sodium selenite (figure 5.22). However on this occasion the sensitivity to 100 μM t-BuOOH was significantly attenuated at 640 nM selenomethionine resulting in a LDH activity retention of 82.1 ± 5.4% (mean ± SD, n=3). At this concentration no significant difference between the protective effect of sodium selenite and selenomethionine was found.

The other contrasting features of these two experiments include the different sodium selenite concentrations at which a significant protective effect against 100 μM t-BuOOH was observed. In the first experiment 10 nM sodium selenite produced a significant protective effect against 100 μM t-BuOOH whilst in the second 40 nM sodium selenite produced a significant response. Also

the increase in sensitivity to 200 μM t-BuOOH observed was shown to be significant in the second experiment with the exception of 160 nM selenomethionine ($p<0.05$).

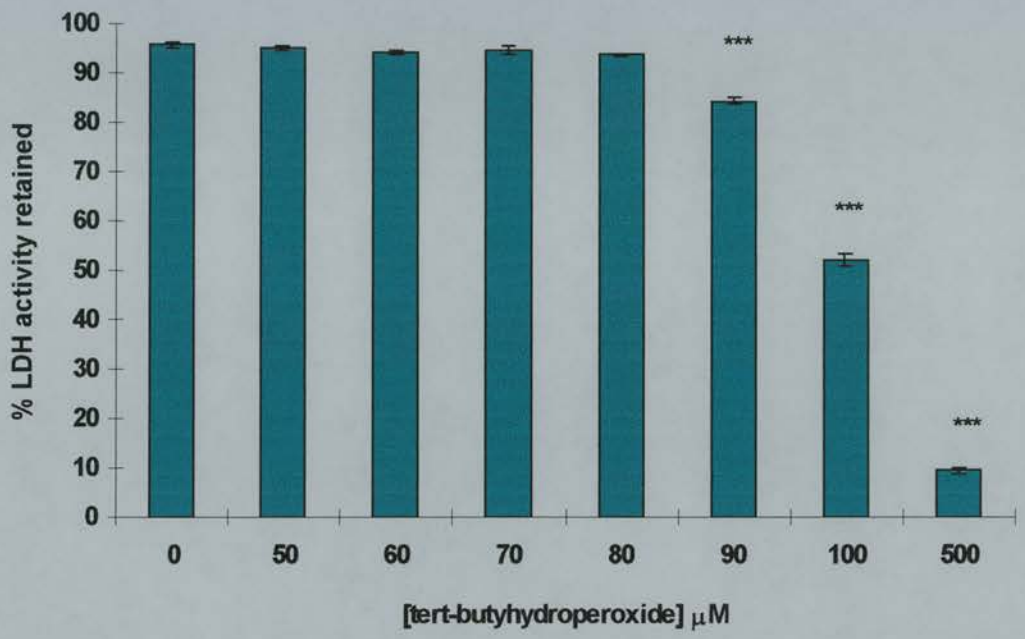


Figure 5.01. %LDH activity retained in HUVEC incubated with various concentrations of tert-butylhydroperoxide (t-BuOOH) for 48 hr. Confluent cultures of HUVEC grown in selenium-sufficient medium were incubated in various concentrations of t-BuOOH (0, 50, 60, 70, 80, 90, 100, 500 μM) for 48 hr. Results shown are the mean of triplicate wells ± SD. Results which are significantly different from untreated cells are denoted as follows:- $p<0.001^{***}$.

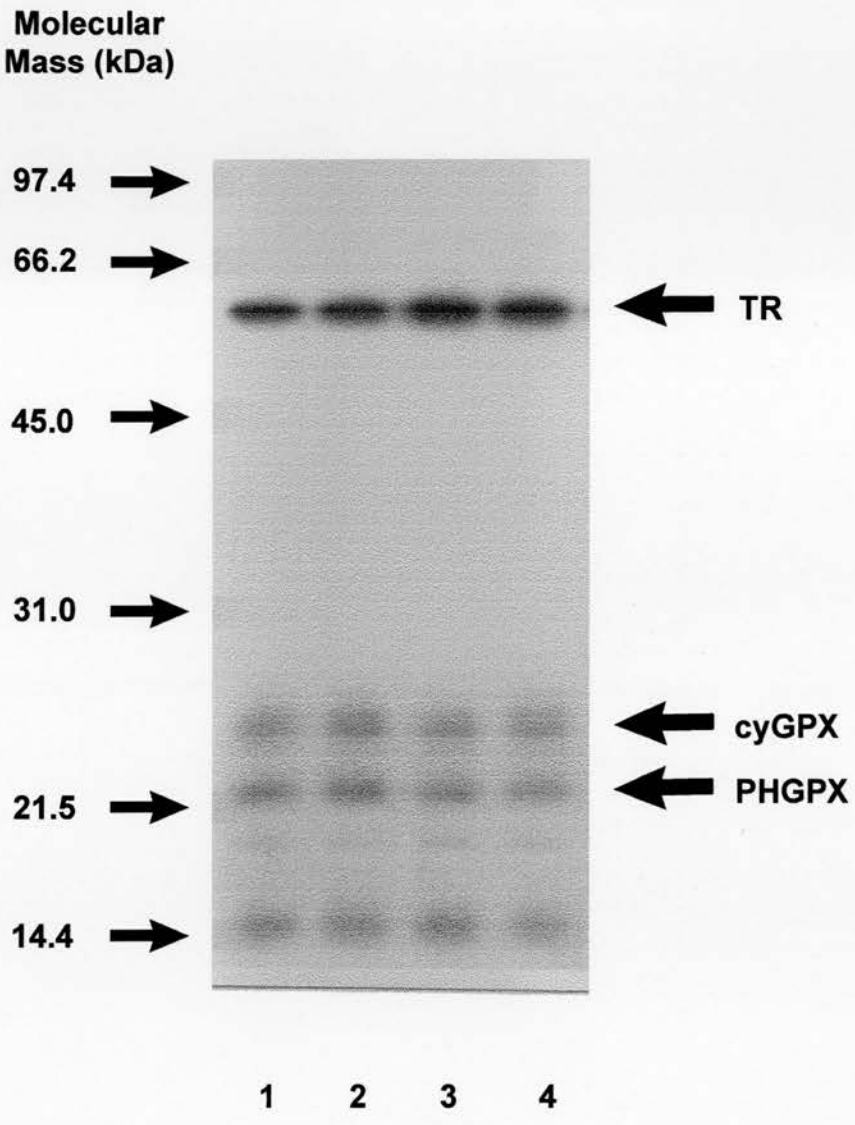


Figure 5.02. Autoradiograph of an SDS-PAGE gel showing the expression of [^{75}Se]-selenoproteins in human umbilical vein endothelial cells (HUVEC) incubated in the continued presence of tert-butylhydroperoxide (t-BuOOH). Confluent cultures of HUVEC were pre-labelled for 48 hr with 0.02 MBq/ml [^{75}Se]-selenite. The effects of t-BuOOH (90 μM) on the expression of [^{75}Se]-selenoproteins was studied by including t-BuOOH in the culture medium for 48 hr in the continued presence of 0.02 MBq/ml [^{75}Se]-selenite. Lanes 1 and 2, no additions; lanes 3 and 4, t-BuOOH (90 μM). Each lane was loaded with 25 μg of protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; phospholipid hydroperoxide glutathione peroxidase, PHGPX.

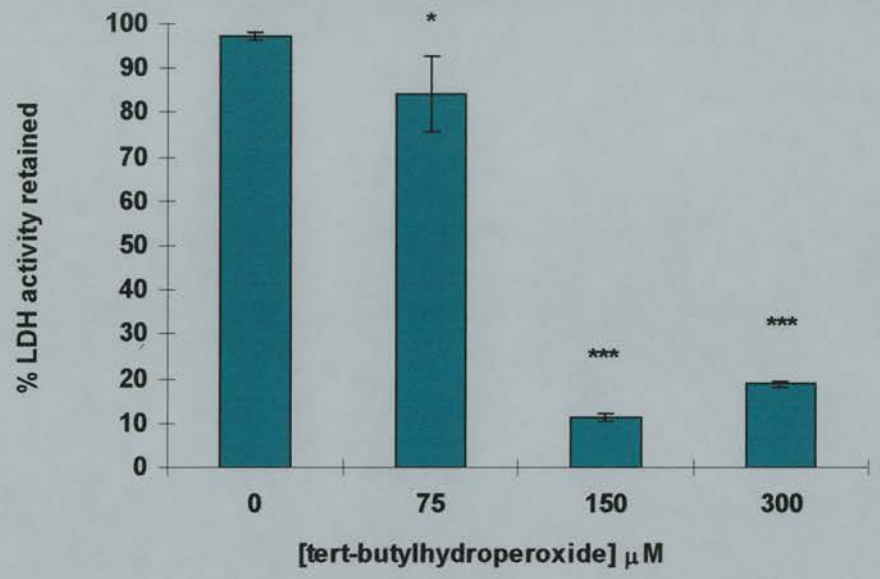


Figure 5.03. %LDH activity retained in HUVEC incubated with various concentrations of tert-butylhydroperoxide (t-BuOOH) for 20 hr. Confluent cultures of HUVEC grown in selenium-deficient medium were incubated in various concentrations of t-BuOOH (0, 75, 150, 300 μM) for 20 hr. Results shown are the mean of triplicate wells ± SD. Results which are significantly different from untreated cells are denoted as follows:- $p<0.05^{*}$, $p<0.001^{***}$.

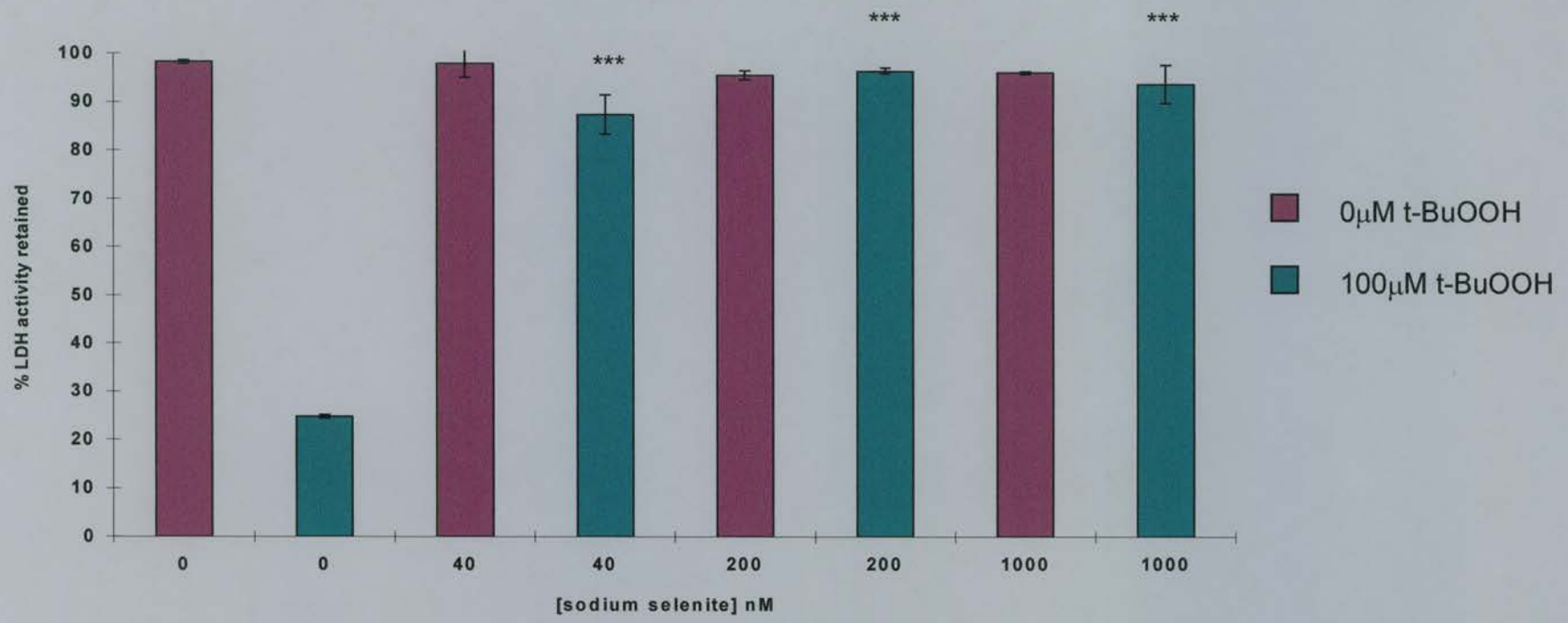


Figure 5.04. The effect of sodium selenite on the sensitivity of HUVEC to tert-butylhydroperoxide (t-BuOOH) induced cell damage. HUVEC were sub-cultured into selenium-deficient medium containing different concentrations of sodium selenite (0, 40, 200, 1000 nM). At confluence HUVEC were incubated in the absence or presence of t-BuOOH (100 μM). After 20 hr cell viability was assessed by determining the **%** LDH activity retained. Results shown are the mean of triplicate wells ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:- $p<0.001^{***}$. One of four experiments on HUVEC.

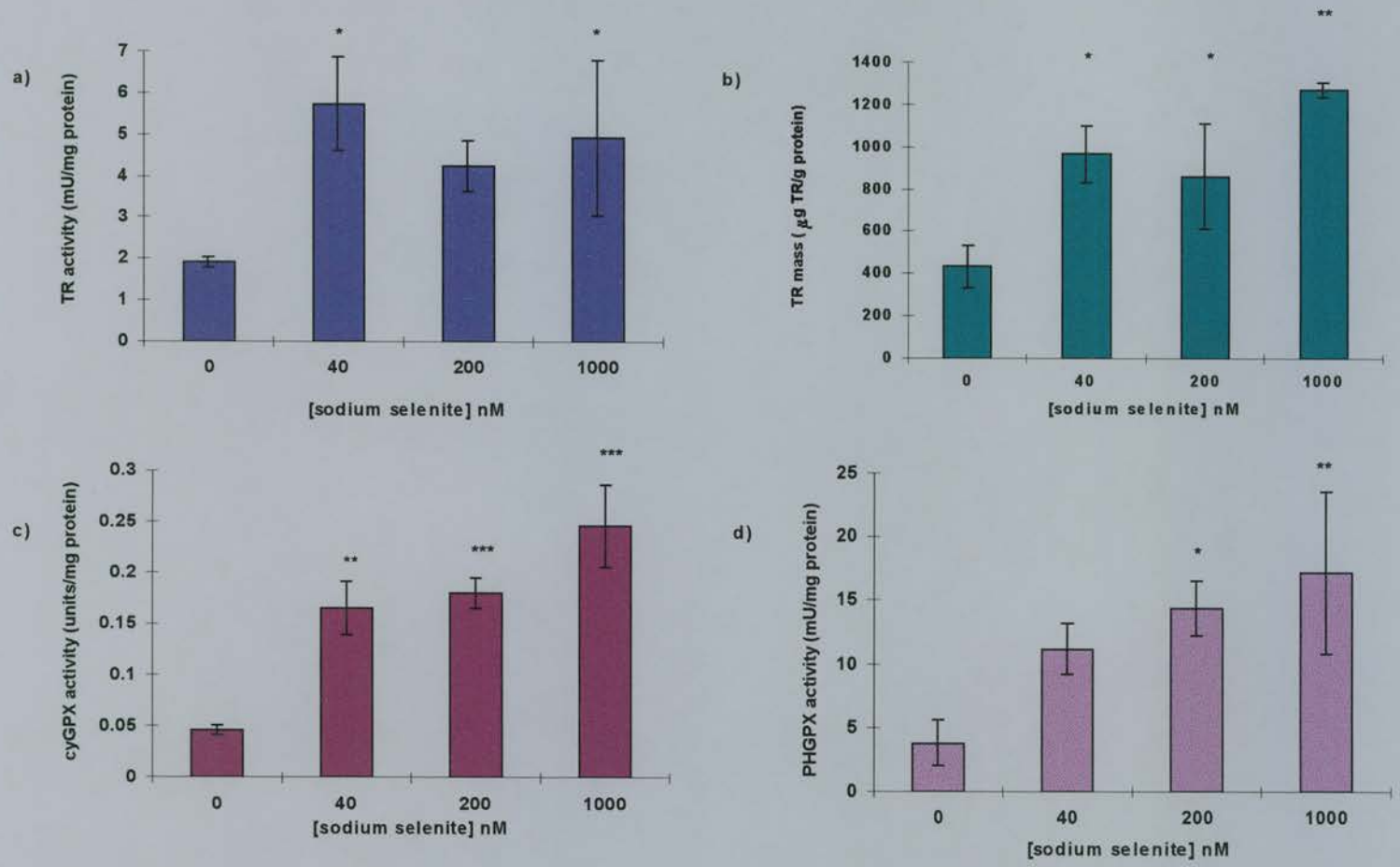


Figure 5.05. The expression and activity of three selenoproteins in HUVEC cultured in selenium-deficient medium containing different concentrations of sodium selenite. HUVEC were sub-cultured into selenium-deficient medium containing different concentrations of sodium selenite (0, 40, 200, 1000 nM). At confluence HUVEC were harvested and then assayed for; a) Thioredoxin reductase (TR) activity; b) TR mass; c) cytoplasmic glutathione peroxidase (cyGPX) activity; d) phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity. Results are shown as the mean of triplicate flasks ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:- $p< 0.05^{*}$. $p< 0.01^{**}$, $p< 0.001^{***}$. One of four experiments using HUVEC.

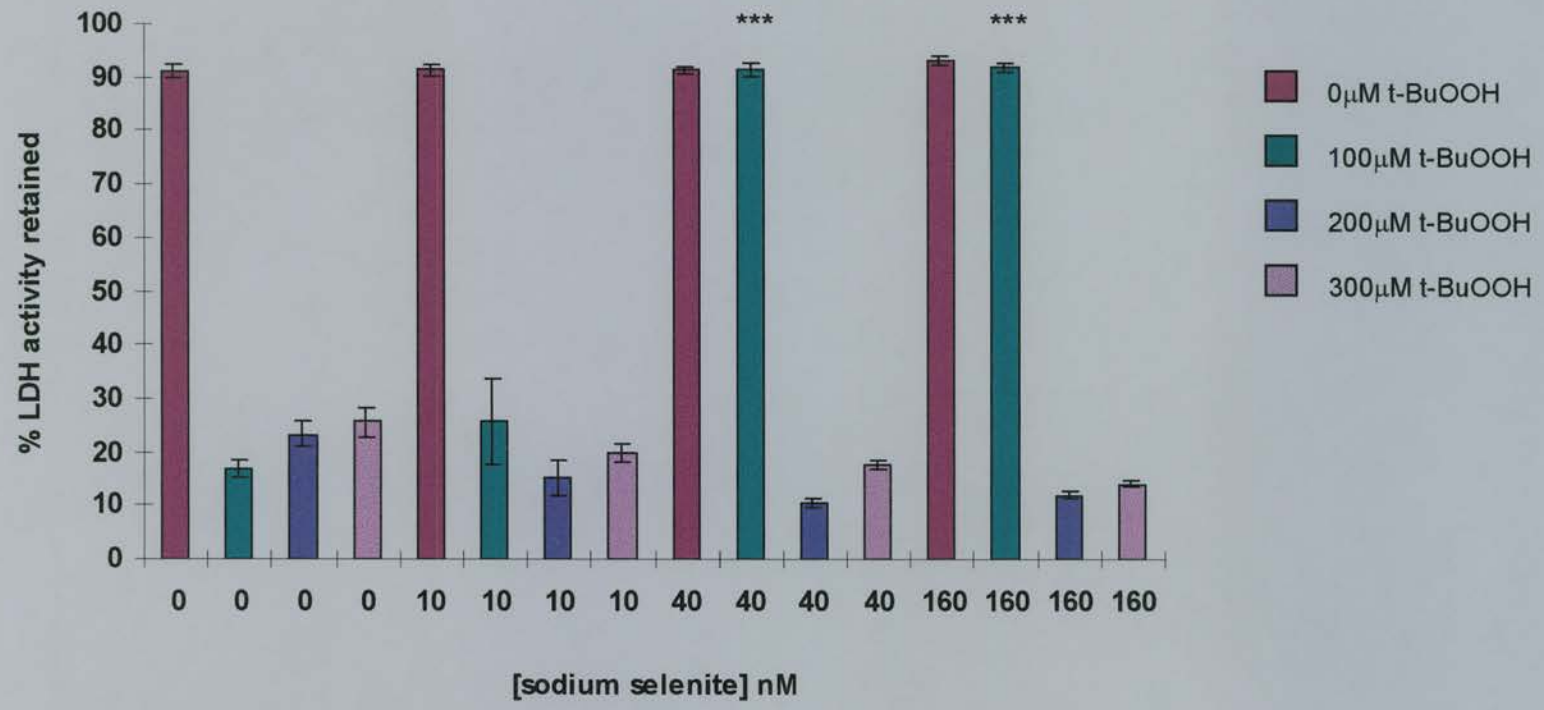


Figure 5.06. The effect of sodium selenite on the sensitivity of HUVEC to tert-butylhydroperoxide (t-BuOOH)-induced cell damage. HUVEC were sub-cultured into selenium-deficient medium containing different concentrations of sodium selenite (0, 10, 40, 160 nM). At confluence HUVEC were incubated in the presence of different concentrations of t-BuOOH (0, 100, 200, 300 μM). After 20 hr cell viability was assessed by determining the % LDH activity retained. Results shown are the mean of triplicate wells ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:-$p<0.001^{***}$. One of four experiments using HUVEC.

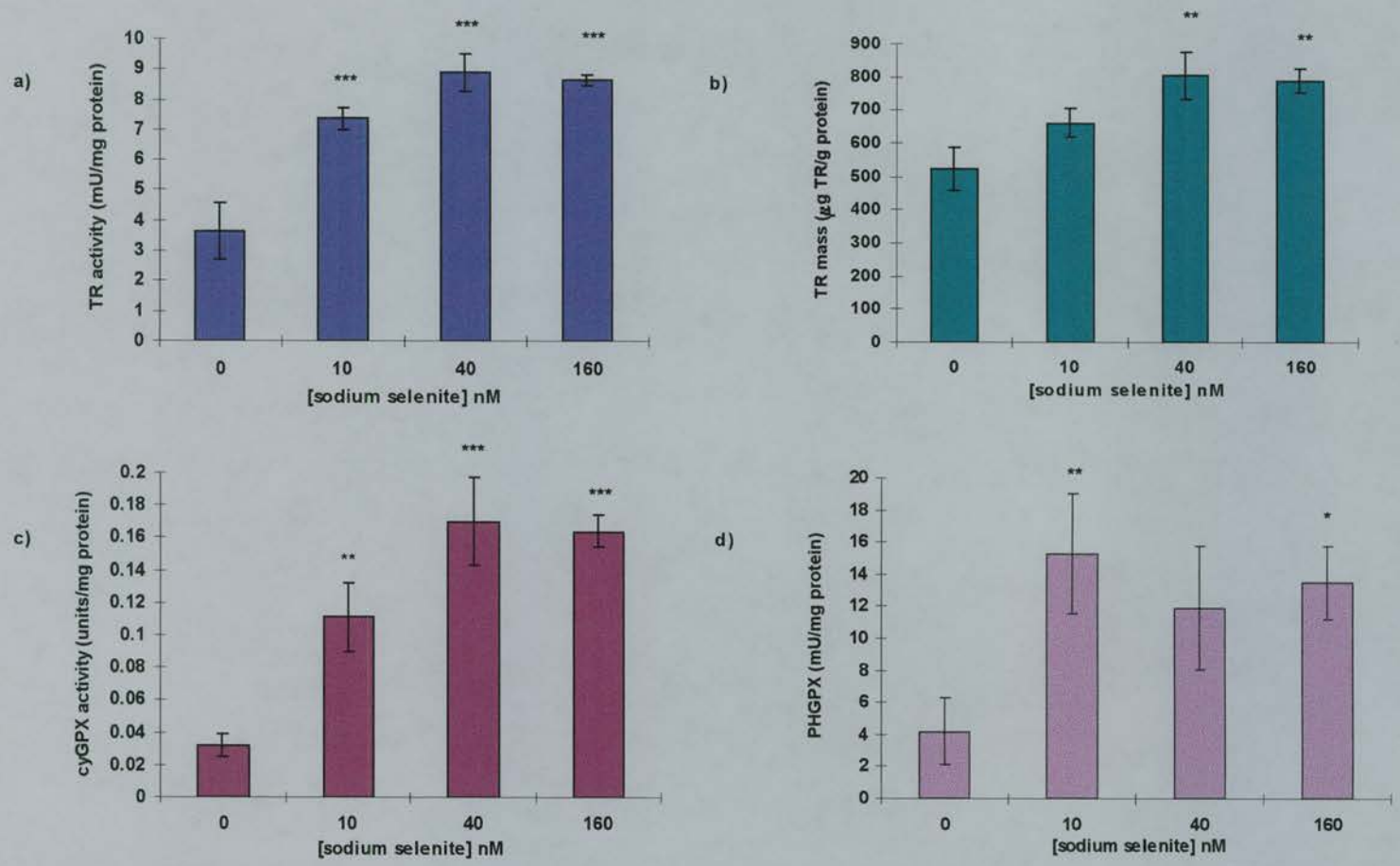


Figure 5.07. The expression and activity of three selenoproteins in HUVEC cultured in selenium-deficient medium containing different concentrations of sodium selenite. HUVEC were sub-cultured into selenium-deficient medium containing different concentrations of sodium selenite (0, 10, 40, 1600nM). At confluence HUVEC were harvested and then assayed for; a) thioredoxin reductase (TR) activity; b) TR mass; c) cytoplasmic glutathione peroxidase (cyGPX) activity; d) phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity. Results are shown as the mean of triplicate flasks ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:-$p< 0.05^{*}$. $p< 0.01^{**}$. $p< 0.001^{***}$. One of four experiments using HUVEC.

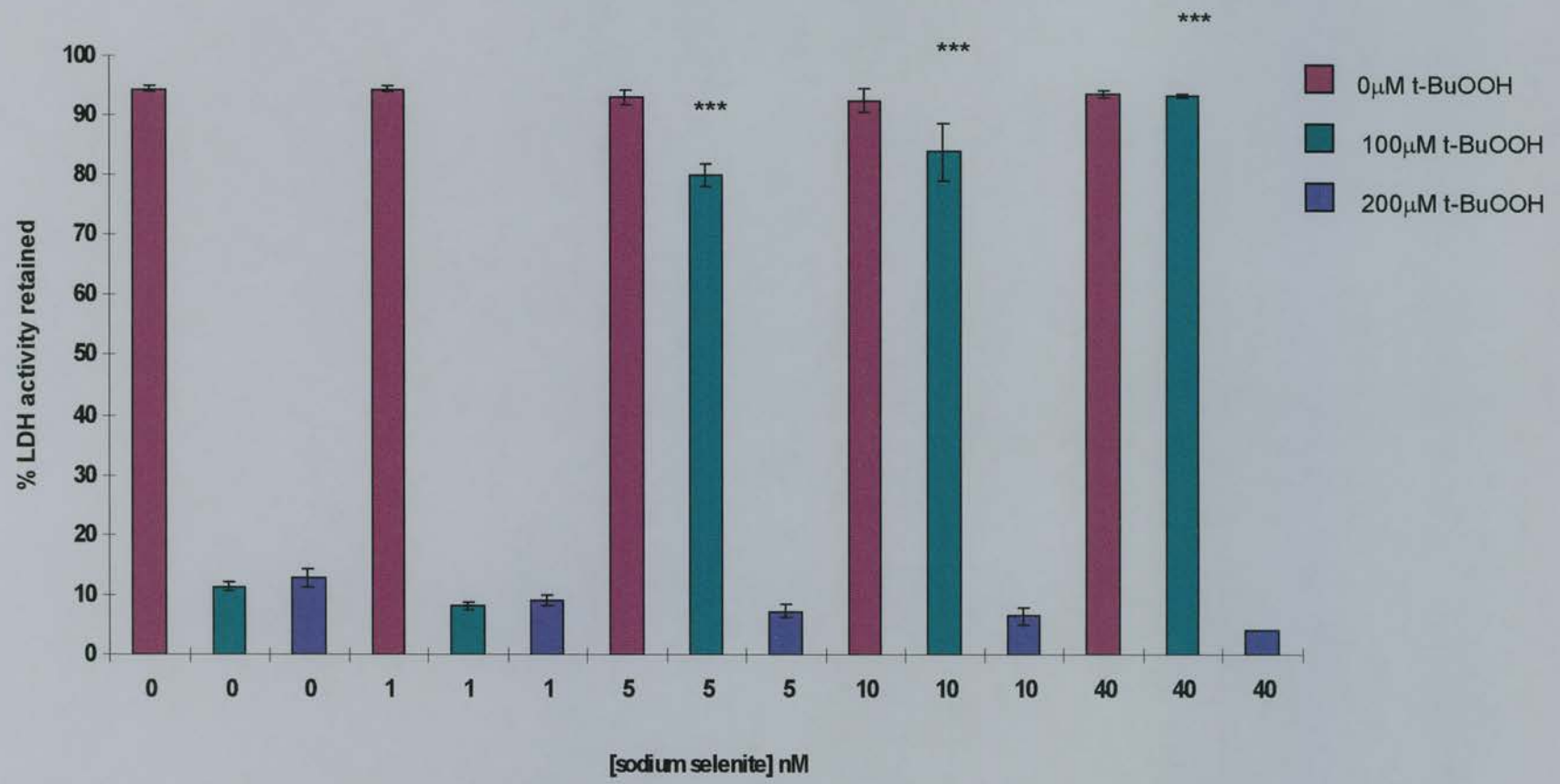


Figure 5.08. The effect of sodium selenite on the sensitivity of HUVEC to tert-butylhydroperoxide (t-BuOOH) induced cell damage. HUVEC were sub-cultured into selenium-deficient medium containing different concentrations of sodium selenite (0, 1, 5, 10, 40 nM). At confluence HUVEC were incubated in the presence of different concentrations of t-BuOOH (0, 100, 200 μM). After 20 hr cell viability was assessed by determining the % LDH activity retained. Results shown are the mean of triplicate wells ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:-$p<0.001^{***}$. One of four experiments using HUVEC.

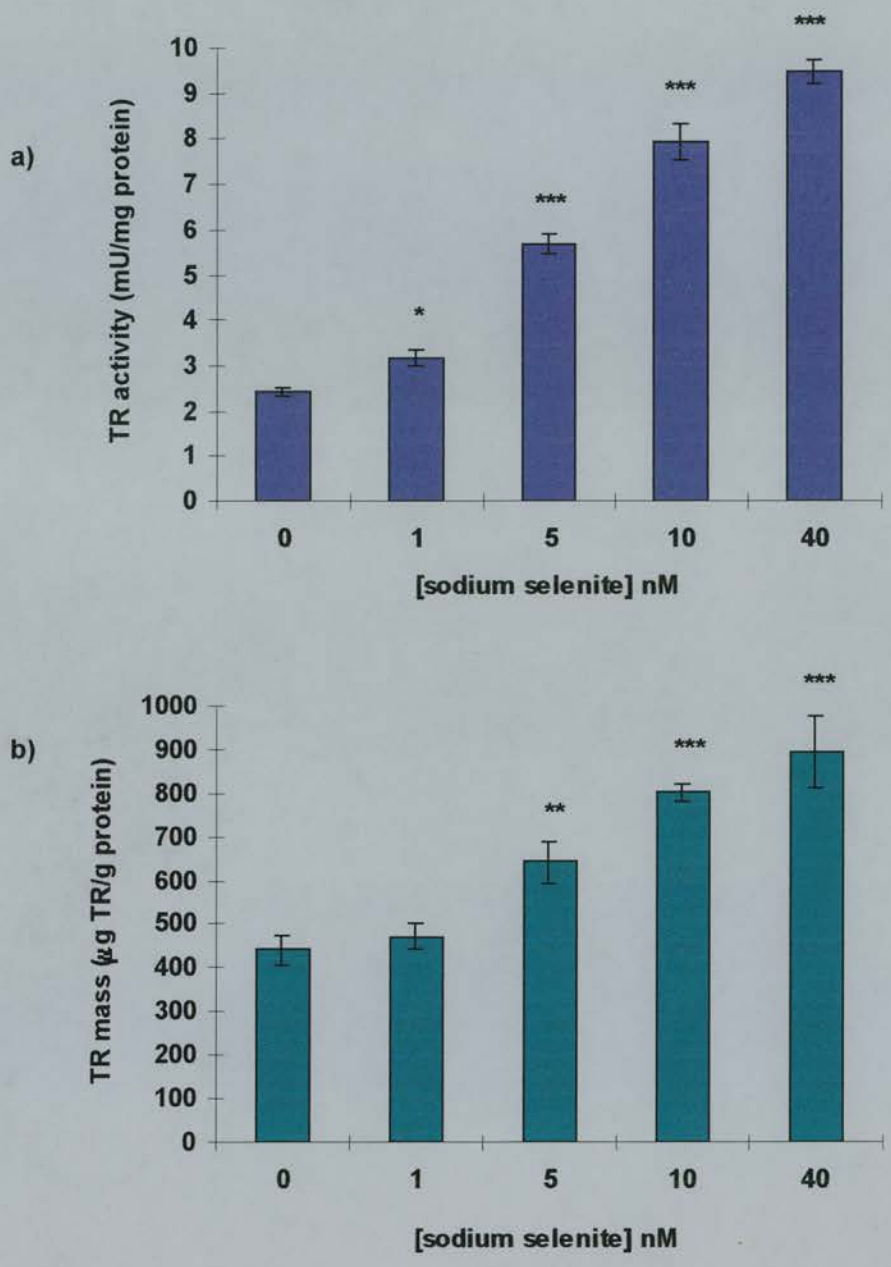


Figure 5.09. Thioredoxin reductase (TR) activity and mass in HUVEC cultured in selenium-deficient medium containing different concentrations of sodium selenite. HUVEC were sub-cultured into selenium-deficient medium containing different concentrations of sodium selenite (0, 1, 5, 10, 40 nM). At confluence HUVEC were harvested and assayed for; a) TR activity; b) TR mass. Results are shown as triplicate flasks ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:-$p< 0.05^{*}$, $p< 0.01^{**}$, $p< 0.001^{***}$. One of four experiments using HUVEC.

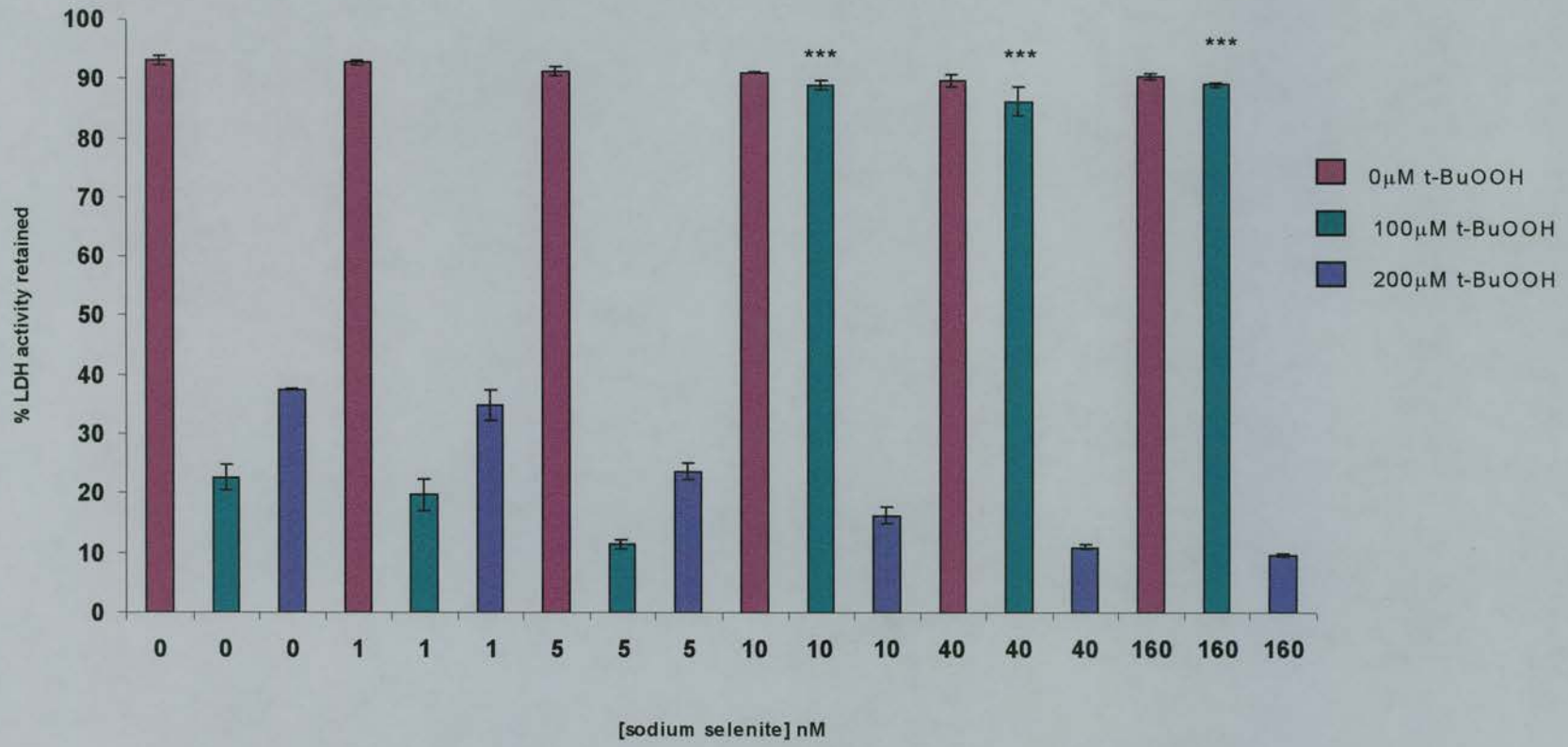


Figure 5.10. The effects of sodium selenite on the sensitivity of HUVEC to tert-butylhydroperoxide (t-BuOOH) induced cell damage. HUVEC were sub-cultured into selenium-deficient medium containing different concentrations of sodium selenite (0, 1, 5, 10, 40, 160 nM). At confluence HUVEC were treated with different concentrations of t-BuOOH (0, 100, 200 μM). After 20 hr, cell viability was assessed by determining the % LDH activity retained by the cells. Results shown are the mean of triplicate wells ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:-$p<0.001^{***}$. One of four experiments using HUVEC.

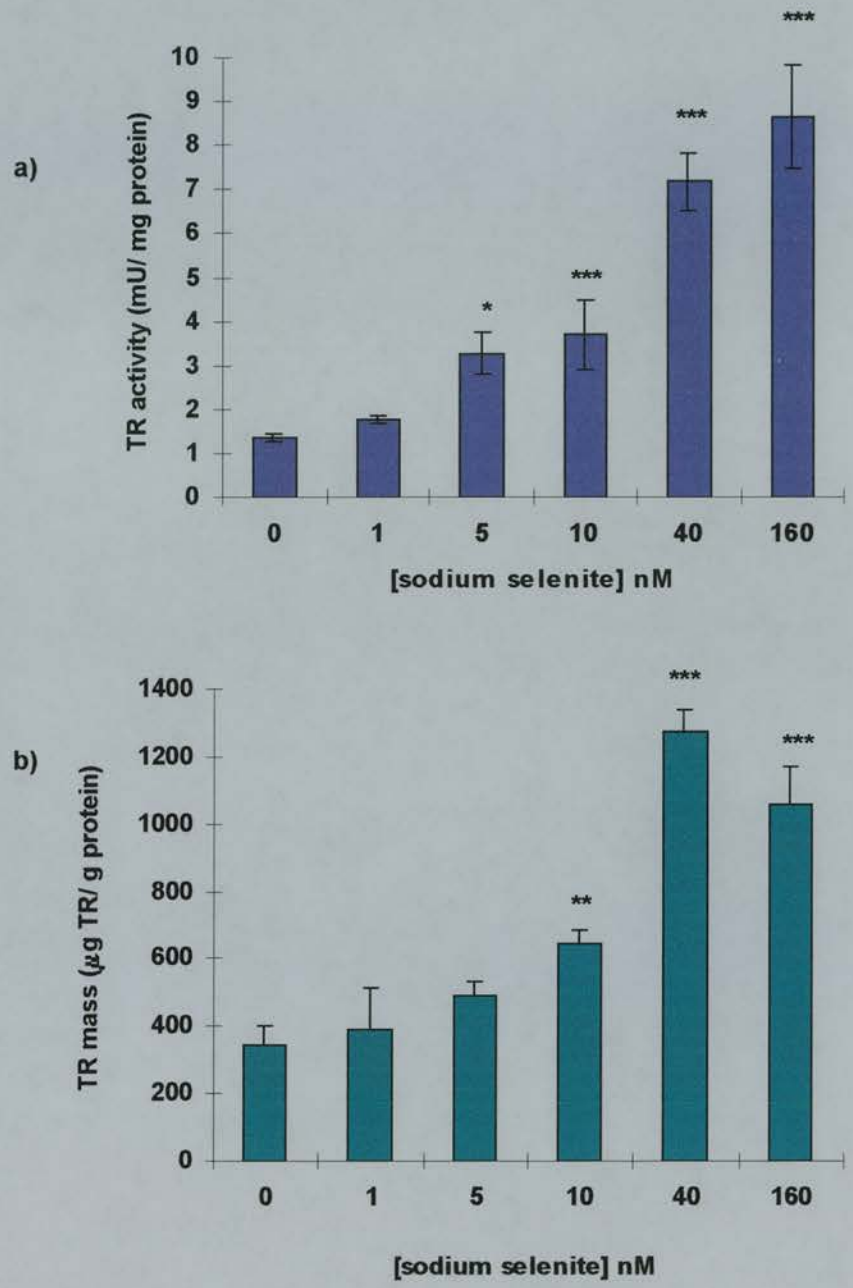


Figure 5.11. Thioredoxin reductase (TR) activity and mass in HUVEC cultured in selenium-deficient medium containing different concentrations of sodium. HUVEC were sub-cultured into selenium-deficient medium containing different concentrations of sodium selenite (0, 1, 5, 10, 40, 160 nM). At confluence HUVEC were harvested and assayed for; a) TR activity and b) TR mass. Results are shown as triplicate flasks ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:-$p< 0.05^{*}$, $p< 0.01^{**}$, $p< 0.001^{***}$. One of four experiments using HUVEC.

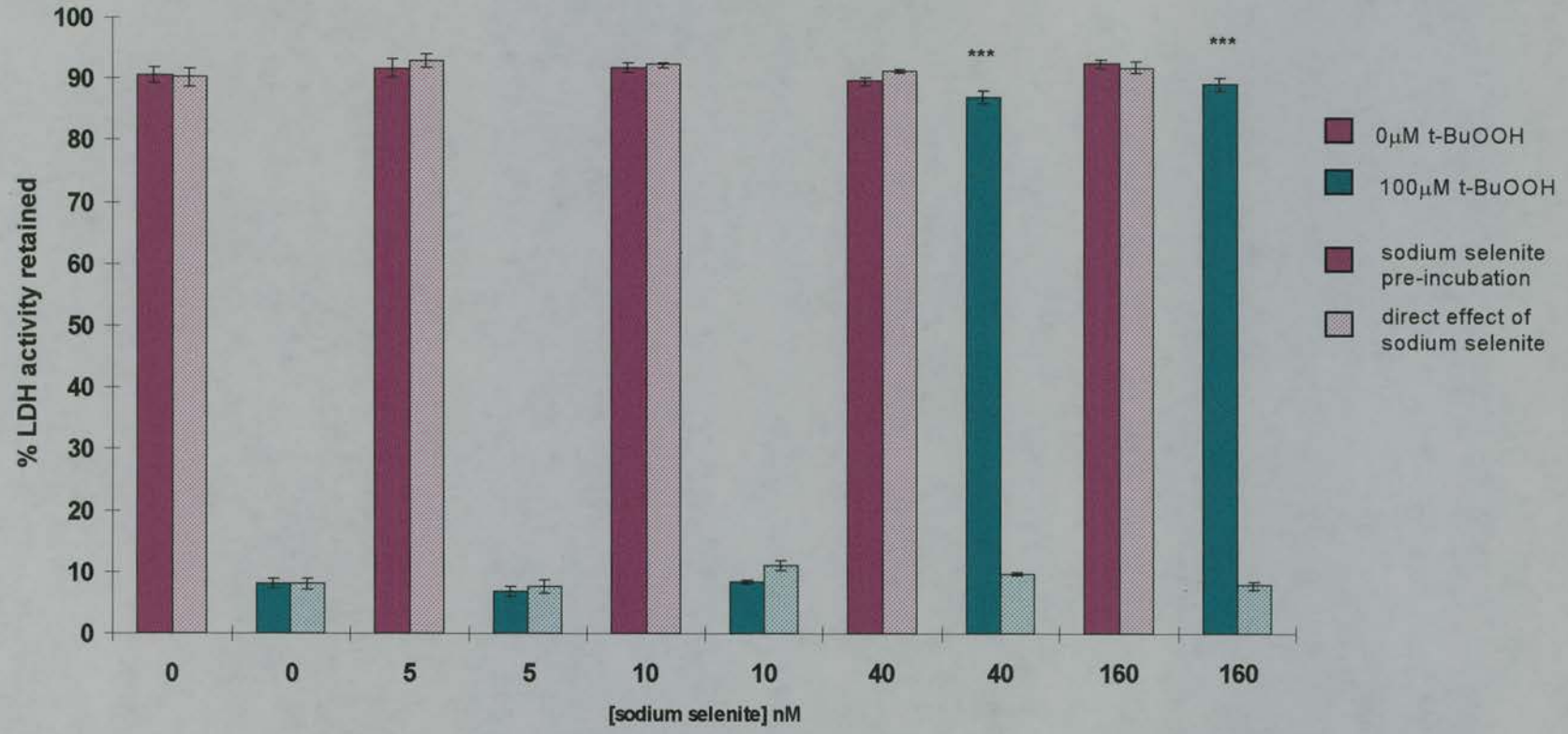


Figure 5.12. The effects of sodium selenite on the sensitivity of HUVEC to tert-butylhydroperoxide (t-BuOOH) added either prior to t-BuOOH treatment or at the same time as t-BuOOH treatment. HUVEC were sub-cultured into either selenium-deficient medium containing different concentrations of sodium selenite (0, 5, 10 ,40, 160 nM) or selenium-deficient medium with no additions. At confluence the medium was removed from all HUVEC and replaced with medium containing sodium selenite concentrations (0, 5, 10, 40, 160 nM) and t-BuOOH (100 μM) for 20 hr. Control cells received no t-BuOOH or selenium supplementation. Results shown are the mean of triplicate wells ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:-$p<0.001^{***}$.

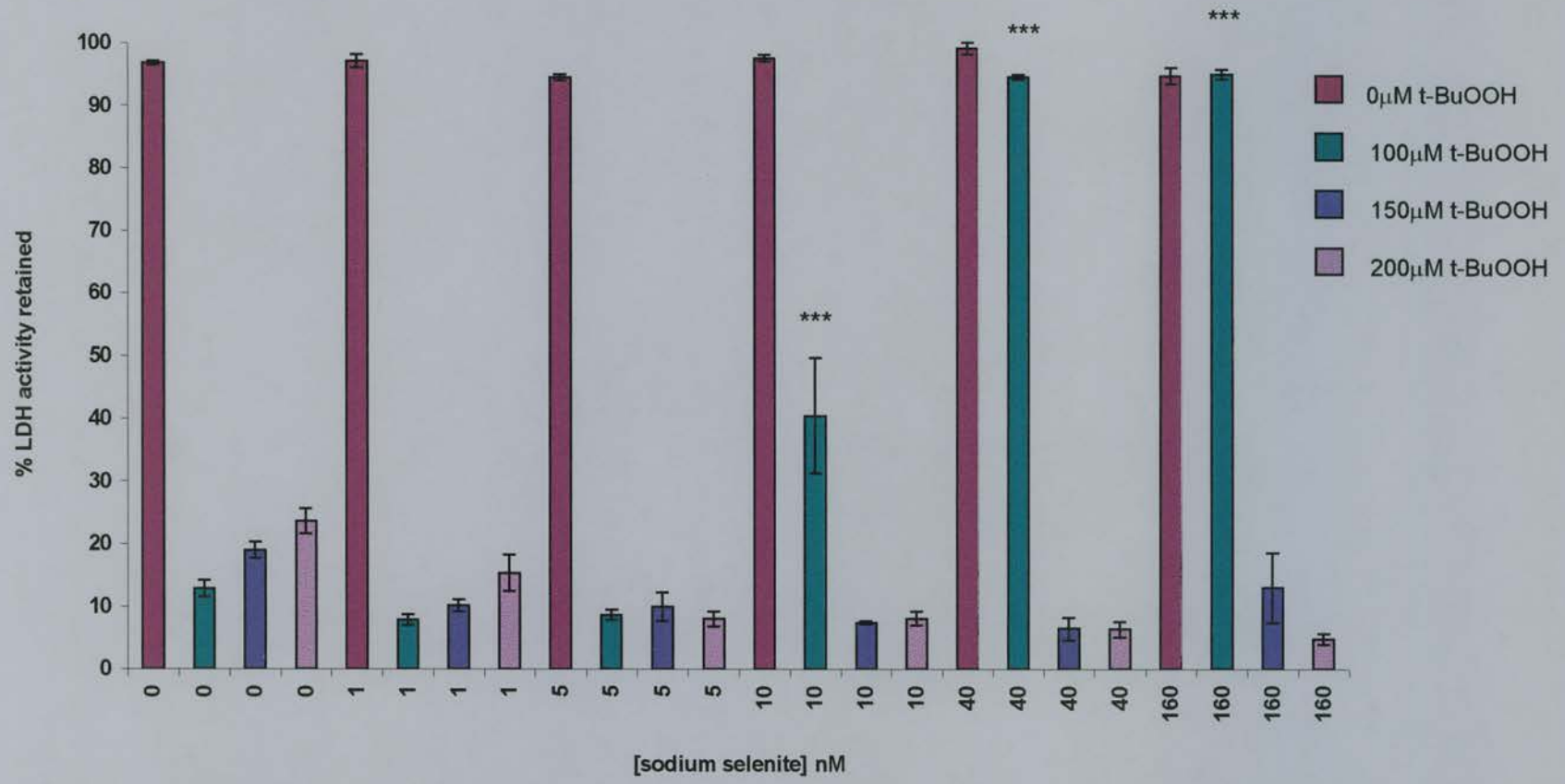


Figure 5.13. The effects of sodium selenite on the sensitivity of human coronary artery endothelial cells (HCAEC) to tert-butylhydroperoxide (t-BuOOH) induced cell damage. HCAEC were sub-cultured into selenium-deficient medium containing different concentrations of sodium selenite (0, 1, 5, 10, 40, 160 nM). At confluence HCAEC were treated with different concentrations of t-BuOOH (0, 100, 150, 200 μM). After 20 hr, cell viability was assessed by determining the % LDH retained by cells. Results shown are the mean of triplicate wells ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:-$p<0.001^{***}$. One of two experiments using HCAEC.

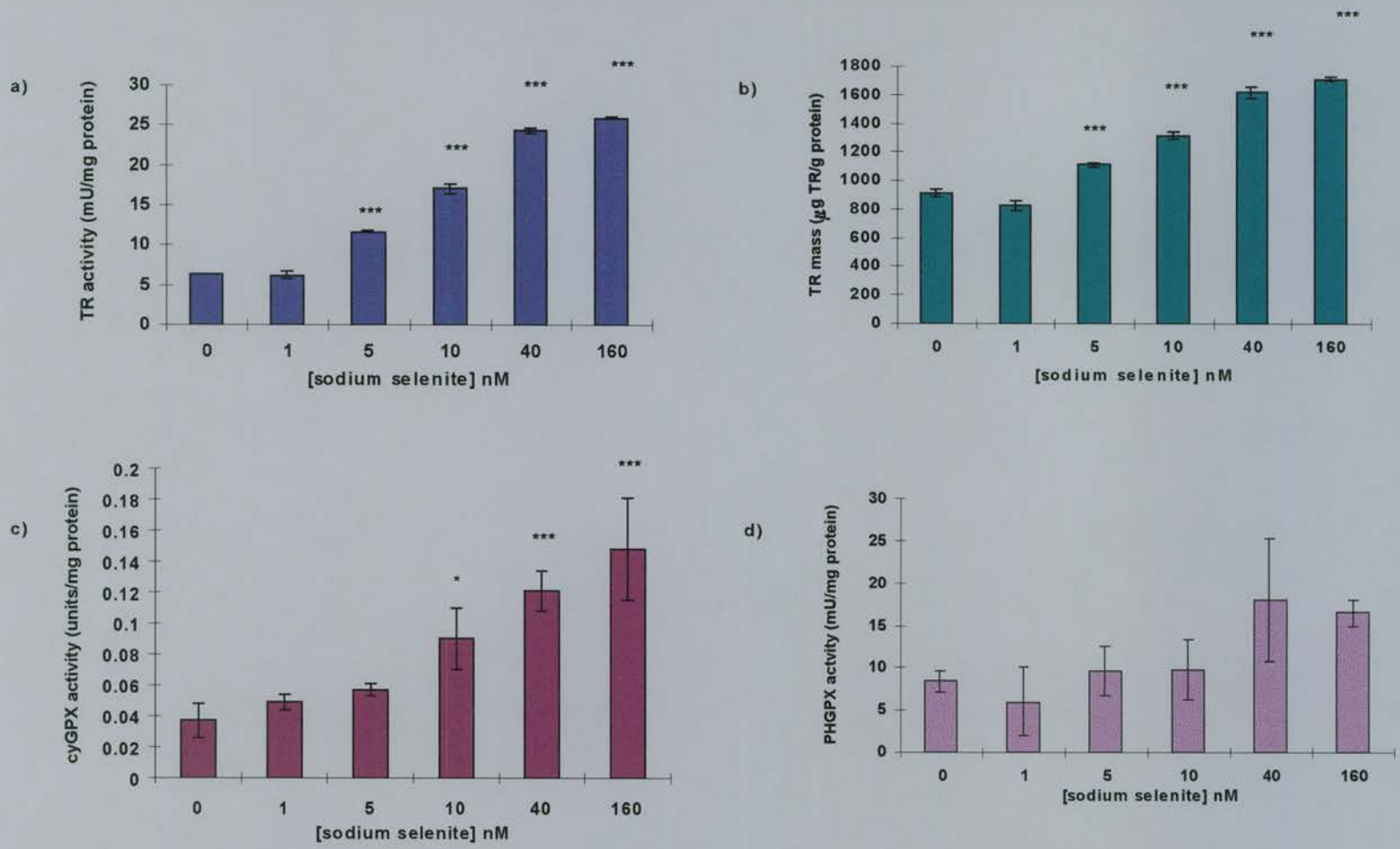


Figure 5.14. The expression and activity of three selenoproteins in human coronary artery endothelial cells (HCAEC) cultured in selenium-deficient medium containing different concentrations of sodium selenite. HCAEC were sub-cultured into selenium-deficient medium containing different concentrations of sodium selenite (0, 1, 5, 10, 40, 160 nM). At confluence HCAEC were harvested then assayed for a) thioredoxin reductase (TR) activity; b) TR mass; c) cytoplasmic glutathione peroxidase (cyGPX) activity; d) phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity. Results are shown as the mean of triplicate flasks ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:-$p< 0.05^{*}$, $p< 0.001^{***}$. No significant difference in PHGPX activity between HUVEC cultured in different concentrations of sodium selenite was shown. One of two experiments using HCAEC.

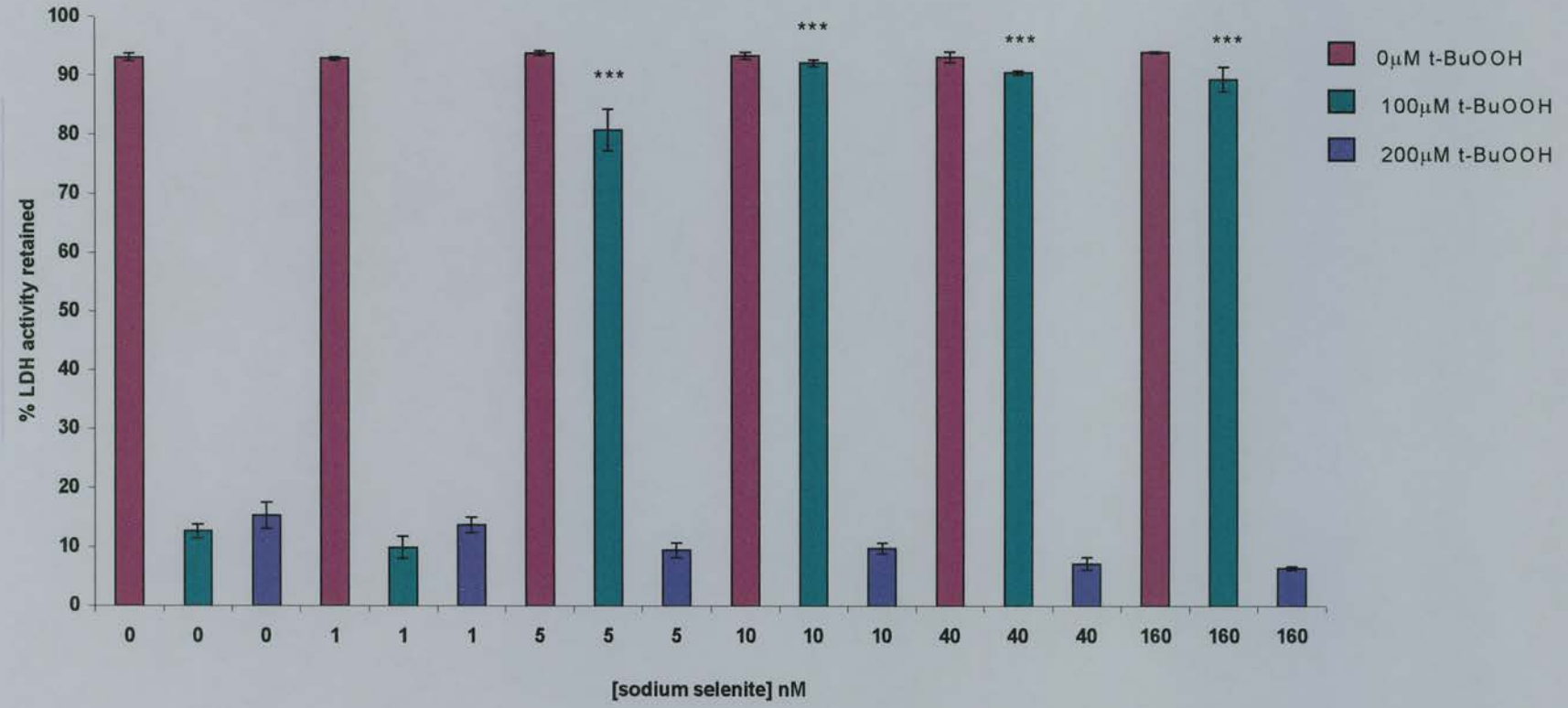


Figure 5.15. The effects of sodium selenite on the sensitivity of human coronary artery endothelial cells (HCAEC) to tert-butylhydroperoxide (t-BuOOH) induced cell damage. HCAEC were sub-cultured into selenium-deficient medium containing different concentrations of sodium selenite (0, 1, 5, 10, 40, 160 nM). At confluence HCAEC were treated with different concentrations of t-BuOOH (0, 100, 200 μM). After 20 hr, cell viability was assessed by determining the % LDH activity retained by the cells. Results shown are the mean of triplicate wells ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:- $p<0.001^{***}$. One of two experiments using HCAEC.

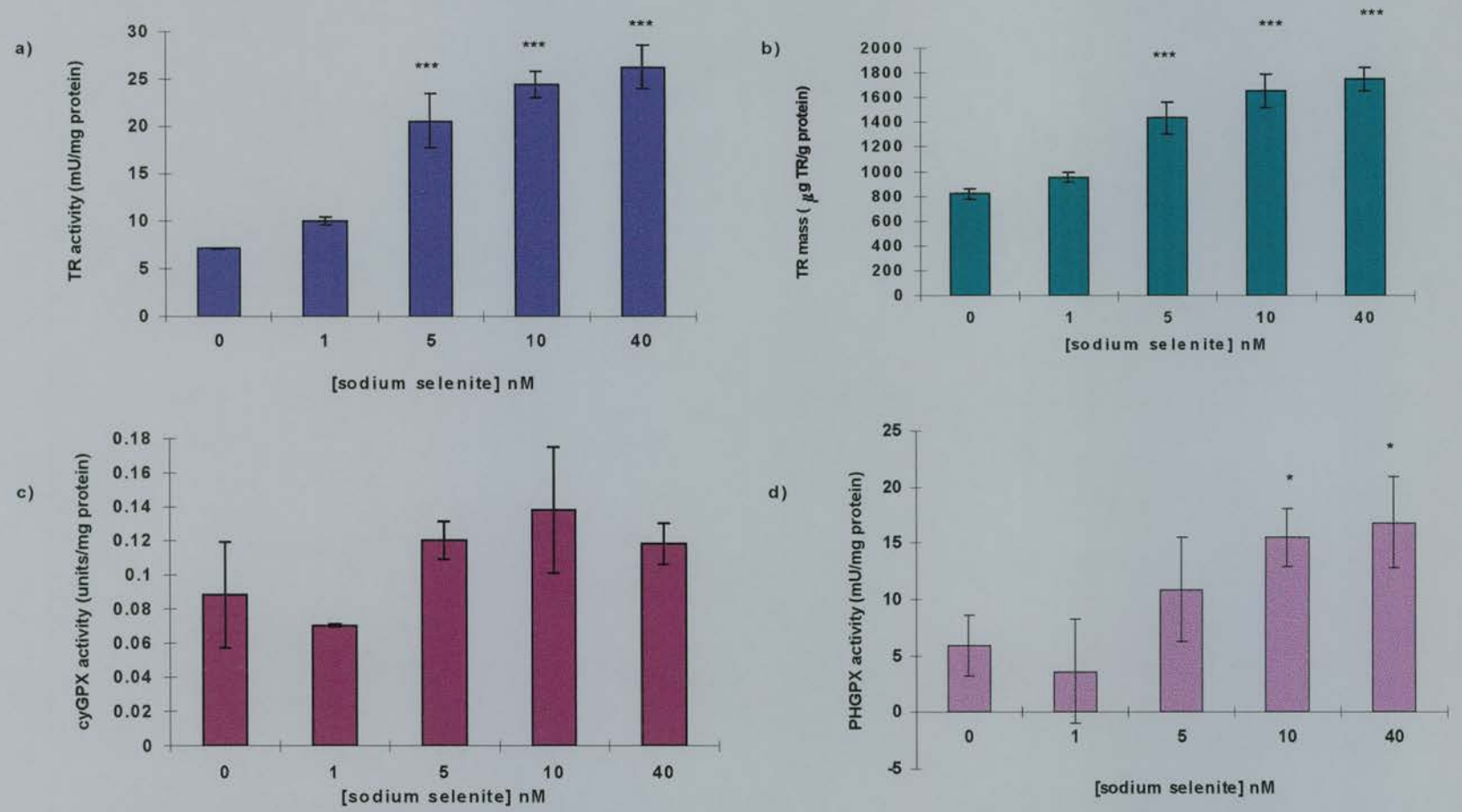


Figure 5.16. The expression and activity of three selenoproteins in human coronary artery endothelial cells (HCAEC) cultured in selenium-deficient medium containing different concentrations of sodium selenite. HCAEC were sub-cultured into selenium-deficient medium containing different concentrations of sodium selenite (0, 1, 5, 10, 40, 160 nM). At confluence HCAEC were harvested and then assayed for a) thioredoxin reductase (TR) activity; b) TR mass; c) cytoplasmic glutathione peroxidase (cyGPX) activity; d) phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity. Results are shown as the mean of triplicate flasks ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:-$p< 0.05^{*}$, $p< 0.001^{***}$. No significant difference in cyGPX activity between HCAEC cultured in different concentrations of sodium selenite was shown. One of two experiments using HCAEC.

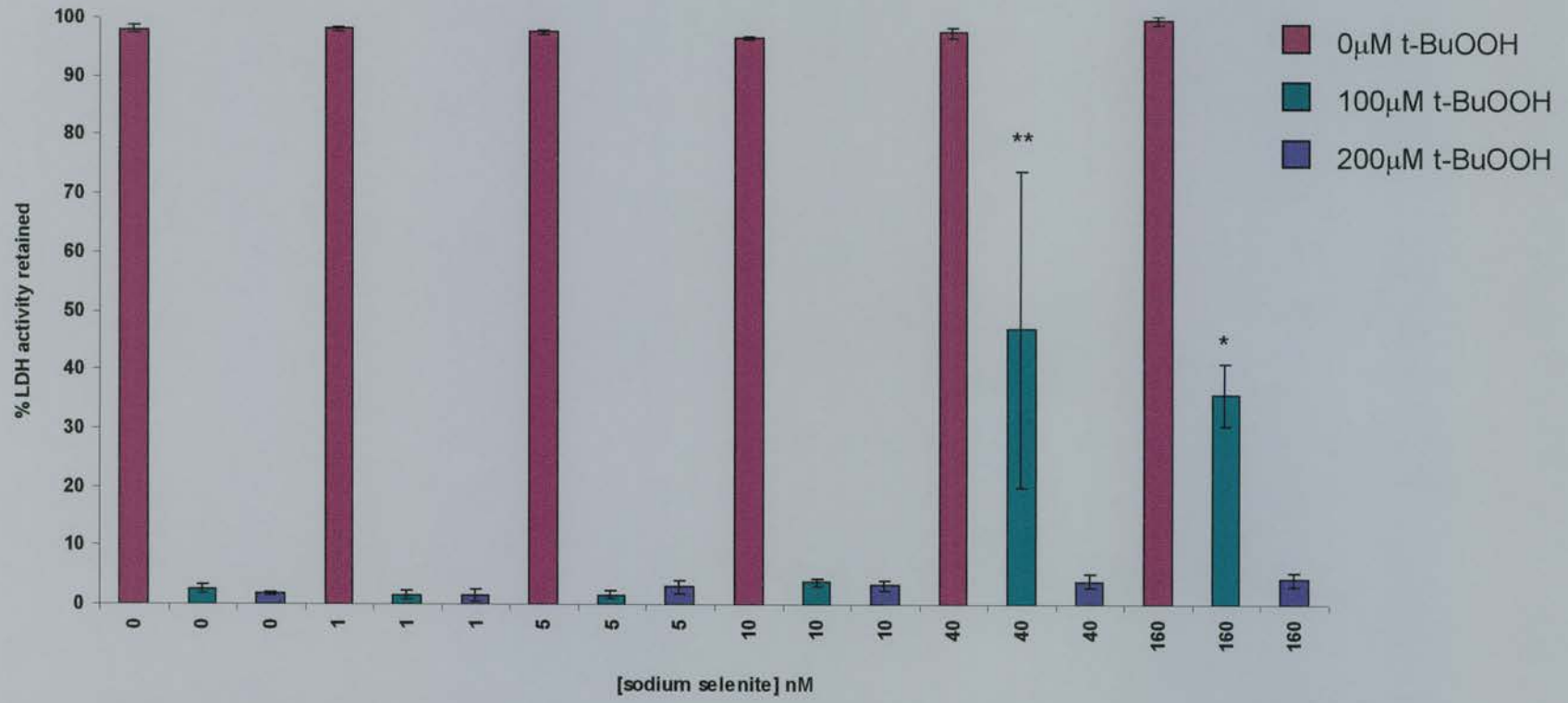


Figure 5.17. The effects of sodium selenite on the sensitivity of bovine aortic endothelial cells (BAEC) to tert-butylhydroperoxide (t-BuOOH) induced cell damage. BAEC were sub-cultured into selenium-deficient medium containing different concentrations of sodium selenite (0, 1, 5, 10, 40, 160 nM). At confluence BAEC were treated with different concentrations of t-BuOOH (0, 100, 200 μM). After 20 hr, cell viability was assessed by determining the % LDH activity retained by the cells. Results shown are the mean of triplicate wells ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:- $p<0.05^{*}$, $p<0.01^{**}$. One of two experiments using BAEC.

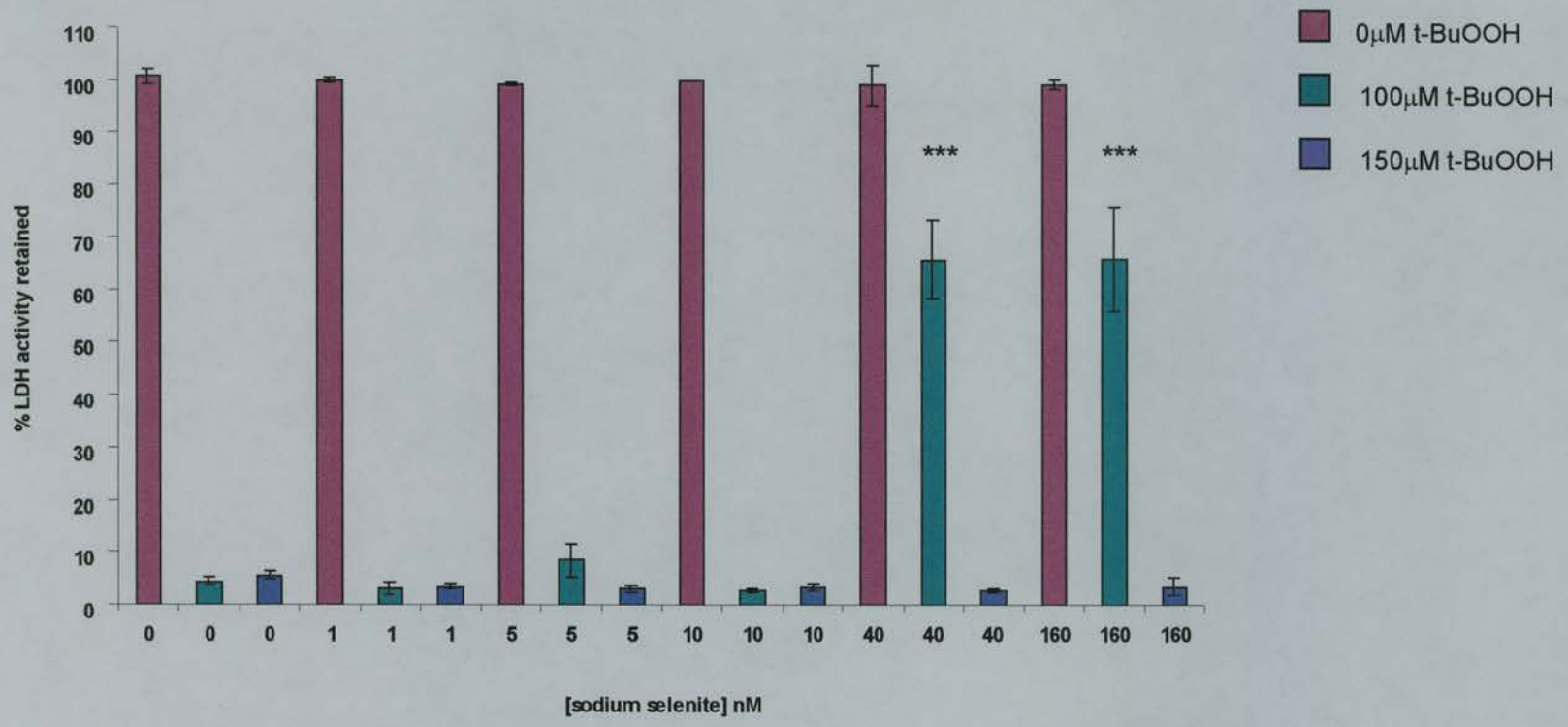


Figure 5.18. The effects of sodium selenite on the sensitivity of bovine aortic endothelial cells (BAEC) to tert-butylhydroperoxide (t-BuOOH) induced cell damage. BAEC were sub-cultured into selenium-deficient medium containing different concentrations of sodium selenite (0, 1, 5, 10, 40, 160 nM). At confluence BAEC were treated with different concentrations of t-BuOOH (0, 100, 150 μM). After 20 hr, cell viability was assessed by determining the % LDH activity retained by the cells. Results shown are the mean of triplicate wells ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:- $p<0.001^{***}$. One of two experiments using BAEC.

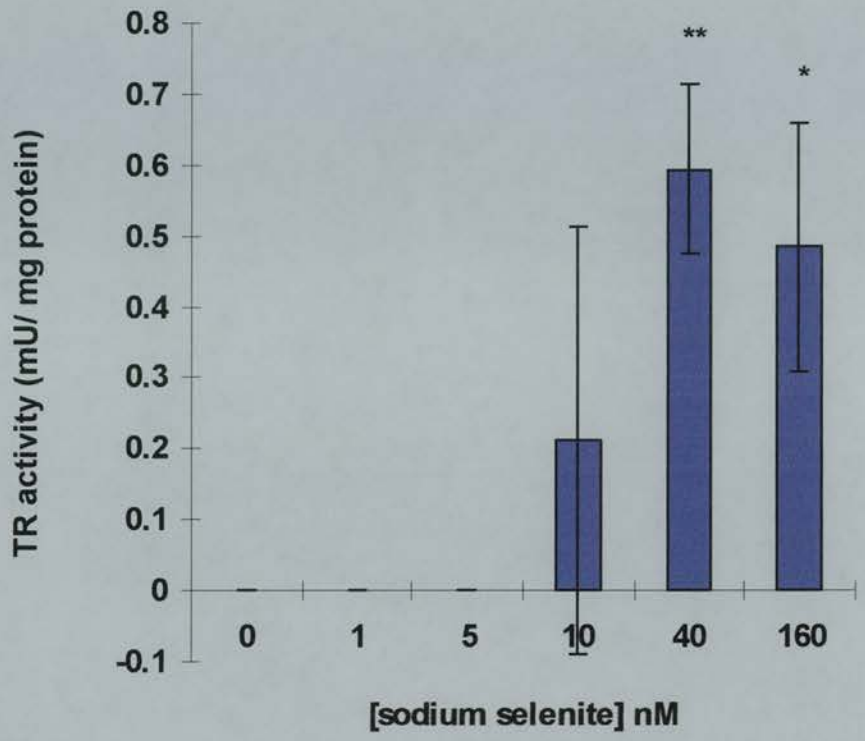


Figure 5.19. Thioredoxin reductase (TR) activity in bovine aortic endothelial cells (BAEC) cultured in selenium-deficient medium containing different concentrations of sodium selenite. BAEC were sub-cultured into selenium-deficient medium containing different concentrations of sodium selenite (0, 1, 5, 10, 40, 160 nM). At confluence BAEC were harvested and then assayed for TR activity. Results shown are the mean of triplicate flasks ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:- $p<0.05^{*}$, $p<0.01^{**}$. One of two experiments using BAEC.

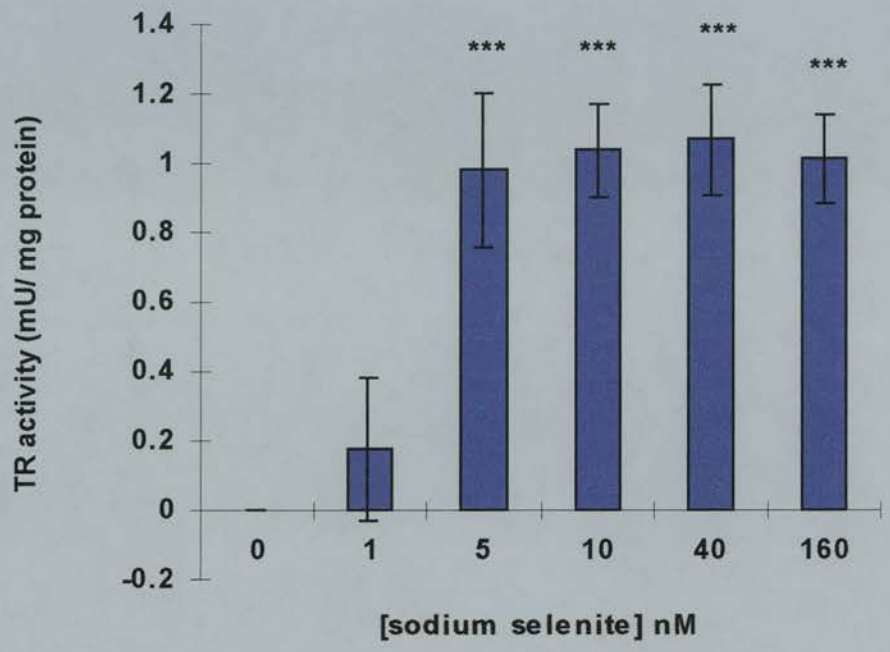


Figure 5.20. Thioredoxin reductase (TR) activity in bovine aortic endothelial cells (BAEC) cultured in selenium-deficient medium containing different concentrations of sodium selenite. BAEC were sub-cultured into selenium-deficient medium containing different concentrations of sodium selenite (0, 1, 5, 10, 40, 160 nM). At confluence BAEC were harvested and then assayed for TR activity. Results shown are the mean of triplicate flasks ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:- $p<0.001^{***}$. One of two experiments using BAEC.

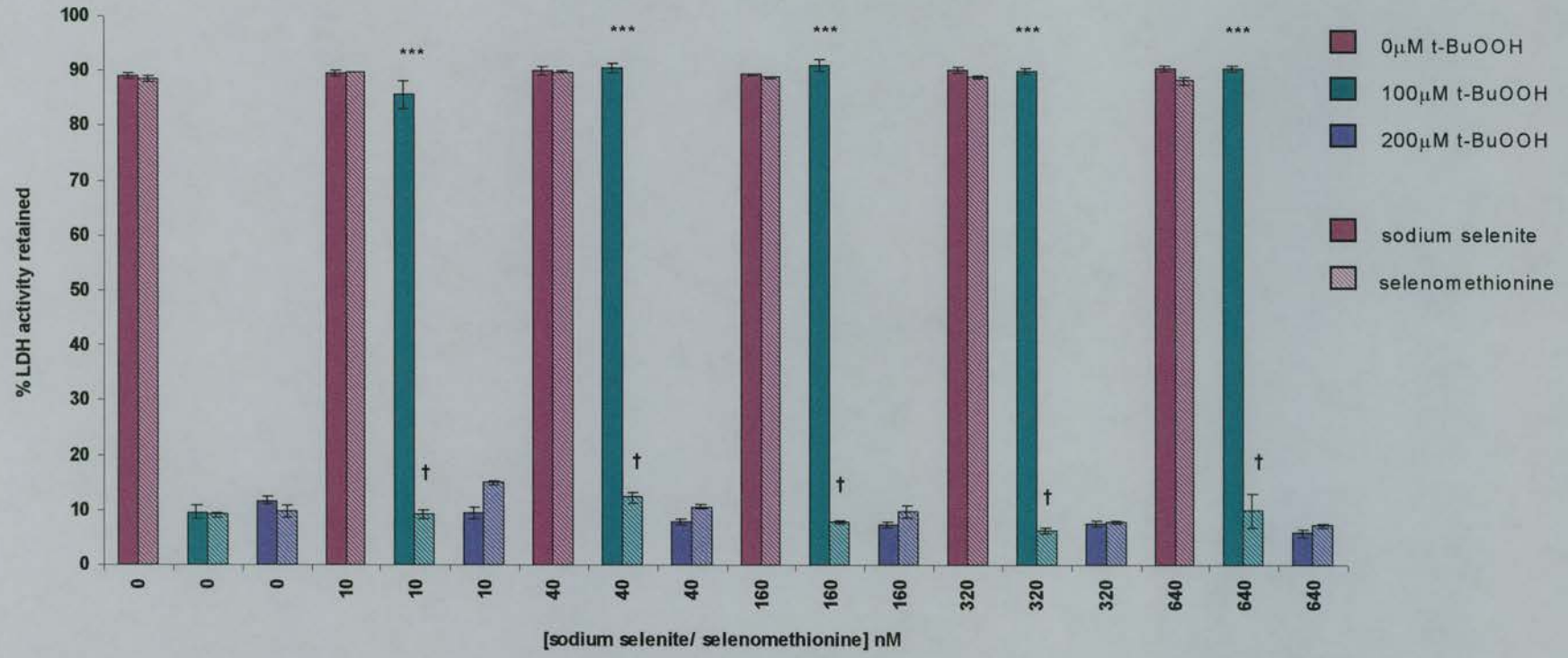


Figure 5.21. A comparison of the effects of sodium selenite and selenomethionine on % LDH retention in HUVEC subject to oxidative stress from tert-butylhydroperoxide (t-BuOOH). HUVEC were sub-cultured into selenium-deficient medium containing different concentrations of either sodium selenite (0, 10 ,40, 160, 320, 640 nM) or selenomethionine (0, 10, 40, 160, 320, 640 nM). At confluence the HUVEC were treated with different concentrations of t-BuOOH (0, 100, 200 μM) for 20 hr. Medium and cells were harvested and analysed to determine the % LDH activity retained. Results shown are the mean of triplicate wells ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:- $p<0.001^{***}$. Significant differences between sodium selenite and selenomethionine results are indicated by † $p<0.001$. One of two experiments comparing sodium selenite and selenomethionine.

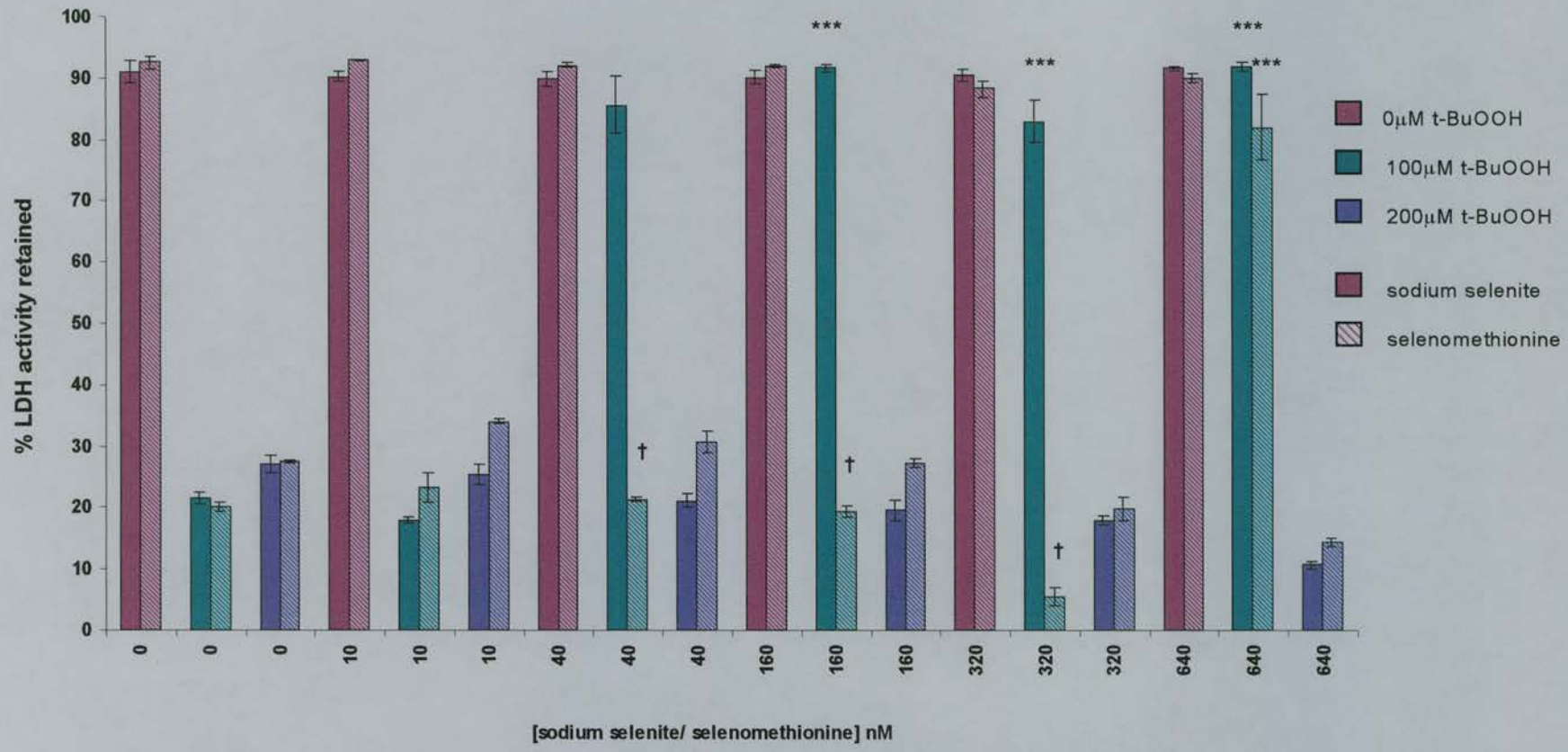


Figure 5.22. A comparison of the effects of sodium selenite and selenomethionine on % LDH retention in HUVEC subject to oxidative stress from tert-butylhydroperoxide (t-BuOOH). HUVEC were sub-cultured into selenium-deficient medium containing different concentrations of either sodium selenite (0, 10 ,40, 160, 320, 640 nM) or selenomethionine (0, 10, 40, 160, 320, 640nM). At confluence the HUVEC were treated with different concentrations of t-BuOOH (0, 100, 200 μM) for 20 hr. Medium and cells were harvested and analysed to determine the % LDH activity retained. Results shown are the mean of triplicate wells ± SD. Results which are significantly different from selenium-deficient cells are denoted as follows:- $p<0.001^{***}$. Significant differences between sodium selenite and selenomethionine results are indicated by † $p<0.001$. One of two experiments comparing sodium selenite and selenomethionine.

5.3 DISCUSSION

Studies *in vitro* have demonstrated that sodium selenite affords resistance to endothelial cells against oxidative damage (Ochi, Morita and Murota, 1992; Thomas, Geiger and Girotti, 1993). The results presented here show that HUVEC cultured in selenium-deficient medium supplemented with sodium selenite (5-40 nM) are significantly protected from the toxic effects of 100 µM t-BuOOH. The protective effect of sodium selenite was observed in all HUVEC tested, though there was some variability in the concentration of sodium selenite which afforded the maximal protective response. For example, with one preparation of HUVEC the lowest concentration of sodium selenite that afforded a significant protective effect against 100 µM t-BuOOH was 40 nM whereas in another preparation a significant effect was observed as low as 5 nM sodium selenite. Thus HUVEC from different preparations display a variable susceptibility to t-BuOOH toxicity. These differences are likely to reflect the variability in endogenous antioxidant defence mechanisms derived from genetic and environmental heterogeneity of these cultured cells. The enzymatic antioxidant defences of the endothelium include selenoproteins, catalase and superoxide dismutase, which all work in concert to maintain the cells redox potential (Michiels, Toussaint and Remacle, 1990; Vercellotti *et al.*, 1988). The varied susceptibility to toxic agents between different isolates of HUVEC and BAEC and passage number within the same isolate has been previously reported (Harlan *et al.*, 1984).

The ability of sodium selenite to protect against oxidative damage from t-BuOOH exhibited in HUVEC was markedly similar to that observed in HCAEC. These findings provide further evidence to support the use of HUVEC as a suitable alternative to HCAEC in the study of the role of selenium in atherosclerosis. In two experiments carried out on the same preparation of HCAEC at different passages, 5 nM and 10 nM sodium selenite, respectively, produced significant protective effects against the cytoxicity from 100 µM t-BuOOH. The difference in susceptibility of these two passages of cells may reflect differences in endogenous antioxidant defense mechanisms which can notably change with the age of cultured cells (Harlan *et al.*,

1984) or with the state of confluence (Miss M Lewin, personal communication). Ideally the protective effect of sodium selenite supplementation should have been studied on a number of HCAEC preparations, but our supply of these cells was limited.

The findings shown here suggest that the protective effect of sodium selenite against oxidative damage from 100 μM t-BuOOH is more potent in HUVEC and HCAEC compared to BAEC. Whilst concentrations of between 5 nM and 40 nM sodium selenite afforded maximal protective effects against t-BuOOH in the HUVEC and HCAEC studied, concentrations of 40 nM and 160 nM sodium selenite protected BAEC only sub-maximally. However, only one preparation of bovine cells were studied here. Differences in the susceptibility to cytotoxic agents resulting from both intra- and inter-species variation has been previously reported (Harlan *et al.*, 1984). To clarify this, experiments need to be repeated using several preparations of BAEC.

Thomas *et al.* have previously investigated the role of selenoproteins in cytoprotection against 0-500 μM t-BuOOH damage in BAEC (Thomas, Geiger and Girotti, 1993). They demonstrated that BAEC cultured in 60 nM sodium selenite were significantly protected against concentrations of t-BuOOH of up to 500 μM. These findings are very much in contrast to our own which found that concentrations of sodium selenite up to 160 nM only sub-maximally protected cells against 100 μM t-BuOOH (as indicated by 50% LDH activity retained compared to control cells) and did not protect at all against higher concentrations of t-BuOOH (200 μM). The clear differences observed between the findings of this study and those of Thomas *et al.* may be possibly explained by the different culture media used. Despite the low selenium status of both media the different formulations of each may potentially alter the overall antioxidant status of the cell. The most likely explanation for these differences probably results from the different preparations of BAEC used. For example, the endothelial cells would have been isolated from different cattle, of different breeds and age, using possibly different methods of isolation.

The difference in susceptibility of selenium-deficient and selenium-sufficient endothelial cells to t-BuOOH was associated with the modification of selenoprotein expression and activity. Our findings show that t-BuOOH is not directly detoxified by sodium selenite in the culture medium (figure 5.12) and that selenoprotein expression and activity can be modified by selenium-supplementation of endothelial cells (figures; 5.05, 5.07, 5.09, 5.11, 5.14, 5.16, 5.19 and 5.20). The modification of selenoprotein expression and activity through selenium-supplementation in cell culture systems is widely reported. Berggren *et al.* demonstrated that supplementation of HT-29 human colon cancer cells with sodium selenite produced a time and dose-dependent increase in intracellular thioredoxin reductase (TR) activity and protein levels (Berggren *et al.*, 1997). Selenium supplementation also enhances also cytoplasmic glutathione peroxidase (cyGPX) and phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity in endothelial cells (Ricetti *et al.*, 1994; Thomas, Geiger and Girotti, 1993).

Our attempts to associate changes in TR activity and mass and cyGPX and PHGPX activity in HUVEC supplemented with sodium selenite with the protection against 100μM t-BuOOH observed in selenium-supplemented cells were not fully conclusive. This study demonstrates that HUVEC cultured to confluence in selenium-deficient medium supplemented with different concentrations of sodium selenite exhibit a dose-dependent increase in both TR activity and mass. This effect was generally consistent in all of the preparations of HUVEC studied. The increase in TR activity and mass consistently occurred at 5 nM sodium selenite. This increase was significant ($p<0.001$) in all but one experiment where 40 nM sodium selenite was the concentration at which TR activity was significantly increased. The concentrations of sodium selenite which resulted in significant changes in TR activity and mass were similar to those which afforded a significant protective effect against t-BuOOH.

cyGPX and PHGPX activities were only measured in two of the HUVEC preparations in which cytoxicity experiments were carried out. Both cyGPX and PHGPX activity exhibited a trend to

increased activity in response to increasing concentrations of sodium selenite although the PHGPX response was not always significant. The absolute values of PHGPX activity assay were generally very small, which may account for poor replication within the assay. The activity of PHGPX needs to be investigated further using an assay system with greater sensitivity.

Short term selenium-deficiency (20 days) in rats results in a 50% decrease in lung CuZn superoxide dismutase activity which is not the result of a diet-induced deficiency of either copper or zinc (Coursin and Cihla, 1996). Therefore the possibility that selenium-deficient cells are more susceptible to oxidative damage because of a selenium-deficiency-induced decrease in superoxide dismutase activity cannot be excluded at this stage.

In summary, it is likely that in the range of 5-40 nM sodium selenite (at which protection against t-BuOOH is generally observed in HUVEC) the activity of TR, cyGPX and PHGPX all change. It is therefore difficult to determine the relative importance of each of these selenoenzymes in the protection of HUVEC against t-BuOOH.

As with HUVEC, the cytoxicity of 100 μM t-BuOOH in HCAEC cultured in different concentrations of sodium selenite could be inversely related to the expression of TR, cyGPX and PHGPX. In HCAEC cultured in selenium-deficient medium supplemented with sodium selenite, dose-dependent increases in TR activity and mass were demonstrated. The activity of cyGPX and PHGPX were either significantly increased or showed a trend towards an increase in HCAEC cultured in increasing concentrations of sodium selenite. The concentrations of sodium selenite which increased TR activity and mass and cyGPX activity (i.e. 10, 40 and 160 nM) also produced a significant protection of HCAEC against 100 μM t-BuOOH.

No TR activity was measurable in BAEC cultured in selenium-deficient medium whereas in both HUVEC and HCAEC levels of between 1-7 mU/ mg protein were measured under these

conditions (table 5.01). Although cyGPX and PHGPX were not measured in this particular preparation of BAEC, we have shown previously that cyGPX and PHGPX activities measured in BAEC grown in selenium-sufficient medium did not differ significantly from those measured in HUVEC also grown in selenium-sufficient medium (figures 3.17 and 3.18). This data implies that the difference in the protective effect of sodium selenite observed between BAEC and HUVEC may be related to the significantly lower TR activity found in BAEC.

Table 5.01: Thioredoxin reductase activity measured in human and bovine endothelial cells cultured in selenium-deficient medium. Results shown are the means of triplicate flasks.

CELL TYPE	TR ACTIVITY (mU/mg protein)
HUVEC	*3.65 2.42 1.36 1.92*
HCAEC	*6.40 7.21*
BAEC	*0 0*

The findings reported in this chapter are important in that they suggest that, in addition to the GPX's, TR may also be important in the protection of endothelial cells against oxidative damage, although, the physiological significance is as yet unclear since t-BuOOH is not an endogenous mediator of oxidative damage in the endothelium. Future studies will need to assess the effect of selenium-supplementation on the protection of cultured endothelial cells against oxLDL, an important endogenous mediator of endothelial cytoxicity and atherogenesis (Nielson, 1999;

Steinberg, 1991; Witzum and Steinberg, 1991). Thomas *et al.* have studied the role of selenoproteins in the protection of endothelial cells against damage from oxLDL, attributing the protective effect of selenium to the GPX's. The data from this study questions the relevance of their findings to humans as we have highlighted distinct differences between the endothelial cells isolated from the two different species.

Studies by Claise *et al.* compared the cytotoxic effect of oxLDL on both HUVEC and the endothelial cell line EAhy926 (Claise *et al.*, 1997). EAhy926 cells were found to be significantly more susceptible to damage from oxLDL than HUVEC, the latter being unaffected by oxLDL concentrations of up to 200 µg/ml. The higher susceptibility of EAhy926 cells to oxLDL cytotoxicity was attributed to the lower antioxidant defenses of these cells compared to HUVEC, particularly as regards the cyGPX activity which was 8% of that of HUVEC. Our studies also found a lower cyGPX activity in EAhy926 cells. The cyGPX activity in our cells was only 27% of that found in HUVEC which may be accounted for by differences in selenium bioavailability in the culture media used for each cell type (see section 3.4). Despite these differences TR activity and mass and PHGPX activity were shown not to be significantly different. Claise's group did not measure the activities of either TR or PHGPX. Therefore, if EAhy926 cells are more susceptible to damage from oxLDL as reported by Claise *et al.* our data would argue against a protective role for TR and PHGPX. Recently data from this laboratory has shown no significant difference in susceptibility to t-BuOOH-induced damage, between HUVEC and EAhy926 cells (Miss M Lewin, personal communication). This would suggest that cyGPX activity is not important in the protection of EAhy926 cells against damage from t-BuOOH.

The pro-oxidant status of sodium selenite at µM concentrations is well documented in both *in vivo* and *in vitro* studies (Dougherty and Hoekstra, 1982; Stewart *et al.*, 1999; Yan and Spallholz, 1993). However there is no data published to date on the ability of sodium selenite to induce cell toxicity at nM concentrations. Our findings show a dose-dependent increase in sensitivity to 200

μM t-BuOOH when both HUVEC and HCAEC were cultured with increasing concentrations of sodium selenite. This response is likely to be a consequence of sodium selenite acting as a pro-oxidant, whereby the antioxidant capabilities of the cells are insufficient to protect against the oxidative stress resulting from the combined insults of both t-BuOOH and sodium selenite.

In the study by Rafferty *et al.* the chemical form of selenium added to cells was shown to be an important factor in determining the ability of selenium to afford protection from ultraviolet B radiation in skin cells (Rafferty *et al.*, 1998). Our results are in agreement with the findings of this study, demonstrating that sodium selenite is more potent than selenomethionine in conferring protection against t-BuOOH in cultured HUVEC. This difference possibly reflects the different abilities of each compound to modify selenoprotein status. Indeed Berggren *et al.* have previously shown that L-selenomethionine favoured a smaller change in TR activity, than sodium selenite in HT-29 human colon cancer cells (Berggren *et al.*, 1997). Therefore inorganic forms of selenium such as sodium selenite may provide a more bioactive form of the selenium supplementation than organic forms such as selenomethionine. An alternative explanation is that L-methionine present in the culture (15 mg/L) may compete with the selenomethionine for its selenium. In rats utilization of dietary selenomethionine was shown to be inversely correlated to methionine uptake (Waschulewski and Sunde, 1988). Consequently the possibility exists that there is less selenomethionine uptake by the cell resulting in a lesser effect. This possibility could be further investigated with the use of a methionine-free M199 medium.

Previous studies have suggested that the GPX's are the important selenoproteins which protect endothelial cells against harmful hydroperoxides which cause oxidative damage (Claise *et al.*, 1997; Hara, 1998; Ochi, Morita and Murota, 1992; Thomas, Geiger and Girotti, 1993). To date no such studies have suggested that TR may be important in this regard. Our results argue that TR maybe important in antioxidant protection. In both HUVEC and HCAEC, the concentrations of sodium selenite which afford a protective effect against the cytoxicity of t-BuOOH are those

which significantly increase TR activity and mass. Also, the incubation of HUVEC with t-BuOOH (90 μM) increases the expression of TR without affecting the expression of the other selenoproteins such as cyGPX or PHGPX.

The relative importance of TR, cyGPX and PHGPX in the protection of endothelial cells against oxidative damage from t-BuOOH is unknown at present. Previous literature suggests that, in BAEC, cyGPX is expressed at much higher relative amounts than PHGPX and is more potent at reducing t-BuOOH (Thomas, Geiger and Girotti, 1993). A comparison of the apparent rate constants for peroxide substrates indicated that PHGPX was approximately one tenth as active as cyGPX against t-BuOOH (Maiorino, Gregolin and Ursini, 1990). Kinetic data is consistent with cyGPX and PHGPX having potentially different roles. In the aqueous phase the reaction of cyGPX with peroxidases is favoured over PHGPX. However, our studies have shown that the 22 kDa selenoprotein (which we characterized as PHGPX) has a much higher relative abundance than cyGPX in BAEC (figure 3.15).

Future studies might include assessing the ability of cells to resist oxidative stress after cells are treated with specific antibodies to TR or other candidate antioxidant selenoproteins.

LDH retention, which quantitates cell death, was used as a measure of cell viability in this thesis. Ideally, LDH retention should be used in tandem with an alternative measurement of cell viability in order to validate the results. The trypan blue exclusion assay, MTT and neutral red assay etc. have all been previously used to quantitate cytoxicity. The more sensitive markers of cell viability, such as the metabolic activity of the cell, provide an appropriate test to use in conjunction with LDH retention.

In conclusion, the protective effect of sodium selenite observed in human endothelial cells is likely to be through the modification of selenoprotein expression rather than a direct antioxidant

effect of sodium selenite. The findings of this study have shown that, in addition to the GPX's, TR may also have an important antioxidant function in the human endothelium, a possibility which has not been previously considered. Our studies were not able to conclusively establish the relative importance of each of the selenoproteins in the protection of the human endothelium against oxidative damage though it is likely that TR, cyGPX and PHGPX all contribute. Under pathological conditions it is possible that one particular selenoprotein however may become more important. Our studies have highlighted the lack of suitability of BAEC as a model for the study of selenoprotein expression and protection from oxidative stress.

CHAPTER SIX CONCLUDING REMARKS

Atherosclerosis is the principle cause of morbidity and mortality of man in Western society. Low plasma selenium levels have been associated with an increased risk of cardiovascular disease including coronary atherosclerosis (Kok *et al.*, 1991; Salonen *et al.*, 1982; Suadicani, Hein and Gyntelberg, 1992). Damage to the endothelium by reactive oxygen species has been shown to favour atherogenesis. Cell culture studies have demonstrated that selenium supplementation confers resistance to bovine endothelial cells from damage by reactive oxygen species possibly through the expression of different antioxidant selenoproteins (Ochi, Morita and Murota, 1992; Thomas, Geiger and Girotti, 1993). This protection has been attributed to the expression of intracellular glutathione peroxidases, though the expression of other selenoproteins with antioxidant properties, such as extracellular glutathione peroxidase, selenoprotein P and TR was not considered in these studies.

The main objective of this thesis was to identify the selenoproteins expressed by endothelial cells and to study the modification of their expression through selenium supply and activation of second messenger systems. The ability of sodium selenite and selenomethionine supplementation to protect cultured endothelial cells against oxidative damage was also studied in order to establish their possible anti-atherosclerotic function.

The work in this thesis has provided evidence to demonstrate the following:-

1. There are distinct differences in [^{75}Se]-selenoprotein expression between cells isolated from different tissues and endothelial cells isolated from different species. In contrast, the differences in [^{75}Se]-selenoprotein expression observed in endothelial cells isolated from different human vascular beds and in the human endothelial cell line EAhy926 were less pronounced.

2. Whilst no extracellular selenoproteins were secreted by HUVEC, four major and several minor intracellular selenoproteins were [^{75}Se]-labelled. Of the major bands, the 22 kDa, 24 kDa and 58 kDa selenoproteins were identified as PHGPX, cyGPX and TR respectively, whilst the fourth major band with a molecular mass of 15 kDa selenoprotein was not identified. TR was the predominantly expressed selenoprotein in HUVEC accounting for approximately 43% of the total intracellular [^{75}Se]-labelled selenoproteins.

3. This study has demonstrated that HUVEC, and possibly the human endothelial cell line EAhy926, could provide a suitable alternative to HCAEC as a cell culture model for the study of selenium expression in human atherosclerotic disease. In contrast, the distinct differences observed in selenoprotein expression by endothelial cells isolated from the bovine and porcine aorta compared to HCAEC suggest that in neither BAEC nor PAEC would provide a suitable cell culture model for such studies.

4. Selenoprotein expression has been shown to be regulated through specific second messenger signalling pathways (Beech *et al.*, 1995; Howie *et al.*, 1998; Howie *et al.*, 1995). In HUVEC we have shown that the phorbol ester PMA, through the activation of PKC, down-regulates the expression of both TR and PHGPX. In contrast, the expression of cyGPX is increased, which may be mediated by the decreased intracellular anti-oxidant capacity resulting from the down-regulation of TR and PHGPX. Since oxLDL has been shown to activate PKC, it is possible that oxLDL may down-regulate the expression of TR and PHGPX in the endothelium which could result in an increased susceptibility to oxidative damage and thus the development of atheroma.

5. The calcium ionophore A23187 was also shown to modify selenoprotein expression. However these effects are likely to be the result of a toxic effect of A23187 rather than the activation of the calcium signalling pathway.

6. The ability of sodium selenite to protect against oxidative damage from t-BuOOH was demonstrated in both HUVEC and HCAEC and is likely to be through the modification of selenoprotein expression rather than a direct antioxidant effect. The relative importance of TR, cyGPX and PHGPX in the protection of endothelial cells against oxidative stress was not established.

7. Selenomethionine was considerably less potent than sodium selenite at inducing protection against oxidative damage from t-BuOOH in HUVEC.

The limitations of the statistical analysis used in this thesis needs to be considered. Both the unpaired 't' test and the ANOVA are parametric tests which assume normal distribution. It was not possible to test whether this was the case, through the use of a normality test, as the sample size was too small. The use of parametric tests on small samples is not powerful and if the population is not Gaussian, the P value can be misleading. Therefore although some p-values give a strong indication that the differences are significant ideally the sample size should be increased.

The protection afforded by sodium selenite against the toxic effects of t-BuOOH may not be widely applicable, as in addition to being a non-physiological oxidative agent, it is also a cyGPX substrate. As such, the upregulation of cyGPX may be a response to an increase in substrate availability. However, previous studies have demonstrated a protective effect of selenium against oxidative damage from UV radiation and menadione treatment in human skin cells. Further studies of interest would be to establish the relative importance of each antioxidant selenoprotein in the protection of endothelial cells against oxidative stress from oxLDL, (15*S*)-HPETE and possibly menadione. TR has not been previously implicated in the protection of endothelial cells against oxidative stress. Human endothelial cells express high levels of TR, which can be modified by selenium supply, PKC activation and oxidative stress. Thus TR may be central to the ability of selenium to protect against oxidative damage and limit the development of atherosclerosis.

CHAPTER SEVEN REFERENCES

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CHAPTER EIGHT PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS

PUBLICATIONS

Anema, S.M., Walker, S.W., Howie, A.F., Arthur, J.R., Nicol, F. and Beckett, G.J. (1999) Thioredoxin reductase expression in human umbilical vein endothelial cells. *Journal of Endocrinology* **160** : P165.

Anema, S.M., Walker, S.W., Howie, A.F., Arthur, J.R., Nicol, F. and Beckett, G.J (1999) Thioredoxin reductase is the major selenoprotein expressed in human umbilical vein endothelial cells and is regulated by protein kinase C. *Biochemical Journal* **342** :111-117.

Miller, S.M., Walker, S.W., Arthur, J.R., Nicol, F., Pickard, K., Lewin, M., Howie, A.F. and Beckett, G.J (2000) Selenite protects human endothelial cells from oxidative damage by tertiary butyl hydroperoxide and induces thioredoxin reductase expression. *Biochemical Journal* (submitted).

PRESENTATIONS

Thioredoxin reductase expression in human umbilical vein endothelial cells: The Scottish Cardiovascular Forum, Edinburgh, October 1998.

Thioredoxin reductase expression in human umbilical vein endothelial cells. Poster presentation at the 18th Joint Meeting of the British Endocrine Society, Bournemouth, 12-15 April 1999.

The role of selenium in the prevention of oxidative damage from tert-butylhydroperoxide in human vascular endothelial cells: The Scottish Cardiovascular Forum, Dundee, January 2000.

P165 THIOREDOXIN REDUCTASE EXPRESSION IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

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Damage to the endothelium by reactive oxygen species, free radicals, oxidised lipids and lipoproteins favours atherosclerosis. Such damage can be prevented by selenium (Se) which is thought to exert its actions through the expression of intracellular and extracellular selenoproteins. The family of glutathione peroxidases (GPX) may have antioxidant roles in the endothelium but other intracellular and extracellular selenoproteins with antioxidant actions may also be important.

We studied the selenoproteins expressed *in vitro* in cultured human umbilical vein endothelial cells (HUVECS). HUVECS were labelled with ^{75}Se-selenite and the labelled selenoproteins separated using SDS-PAGE. No extracellular selenoproteins were secreted by HUVECS. The intracellular selenoprotein profile of ^{75}Se-labelled HUVECS was quite distinct from that observed in other tissues. A single selenoprotein with a molecular mass of 58kDa accounted for approximately 36% of the intracellular ^{75}Se-labelled proteins in HUVECS and this was identified by western blotting as thioredoxin reductase (TR). Treatment with the calcium ionophore A23187 ($0.5x10^{-6}$M) increased TR expression 8.45-fold over basal, whilst the phorbol ester phorbol-12-myristate 13- acetate ($0.5x10^{-6}$M) decreased TR expression 3-fold compared to basal.

HUVECS express high levels of TR and this expression is under the regulation of the calcium / phosphoinositol signalling cascade. *In vitro* this selenoenzyme appears to be more potent than the GPX's at detoxifying hydrogen peroxide and harmful lipid hydroperoxides. TR expression may be an important factor in the known ability of Se to protect HUVECS from oxidative damage.

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Thioredoxin reductase is the major selenoprotein expressed in human umbilical-vein endothelial cells and is regulated by protein kinase C

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Damage to the endothelium by reactive oxygen species favours atherogenesis. Such damage can be prevented by selenium, which is thought to exert its actions through the expression of selenoproteins. The family of glutathione peroxidases (GPXs) may have antioxidant roles in the endothelium but other intracellular and extracellular selenoproteins with antioxidant actions may also be important. The selenoproteins expressed by cultured human umbilical-vein endothelial cells (HUVECs) were labelled with [^{75}Se]selenite and separated using SDS/PAGE. HUVECs secreted no extracellular selenoproteins. There were distinct differences between the intracellular selenoprotein profile of ^{75}Se-labelled HUVECs and those of other tissues. A single selenoprotein with a molecular mass of 58 kDa accounted for approx. 43 % of the intracellular ^{75}Se-labelled proteins in HUVECs. This protein was identified by Western blotting as the redox-active lipid-hydroperoxide-detoxifying selenoprotein, thioredoxin reductase (TR). TR expression in HUVECs was down-regulated by transiently exposing cells to the phorbol ester PMA for periods as short as 1 min. However, there was a delay of 48 h after PMA exposure before maximal down-regulation of TR was observed. The protein kinase C (PKC) inhibitor bisindolylmaleimide I hydrochloride had no effect on TR expression when added alone, but the agent prevented the down-regulation of TR expression seen with PMA. The calcium ionophore A23187 increased TR expression in HUVECs after a 12-h exposure, but the maximal effect was only observed after a 35-h exposure. These findings suggest that TR may be an important factor in the known ability of Se to protect HUVECs from peroxidative damage. Furthermore, the results also suggest that TR expression can be negatively regulated through PKC. It is possible that TR expression may be positively regulated by the calcium-signalling cascade, although TR induction by A23187 may be due to toxicity.

Key words: atherogenesis, endothelium, oxidative damage, selenium.

INTRODUCTION

The endothelium of the vascular system regulates vessel tone, smooth-muscle proliferation and blood coagulability. Damage to the endothelium by reactive oxygen species, free radicals and oxidized lipids and lipoproteins favours atherogenesis [1]. Selenium is essential for the expression of several peroxidase and redox enzyme systems, which protect cells from oxidative stress and, as shown *in vitro*, selenium supplementation can protect human endothelial cells (ECs) from oxidative injury [2]. A possible mechanism for this protection is through the selenium-containing glutathione peroxidase (GPX) enzyme family [2]. Indeed, treatment of human ECs with selenium enhances GPX activity [3,4]. Furthermore, the activity of GPX in human ECs is induced by $n-3$ polyunsaturated fatty acids, which may have a role in the prevention of vascular disease [5].

Selenium exerts many of its effects through the expression of both extracellular and intracellular selenoproteins [6]. At least 30 such selenoproteins have been identified by SDS/PAGE of ^{75}Se-labelled tissue, but only approx. 12 have been characterized by purification and cloning. The intracellular selenoproteins identified to date that are most likely to protect against oxidative damage include cytoplasmic GPX (cyGPX), phospholipid hydroperoxide GPX (PHGPX) and thioredoxin reductase (TR).

Extracellular selenoproteins, including extracellular GPX (EGPX) and selenoprotein P, are secreted by many tissues and may also have an antioxidant action. It is not known if ECs synthesize and secrete these extracellular selenoproteins, although selenoprotein P is associated with vascular ECs in brain, capillary ECs in the renal glomeruli and ECs of the liver [7].

The expression of some selenoenzymes is regulated through second-messenger systems. For example, in human thyrocytes and in the human fetal liver cell line, HepG2, the expression of TR is greatly increased by the addition of the calcium ionophore A23187 and PMA [8]. In contrast, EGPX secretion is inhibited by A23187, whereas PMA has little or no effect [9]. These observations have led to the suggestion that the Ca^{2+}/phosphoinositol signalling cascade may be an important modulator of selenoprotein expression in certain tissues [8]. Such studies have not been performed in ECs, although phorbol ester has been reported to increase cyGPX mRNA levels in human umbilical-vein endothelial cells (HUVECs) [10].

Labelling with [^{75}Se]selenite demonstrates clear tissue differences in the pattern of selenoprotein expression [11] but little information of this nature is available for ECs. Although studies *in vitro* have found that selenite supplementation confers resistance on ECs to oxidative injury, the mechanisms by which selenium acts are not clear [2]. Resistance to oxidative damage may arise from increased expression of cyGPX and PHGPX [2], but the expression of other selenoproteins with antioxidant properties, such as TR, EGPX and selenoprotein P, was not investigated. Here we describe experiments performed to examine the range of intracellular and extracellular selenoproteins labelled

Abbreviations used: HUVECs, human umbilical-vein endothelial cells; TR, thioredoxin reductase; PKC, protein kinase C; EC, endothelial cell; GPX, glutathione peroxidase; cyGPX, cytoplasmic GPX; PHGPX, phospholipid hydroperoxide GPX; EGPX, extracellular GPX; EBS, Earle's balanced salt solution; GF109203X, bisindolylmaleimide I hydrochloride; (15*S*)-HPETE, (15*S*)-hydroperoxy-(5*Z*),(8*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid; EGM-2, endothelial-cell growth medium 2; DMEM, Dulbecco's modified Eagle's medium.

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by [^{75}Se]selenite and report on the effects that PMA and A23187 exert on the expression of TR.

EXPERIMENTAL

Materials

Endothelial-cell growth medium 2 (EGM-2) bullet-kit and endothelial-cell basal medium (EBM-2) were both supplied by BioWhittaker UK (Wokingham, Berks., U.K.). Earle's balanced salt solution (EBS), M199, Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 nutrient mix, fetal bovine serum, glutamine, penicillin, streptomycin, amphotericin B and Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution were obtained from Life Technologies (Paisley, Scotland, U.K.). Collagenase was purchased from Worthington Biochemicals via Lorne Laboratories (Twyford, Berks., U.K.). [^{75}Se]Selenite (specific radioactivity, 16 MBq/nmol) was obtained from the Reactor Center, University of Missouri (Columbia, MO, U.S.A.). Low-range SDS/PAGE molecular-mass standards were purchased from Bio-Rad (Hemel Hempstead, Herts., U.K.). EDTA was supplied by Boehringer Mannheim UK (Lewes, East Sussex, U.K.). Glycerol and poly(ethylene glycol) were both purchased from Merck (Lutterworth, Leics., U.K.). Antisera to human and rat TR were raised in rabbits to TR purified from rat liver and human placenta as described previously [8,12]. Bisindolylmaleimide I hydrochloride (GF109203X) was supplied by Calbiochem Novabiochem UK (Nottingham, U.K.). Antisera to rat PHGPX were raised in rabbits to PHGPX purified from rat liver. Antiserum to human cyGPX was obtained from The Binding Site (Birmingham, U.K.). Sigma (Poole, Dorset, U.K.) supplied all other reagents.

Isolation and culture of HUVECs and human thyrocytes

Human umbilical cords (> 100 mm in length) were obtained at normal deliveries or Caesarean sections from non-smoking women. Immediately after delivery, the cords were placed into sterile EBS containing penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml) at 4 °C. ECs were isolated within 20 h of delivery using a method adapted from that described previously by Jaffe et al. [13]. Briefly, the umbilical vein was cannulated with a Venflon (17 gauge, 45 mm) and the vein was washed with 100 ml of EBS (pre-warmed to 37 °C). One end of the vein was clamped shut and the opposite end infused with 0.07% (w/v) collagenase in EBS (5–15 ml). The cord was then incubated at 37 °C in an atmosphere of 5% CO_2/95% air. After 10 min the cord was removed and massaged gently, and the contents of the umbilical vein were flushed out with 30 ml of Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution. The resulting cell suspension was collected, centrifuged at 450 *g* for 10 min and the pellet washed once with EGM-2 containing penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml). The cells were resuspended in 15 ml of EGM-2, plated out into one 75-cm^2 flask and cultured at 37 °C in an atmosphere of 5% CO_2/95% air.

After 24 h, and then on alternate days, the culture medium was changed and replaced with a further 15 ml of EGM-2. Cells reached confluence in 3–7 days, and were then subcultured as required, seeding at a density of approx. 3000 cells/cm^2. HUVECs were identified by their cobblestone appearance under phase-contrast microscopy and by the presence of factor-VIII-related antigen detected by a specific antibody using immunofluorescent microscopy.

Human thyrocytes were isolated as described previously using thyroid tissue (surplus to routine histopathological examination) obtained from a patient with Graves' disease [14,15].

Maintenance of HepG2 cell line

HepG2 cells, a human fetal liver-derived cell line, were obtained from the European Collection of Cell Cultures (Salisbury, Wilts., U.K.) and maintained in DMEM/Ham's F-12 nutrient mix plus L-glutamine and 15 mM Hepes containing 5% (v/v) fetal bovine serum. The cells were cultured in 75-cm^2 culture flasks and incubated at 37 °C in an atmosphere of 5% CO_2/95% air.

Monitoring selenoprotein expression

Intracellular and extracellular selenoproteins expressed by HUVECs were detected by labelling cells with [^{75}Se]selenite as described previously for thyrocytes [9]. Labelling with [^{75}Se]selenite provides a reliable, precise and sensitive method for assessing the expression of selenoproteins, where the incorporation of ^{75}Se is directed to specific selenocysteine residues by a UGA codon. However, equilibration of exogenous [^{75}Se]selenite with the endogenous pool of selenium and selenoproteins can take in excess of 27 h [16] and therefore cells are usually labelled for between 32 and 48 h to ensure that a steady state of labelling has been achieved. If it is required to examine changes in selenoprotein expression over short time periods it is essential to use cells pre-labelled with [^{75}Se]selenite. In the time-course experiments described in this article, cells pre-labelled for 48 h with [^{75}Se]selenite were used. The mass of exogenous selenium present in the culture medium was maintained at adequate levels over the labelling period by using media containing selenite at a concentration of 30 nM.

Therefore, to investigate intracellular selenoprotein expression in the steady state, confluent cultures of HUVECs or other cell types were incubated for 48 h with EGM-2 (which contains 30 nM selenite) in the presence of 0.02 MBq/ml [^{75}Se]selenite. After labelling, the culture medium was removed and the cells were washed three times with EBS and harvested into 20 ml of EBS by scraping followed by centrifugation at 2000 *g* for 10 min. The cells were resuspended in 1 ml of 60 mM Tris buffer, pH 7.8, containing 1 mM EDTA and 1 mM dithiothreitol and lysed by sonication at 4 °C.

After dilution to a common final protein concentration, the HUVECs were diluted 2:1 with 'boiling mix' (35 mM SDS/1.4 mM glycerol/0.3 mM 2-mercaptoethanol/15 mM Bromophenol Blue) and heat-treated at 90 °C for 10 min.

To monitor the extracellular selenoprotein expression, HUVEC cultures were incubated for 48 h in the presence of 0.02 MBq/ml [^{75}Se]selenite. The culture medium was changed from EGM-2 to protein-free EBM-2 for the last 18 h of the incubation period, but the [^{75}Se]selenite concentration was maintained throughout.

After the final 18-h labelling period, the culture medium was removed, centrifuged at 2000 *g* for 20 min and dialysed over 2 days to remove the unbound [^{75}Se]selenite. Dialysis was against 2 litres of 60 mM Tris buffer, pH 7.8, containing 1 mM EDTA and 1 mM dithiothreitol at 4 °C (which was changed four times over the 2-day period). The dialysed solution was then concentrated by placing the dialysis sac into a saturated solution of poly(ethylene glycol) prepared in the dialysis buffer. The protein content of the dialysed medium was then measured and the medium treated with boiling mix as described above.

The [^{75}Se]selenoproteins present in 25 μg of protein in the HUVECs and in 12.5 μg of protein in the culture medium were separated by SDS/PAGE on a 12% gel, the resulting gel was dried and the ^{75}Se-labelled selenoproteins were visualized by autoradiography using Kodak X-OMAT XAR-5 film.

The gels were scanned using a Bio-Rad model GS-525 Molecular Imager System to create a digitized image. The radio-

activity in each band was quantified using the Bio-Rad Molecular Analyst/PC image-analysis software. The data were presented as a percentage of the total intracellular ^{75}Se-labelled proteins expressed. Quantification of TR in the Western blots was carried out on an Epson$_{GT}$-9500 scanner using Phoretix software.

The intracellular selenoproteins expressed by human thyrocytes and HepG2 cells were determined in an identical fashion to the HUVECs.

To investigate the changes in selenoprotein expression that occurred over time in response to various agents, HUVECs pre-labelled to a steady state with [^{75}Se]selenite (0.02 MBq/ml) for 48 h were used. Monitoring of selenoprotein expression in these cells was then carried out as described above. These experiments are described in detail below.

Western-blot analysis

Proteins (20 μg per sample) resolved by SDS/PAGE were transferred to Immobilon P membranes that were then blocked using 10% (v/v) horse serum in 0.025 M Tris buffer/0.5M NaCl (pH 7.5) containing 0.05% (v/v) Tween before being probed with affinity-purified anti-rat TR antibody at a final dilution of 1:500. Chemiluminescence was used to visualize the immunoreactive proteins [8].

Previous studies that we have performed with this antiserum and the purified 58-kDa selenoprotein isolated from ^{75}Se-labelled HepG2 cells confirmed that the antiserum reacted with TR and not another protein of similar molecular mass [8]. Western blotting for PHGPX was performed as for TR, using an antibody raised to rat testes PHGPX.

Changes in expression of the 58-kDa selenoprotein in HUVECs following exposure to PMA and A23187 for different times

Selenoprotein expression in cells can be modified by PMA and A23187 [8,9]. Whereas these agents may produce a rapid effect on signalling systems in the cell, the changes in selenoprotein expression that they elicit may only be observable after several hours. The following experiments were performed to determine (i) the time at which maximal changes in the expression of the 58-kDa selenoprotein occur when HUVECs are exposed continually to PMA and A23187 and (ii) the influence of short 'pulsed' exposure times to PMA and A23187 on the subsequent maximal changes observed in the expression of the 58-kDa selenoprotein.

To investigate (i) above, confluent cultures of HUVECs were pre-labelled to a steady state for 48 h in the presence of EGM-2 containing 0.02 MBq/ml [^{75}Se]selenite. The culture medium was then removed and replaced with a further 15 ml of medium containing 0.02 MBq/ml [^{75}Se]selenite to ensure that nutrient and selenium supply were not exhausted. The effect of PMA (0.5×10^{-6}M) or A23187 (0.5×10^{-6} M) on the expression of the 58-kDa selenoprotein was studied by including the compounds in the culture medium for 1 min, 12 h, 24 h, 48 h, 72 h or 96 h in the continuing presence of 0.02 MBq/ml [^{75}Se]selenite. The timing of the additions were made to ensure that the cells for each data point were harvested immediately at the end of the drug-incubation period and at the same overall time point (i.e. after a total culture time of 146 h, which includes the 48-h pre-incubation interval). After harvesting the HUVECs, the intracellular [^{75}Se]selenoproteins were monitored as described above.

The concentrations of PMA and A23187 were selected on the basis of previous studies, which had shown optimal effects of these agents on the expression of the 58-kDa selenoprotein (results not shown).

To address (ii) above, confluent cultures of HUVECs were used that had been pre-labelled to a steady state with [^{75}Se]selenite (0.02 MBq/ml) for 48 h. The addition of PMA (0.5×10^{-6} M) or A23187 (0.5×10^{-6} M) was then made to these pre-labelled cells for 1 min, 10 min, 1 h or 12 h in the continued presence of [^{75}Se]selenite (0.02 MBq/ml). Additions were made at the same time and after each time point the culture medium was removed and the cells were washed. Fresh culture medium containing [^{75}Se]selenite (0.02 MBq/ml) but without PMA or A23187 was then added and the cells were incubated for a total incubation time of either 48 h in the case of PMA or 35 h in the case of A23187. These total incubation times were selected because the experiments performed in (i) above showed that they gave maximal changes in the expression of the 58-kDa selenoprotein. Cells were then harvested and the expression of the 58-kDa selenoprotein was monitored by autoradiography.

To control for any possible effects of DMSO, in which the PMA and A23187 were dissolved, HUVECs were also incubated with 0.05% DMSO at each time point.

Effects of PMA and A23187 added alone and in combination on the expression of the 58-kDa selenoprotein

The effects of PMA (0.5×10^{-6} M) and A23187 (0.5×10^{-6} M), added individually or in combination, on the expression of the 58-kDa selenoprotein in HUVECs were investigated by the inclusion of the compounds in the culture medium while the cells were being labelled with [^{75}Se]selenite for 35 h, as described above. These conditions were chosen because they produced the maximum changes in expression of the 58-kDa selenoprotein in the presence of A23187. It was not possible to use time points in excess of 35 h for these experiments as A23187 occasionally caused some cell detachment if time periods were longer than this. Pre-labelled cells were not used for this experiment, as the incubation time (35 h) was sufficient to ensure steady-state labelling of the selenoproteins.

Effects of GF109203X and PMA on expression of the 58-kDa selenoprotein

Using cells pre-labelled to a steady state for 48 h with [^{75}Se]selenite (0.02 MBq/ml), the effect of the specific protein kinase C (PKC) inhibitor GF109203X (4.5×10^{-7} M) on the 58-kDa selenoprotein expressed by HUVECs was studied in the presence and absence of PMA (0.5×10^{-6}M). After pre-labelling for 48 h, the culture medium was removed from HUVECs and replaced with fresh medium containing 0.02 MBq/ml [^{75}Se]selenite. The GF109203X (4.5×10^{-7}M) was added to the culture medium 1 h prior to any PMA additions. Previous studies have shown that PKC activity is inhibited using these conditions [17]. After the PMA (0.5×10^{-6} M) addition, the HUVECs were incubated for a further 48 h before the cells were harvested for detection of selenoprotein expression by autoradiography.

Determination of protein

The protein content of the sonicated cells and extracellular proteins was determined using the Bradford dye-binding method using BSA standards [18].

RESULTS

Selenoproteins expressed by cell types

There were distinct differences in the ratios of the various intracellular selenoproteins expressed between the cell types

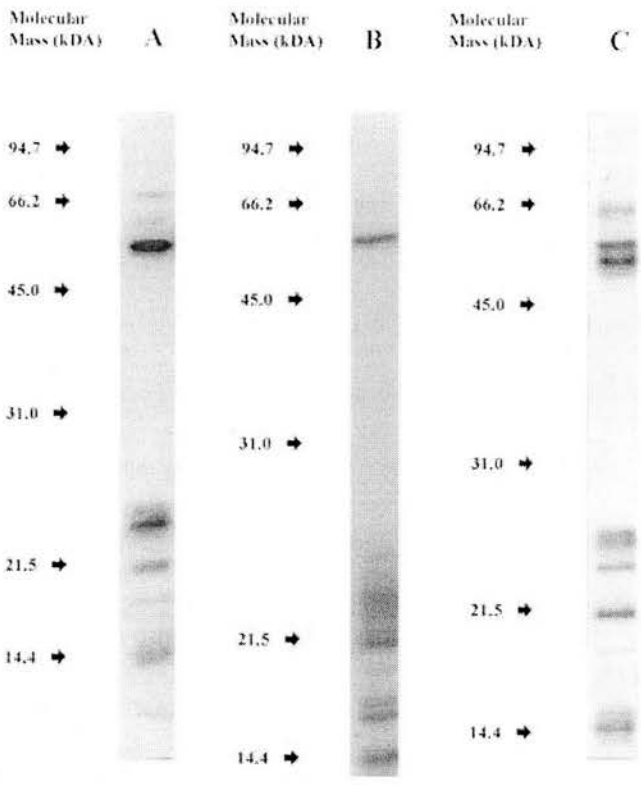

Figure 1 Autoradiograph of an SDS/PAGE gel showing various human cell types grown under basal conditions labelled with [^{75}Se]selenite (0.02 MBq/ml) for 48 h

Lane A, HUVECs; lane B, human thyrocytes; lane C, HepG2 cells. Each lane was loaded with 25 μg of protein.

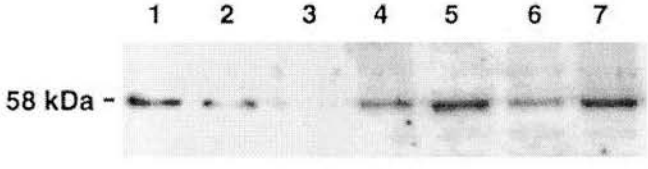

Figure 2 Western blot of HUVECs using antiserum to TR

Cells were incubated for 35 h in the presence and absence of various agents, as specified, prior to the Western blotting of cell lysates. Lanes 1–3, purified human TR standard (4, 2 and 1 ng respectively); lane 4, no additions; lane 5, calcium ionophore A23187 (0.5×10^{-6} M); lane 6, PMA (0.5×10^{-6} M); lane 7, A23187 (0.5×10^{-6} M) with PMA (0.5×10^{-6} M).

studied (Figure 1). In HUVEC preparations a single selenoprotein with a molecular mass of 58.1 ± 1.0 kDa (mean $\pm$ S.E.M., $n = 5$) dominated the selenoprotein profile accounting for $42.9 \pm 1.4\%$ ($n = 3$) of the total intracellular ^{75}Se-labelled proteins. Two other selenoproteins showed quite pronounced labelling with [^{75}Se]selenite, with calculated molecular masses of 21.7 ± 0.5 ($n = 5$) and 24.4 ± 0.5 kDa ($n = 5$). These two selenoproteins accounted for $14.6 \pm 1.0\%$ ($n = 3$) and $15.1 \pm 2.8\%$ ($n = 3$) of the intracellular ^{75}Se-labelled selenoproteins, respectively. Western blotting identified the 21.7-kDa selenoprotein as PHGPX (results not shown). Western blotting was also attempted using antiserum to cyGPX but no immunoreactive band was observed.

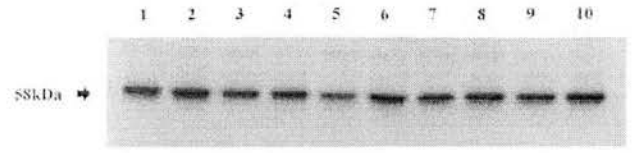

Figure 3 Autoradiograph of an SDS/PAGE gel showing the expression of the 58-kDa selenoprotein in HUVECs incubated in the continued presence of PMA (0.5×10^{-6} M) for various times

Confluent cultures of HUVECs pre-labelled for 48 h with 0.02 MBq/ml [^{75}Se]selenite were used. The effect of PMA on the expression of the 58-kDa selenoprotein was studied by including either PMA (0.5×10^{-6} M) or DMSO (0.05%) in the culture medium for 1 min, 12 h, 48 h, 72 h or 96 h in the continued presence of 0.02 MBq/ml [^{75}Se]selenite. Lane 1, PMA, 1 min; lane 2, DMSO, 1 min; lane 3, PMA, 12 h; lane 4, DMSO, 12 h; lane 5, PMA, 48 h; lane 6, DMSO, 48 h; lane 7, PMA, 72 h; lane 8, DMSO, 72 h; lane 9, PMA, 96 h; lane 10, DMSO, 96 h. Each lane was loaded with 25 μg of protein.

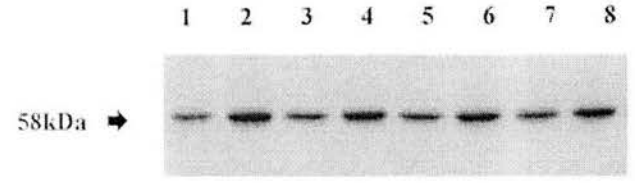

Figure 4 Autoradiograph of an SDS/PAGE gel showing the expression of the 58-kDa selenoprotein in HUVECs incubated with PMA (0.5×10^{-6} M) for various times

Confluent cultures of HUVECs pre-labelled for 48 h with 0.02 MBq/ml [^{75}Se]selenite were used. The effect of PMA on the expression of the 58-kDa selenoprotein was studied by including either PMA (0.5×10^{-6} M) or DMSO (0.05%) in the culture medium for 1 min, 10 min, 1 h or 12 h in the continued presence of 0.02 MBq/ml [^{75}Se]selenite. After each time point the medium was removed, the cells were washed and fresh culture medium containing 0.02 MBq/ml [^{75}Se]selenite without PMA or DMSO was added. The cells were incubated for a total incubation time of 48 h. Lane 1, PMA, 1 min; lane 2, DMSO, 1 min; lane 3, PMA, 10 min; lane 4, DMSO, 10 min; lane 5, PMA, 1 h; lane 6, DMSO, 1 h; lane 7, PMA, 12 h; lane 8, DMSO, 12 h. Each lane was loaded with 25 μg of protein.

In the human thyrocytes and HepG2 cells the ^{75}Se-labelled 58-kDa selenoprotein was not as prominent as that observed in the ^{75}Se-labelled HUVECs (Figure 1). No significant ^{75}Se-labelling of any extracellular proteins was detected (results not shown).

Identification of the 58-kDa selenoprotein by Western blotting

Using antiserum to rat TR, the 58-kDa selenoprotein expressed by HUVECs was identified by Western blotting as TR. HUVECs expressed high concentrations of TR under basal conditions (4.36 ± 0.63 μg/mg, $n = 3$; Figure 2). TR concentrations in HepG2 cells were approximately one-tenth of those found in HUVECs. The Western blot was insufficiently sensitive to detect TR in the thyrocytes grown in the basal state.

Time-dependent changes in expression of the 58-kDa selenoprotein in HUVECs following exposure to PMA and A23187 for different times

The effects of both PMA and A23187 on the expression of the 58-kDa selenoprotein were time-dependent. The continued presence of PMA (0.5×10^{-6} M) significantly decreased the expression of the 58-kDa selenoprotein. The down-regulation of this protein was first apparent at 12 h, with the lowest level of expression seen at 48 h (Figure 3). After 72 and 96 h of exposure, the expression

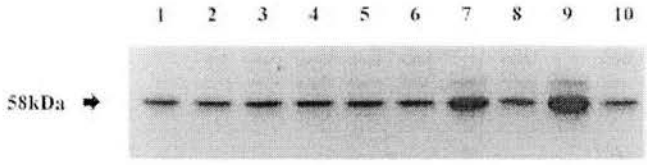

Figure 5 Autoradiograph of an SDS/PAGE gel showing the expression of the 58-kDa selenoprotein in HUVECs incubated with A23187 (0.5×10^{-6} M) for various times

Confluent cultures of HUVECs were pre-labelled for 48 h with 0.02 MBq/ml [^{75}Se]selenite. The effect of A23187 on the expression of the 58-kDa selenoprotein was studied by including either A23187 (0.5×10^{-6} M) or DMSO (0.05%) in the culture medium for 1 min, 10 min, 1 h, 12 h or 35 h in the continued presence of 0.02 MBq/ml [^{75}Se]selenite. After each time point the medium was removed, the cells were washed and fresh culture medium containing 0.02 MBq/ml [^{75}Se]selenite without A23187 or DMSO was added. The cells were incubated for a total incubation time of 35 h. Lane 1, A23187, 1 min; lane 2, DMSO, 1 min; lane 3, A23187, 10 min; lane 4, DMSO, 10 min; lane 5, A23187, 1 h; lane 6, DMSO, 1 h; lane 7, A23187, 12 h; lane 8, DMSO, 12 h; lane 9, A23187, 35 h; lane 10, DMSO, 35 h. Into each lane, 25 μg of protein was loaded.

of the 58-kDa selenoprotein started to rise but the level did not return to the basal levels observed in the control DMSO-treated HUVECs.

The continued presence of A23187 (0.5×10^{-6} M) in the culture medium resulted in a significant induction of the 58-kDa ^{75}Se-labelled selenoprotein at 24 and 38 h (results not shown). However, in some experiments, where HUVECs were exposed to A23187 for 38 h and longer, cells started to detach from the monolayer, suggesting possible toxic effects of the ionophore. For all further experiments, a total incubation period of 35 h was therefore chosen, as this allowed maximal induction of the 58-kDa selenoprotein without any observable cell detachment. Controls containing DMSO showed no significant change in expression of the 58-kDa selenoprotein over the duration of the experiments.

The effects of the shorter pulse times of exposure to PMA and A23187 are shown in Figures 4 and 5. In HUVECs transiently exposed to PMA (0.5×10^{-6} M) for 1 min–12 h, a decrease in the expression of the 58-kDa selenoprotein was measured 48 h after the initial exposure to PMA (Figure 4).

HUVECs exposed to A23187 (0.5×10^{-6} M) for 1 h showed a slightly increased expression of the 58-kDa selenoprotein 35 h later (Figure 5). The observed induction of this 58-kDa selenoprotein by A23187 was increased with exposure time such that clear increases were observed after 12 h, with maximal induction after 35 h of exposure.

Effects of PMA and A23187, added alone and in combination, on the expression of the 58-kDa selenoprotein

The addition of PMA down-regulated the expression of the 58-kDa [^{75}Se]selenoprotein in HUVECs, whereas the addition of the calcium ionophore A23187 alone significantly induced its expression, as visualized by autoradiography (Figure 6). PMA and A23187 added in combination produced an overall net increase in the expression of the 58-kDa protein but the expression was lower than that seen when A23187 was added alone.

Quantification of the ^{75}Se-labelled 58-kDa selenoprotein using the Molecular Imager System showed that treatment with PMA decreased the expression of the 58-kDa band by between 43.5 and 50.7% of basal levels ($n = 2$), whereas treatment with A23187 increased the expression of the same band by 2.39–2.60-fold over basal levels ($n = 2$). PMA and A23187 added in combination produced an overall increase in expression of between 1.44- and 1.64-fold over basal levels ($n = 2$).

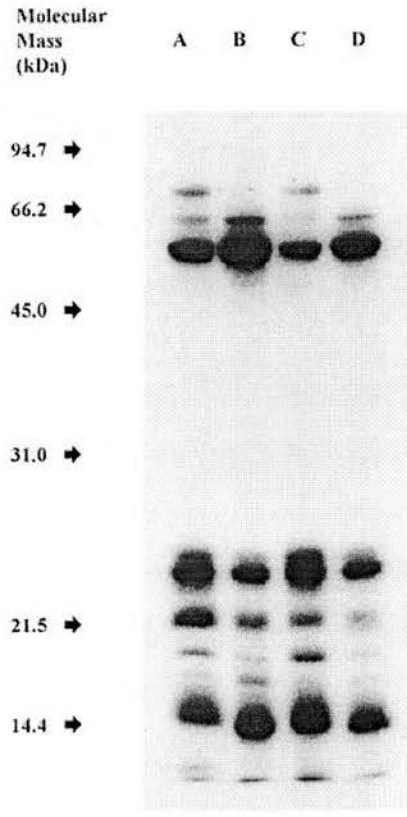

Figure 6 Autoradiograph of an SDS/PAGE gel of HUVECs labelled with [^{75}Se]selenite (0.02 MBq/ml) for 35 h in the presence and absence of various agents

Lane A, no additions; lane B, calcium ionophore A23187 (0.5×10^{-6} M); lane C, PMA (0.5×10^{-6} M); lane D, A23187 (0.5×10^{-6} M) with PMA (0.5×10^{-6} M). Each lane was loaded with 25 μg of protein.

The effects of PMA and A23187 on the expression of the 58-kDa selenoprotein were confirmed by Western blotting using the anti-TR antibody (Figure 2). Western-blot analysis showed that PMA significantly ($P < 0.05$, analysis of variance and test of least significant difference) decreased TR expression in HUVECs from 4.36 ± 0.63 μg/mg of protein (mean $\pm$ S.E.M., $n = 3$), expressed under basal conditions, to 2.97 ± 0.27 μg/mg of protein ($n = 3$), a decrease of 29.6 ± 9.7%. In contrast, A23187 significantly ($P < 0.01$) increased TR expression to 8.38 ± 0.69 μg/mg of protein ($n = 3$), a 1.96 ± 0.13-fold increase over basal levels. When HUVECs were treated with PMA and A23187 in combination there was a net increase in TR expression to 6.58 ± 0.42 μg/mg of protein ($n = 3$), a 1.57 ± 0.24-fold increase over basal levels.

The addition of PMA and A23187 also had effects on some of the other ^{75}Se-labelled selenoproteins in HUVECs. For example, treatment with PMA decreased the labelling of the 64- and 72-kDa [^{75}Se]selenoproteins. However, in the presence of A23187, ^{75}Se-labelling of the 64-kDa selenoprotein increased, whereas that of the 72-kDa selenoprotein was significantly attenuated. Both PMA and A23187 down-regulated the expression of the 22-kDa selenoprotein. The expression of the 24-kDa selenoprotein was increased by PMA, whereas A23187 had no effect on the expression of this selenoprotein (Figure 6).

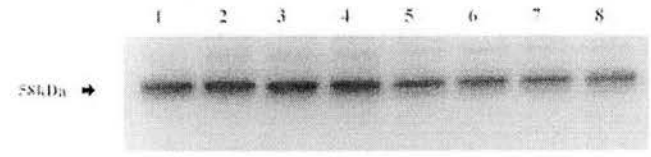

Figure 7 Autoradiograph of an SDS/PAGE gel showing the expression of the 58-kDa selenoprotein in HUVECs incubated with the PKC inhibitor GF109203X (7.5×10^{-7} M) and/or PMA (0.5×10^{-6} M)

Confluent cultures of HUVECs pre-labelled for 48 h with 0.02 MBq/ml [^{75}Se]selenite were used. The effect of GF109203X and PMA on the expression of the 58-kDa selenoprotein was studied by including GF109203X and PMA in the culture medium alone or in combination. GF109203X was added 1 h prior to the 48-h PMA incubation in the continued presence of 0.02 MBq/ml [^{75}Se]selenite. Lanes 1 and 2, DMSO; lanes 3 and 4, GF109203X alone; lanes 5 and 6, GF109203X and PMA; lanes 7 and 8, PMA alone. Each lane was loaded with 25 μg of protein.

Effects of GF109203X and PMA on the expression of the 58-kDa selenoprotein

Treatment of HUVECs with PMA (0.5×10^{-6} M) for 48 h decreased the expression of the 58-kDa selenoprotein, as shown in the previous experiments. Pre-incubation of HUVECs with the PKC inhibitor GF109203X (4.5×10^{-7}M) for 1 h prior to the PMA addition attenuated the down-regulation of the 58-kDa [^{75}Se]selenite-labelled selenoprotein produced by PMA (Figure 7). No change in the expression of the 58-kDa selenoprotein was observed when HUVECs were incubated with GF109203X alone.

DISCUSSION

HUVECs exhibit an intracellular selenoprotein profile that is quite distinct from the other cells studied. We have found that HUVECs show dominant expression of a [^{75}Se]selenoprotein with a molecular mass of 58 kDa (Figure 1). Using Western blotting with antiserum characterized previously [8], we have demonstrated that this 58-kDa selenoprotein is TR (Figure 2). The expression of TR in HepG2 cells was found to be only approx. one-tenth of that found in HUVECs. In human thyrocytes, its expression was so low that it could not be detected by Western blotting, but ^{75}Se-labelling experiments suggested that TR expression in thyrocytes was approx. half of that seen in HepG2 cells (Figure 1). These different patterns of expression presumably underlie the requirement for different selenoproteins to contribute to the specific functions of the different cell types.

TR is an FAD-containing homodimeric selenoenzyme, which, together with thioredoxin as a substrate and NADPH as a cofactor, forms a powerful dithiol-disulphide oxidoreductase system that has multiple roles. The TR/thioredoxin system has been associated with a number of cellular processes, including regulation of cell growth and the modification of the activity of transcription factors and receptors [19–21]. TR expression is regulated in cancer cells through selenium supply, which stabilizes TR mRNA [21].

In addition, TR can reduce and detoxify lipid hydroperoxides, hydrogen peroxides and organic hydroperoxides [22]. The accumulation of these compounds in tissues exerts deleterious effects. For example the hydroperoxide (15*S*)-hydroperoxy-(5*Z*),(8*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid [(15*S*)-HPETE] oxidizes low-density lipoprotein to a form that under some conditions renders the particle more toxic to the endothelium, with implications in the pathogenesis of atherosclerosis [22]. TR also appears to be more potent than the GPX system at detoxifying (15*S*)-HPETE and other lipid hydroperoxides [22].

The data we have presented here show that the expression of TR is far greater in HUVECs than the expression of the selenoproteins with molecular masses of 22 and 24 kDa (Figure 1). The 22-kDa band was identified as PHGPX using Western-blot analysis (results not shown). It was not possible to visualize an immunoreactive band in HUVECs using antisera to cyGPX. However, other groups have shown that HUVECs express cyGPX [10] and exhibit GPX activity [2,3,5]. The molecular mass of cyGPX has been reported to be in the range of 21–26 kDa [23,24], which corresponds to the 24-kDa selenoprotein band we have found in HUVECs. These observations suggest that the 24-kDa selenoprotein expressed by HUVECs is cyGPX.

Thomas et al. [2] have suggested that selenite confers resistance on ECs to oxidative injury by increasing expression of cyGPX and PHGPX [2]. Whereas it is likely that these selenoperoxidases do have an important role in preventing oxidative injury to the ECs, our observations concerning TR expression raise the possibility that this reductase may be more important than the peroxidases in the protection of the endothelium against oxidative damage. The molecular mass of TR appears greater in HUVECs than that of PHGPX (22-kDa selenoprotein) and cyGPX (24-kDa selenoprotein) (Figure 1) and TR can detoxify many lipid hydroperoxides more efficiently than the GPXs [22]. TR may also have effects on other antioxidant systems in ECs; it can induce manganese superoxide dismutase [25] and regenerate ascorbate from dehydroascorbate [26].

We have shown that the addition of the phorbol ester PMA and the calcium ionophore A23187 have significant effects on the expression of TR. Exposure of HUVECs to PMA for times as short as 1 min decreased TR expression by approx. 30%. However, changes in TR expression were only seen 48 h after the short exposure to PMA. A similar degree of down-regulation of TR was seen when the exposure time of HUVECs to PMA was as long as 48 h. However, if cells were exposed to PMA for times in excess of 48 h the effect of the PMA on TR expression was diminished. These observations suggest that the down-regulation of TR by PMA arises by activation of PKC rather than resulting from desensitization of PKC. However, if PMA was present for times in excess of 48 h it seems likely that PKC desensitization does occur, as reported previously in studies using long-term exposures to PMA [27,28].

Our experiments using the PKC inhibitor GF109203X produced further evidence that increased TR expression in response to PMA is not the result of desensitization of PKC. GF109203X added alone had no effect on TR expression but when added with PMA the PKC inhibitor attenuated the effect of PMA on down-regulating TR. Overall, our results suggest that TR expression in HUVECs can be down-regulated by activation of PKC by PMA. This contrasts with human thyrocytes and HepG2 cells where PMA produces an increase in TR expression [8].

The addition of A23187 increased the expression of TR 1.96 ± 0.13-fold over basal levels in HUVECs (Figure 2). Similar effects of the ionophore on TR expression have also been reported in human thyrocytes and HepG2 cells [8]. A23187 increases intracellular calcium levels but it may also act as an uncoupler of oxidative phosphorylation and as an inhibitor of mitochondrial ATPase activity. The A23187-induced cGMP response in ECs occurs rapidly following treatment with A23187 and reaches a plateau within 3 min [29]. However, our studies have shown that clear increases in the expression of TR only occur when A23187 is present in the culture medium for periods in excess of 12 h. This suggests that regulation of TR in the presence of A23187 in HUVECs may not be the result of calcium signalling but rather the result of a stress effect produced by A23187 on the HUVECs.

If A23187 was acting through a calcium response it would appear that, in HUVECs, the two branches of the Ca^{2+}/phosphinositol signalling pathway are acting antagonistically. There are other examples in ECs where this is also the case. For example, constitutive nitric oxide synthase activity in ECs is stimulated by A23187, but the subsequent A23187-induced nitrite release is inhibited by PKC activation by the phorbol ester PMA [30].

The activation of PKC through oxidized low-density lipoprotein has been implicated in vascular disease [31] and we have shown that PKC activation may also give rise to a decrease in TR expression. Since TR may have an antioxidant function within the EC, down-regulation of TR by this mechanism may diminish the antioxidant capacity of ECs and render the cell prone to oxidative damage and to the development of atheroma.

In our culture system, HUVECs do not secrete extracellular selenoproteins. This contrasts with human thyrocytes, which secrete a 24-kDa selenoprotein identified as EGPX [9]. The vascular endothelium thus appears to make no contribution to the circulating pool of EGPX or selenoprotein P, another extracellular selenoprotein that may have antioxidant properties [32]. Thus it would seem that the selenoprotein P found bound to ECs is unlikely to have originated from synthesis within these cells, but rather that it is synthesized in other tissues such as kidney and released into plasma [7].

In conclusion, HUVECs, unlike other human cell types examined, express high levels of TR, a selenoprotein that has the potential to detoxify hydrogen peroxide and lipid hydroperoxides [22], regenerate ascorbate [26], promote superoxide dismutase activity, maintain cell redox state and influence the activity of the transcription factors of a number of important genes [19–21]. TR may thus be an important factor in the known ability of selenium to protect HUVECs from oxidative damage.

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