Effects of Oxidative Stress Induced by Glutathione Depletion on the Expression of Endothelial-Derived Vasomediator Genes

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ABSTRACT

The endothelium plays an important homeostatic role in vascular physiology. Important diseases of the blood vessel, such as atherosclerosis and diabetes mellitus, evidence a common phenotype best summarized as a dysfunctional endothelium. A major component of this endothelial phenotype is an impaired ability to appropriately regulate local vascular tone. It is of interest that these diseases are associated with oxidative stress. Surprisingly however, the vascular endothelium is richly endowed with antioxidant defense mechanisms. The aim of this thesis is to address the role of oxidative stress in perturbations in the gene expression of endothelial-derived vasomediators. Intracellular glutathione is a prominent antioxidant defense against oxidative stress. We hypothesized that depletion of glutathione, a non-protein sulfhydryl, would lead to decreased vasodilator and enhanced vasoconstrictor gene expression. We found that chronic diethyl maleate (DEM), known to deplete glutathione, decreased steady-state endothelial nitric oxide synthase (eNOS) mRNA in vascular endothelium in a time- and concentration-dependent fashion. DEM also increased heme oxygenase-1 (HO-1) mRNA, decreased preproendothelin-1 (ppET-1) mRNA but had no effect on steady-state endothelin-converting enzyme-1 (ECE-1) mRNA expression. DEM also decreased steady-state eNOS protein and functional studies indicated the decrease was reflected in less NO synthase enzymatic activity. Basal and calcium-dependent conversion of L-arginine to L-citrulline were blunted by DEM. We further demonstrated that DEM decreased eNOS mRNA and protein in a variety of endothelial cells, both *in vitro* and *in vivo*. *In vivo* studies showed decreased eNOS mRNA and immunoreactive protein in lung and kidney of DEM-treated mice. Furthermore, the *in vitro* effect of DEM on steady-state eNOS mRNA expression was recapitulated with other agents known to decrease intracellular GSH content in vascular endothelium, namely phorone and buthionine sulfoximine (BSO). To examine the molecular mechanisms implicated in DEM- mediated changes in eNOS mRNA expression we evaluated the relative contributions of transcriptional and post-transcriptional processes. Though minor changes were observed in the loading of RNA polymerase II at 5'-regions of the human eNOS gene, nuclear run-on assays indicated that full-length eNOS pre-mRNA transcripts did not decrease with

acute or chronic DEM-treatment. We take these data to indicate that chronic DEM treatment increased the rate of degradation of eNOS mRNA transcripts in vascular endothelial cells. This molecular mechanism is a novel finding with respect to how oxidative stress affects endothelial genotype and phenotype and suggests that chronic oxidant stress may modulate the expression of other genes at the post-transcriptional level.

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Ach = acetylcholine

AGEs = advanced glycosylation endproducts

ALS = amyotrophic lateral sclerosis

AP-1 = activator protein-1

BAEC = bovine aortic endothelial cells

BSO = buthionine sulfoximine

Ca^{2+} = calcium

Cu/Zn = copper/zinc

cGMP = cyclic guanosine monophosphate

CO = carbon monoxide

COOH = carboxy

CTP = cytidine triphosphate

DAG = diacyl glycerol

dCTP = deoxycytidine triphosphate

DEM = diethyl maleate

DTNB = 5,5'-dithiobis-(2-nitrobenzoic acid)

ECE-1 = endothelin-converting enzyme-1

eNOS = endothelial nitric oxide synthase

ET-1 = endothelin-1

Fe = iron

fMLP = formyl-methionyl-leucyl-phenylalanine

GAPDH = glyceraldehyde-3-phosphate

GSSG = glutathione disulfide (oxidized form)

GSH = glutathione (reduced form)

GPx = glutathione peroxidase

H_2O_2 = hydrogen peroxide

HIF-1 = hypoxia-inducible factor-1

HO = heme oxygenase

HUVEC = human umbilical vein endothelial cells

ICAM = intercellular cellular adhesion molecule

IP_3 = inositol triphosphate

IRE = iron-responsive element

IRP = iron regulatory protein

L-arg = L-arginine

L-cit = L-citrulline

LF-L = lactoferrin-like polypeptide

LPS = lipopolysaccharide

LTP = long-term potentiation

MAPK = mitogen-activated protein kinase

Mn = manganese

mRNA = messenger ribonucleic acid

NAC = N-acetylcysteine

NH_2 = amino

NF-1 = nuclear factor-1

NF-κB = nuclear factor kappa B

NO = nitric oxide

$^1O_2$ = singlet oxygen

O_2^- = superoxide anion

$ONOO^-$ = peroxynitrite •OH = hydroxyl radical

oxLDL = oxidized low-density lipoprotein

PGE_2 = prostaglandin E_2

PGI_2 = prostaglandin I_2

PMN = polymorphic neutrophils

ppET-1 = preproendothelin-1

RAGE = receptor for advanced glycosylation endproducts

ROI = reactive oxygen intermediates

SOD = superoxide dismutase

TBARS = thiobarbituric acid reactive substances

TRX = thioredoxin

UTP = uridine triphosphate

UTR = untranslated region

VCAM = vascular cellular adhesion molecule

VEGF = vascular endothelial growth factor

VSMC = vascular smooth muscle cell

I. INTRODUCTION

I.1 CLINICAL RELEVANCE OF ENDOTHELIAL DAMAGE

It is now appreciated that the endothelium plays an important role in vascular physiology and pathophysiology. Indeed, current concepts suggest that the endothelium should rightly be considered as a distinct organ. Unique features important to highlight include the distinct position of the endothelium and its barrier function. Endothelial cells lining the inner surface of blood vessels occupy a pivotal position between the circulating humoral and cellular elements of the blood and tissues. In this unique position, endothelial cells regulate protean processes, such as leukocyte adherence and transit through the blood vessel wall, modulation of the immune response and the balance between thrombosis and thrombolysis (1). Endothelial cells can respond to varied exogenous stimuli. This thesis focuses upon one of the newly appreciated biological roles of the endothelium, namely the control of vascular tone. The endothelium releases diverse vasoactive substances that regulate local blood flow through their direct effects on vascular smooth muscle cell (VSMC) contraction. Endothelial-derived vasomediators include vasodilators, such as nitric oxide (NO) (2) and vasoconstrictors, such as endothelin-1 (ET-1) (3). As a result of their unique position and involvement in several crucial vascular functions, endothelial injury affects a variety of physiologic processes. Especially prominent in this change in function is the changes that are known to occur in paracrine control of the circulation.

Endothelial dysfunction

Endothelial dysfunction comprises a major feature of important clinical disorders of man. Local or diffuse abnormalities in endothelial biology have been modelled. Especially of interest, are studies addressing the role of the vascular endothelium in atherosclerosis. Even the earliest studies concluded that the endothelium was important in the initial stages in the disease process.

Indeed, experiments in hypercholesterolemic rabbits, showed that removing the endothelium accelerated the formation of atherosclerotic lesions, suggesting that loss of the endothelial monolayer contributed to the disease state (4). Pathologists posited that the endothelium was even physically absent. However, subsequent detailed observations indicated that this belief was naive. Endothelial cells are in fact present overlying atherosclerotic lesions in fewer numbers. Though they are present, clearly they evidence an altered phenotype (5). Therefore, the endothelium is altered in response to injury, not absent. A key aspect of this dysfunction of the endothelium is the improper response to physiologic stimuli. It is remarkable how many diverse environmental stimuli the endothelial cell can respond to. Endothelial dysfunction has been variably defined, with most models stressing important imbalances between relaxing and contracting factors, between procoagulant and anticoagulant mediators or between growth-inhibiting and growth-promoting substances (6).

Contributions of Endothelium to Clinical Disorders

In diseases such as diabetes and atherosclerosis, patients have impaired vasomediator responses (7-11). Acetylcholine (Ach) normally causes vasodilatation of blood vessels by promoting the release of vasorelaxant substances from the endothelium, such as endothelium-derived relaxing factor (EDRF) (12-14). In normal vessels, if the endothelium is removed experimentally, Ach constricts blood vessels. In diseased human vessels, Ach induces a paradoxic vasoconstriction. For example, in coronary atherosclerosis cholinergic vasodilation is impaired (15). Diseased vessels with moderate or advanced coronary atherosclerosis have a constrictive response to Ach. Even though there are minimal gross structural features of atherosclerosis, the impaired response to Ach is common to a range of stages in atherosclerosis (15). Likewise, in epicardial coronary arteries of hypertensive patients, the response to Ach is impaired (11, 16). However, these vessels were still able to respond to nitroglycerin, an NO-donating compound, indicating that the smooth muscle still has the capacity to dilate. The inappropriate response to Ach

suggests that the endothelium is not functioning properly and its production of vasomediators is impaired. Other clinical disorders exhibiting impaired vasomediation include ischemia-reperfusion injury (17, 18), aging and preeclampsia (19), among others. A theme that has emerged from studies addressing mechanisms of blood vessel perturbations in these diverse clinical settings is the prominent role of oxidative stress and redox-based mechanisms for changes in vascular wall homeostasis (20). Oxidative stress induced by free radicals are thought to play a role in these diseases (21). The endothelium consequently emerges as a target organ for damage and dysfunction. Thus, endothelial dysfunction plays a critical role in the pathology of several major disease states, and oxidative stress has been implicated as a cause of endothelial injury. In this thesis, focus will be maintained on atherosclerosis and diabetes. The idea that oxidative injury of endothelial cells may lead to imbalances in vasomediator production, characteristic of dysfunctional endothelium, has not been extensively explored.

I.2 ENDOTHELIAL-DERIVED VASOMEDIATORS

The exciting discovery of endothelial-derived vasomediators has led to conceptual advances necessitating a reevaluation of blood vessel biology at the local level. Formerly viewed as a passive barrier, the endothelium is now recognized as a dynamic component responding to physiologic stimuli and contributing to vascular tone. The fascinating picture emerged that, in both health and disease, the endothelial organ releases both vasorelaxants and vasoconstrictors. The balance of local production and action of these substances determines, to a major extent, overall vasomotor tone of the underlying vascular smooth muscle cell or pericyte. Prior to this conceptual advance, peripheral vascular tone was perceived to represent static influences from endocrine glands, the autonomic nervous system and intrinsic myogenic tone. Since the field emerged, it is a fair statement that our understanding of the physiologic regulation and action of endothelial-derived vasomediators has evolved substantively. This claim is, arguably, less valid for our understanding of how these pathways and paradigms go awry in disease. This thesis work addresses a focused mechanism that may be relevant to long-term control of vasomediator production.

I.2.1 Endothelial vasodilators

Endothelial-derived relaxing factor (EDRF) is a labile humoral agent that explains much of the action of endothelium-dependent vasodilators. It was Furchgott and Zawadaski who proposed an explanation for differences in the relaxation capacity of independent smooth muscle aortic rings in response to Ach. The differences reflected the presence or absence of the endothelium. When denuded of endothelial cells, Ach did not induce relaxation of aortic rings (2). These researchers coined the term EDRF. Subsequently, NO was found to be the main agent comprising EDRF, as both were labile, humoral agents (14, 22, 23). Nitroglycerin and nitroprusside were known to have vasodilatory effects and had been used in the treatment of cardiovascular disease for over a century. However, it has only been in the past ten years, that the molecular basis of their action

has been discerned. These pharmaceuticals release NO at the level of the vascular smooth muscle cell (VSMC). NO plays a vital role in cell-cell signalling in the blood vessel wall. The researchers acknowledged for their contributions in the recognition that NO is an important signalling molecule in the cardiovascular system were recently awarded the 1998 Nobel Prize in Physiology or Medicine: Robert Furchgott, Ferid Murad and Louis Ignarro. It was a remarkable discovery, that a simple molecule had potent vascular effects.

NO, though an endothelial-derived vasomediator, has physiological functions as a messenger molecule in a wide range of physiologic systems (23). NO influences other events besides vasomediation, participating in cytotoxic activity of macrophages during inflammatory responses towards viruses, intracellular microbes and tumours (24). As well, NO participates in neurotransmission and neuronal development (25).

I.2.1.1 Nitric oxide synthases

A family of enzymes, nitric oxide synthases (NOS), catalyze the formation of NO. Two constitutive enzymes exist, endothelial (eNOS) and neuronal (nNOS). There is a third enzyme that is inducible (iNOS). It was first identified in monocyte / macrophages. These isoforms, eNOS, nNOS and iNOS, were mapped to human chromosomal regions 7q35-36, 12q24.2 and 17q11.2-12, respectively (26-36). The genomic organization of the individual isoforms exhibit a high degree of relatedness and all three enzymes display homology with cytochrome P450 reductase (37). While nNOS and iNOS are widely expressed in several cell types and tissues, the eNOS isoform is relatively restricted to vascular endothelium. However, there are some notable exceptions. eNOS has been identified in the pyramidal neurons of the CA1 region of the hippocampus (38, 39). Syncytiotrophoblasts from placental villi also express eNOS (40). eNOS has been found in cardiac myocytes (41) and possibly, in epithelium (42).

I.2.1.2 Physiology and biochemistry

The following section discusses the physiology and biochemistry of eNOS, the diverse roles of endothelial-derived NO and how various stimuli enhance its production. NO plays a number of roles in vascular biology, including maintenance of pressure and body fluid homeostasis, modulation of platelet aggregation/adhesion and leukocyte adhesion, cardiac myocyte / myoblast biology and the regulation of vascular cell migration, proliferation and apoptosis (43). Various agents and mechanical stress are known to induce the production of endothelial-derived NO. Humoral agents, including Ach, bradykinin or histamine exert vasodilatory effects through stimulation of eNOS and increased production of NO (2, 44, 45). Hence their designation as endothelial-dependent vasodilators. Bradykinin, Ach and histamine bind to high-affinity cell-surface receptors and activate guanine nucleotide-binding protein. This, in turn, stimulates phospholipase C to produce inositol trisphosphate (IP_3) and diacylglycerol (DAG) (46). IP_3 elevates intracellular calcium levels, while DAG activates protein kinase C (PKC) (47). The increase in calcium stimulates eNOS to produce NO. NO activates soluble guanylate cyclase thereby increasing cyclic guanosine monophosphate (cGMP) levels in VSMC (2, 44, 46). NO mediates vascular relaxation in response to stimulation of eNOS activity by other stimuli, notably mechanical stress. Pulsatile stretch is important under normal circumstances; however, changes in heme oxygen content are also are known to increase the production of NO.

The production of NO by endothelial cells depends on critical subcellular localization of eNOS. eNOS is localized to the cell membrane, an event facilitated by post-translation acylation: myristoylation and palmitoylation. Two acylation sites in the NH_2-terminal domain, for myristoylation and palmitoylation, facilitate this targeting of eNOS to the plasma membrane (48-52). The myristoylation site is unique to eNOS and does not appear in nNOS or iNOS.

Myristoylation targets eNOS to the plasma membrane (48, 49), while palmitoylation stabilizes eNOS in the plasmalemmal caveolae (50-52).

Just as eNOS require specific post-translational modifications for proper localization, eNOS also requires many cofactors to function. Consistent with this, the eNOS apoenzyme possesses specific domains to ensure proper activation. Cofactors facilitate the enzymes to catalyze the five-electron oxidation of the terminal guanidino nitrogen of the amino acid L-arginine (L-arg) to produce NO and L-citrulline (L-cit) (53). NOSs are best characterized as cytochrome P450-like heme proteins requiring molecular oxygen, NADPH, FMN, FAD, enzyme-bound heme and tetrahydrobiopterin for their activation (37). NOSs function as dimers and tetrahydrobiopterin is required for dimerization (54). eNOS also contains a putative phosphorylation site for protein kinase A (37). As expected, as calcium is required for eNOS activation and increases in intracellular calcium facilitate calmodulin binding, which activates eNOS. As well, changes in calcium affect subcellular localization during cellular activation. The calmodulin binding domain controls electron transfer from the COOH-terminal reductase domain to the NH_2-terminal oxidative domain (55). NADPH supplies these electrons which are transferred along flavins and calmodulin to the catalytic heme centre, at the NH_2-terminus (54). Thus, the domains involved in electron transfer affect catalytic activity of eNOS, while the N-terminal domain plays a role in localization of the enzyme.

The membrane localization of eNOS facilitates an immediate response to the various stimuli which promote endothelial-derived NO production. eNOS has been localized to plasmalemmal caveolae, and associates with a protein known as caveolin-1. Molecular chaperones may play a role in subcellular targeting during activation. Caveolin-1 attenuates enzyme activity in the resting endothelial cell. Upon stimulation, calcium levels increase allowing calmodulin to bind eNOS, displacing caveolin-1 and activating eNOS. (44, 56). Recent studies have demonstrated that upon stimulation, the molecular chaperone Hsp 90 is recruited to eNOS (57).

I.2.1.3 Murine genetic models

Genetic studies on NOSs have revealed the role of the individual enzymes with more specificity than the actions of pharmacologic blocking agents, especially since mutating and inactivating each isoform's gene does not produce lethality. Targeted inactivation of each NOS has confirmed the diverse roles of the varied isoforms. Studies with eNOS (-/-) knockout mice have substantiated the viewpoint that endothelial-derived NO plays a role in maintenance of vascular tone, endothelial-mediated vasodilatation and modulation of proliferation of VSMC. eNOS (-/-) knockout mice are viable and fertile in the absence of detectable eNOS mRNA and protein. They exhibit a hypertensive phenotype with an increased susceptibility to organ damage upon focal ischemia (58-60). These studies also indicate a role for NO in maintenance of blood pressure. eNOS (-/-) mice are deficient in EDRF, as the aorta loses its response to Ach. There does not appear to be any vasoconstriction. The response of VSMC to sodium nitroprusside is still maintained (58).

Studies using a rabbit model of hindlimb ischemia demonstrated eNOS as an important modulator of angiogenesis. In this model of limb ischemia, L-arg augmented endogenous production of NO and stimulated angiogenesis in the ischemic appendage, as measured by limb perfusion, capillary density and vasomotor reactivity (61). eNOS (-/-) mice exhibited impaired angiogenesis in the setting of ischemia (61). Administration of vascular endothelial growth factor (VEGF) did not improve the impairment in eNOS (-/-) mice, suggesting that eNOS acts downstream of VEGF (61). This was surprising given that VEGF is such a potent modifier of new blood vessel formation in wild-type ischemic animals.

In summary, endothelial-derived NO maintains blood pressure and acts as a vasorelaxant. The effects of NO on proliferation serve to maintain and remodel constituents of the vascular wall

in response to changes in blood flow and influence angiogenesis. Complicating the interpretation of eNOS's contribution during development is nNOS in eNOS (-/-) animals. nNOS may compensate during periods of deficiency of endothelial-derived NO. These studies provide genetic evidence that eNOS is responsible for EDRF. Studies have shown that nNOS may also contribute to vascular tone. This idea was confirmed by a surprising finding in pial arterioles. These vessels maintain a response to Ach in eNOS (-/-) mice. Experiments delving into the cause of this phenomenon suggest that nNOS, as it is constitutively expressed in eNOS (-/-) mice, compensates for the loss of endothelial-derived NO (62). eNOS (-/-) mice also exhibit other characteristics, including disrupted vascular remodeling (63). Loss of endothelial-derived NO leads to an increase in vessel wall thickness in response to external carotid artery ligation, reflected by smooth muscle cell proliferation (63). This study, among others, has underscored the important role of NO as a suppressor of smooth muscle cell proliferation.

Considering its short half-life, NO has the capacity to exhibit fine-tuned control over developmental processes and structural organization of the vascular wall, through its effects on proliferation. As with eNOS (-/-) animals, nNOS (-/-) animals are viable, fertile and grossly normal with respect to neuroanatomy. Many cell types are now known to express nNOS: neurons of both the central and peripheral nervous system, skeletal muscle, macula densa, neuroendocrine tissues and gonadal tissues (64-70). nNOS (-/-) mice evidence pyloric stenosis. Enlarged stomachs are likely secondary to abnormal expression and action of nNOS in the non-adrenergic non-cholinergic peripheral nervous system (71). Interestingly, the candidate's laboratory has provided genetic epidemiologic evidence that nNOS is also a molecular determinant in humans with inherited pyloric stenosis (72). The loss of nNOS is also known to be relevant to skeletal muscle function in nNOS (-/-) mice, especially in models of muscular dystrophy (73-75). nNOS (-/-) mice studies have focussed upon the central nervous system for the most part. The brain is particularly susceptible to damage by excess production of NO, contributing to nitrosative and oxidative stress (76, 77). NO interacts with superoxide anion ($O_2^{\cdot}$) to form peroxynitrite ($ONOO^{\cdot}$),

a very reactive compound that is responsible for the damage during ischemia-reperfusion. nNOS (-/-) mice suffer less ischemic damage in models of ischemia-reperfusion injury, sustaining smaller cerebral infarcts following middle cerebral artery occlusion (78-81). These studies show that deficient NO production by nNOS leads to developmental problems, in the intestine and dystrophy of skeletal muscle. In contrast, excess NO exacerbates neuronal injury.

Within vascular tissues, iNOS can contribute to the formation of NO; however, its major contribution to NO production occurs in the immune system. Excessive production of NO leads to severe hypotension and tissue damage in the setting of overwhelming sepsis. iNOS (-/-) mice have peritoneal macrophages which do not express iNOS. In response to lipopolysaccharide (LPS), iNOS (-/-) mice have an attenuated hypotensive response, but little difference in mortality compared to wild type mice (82). Mutant animals are, however, more sensitive to *Listeria monocytogenes* (83) and *Leishmania major* (84). iNOS (-/-) mice have increased susceptibility to intracellular parasites, viruses and tumours. These observations substantiate the widely held belief that NO is important in cell-mediated immunity while providing genetic evidence for this isoform's role in sepsis. In transgenic mice overexpressing iNOS in pancreatic beta cells, mice develop insulin-dependent diabetes (85). Excess production of NO may result in free radical-like injury to pancreatic cells.

I.2.1.4 Structure of the gene

Human eNOS shares 94% amino acid sequence identity with bovine eNOS (37) and 93% identity with murine eNOS (86) but only 60% and 50% identity with human nNOS and iNOS, respectively. The human eNOS gene spans 21 kB of genomic DNA, contains 26 exons and encodes a mRNA of 4052 nt. It exists as a single copy in the haploid genome. Molecular characterization revealed that the mRNA has a short 5'-untranslated region (UTR) of 22 nt in length, the open reading frame (ORF) has 3609 nt and the known 3'-UTR is 419 nt in length. The

promoter is "TATA-less" and contains putative cis-elements for Sp1, GATA, AP-1 (activator protein-1), AP-2 and NF-1 (nuclear factor-1). The promoter also bears putative heavy-metal, acute-phase, shear stress and sterol-regulatory elements (31, 87). The core promoter regulates baseline eNOS mRNA transcription and contains two positive regulatory domains: positive regulatory domain I (PRD I) (-104/-95) relative to transcription initiation) and PRD II (-144/-115). These two clustered cis-regulatory regions were characterized in the proximal promoter using deletion analysis and linker-scanning mutagenesis in the candidate's laboratory (88). Functional expression and analysis of trans-factor binding studies revealed that multiprotein complexes form upon these regions in the nucleus of endothelial cells. The complexes consist of Ets family members, Sp1 (88, 89), variants of Sp3, MAZ, and YY1, as assessed by transfection and electrophoretic mobility shift assays (88). A high-affinity Sp1 site is found within PRD I, while a low affinity site for Sp1 in PRD II allows variants of Sp3 and MAZ to interact. In addition, Elf-1, Ets-1 and YY1 interact with PRD II (88). These activator regions act as an enhanceosome to confer basal eNOS transcription in vascular endothelium.

I.2.1.5 Regulation of eNOS mRNA expression, transcription and mRNA stability

Considering the detrimental effects of deficient and excess production of NO in blood vessels, understanding the regulated production in vascular tissues will provide insight into disease and potentially offer novel therapeutic uses. Though NO itself has a very short half-life of approximately 6 seconds (23), the same is not true for eNOS mRNA and protein. Longer-term regulation of endothelial-derived NO occurs at the level of the enzyme's expression, especially when considering the half-life of eNOS mRNA under baseline conditions is 24 - 48 hours (90). Expression of eNOS mRNA and protein is altered in endothelial activation. Moreover, detailed in situ cRNA hybridization and immunohistochemistry studies in diseased human tissues revealed important changes in the expression of eNOS mRNA and protein in atherosclerotic blood vessels

(91) and pulmonary hypertension (92). Studies in cultured vascular endothelium have provided important new insight into the molecular basis and, though they are merely models for the in vivo setting, suggested mechanisms that may be operative in disease. TGF-β1 (93), estrogen (94), shear stress (28, 95, 96), and lysophosphatidylcholine (97) enhance transcription of eNOS. Tumour necrosis factor-alpha (TNF-α) (31, 90), hypoxia (98), proliferation-injury (99) and oxidized LDL (100) decrease eNOS mRNA by decreasing mRNA stability. Hypoxia downregulates eNOS expression in pulmonary artery endothelial cells and in HUVEC. This change has been attributed, in major part, to decreased mRNA stability (101, 102). Proliferation and injury lowered eNOS steady-state mRNA expression by decreasing mRNA stability. TNF-α decreased eNOS mRNA and protein in endothelial cells, due to a decrease in mRNA stability. TNF-α altered eNOS mRNA half-life from 24 - 48 h to 3 - 4 h (90). mRNA stability is a major contributor to steady-state eNOS mRNA levels. Therefore, transcriptional and post-transcriptional regulation contribute to changes in levels of steady-state eNOS mRNA in response to exogenous cellular stimuli.

I.2.2 Endothelins

In 1988, a startling discovery was reported (3). The conditioned medium of cultured endothelial cells contained a potent vasoconstrictor peptide termed endothelin-1 (ET-1). ET-1 is the most potent vasoconstrictor known in humans. It is now known to have protean cellular effects. Indeed, this short peptide of 21 amino acids can also produce vasorelaxation under specific circumstances. Endothelin-converting enzymes (ECE) are expressed in endothelial cells. They play a key role in processing and maturation of endothelin.

Three isoforms of endothelin (ET) are now known to exist: all are 21 amino acids (aa) in length with four cysteine residues, which form two intramolecular disulfide bonds. ET-1 is the

isoform produced by vascular endothelium. All three isoforms have an amino acid sequence similar to the sarafotoxin S6b of Attractaspis engaddensis. ET-1, ET-2 and ET-3 have been localized to human chromosomes 6p23-24, 1p34 and 20q13.2-13.3, respectively (103, 104). These three peptides most likely originate from a common ancestral gene. There are no differences in the aa composition of ET-1, human, rat, dog, rabbit, porcine and bovine species (105). Processing of all three endothelins to mature peptides occurs in a similar fashion. Human ET-1 is translated as a 212-aa precursor, preproendothelin-1, (porcine as a 203-aa precursor). A dibasic-specific endopeptidase of the subtilisin family, most likely furin, cleaves preproendothelin-1 at Lys_{53}-Arg_{54} and at Lys_{90}-Arg_{91} (106). Endothelin-converting enzyme-1 (ECE-1) cleaves between a Trp_{21} and Val_{22} residue rendering the biologically active 21-aa peptide, ET-1 (3, 107, 108).

Endothelins have a broad tissue distribution. In vitro and in vivo observations indicate that ET-1 differs from ET-2 and ET-3, as this isoform is the only endothelin expressed in the vascular endothelium (3, 107, 108). Vascular smooth muscle cells, neurons, epithelial and mesangial cells in the kidney, cardiac myocytes in the heart, and resident cell types of the eye, adrenal gland, pancreas, spleen, lung and intestine all express ET-1 mRNA and peptide (109, 110). Endothelin receptors demonstrate a similar distribution pattern as endothelin expression in tissues, suggesting that endothelin acts as a local paracrine mediator. Endothelin receptor type A (ET_A) has a high affinity for ET-1 and ET-2 compared to ET-3, whereas ET_B receptors exhibit equal affinity for all three endothelins (111-115). ET_B receptors are important physiologically in clearance. Upon binding, these 7-transmembrane domain receptors interact with GTP binding proteins to modulate activity of phospholipase C, D and A2, adenylyl cyclase, varied K^+ channels, the Na^+/ H^+ exchange ion channels, as well as tyrosine kinases and mitogen-activated protein kinases (111, 116). ET_A receptors are thought to mediate vasoconstriction, bronchoconstriction and aldosterone secretion, whereas vasodilation and platelet aggregation are ascribed to the action of the ET_B receptors (105). For the most part, VSMC express the ET_A receptor and endothelial cells express the ET_B receptor.

ET-1 induces enduring vasoconstriction in vitro and in vivo (103, 104, 117). However, animals initially respond to injected ET-1 with a fall in blood pressure (118), which is thought to be mediated by ETB receptors and the effects of NO, prostaglandins (PGI_2 and PGE_2) and atrial natriuretic peptide (119-121). ET-1 also acts as a mitogen and can suppress apoptosis in vivo (122). In atherosclerotic patients, endothelial injury leads to VSMC proliferation. Patients with advanced atherosclerosis have enhanced levels of circulating and tissue ET-1 (123). Increased levels of endothelin-1 post-myocardial infarction are associated with 1-year mortality rates. Coronary artery ligation-induced heart failure (124) and rat balloon injury result in marked upregulation of ET-1 levels (125). Thus, ET-1 is important in the cardiovascular response to injury, and ET-1 blockers are of great interest in these disorders.

ET-1 plays a role in development, and influences the ontogeny of neural crest-derived cells. Homozygous mutant ET-1 (-/-) mice have a lethal phenotype in which both cardiovascular malformations, including interruption of the aortic arch and ventricular septal defects, and abnormalities in craniofacial tissues are observed (126, 127). The animals die at birth. The phenotype of ETA receptor (-/-) mice have identical malformations in cardiovasculature and in craniofacial structure (128), providing genetic evidence indicative of paracrine-mediated signalling of ET-1 through ETA in development. It is important to note that ET-3 (-/-) and ETB (-/-) mice exhibit a shared phenotype characterized by the absence of epidermal melanocytes and enteric neurons (129, 130) indicating their role in neural crest-derived tissues. These mice develop colonic obstruction syndromes reminiscent of Hirschsprung's disease in humans. Families with Hirschsprung's disease, exhibit mutations either in the ET-3 ligand or the ETB receptor (131, 132). This is a fascinating example of the relevance of endothelins to human disease.

I.2.2.2 Regulation of endothelin-1 expression

Steady-state preproendothelin-1 (ppET-1) mRNA expression determines ET-1 peptide levels: both secreted and cell-associated levels. The mRNA is extremely labile with a half-life of approximately 15 - 30 minutes (110, 133). Transcriptional and post-transcriptional mechanisms regulate ppET-1 expression, and further regulation by endothelin-converting enzyme (ECE-1), which processes ppET-1 to its mature form, may occur. The ppET-1 promoter is well-characterized and contains NF-1, GATA-2 and AP-1 cis elements. Proliferation, hypoxia and TNF-α upregulate ppET-1 mRNA expression by enhancing transcriptional activity, while IFN-γ potentiates the effects of TNF-α on ppET-1 expression (99, 110, 133, 134). ET-1 is upregulated by factors associated with atherosclerosis including TGF-β (135), IL-1β (136), TNF-α (110) and oxLDL (137). TGF- β modulates function via NF-1 binding sites (135). Thrombin enhances polyphosphoinositide hydrolysis by activating phospholipase C, and may also enhance transcription of ppET-1. Phorbol esters and protein kinase C can also upregulate ppET-1, by interacting with TPA-responsive elements, also known as AP-1 cis elements. ppET-1 transcription is regulated by AP-1, a transcription factor regulated by many signalling paradigms, especially redox-based mechanisms and is upregulated by cytokines (138). Recently, verotoxins have been shown to modulate ET-1 expression (139). Verotoxins increase ET-1 expression via increasing mRNA stability of ppET-1 without affecting transcription (139). Verotoxins are bacterial-derived exotoxins implicated in E. coli O157: H7-associated hemolytic uremic syndrome.

I.2.2.3 Endothelin-converting enzymes

Endothelin-converting enzyme is the rate-limiting enzyme for conversion of big ET-1 to a biologically active species. ECE-1 expression closely resembles the expression of ET-1's and ET_A (the receptor mentioned) during development. ECE-1 (-/-) mice exhibit all the characteristics of the above phenotypes, both the craniofacial malformations and cardiac abnormalities, characteristic of

ET_A (-/-) and ET-1 (-/-) mice, and the absence of enteric neurons and epidermal melanocytes, seen in ET-3 (-/-) and ET_B (-/-) mice (129, 130). These studies provide genetic evidence that ECE-1 is physiologically relevant for both big ET-1 and big ET-3 in vivo. ECE-1 cleaves big ET-1 more efficiently than big ET-2 and big ET-3 (140, 141). The activity of ECE-1 is optimal at neutral pH, inhibited by metal chelators, sensitive to phosphoramidon (142-144) and membrane-associated (143). The human ECE-1 gene spans 80 kB of genomic DNA, representing some 18 or 19 exons (145). ECE-1 has been localized to 1p35-36 in the human chromosome (146). Two major poly(A) cleavage and addition signals (AATAAA) exist in bovine species (147). Recent evidence suggests that 5 independent promoters regulate expression of the human gene. Each of these promoters is active in endothelial cells (148). ECE-1 is homologous with neutral endopeptidase 24.11 (NEP) , a major neuropeptide-degrading ectoenzyme in the brain and other tissues, and the Kell blood group protein (149). These proteins exhibit 40% and 20% identity, respectively. ECE-1 is a type II membrane protein of 120-130 kDa, found in intracellular membrane and ectomembrane fractions in many cell types (147). It contains one transmembrane-spanning domain near its NH_2-terminus and a consensus sequence (HEXXH) for zinc binding at the COOH-terminus. The enzyme exists as a disulphide-linked dimer, however, the dimeric structure is not essential for conversion of big ET-1 to the mature ET-1 peptide (150). ECE-1 exists as a highly glycosylated enzyme with numerous consensus sites for N-linked glycosylation and glycosylation of specific amino acid residues affects its functional activity (151). In these studies, the specific mutation of two asparagine (Asn) residues rendered the enzyme inactive.

Recent studies have shown that vascular endothelial growth factor (VEGF), an angiogenic factor and growth factor specific for endothelial cells, regulates ECE-1 by increasing its mRNA and protein expression (144). It will be interesting to see if ECE-1 plays a role in these pathological cellular responses. Physiologic shear stress downregulates ECE-1 mRNA expression in endothelial cells in an intensity-dependent manner (152). This may explain the decreased levels of ET-1 peptide during vasodilation induced by shear stress, possibly through a feedback

mechanism (153-155). Vascular endothelial cells and macrophages of myocardial tissue also express elevated ECE-1 mRNA and protein levels in a disease setting such as congestive heart failure (156). Also, rats with induced congestive heart failure express higher levels of ECE-1 in the renal cortex (157). This also occurs in vascular injury models (158). Increased ECE-1 immunoreactivity was reported in VSMC and macrophages within human atherosclerotic lesions (158) and in the atheroma of hyperlipidemic rabbit aorta (159). Furthermore, ECE-1 plays a role in inflammatory processes, as demonstrated by the elevated levels of ET-1 and ECE-1 peptide in a rat lung allograft model for chronic rejection during the early post-transplantation period (160).

I.3 OXIDATIVE STRESS AND ENDOTHELIAL DYSFUNCTION

Disturbances in production of vasomediators represent a major feature of the dysfunctional endothelium. Efforts to determine the underlying causes of impaired endothelial function indicate a prominent role for oxidative stress. Oxidative stress is defined as a disturbance in the prooxidant-antioxidant balance in favour of the former (161). It is also defined as a dynamic condition influenced by external factors ... [in which] oxidative damage [is] a resultant of oxidative stress and in many cases, the damaged biomolecules then further contribute to oxidative stress (162). The term oxidative stress also refers to a state in which a cell cannot maintain redox cellular homeostasis. Changes in intracellular redox state may characterize a common feature of the altered phenotype observed in dysfunctional endothelium. It is believed that the broad range of insults that initiate vascular disease may share a common facet. Imbalances in the endogenous oxidant-antioxidant balance, within endothelial cells, may account for the range of insults that initiate vascular damage associated with atherosclerosis, hypertension and diabetes, among others (21).

Taken together as a whole, evidence indicates that oxidation-reduction chemistry plays a role in endothelial dysfunction. This section highlights key observations during endothelial cell injury that substantiate the viewpoint that oxidative stress is relevant to endothelial injury. For instance, physiologic studies demonstrated that, in the presence of endothelium, increased free radical formation occurs in hypercholesterolemic rabbits. However, if the endothelium was removed, oxidant production did not increase. These results imply that the vessel wall releases free radicals during endothelial injury (163). Endothelial cell injury resulting from a wide range of exogenous stimuli can induce increases in oxidant production or block endogenous antioxidant defense mechanisms within endothelial cells. These stimuli include cytokines, such as TNF-α, IL-1β and IFN-γ, as well as shear stress, hypoxia, interactions with phagocytes and exposure to products of the coagulation or complement cascades. This overall disturbance in oxidant-

antioxidant balance alters the endothelial phenotype (164, 165). Moreover, endothelial cell injury can enhance oxidant production not only directly, but also indirectly, by inducing neighbouring cells to release oxidants. Therefore, endothelial injury can affect oxidant production in the underlying VSMC or pericyte. Finally, the endothelial cell can modify exogenous stimuli. In doing so, endothelial cells contribute to the formation of atherosclerotic lesions. Macrophages phagocytose oxidized low-density lipoprotein (oxLDL) more readily than native LDL, as oxLDL enters through the less regulated scavenger receptors. LDL uptake is more controlled, as LDL receptors are tightly regulated through feedback inhibition by sterols. As macrophages phagocytose oxLDL, they form the foam cells characteristic of atherosclerotic lesions (166). This observation explains, in part, the cellular link between hyperlipidemia and atherosclerosis. Endothelial cells play a critical role in the oxidative modification of LDL (167). Therefore, endothelial cells are crucial to the formation of oxLDL from native LDL. Thus, endothelial cells provide oxLDL, which is a major stimulus for foam cell development. This observation, in combination with the above study of hypercholesterolemic rabbits, reinforces the conclusion that oxidizing conditions occur in response to endothelial injury. Oxidizing conditions represent a unifying theme in endothelial dysfunction. In summary, endothelial cells can produce oxidants, can induce other cells to produce oxidants and can also modify key intercellular components of oxidant-mediated injury.

I.4 REACTIVE OXYGEN INTERMEDIATES

The reactive properties of molecular oxygen, which aerobic organisms exploit, benefit energy production. However, the very same advantageous properties can harm cellular components. Oxygen's powerful capacity to accept electrons allows for highly efficient metabolism. Although oxygen, in becoming reduced, maximizes ATP production from glucose, its use presents a paradox. In accepting electrons, oxygen can form potentially toxic intermediates,

highly reactive byproducts. These deleterious byproducts, called reactive oxygen intermediates (ROI), act as oxidizing agents and contribute to oxidative stress. In aerobic metabolism, intracellular formation of ROI creates a challenge for the cell. For instance, when use of oxygen is not controlled, many levels of "leaks" lead to imbalances in the orderly flow of electrons. The major source of intracellular ROI is aerobic metabolism. However, ROI also form when oxygen is used in controlled oxidation reactions during the synthesis of essential biologic molecules, such as neurotransmitters, hormones, and eicosanoids, as well as in the detoxification of endogenous and exogenous compounds. The processes of detoxification of exogenous compounds and foreign particles can use extracellular sources of ROI (168). The immune system has evolved to take advantage of the reactive properties of oxygen. Phagocytes produce ROI as an endproduct to destroy invading organisms, localize infection and enhance immunity. Though normal byproducts of metabolism and inflammatory responses, in excess, ROI cause damage. ROI react upon contact, oxidizing and impairing biological molecules. ROI-mediated injury targets lipids, nucleic acids and protein. These deleterious chemical alterations lead to profound physiological consequences.

ROI are volatile, reactive species which include singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH). Of these four ROI, superoxide anion and hydroxyl radical are free radicals. Interestingly, NO is also a free radical. Free radicals are molecules containing one or more unpaired electrons. ROI form both inside a cell and outside a cell. Of the ROI, singlet oxygen resembles molecular oxygen in numbers of electrons; however, it differs from molecular oxygen in that it contains an empty orbital. As a result, singlet oxygen is more prepared to receive electrons than molecular oxygen, becoming reduced to superoxide anion. Enzymatic catalysis results in intracellular formation of superoxide anion, a byproduct of aerobic metabolism in mitochondria. In phagocytes and VSMC, NADPH oxidase and NADH oxidase respectively, catalyze the production of superoxide anion. The unpaired electron of superoxide anion reacts readily with other molecules, forming hydrogen peroxide. This is a slow inorganic

chemical reaction. The reaction can be efficiently catalyzed by the antioxidant enzyme, superoxide dismutase (SOD). Hydrogen peroxide readily forms hydroxyl radical in the presence of transition metals, copper (I) and iron (II), through the Fenton or Haber-Weiss reactions. The next section describes sources of ROI, their formation and the biological consequences of their formation.

I.4.1 Formation through aerobic metabolism

Mitochondria comprise the main intracellular source of ROI, where they form through the oxidative phosphorylation process during aerobic metabolism. Molecular oxygen acts as a terminal electron acceptor and the reduction of molecular oxygen leads to the potentially harmful byproducts, known as ROI. The electron transport chain transfers four electrons to O_2. O_2 accepts these electrons one at a time forming $O_2^{\cdot}$ (169), H_2O_2 (170) and finally water. This process engages five protein complexes to transfer electrons and generate ATP. Leaks or openings in the five-complex transport pathway allow for electron loss. The leaks are estimated to form $O_2^{\cdot}$ at a rate equivalent to 1 - 2% of the total rate of oxygen consumption (171, 172). SOD reduces $O_2^{\cdot}$ to H_2O_2. However, mitochondria only account for approximately 15% of H_2O_2 generated in a cell (173). Microsomes and peroxisomes account for the majority of H_2O_2 produced in the cell. Mitochondria have "leaks" of ROI, whereas one of the functions of microsomes and peroxisomes is to detoxify ROI within the organelle.

Excess formation of ROI can occur, especially under circumstances in which certain stimuli cause imbalances in the orderly electron flow in mitochondrial metabolism. The imbalances lead to an increase in "leaks" of ROI that can cause cell death. For instance, ischemia-reperfusion injury leads to elevated levels of Ca^{2+}. Perhaps because the lack of ATP disrupts membrane ionic balance and leads to increases in intracellular calcium. The increase in intracellular Ca^{2+}, in turn, stimulates production of ROI damaging tissues through oxygen- and calcium-dependent processes. In

ischemic conditions or hyperoxic conditions during reperfusion, the formation of ROI is augmented (174) and this leads to membrane leakage and necrosis. Although the dominant form of cell death is necrosis in response to ischemia-reperfusion injury, apoptosis can also take place. Glutathione, an endogenous antioxidant plays a role in this process. Accumulation of $O_2^{\cdot}$ leads to cytochrome c inactivation, mitochondrial swelling, release of cytochrome c, activation of caspases and finally, apoptosis. N-acetylcysteine (NAC), an antioxidant (175) and expression of bcl-2, an antioxidant gene attenuate the deleterious effects of $O_2^{\cdot}$ thus, preventing cytotoxicity (176, 177). Bcl-2 protein protects cytochrome c from inactivation enhancing cell survival (178). Bcl-2 does this by sequestering glutathione to the nucleus. The endogenous antioxidant glutathione modulates nuclear redox, blocking caspase activity and attenuating nuclear alterations characteristic of apoptosis (179). Overall, ischemia-reperfusion promotes increases in intracellular Ca^{2+}, stimulating $O_2^{\cdot}$ production, that in turn, causes inactivation and release of cytochrome c. Thus, excess ROI production can lead to cell death by disrupting mitochondrial function.

Cellular enzymes, other than those in the electron transport chain implicated as intracellular sources of ROI are protean: NADPH oxidase (180), NADH oxidase (181), xanthine oxidase (182), cyclooxygenase (183), NOS (184) and lipoxygenase (185), among others. Each of these pathways is characterized as allowing electrons to reach molecular oxygen through routes outside the major cellular end point, the mitochondria. A useful example can be offered. Ischemic injury leads to xanthine oxidase activation. Xanthine oxidase generates $O_2^{\cdot}$ through the univalent reduction of molecular oxygen. The rise in intracellular calcium, in ischemia, activates Ca^{2+}-dependent proteases, which in turn, attack the innocuous enzyme xanthine dehydrogenase, converting it by limited proteolysis to a protein with a different enzymatic product. Once the conversion of the dehydrogenase to oxidase occurs, the availability of molecular oxygen will result in the production of ROI (168). This pathway has been proposed to play a quintessential role in ischemia-reperfusion injury. The capacity for exogenous xanthine oxidase and hypoxanthine to mimic many aspects of ROI-induced tissue injury has lent support to this concept (168). Other

sources of one ROI, H_2O_2 include urate, acyl coA, L-gluconolactone and monoamine oxidases, which catalyze the formation of H_2O_2 by a 2-electron transfer to molecular oxygen. Monoamine oxidases catalyze the oxidative deamination of biogenic amines to aldehydes, ammonium ion and H_2O_2. The enzymes discussed above actively form ROI within the endothelial cell, with the exception of NADPH oxidase.

I.4.2 Formation through inflammatory responses

Organisms use systems to exploit cellular production of ROI in host defense as destructive agents against invading pathogens. NADPH oxidase plays a major role in polymorphonuclear neutrophils (PMN) in the one-electron reduction of molecular oxygen to $O_2^{\cdot}$. In this case, $O_2^{\cdot}$ is the product of the reaction, whereas it is a byproduct of mitochondrial metabolism. A transient rise in oxygen consumption, called the respiratory burst, accompanies the production of ROI following PMN activation. NADPH acts as an electron donor in NADPH oxidase. Flavins act as an electron carrier, and O_2, as the electron acceptor. The complex has multiple components, is membrane-associated, and is tightly regulated so that harmful oxidants are localized.

Predictably, patients with chronic granulomatous disease have genetic deficiencies in PMN NADPH oxidase. They suffer from chronic life-threatening bacterial infection. Study of the inherited disorders of the NADPH oxidases has enabled researchers to dissect out the major structural components of this enzyme, and their function. NADPH oxidase in phagocytes consists of two membrane-bound components and two cytosolic components. The membrane-bound components include $gp91^{phox}$ and $p22^{phox}$, which together make up cytochrome b_{558} (186). Cytochrome b_{558} is bound in membranes of specific granules and tetranectin-containing intracellular vesicles that fuse to the plasma membrane during cellular activation. The small cytosolic G protein, Rac, is also involved in NADPH oxidase activation (187). A flavoprotein links cyt b_{558} to NADPH. The two cytosolic components, $p47^{phox}$ and $p67^{phox}$, translocate to the

membrane and interact with cyt b_{558} following cellular activation by stimuli such as leukotriene B_4 and GM-CSF. Phosphorylation plays a role in allowing specific domains of the proteins to interact (188, 189). Regulatory proteins control the interaction of the various components, such that $O_2^{\cdot}$ production occurs following cellular activation. This respiratory burst is important for protection against infection. Receptor-dependent activation occurs in response to diverse stimuli: C5a, FMLP (N-formyl-met-leu-phe) and immune complexes that bind the FcRII receptor. Receptor-independent activation can be achieved using phorbol myristate acetate, oleic acid and arachidonic acid (190).

It is important to mention that VSMC express NADH oxidase. This membrane-bound enzyme system, in contrast to NADPH oxidase, seems to contain only $p22^{phox}$. NADH oxidase contributes to oxidative stress in the arterial wall and in cardiac myocytes through the production of $O_2^{\cdot}$ and H_2O_2. Angiotensin II and vascular injury both stimulate ROI production in VSMC (181).

I.4.3 Markers of oxidative stress

Lipid peroxidation: ROI readily react with lipids of cell and organelle membranes. The products of these interactions can be used as a marker of oxidative stress. Free radicals abstract hydrogen molecules from double bonds of polyunsaturated fatty acids, forming alkyl radicals (191). These react with oxygen generating hydroperoxides which form lipid peroxyl, alkyl radical and alkenal, or endoperoxides (192). These reactive compounds form conjugated dienes, lipid peroxyl, malondialdeyde and other radicals that propagate chain reactions of lipid peroxidation.

The chemical changes caused by lipid peroxidation are associated with important cellular effects: decreased membrane fluidity, disrupted bilayer presentation of membrane-bound proteins, generation of cyclic endoperoxides and aldehydes capable of acting as mutagens. Lipid

peroxidation products can activate or inactivate enzymes, cross-link proteins and nucleic acid. Overall, cellular and plasma membranes lose integrity of lipid bilayers (193). A specific product of lipid peroxidation, 4-hydroxynonenal (HNE) prevents calcium release from rat liver mitochondria by covalently attaching to thiolate anions (194). The retention of calcium precedes a loss of mitochondrial function, as discussed earlier. Thus, lipid peroxidation products can lead to cell death, in part, through damaging effects on mitochondria. Destruction of lipid bilayers and structural proteins lead to an influx of calcium and sodium, such that there is a loss of normal ionic gradients, homeostasis leading to cell death. Lipofuscin and thiobarbiturc acid reactive substances (TBARS) assays conveniently measure pools of molecules damaged by lipid peroxidation and therefore useful assay tools for assessing damage by oxidative stress.

Nucleic acid damage: Chemical changes and damage to DNA often indicate oxidative stress. Modification of bases and breakage of DNA represent chemical and physical alterations in DNA, respectively. 8-hydroxydeoxyguanosine (8-hDG), a base modification, is commonly used as a marker of oxidative stress. For instance, excreted urinary 8-hDG is used as a marker of oxidative stress in humans (195). This base modification serves as a molecular "fingerprint" of •OH action (196). Common base modifications occur from •OH addition to the pi bonds of the bases (197, 198), which result in dihydrothymine (199), thymine glycol, 5-hydroxycytosine, 5-hydroxyuracil from hydrolytic deamination of cytosine, 8-hydroxydeoxyguanosine, 8-oxoguanine (200), 8-oxoadenine (201) and formamidopyrimidine. NO, also a free radical, deaminates adenosine to hypoxanthine (inosine). •OH can modify purine and pyrimidine bases. •OH can also react with sugar moieties of the phosphodiester bond causing DNA breakage (202). Single strand breakage leads to the activation of enzymatic repair, which can further exacerbate cells and lead to dysfunction. Single-strand breaks occur when hydroxyl radical attacks the -OH groups of the sugar portion of DNA, resulting in scission and cleavage of the backbone and breakage (202, 203).

Two major consequences of DNA damage are mutations and oxidant-mediated cell death due to NAD and ATP depletion. The consequences of modification of bases or cleavage of the phosphodiester backbone, depends on the efficiency of DNA repair and the interaction of unrepaired lesions with the DNA replication apparatus (204): lethality and mutagenicity are the results of inefficient "base excision repair" or overwhelming nucleic acid damage. Repair enzymes must recognize and repair DNA modifications prior to metaphase to avoid permanent alteration of the genetic code of daughter cells (168). Some modifications, on the other hand, lead to the loss of a base and are lethal. This is because abasic sites block DNA polymerases. Thymine glycol and formamidopyrimidine are abasic sites that cause DNA polymerases to be blocked. Other modifications such as dihydrothymine, 8-oxoadenine and 8-oxoguanine do not block polymerases and are not lethal. Of the latter non-lethal modifications, 8-oxoguanine is known to be mutagenic. "Base excision repair" processes, which recognize oxidative DNA lesions (205), comprise two groups of enzymes: DNA N-glycosylases and apurinic endonucleases. DNA N-glycosylases recognize damaged purines and pyrimidines and cleave both sides of the nucleotide. Thus, exonucleases can release the base to prepare the site for polymerase repair. Apurinic endonucleases recognize sites of base loss of purines, remove the remaining unpaired base to leave a single-base gap and therefore, clear up single strand breaks. Thus, polymerization can proceed (Warner 1990). Both of the enzymes create single-base gaps so that DNA polymerase II can repair damage and ligases proceed to seal nicks. Single-strand breaks activate another polymerase during oxidative injury: poly ADP-ribose polymerase (PARP) (206). This repair enzyme's unchecked activity can lead to cellular dysfunction, as it consumes NAD. In depleting NAD, continuous PARP activation inhibits glycolysis and thus, depletes ATP (206). Consistent with the key role for this enzyme, inhibition of PARP prevents oxidant-mediated cell death in endothelial cells (207, 208).

Protein damage: Oxidative stress can inactivate and destroy proteins via thiol oxidation, disulfide formation and protein carbonylation. By altering the redox state of critical thiols,

oxidizing agents can inactivate enzymes, damage structural proteins and modulate protein-protein or protein-nucleic acid interactions. An important indicator of oxidative stress is a decrease in glucose-6-phosphate dehydrogenase (G6PD) activity. Hydroxynenal (HNE), a lipid peroxidation product is implicated in the inactivation of this key cellular enzyme. An intriguing theme is the correlation between ROI-induced protein damage and the process of aging. Specifically, decreased G6PD activity is associated with age. Carbonylation of proteins is also observed in aging. In addition, oxidation of thiol groups occurs in the aging brain (209). This results in in activation of G6PD (209). Carbonylated proteins accumulate with age (210) and fibroblasts from patients with premature aging conditions, such as progeria or Wernerís syndrome, exhibit a higher degree of oxidation of proteins (211). One type of carbonylation, advanced glycosylation will also be treated as an individual section because of its relevance to oxidative stress in the vascular wall. Besides oxidation, nitration of proteins can also occur. Due to its special relevance to endothelial cells, peroxynitrite and nitration are discussed in a separate section.

I.4.4 Peroxynitrite

The reaction between $O_2^{\cdot}$ and NO produces peroxynitrite (ONOO-) with a rate constant of $6.7 \times 10^9\ M^{-1}s^{-1}$, effectively equivalent to a diffusion-limited reaction. (212). Its formation proceeds at a faster rate than the dismutation of $O_2^{\cdot}$ by SOD. $ONOO^-$ has a half-life of approximately 1.9 seconds at pH 7.4 (213). It readily reacts with protons to form ONOOH at physiological pH (214). ONOOH, in turn, spontaneously generates (•OH and NO_2). Especially prominent in $ONOO^-$-mediated injury is nitration at critical tyrosine residues.

In macrophages, the reactive properties of $ONOO^-$ are exploited for bacterial killing (215, 216). However, excessive production of $ONOO^-$ also damages host tissues. For example, peroxynitrite participates in membrane lipid peroxidation in oxidative stress-induced apoptosis. Excessive nitration of tissues in atherosclerosis has been observed with a monoclonal antibody to

nitrotyrosine residues (217). At high levels, $ONOO^-$ damages cellular components, inhibits glycolysis and disturbs metabolism.

High levels of $ONOO^-$ lead to the inactivation of endogenous defense mechanisms. For instance, $ONOO^-$ targets manganese SOD (MnSOD), by inducing three (Y34, Y45 and Y193) of nine tyrosine sites to become nitrated. Peroxynitrite plays a role in neuronal apoptosis through inactivation of MnSOD. Overexpressing MnSOD, in contrast, prevents apoptosis induced by NO-generating agents and attenuates accumulation of peroxynitrite, nitrated proteins, and membrane lipid peroxidation (218).

Although the principal source of $ONOO^-$ is the macrophage, the endothelial cell can also produce $ONOO^-$ at physiologically relevant levels (219, 220). The formation of $ONOO^-$ depletes biological pools of NO (44). This molecule is as reactive as •OH and may participate in endothelial injury. As mentioned above, $ONOO^-$, is implicated in the damage observed in ischemia-reperfusion. O_2^- or $ONOO^-$ are thought to inhibit NO-mediated vasorelaxation (221, 222). We have been discussing the effects of $ONOO^-$ at high levels. Surprisingly, peroxynitrite, at low levels, can actually prolong the vascular relaxation response to NO (223). Nitration of thiols may play a part in peroxynitrite's ability to act as a signal in prolonging vascular relaxation responses (224). In opening potassium channels, $ONOO^-$ may also have a direct effect on VSMC (225). At low levels, $ONOO^-$ acts as a signalling molecule. It is thought that NO_2, a compound generated at neutral pH, may provide time-dependent release of NO, thus activating soluble guanylate cyclase and elevating cGMP levels (226). However, high levels of $ONOO^-$ cause excessive nitrosation and oxidation leading to damage. Nitrosative and oxidative stress play a role in disease, such as causing the formation of nitrotyrosine residues in atherosclerosis.

I.4.5 Advanced glycation end-products generate free radical damage

As with atherosclerosis, oxidative stress has been implicated in the pathophysiology of intriguing aspects of diabetic vascular disease. Advanced glycation endproducts (AGEs) represent a fundamental advance in our understanding of the pathophysiology of hyperglycemia (227). High glucose leads to the formation of advanced glycation endproducts (AGEs) in the presence of molecular oxygen through non-enzymatic processes (228). Glycoxidation and lipid peroxidation promote the formation of AGEs (229). These molecules themselves, AGEs, enhance oxidative stress as a consequence (230). AGEs can activate cells and can damage tissues by slow release of oxidant molecules. AGEs are an oxidative biomarker found at higher levels in diabetes. Intriguingly, they also accumulate with age. This has been observed in rats, dogs, pigs, monkeys and humans (231). AGEs generate oxidative stress through release of oxidant molecules; and through their interactions with specific receptors. AGEs generate H_2O_2 and •OH by auto-oxidation (232). Thus, AGEs generate ROI directly. They also interact with specific receptors producing oxidative stress. AGEs generate ROI in the presence of iron and the LF-L (lactoferrin-like polypeptide) receptor (233) or receptor for AGEs (RAGE) (234) causing oxidative stress (233). In generating ROI directly and upon interaction with receptors, such as RAGE (233), AGEs cause a decrease in intracellular glutathione and in ascorbic acid levels in bovine aortic endothelial cells (235). The decrease in endogenous cellular antioxidants leads to oxidative stress, as assessed by TBARS and malondialdehyde (233).

High glucose levels perturb endothelial function and increase the risk of vascular complications. Disturbed endothelial barrier function and increased procoagulant and prothrombotic activity are consequences of endothelial dysfunction in hyperglycemia. AGEs diminish endothelial adherence, enhance the trapping of molecules and activate the expression of redox-sensitive genes. The process begins with endothelial cells having difficulties adhering to a

glycated matrix (236). This leads to the loss of some endothelial cells and dysfunction of the remaining cells. Endothelial cells interact with AGEs and this interaction results in increased vascular permeability (237). Also, AGE modification reduces the degradation of extracellular matrix by endothelial-derived proteases and is associated with AGE-induced protein crosslinking (238). Molecules such as immunoglobulins and LDLs tend to accumulate. When cholesterol accumulates, these deposits can serve as an initiating nidus for the atherosclerotic process and thus, increase the possibility for LDLs to be oxidized (239). The resulting reduction in endothelial barrier function allows cells and particles to enter the subendothelium.

AGEs and oxidative stress can lead to altered gene expression in endothelial cells. Especially well studied are cellular adhesion molecules, such as those that are responsible for endothelial-leukocyte interaction (240). AGEs interact with endothelial cells to enhance expression of vascular cellular adhesion molecule (VCAM-1) by a redox-sensitive pathway. Endothelial expression of VCAM-1 attracts lymphocytes and monocyte/macrophages (234). These immune cells release IL-1β and TNF-α in response to AGE exposure. IL-1β and TNF-α increase procoagulant activity, another feature of endothelial dysfunction. AGEs activate endothelium to express VCAM-1 and intercellular cellular adhesion molecule (ICAM-1) (241), receptor for AGEs (RAGE) (242) and heme-oxygenase-1 (HO-1) (233). AGEs also enhance p21/ras-mediated mitogen-activated protein kinase (MAPK) activity. Glutathione depletion augments this effect (243, 244). Both of the above events may contribute to the induction of nuclear factor kappaB (NF-κB), the redox-sensitive transcription factor responsible for enhanced expression of the above genes in endothelial cells. The activation of signalling cascades mediate alterations in gene expression that result from AGEs interacting with their specific receptors. All of the above genes are also induced by oxidative stress (245-253).

Endothelial dysfunction is associated with imbalances in vasomediator production, as discussed earlier. Endothelial dysfunction is associated with prolonged exposure to high glucose,

prompting the question as to whether NO and ET-1 function is disturbed. Reports suggest a decrease in EDRF bioactivity in diabetic patients (254). Initial studies investigated the effects of AGEs on NO. The first study on AGEs and NO showed that accelerated formation of AGEs resulted in impaired endothelium-dependent vasodilation and in vitro studies suggested that AGEs quench NO (255). AGEs do form through radical intermediates that react with NO, and once formed generate ROI that could quench the gaseous molecule. AGEs form on the collagen of connective tissue, which separates endothelial cells from the underlying VSMC. Endothelium-dependent relaxation is markedly affected at two months of AGE exposure in mice (255). It is important to note that NO circulates in blood attached via S-nitrosothiols to albumin and the protein component of hemoglobin. Later studies showed that AGE-modified proteins lose the capacity to carry NO. For example, AGE-modified albumin has a decreased carrying capacity for NO (256). The conclusions of these in vitro and in vivo studies, indicated that AGEs modulate vascular tone via NO.

AGEs induce the expression of TNF-α, enhance proliferation (257) and promote the formation of oxLDL (258). All three of these stimuli decrease eNOS mRNA expression. Having stated this, a relationship between AGEs and eNOS mRNA and protein expression has not been explored, until recently. AGE-modified albumin suppress eNOS expression after 10 days of exposure in retinal vascular endothelial cells (259). At the same time, AGEs increase IL-1β and TGF-β production and release, two substances known to enhance ppET-1 expression. AGEs have been found to enhance transcription of ppET-1 (235). These changes in vasomediator expression may account for some of the problems with vasodilation associated with diabetes.

I.5 ANTIOXIDANT DEFENSE MECHANISMS

Numerous antioxidant defense mechanisms have evolved to deal with oxidative stress. Catalytic enzymes and antioxidant molecules detoxify and eliminate ROI. One aspect of this stress

is induction of a genetic program to limit further stress. For instance, ROI induce the expression of heat shock proteins, one of which is Hsp32. Hsp32 is also known as heme oxygenase-1 (HO-1).

Endogenous antioxidants represent molecules that have evolved to defend against ROI, whereas extracellular antioxidants act in conjunction to localize and minimize oxidative damage. The catalytic enzymes, SOD, glutathione peroxidase (GPx) and catalase act in concert to detoxify and eliminate reactive oxygen intermediates. SOD dismutes $O_2^{\cdot}$ to H_2O_2. GPx and catalase eliminate H_2O_2. The removal of superoxide anion and hydrogen peroxide prevents formation of hydroxyl radical and oxidative injury. Lipophilic antioxidants and hydrophilic antioxidants scavenge ROI directly to detoxify and eliminate them.

I.5.1 Catalytic Enzymes

Varied SOD isoforms function extracellularly, within the cell, and in the mitochondria to reduce oxidative stress cause by $O_2^{\cdot}$. The extracellular and intracellular enzymes are copper/zinc forms (Cu/ZnSOD), whereas the mitochondrial enzyme is a manganese-dependent enzyme (MnSOD). The Cu/ZnSODs have different evolutionary origins from the dimeric MnSOD and comprise two distinct phylogenetic families (260). Extracellular SOD is a tetramer and is glycosylated. It binds acidic extracellular matrix glycosamino-glycans and minimizes cellular exposure to $O_2^{\cdot}$ (261). This enzyme is normally expressed in extracellular fluids (262). However, it is also found in smooth muscle cells and macrophages of rabbit and human atherosclerotic lesions (263). Extracellular SOD observed in lipid-laden macrophages may play a part in the development of atherosclerosis (264)

Three separate genes code for these enzymes in humans. Only MnSOD is known to be inducible (265). TNF-α and IL-1β enhance expression of MnSOD (266). When MnSOD is

overexpressed in cells, or cells are exposed to hypoxic conditions (3% oxygen) there is a decrease in IL-1α protein and mRNA (267). Although oxidant injury initiates an inflammatory response by stimulating TNF-α release, the resulting protective response enhances MnSOD production couteracting oxidant activity.

Deficient SOD production can result in tissue damage; on the other hand, excess expression can also be deleterious. Mutations of the SOD-1 gene, encoding for intracellular Cu/ZnSOD, can result in amyotrophic lateral sclerosis (ALS). It is important to note that only 25% of familial ALS cases are inherited or caused by mutations in SOD1, whereas the other 75% remain unidentified (268). In the cases where ALS is inherited, the disease is autosomal dominant. The disease is associated with accelerated apoptosis in motor neurons. However, Cu/ZnSOD (-/-) knockout mice have no overt signs of disease. Moreover, overexpressing transgenic mice have an ALS phenotype. It has been suggested that increases in SOD reverse the dismutation reaction such that O_2^- accumulates to cytotoxic levels. Although, overexpression of Cu/ZnSOD can be harmful, transgenic mice overexpressing SOD-1 (Cu/ZnSOD) do exhibit less injury after focal cerebral ischemia with reduced brain edema and blood-brain barrier permeability (76). Chronic neurological deficits are significantly improved in these transgenic animals following traumatic brain injury (76). In transgenic studies of Drosophila overexpressing Cu/Zn SOD, increased resistance to oxidative stress was observed and there was an increase in life span (269, 270). Therefore, levels of this antioxidant enzyme, SOD, need to be regulated tightly.

Once SOD has converted O_2^- to H_2O_2, enzymes detoxify H_2O_2 preventing the generation of hydroxyl radical. These enzymes include catalase and GPx. Catalase catalyzes the divalent reduction of H_2O_2 to water. The degradation of H_2O_2 to water in tissues was described in 1818 by Thénard. In 1901, Loew presented forth the idea that an enzyme catalyzed this process of peroxide degradation (271) and cyanide was shown to inhibit its activity, suggesting the enzyme contained iron. It was purified from beef liver in 1937 (272) and has been shown to have molecular

symmetry (273). The enzyme does not require a substrate, but binds NADPH. The interactions of heme and NADPH facilitate electron transfer. Oxidative stress increases this enzyme's expression (274) and it is found at high levels in mitochondria of myocardial cells, because of the intense aerobic metabolism of these cells. Interestingly, catalase markedly stimulates soluble guanylate cyclase also (275, 276). Although activities and expression differ dependending on the type of stress, MnSOD generally is elevated, while catalase remains unchanged and Gpx is decreased by oxidative stress in atherosclerosis or diabetes (277, 278).

In addition to catalase, GPx can also detoxify H_2O_2. Three types of the enzyme exist. One form of GPx is a selenuim-containing protein that is found in the cytoplasmic and mitochondrial matrix. A second isoform is a highly glycosylated secreted protein found at high levels in plasma. A final isoform is membrane-associated that lacks selenium. Selenoprotein GPx also catalyzes the divalent reduction of hydrogen peroxide to water with a rate constant of 5×10^7 $M^{-1}s^{-1}$ and has a higher affinity for hydrogen peroxide than catalase. In liver, GPx and catalase concentrations are similar. GPx, in contrast to catalase, requires a substrate to perform its functions. The substrate GSH donates electrons in reactions to reduce H_2O_2. 2 GSH molecules donate one electron each to H_2O_2 such that the 2 molecules of GSH interact to form glutathione disulfide (GSSG). GPx promotes S-thiolation of proteins in its action. This enzyme's expression is also induced by oxidative stress (279); however O_2^- can inactivate GPx (280). Selenium deficiency is associated with ROI injury and can decrease GPx mRNA expression (281).

I.5.2 Lipid-soluble antioxidants

Lipid-soluble antioxidants play an important role in maintaining other antioxidants and in preventing lipid peroxidation of cell and organ membranes. Vitamin E, also known as α-tocopherol, is a lipid-soluble antioxidant. This antioxidant molecule scavenges oxidant molecules and maintains ascorbic acid in its reduced form (282). α-tocopherol and β-carotene, another lipid-

soluble antioxidant, inhibit singlet oxygen-dependent peroxidation (283). Lipid-soluble antioxidants, α-tocopherol and β-carotene, are significantly decreased in juvenile rheumatoid arthritis, whereas while lipid peroxidation products are increased in these patients (284, 285).

I.5.3 Water-soluble antioxidants

Extracellular water-soluble antioxidants include albumin, metal chelators, uric acid and ascorbic acid. Transition metals, Cu^{+} and Fe^{2+}, play key roles through the Fenton and Haber-Weiss reactions in forming •OH. Albumin present in serum, scavenges hypochlorous acid and binds free copper, making this protein an important extracellular antioxidant (286). In binding copper, this antioxidant can localize •OH formation. Ceruloplasmin, transferrin and lactoferrin also prevent formation of the hydroxyl radical. Ceruloplasmin converts Fe^{2+} to Fe^{3+}, a less reactive form of the transition metal. Transferrin and lactoferrin bind Fe^{3+}, and remove it (287). Iron plays a major role in lipid peroxidation and thus, proteins capable of removing free iron are extremely important in limiting cellular damage. In addition, uric acid also inhibits lipid peroxidation by scavenging free radicals (282). Uric acid, or urate, was traditionally considered a metabolically inert end product of purine metabolism in humans. Current concepts suggest that uric acid is a selective antioxidant, capable of reaction with •OH and ONOO- as mentioned earlier. Vitamin C (ascorbic acid) scavenges oxidants (282). GSH, an endogenous antioxidant, maintains ascorbic acid in its reduced form.

Having discussed the extracellular lipid-soluble and water-soluble antioxidants, a major intracellular antioxidant is thioredoxin. This molecule acts in conjunction with thioredoxin reductase, a protein oxidoreductase and is overexpressed in some tumours (288, 289). Thioredoxin (TRX) / thioredoxin reductase function in a wide range of organisms, from prokaryotes to eukaryotes. TRX reductase catalyzes the transfer of electrons from NADPH to TRX, which then reduces protein thiols. Human thioredoxin consists of 104 amino acids with a

conserved region Trp_{31}-Cys_{32}-Gly_{33}-Pro_{34}-Cys_{35} which comprises the active site shared between E. coli and human thioredoxin (290). Thioredoxin reductase functions as a dimer and contains 497 amino acids. This enzyme has a final amino acid of selenocysteine which is encoded by the mRNA stop codon (UGA), due to a SeCys incorporation sequence stem-loop element in the 3'UTR (291). This phenomenon occurs in type 1 deiodinase also (292). Depending on selenium availability, the mRNA encodes SeCys or a stop signal. The G following UGA creates a site for termination to be terminated when selenocysteinyl-t RNA is limiting (290, 293, 294). Thus, levels of TRX are controlled by selenium levels.

One of its main roles is that of maintaining nuclear redox conditions. TRX maintains transcription factors, enzymes and other proteins though its effects on critical thiol groups. For instance, TRX maintains thiols of NF-κB (295). In addition, it acts through a nuclear redox factor, Ref-1/HAP1, to regulate AP-1 (jun and fos) (296). This protein conserves protein activity of essential enzymes. TRX plays a role in ribonucleotide reductase redox reactions, critical for DNA synthesis (297). It also regulates tyrosinase activity, a key enzyme in melanin biosynthesis (297). TRX also plays a role in maintaining the glucocorticoid receptors, receptors which can induce transcription when stimulated (298). Finally, TRX prevents apoptosis by protecting redox state in the cell and maintaining calcium homeostasis (290).

I. 5 .4 Glutathione redox cycle

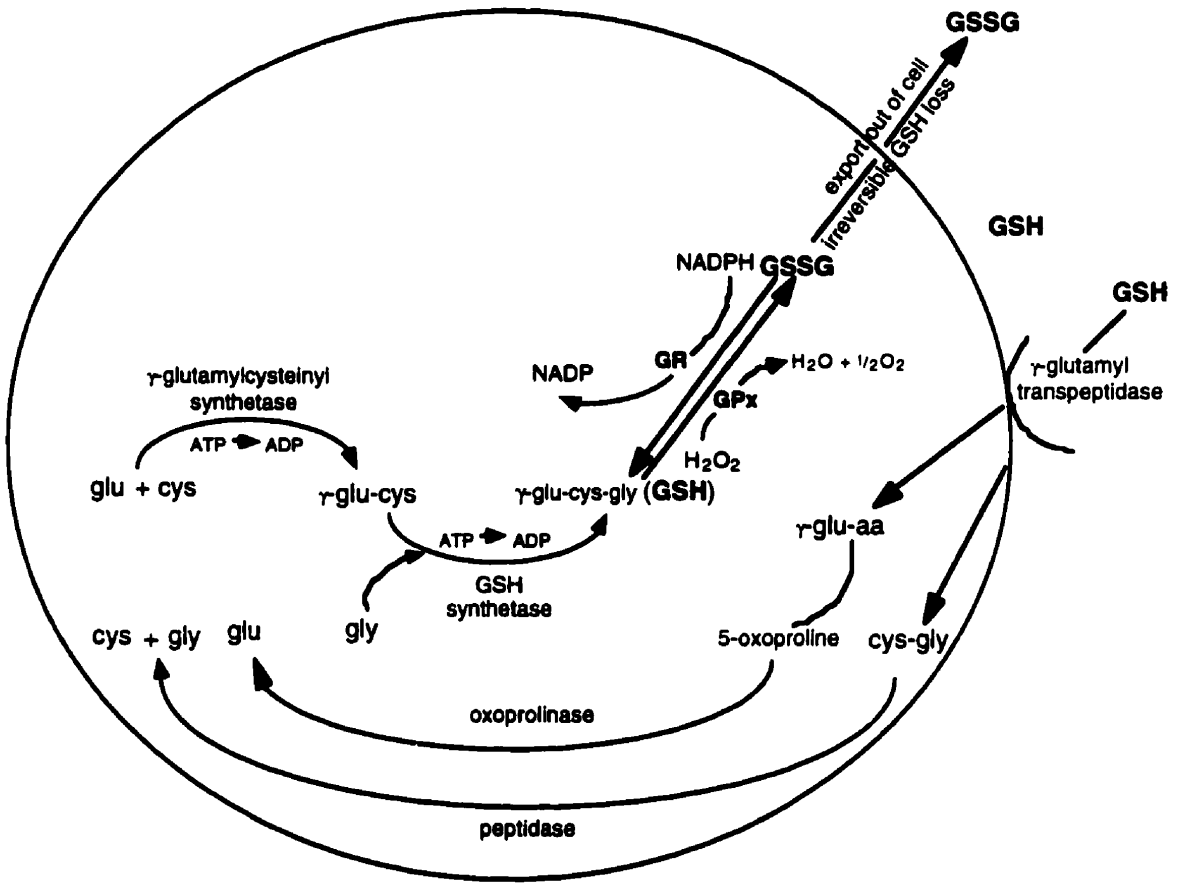
Glutathione (GSH) is the name for the tripeptide comprising γ-glutamylcysteinylglycine. It is the major endogenous cellular antioxidant and is present in significant amounts within cells (0.5 - 10 mM). Its oxidized form, GSSG, exists at 5 - 50 mM (299). Glutathione-S-conjugates represent 1-10% of total cellular glutathione content. Two enzymes synthesize GSH in an ATP-dependent manner. γ-glutamylcysteinyl synthetase catalyzes the reaction of glutatamate and cysteine to form γ-glu-cys, while glutathione synthetase catalyzes the reaction of γ-glu-cys with

glycine to form GSH. GSH acts in conjunction with GSH peroxidase to detoxify H_2O_2; however, this molecule also scavenges oxidants and ROI by itself. It takes 2 GSH molecules to react with H_2O_2 in the presence of GSH peroxidase to form an oxidized form of glutathione, GSSG, and water. GSH can also react with other peroxides, such as lipid hydroperoxides that are formed during oxidative stress. GSH can be reduced to be utilized again in reactions with glutathione peroxidase catalyzing the reduction of H_2O_2. GSSG reductase uses 2 NADPH molecules as electron donors to reduce both sulphur residues, thus restoring two GSH molecules for reutilization. Besides detoxifying H_2O_2, GSH also can inactivate lipid peroxides and aldehydes through the action of glutathione-S-transferases, resulting in an irreversible oxidation of glutathione (300). During oxidative stress, NADPH is depleted and GSSG is exported from the cell depleting intracellular glutathione pools. Glutathione conjugates form mercapturic acids which are excreted in the urine (301).

Generally, intracellular GSH is exported, especially from the liver, and transported to other organs. GSH is not taken up efficiently in the absence of accessory pathways. Cells of certain tissues express an enzyme on the cell surface to break down GSH and import the smaller fragments of GSH. Thus, GSH can be degraded and recycled. It is the enzyme, γ-glutamyltranspeptidase that degrades GSH. This allows amino acids to be recycled (302). Organs such as the kidney, the choroid plexus, lymphocytes, biliary duct, ciliary body, intestine and pancreas are especially enriched in this enzyme (302). γ-glutamyltranspeptidase cleaves the γ-peptide bond, while incorporating an amino acid. This results in γ-glutamyl-aa and cys-gly. γ-glutamyl cyclotransferase cleaves γ-glutamyl-aa forming oxoproline, a cyclic form of glutamate. Oxoprolinase converts 5-oxoproline to glutamate in an ATP-dependent step. A peptidase breaks Cys-Gly down into individual amino acids. Thus, these amino acids can be recycled to reform GSH (303).

Table 1. Enzymes involved in GSH synthesis and function.

Substrates	Enzymes	Products
[illegible]		γ-glu-cys + [illegible]
γ-glu-cys + gly	GSH synthetase	γ-glu-cys-gly (GSH)
2GSH [illegible]		GSSG + H_2O + $^1/_2O_2$
GSSG + 2 NADPH	GSSG reductase	2 GSH + 2 NADP
GSH [illegible]		γ-glu-aa + cys-gly
γ-glu-aa	γ-glutamylcyclotransferase	5-oxoproline
5-oxoproline [illegible]		glu
cys-gly	peptidase	cys + gly



Biosynthesis and degradation of GSH. GSH=reduced form of glutathione, GSSG=oxidized form, GR=GSH reductase, GPx=GSH peroxidase.

I.5.5 Heat shock proteins and heme oxygenases

Heat shock or stress proteins are a set of proteins which transiently increase during sublethal heat stress, allowing a cell to withstand subsequent heat challenge which is lethal in the absence of prior and continued expression of heat shock proteins. Ritossa discovered that heat shock generated puffs in chromosome of salivary gland cells of Drosophila (304). Subsequent studies demonstrated a similar set of protective genes were engaged by hypoxia or ischemia, heavy metals, amino acid analogues, inflammation and oxidative stress (304). Heat shock proteins are characterized by the absence of introns, which allows for quick expression and an efficient response to potentially damaging stimuli. Varied classes of stress proteins are recognized by their molecular weights include Hsp110, Hsp90, Hsp70, small Hsps. Hsps are inducible heat shock proteins, whereas Hscs are constitutively expressed proteins that function in heat shock. Hsp110 is found in the nucleolus and cytoplasm and associates with Hsc70 for thermotolerance. Two Hsp90 proteins are located in the cytoplasm. Hsp90α, an inducible protein, binds Hsp70, Hsp56 and p23 during heat stress and participates as an aporeceptor for ligand-activated nuclear receptors. These are regulated by estrogen and progesterone. Hsp90β, a constitutively expressed protein acts with immunophilin to play a role in immunosuppresion (304). Recently, constitutively expressed proteins have been found to interact with inducible heat shock proteins and play a role in protein folding in the cytoplasm and nucleus. Some small Hsps are important in actin dynamics. Small heat shock proteins include Hsp32. Hsp32 induces hypoxia inducible factor, a transcription factor and its expression is significantly induced during oxidative stress. Hsp32 is also known as heme oxygenase-1 (HO-1) (305).

HO-1 breaks heme down: 1) to remove free Fe^{2+} in concert with ferritin (a protein that stores iron), the major transition metal which contributes to the deleterious formation of •OH, and 2) to form two antioxidants from the porphyrin ring of heme. HO-1 is a major stress protein

induced in human cells following induction of oxidative stress (305). It is transcriptionally induced (305). Itis thought that NF-κB mediates this response. 2 NF-κB sites occur in the promoter (306). HO-1 is an inducible isoform, whereas heme oxygenase-2 (HO-2) is a constitutively expressed form. Both enzymes degrade heme into biliverdin, carbon monoxide, and free iron. Embryonic fibroblasts lacking HO-1 have increased free radical production and cytotoxicity in response to hemin and H_2O_2 (307). HO-1 knockout mice undergo hepatic necrosis and increased mortality when challenged with lipopolysaccharide (308). Mice with null mutations of HO-2 were sensitized to hyperoxia-induced mortality due to overwhelming oxidative stress (309). GSH levels are twice as high prior to oxygen exposure, suggesting the animals may be compensating with other endogenous antioxidants. Although HO-1 was upregulated even prior to exposure to oxygen, ferritin was not. The lack of compensatory ferritin allowed for heme accumulation, particularly in the lungs (309). It seems that HO-2 localizes with nNOS in the myenteric plexus and produces CO, which acts on co-localizing soluble guanylyl cyclase. nNOS controls penile erection. HO-2 knockout mice evidence dysfunction in penile ejaculatory behaviour (310).

I.6 REGULATION OF GENE EXPRESSION BY REDOX-BASED MECHANISMS

ROI can exert their effects on expression of genes at varied levels: chromatin structure, transcription initiation, elongation, mRNA stability and translational efficiency. Here, we will discuss the latter four.

I.6.1 Transcription initiation

ROI affect the redox state of critical thiols of transcription factors. These changes can alter patterns of gene expression. For instance, oxidative stress affects the endothelium to alter gene expression. Endothelial cells express higher levels of vascular and inducible cellular adhesion molecules (VCAM-1, ICAM-1) (311) on the cell surface in the presence of varied stimuli. A broad range of agents can alter endothelial expression of VCAM-1 and ICAM-1, including cytokines (312), lipopolysaccharide (247), antioxidants (245, 313), ROI (246, 314), metal chelators (312) and thiol-modifying agents (245). This implicates specific transcription factors transducing a myriad of activating stimuli. Differences in redox state represent an underlying cause of transactivation due to the breadth of stimulating agents. For instance, cytokines achieve their effects by modulating redox state, which in turn activates transcription factors to induce expression of VCAM-1 and ICAM-1. Endothelial cells respond to leukocyte-derived cytokines, such as TNF-α or IL-1β, by reversibly altering levels of expression of specific gene products that promote inflammation. Upon further inspection, the redox-sensitive transcription factor NF-κB was found to be responsible for the induction of ICAM-1 and VCAM-1 gene expression (315, 316). Changes in redox state of this transcription factor allow it to be released from a protein in the cytoplasm, translocate to the nucleus and activate transcription at sites of DNA containing NF-κB cis-elements. Viruses, cytokines, lipopolysaccharide and ROI activate translocation of NF-κB to the nucleus. An early investigation revealed that lysophosphatidylcholine, a component of oxLDL, enhances

expression of VCAM-1 (241). Later studies showed that antioxidants abolish its induction by IL-1 (245). Finally, ROI induce expression of ICAM-1 (317).

Increased ROI formation is a result of endothelial dysfunction and has an effect on critical thiol groups of the redox-sensitive transcription factor. As discussed in section I.3, the endothelium can act as a source of free radicals, especially when injured. Dysfunctional endothelium has different patterns in gene expression. The example here is expression of specific cellular adhesion molecules that increase, attracting leukocytes to adhere to endothelial cells. Normally, cellular adhesion molecules facilitate cell-cell interactions, between endothelial cells and between endothelial cells and leukocytes during inflammatory responses as a protective response to localize infection. However, prolonged exposure to products of activated leukocytes may alter vascular endothelial gene expression. The effects discussed are dependent on protein binding of nucleic acids to mediate regulation of gene expression. For instance, NF-κB is composed of different subunits, often p65 and p50, but other variants exist, including c-Rel and p52. I-κB also binds these inhibiting nuclear translocation. Upon activation, IkB kinases phosphorylate I-κB allowing it to be ubiquitinized by a proteasome, while NF-κB translocates to the nucleus. Cys_{62} of p50 is critical for DNA binding, and oxidation of this thiol group abolishes binding, while reducing agents enhance binding (318).

Oxidizing conditions also increase transcription of the HO-1 gene (319). HO-1 contains two NF-κB sites in its promoter (320-323). This enzyme has a protective effect in inducing the production of cellular antioxidants, in an attempt to maintain cellular homeostasis. Oxidizing conditions induce the expression of cytokines, such as TNF-α and IL-1β through their effects on NF-κB. Pyrrolidine dithiocarbamate, an antioxidant, can suppress LPS-induced translocation of NF-κB, and attenuate expression of TNF-α. Oxidants enhance expression of TNF-α, whereas free radical scavengers inhibit expression of TNF-α in vitro and in vivo (324, 325). Although this transcription factor plays an important role in the activation of cytokines, those cytokines can also

stimulate NF-κB activity. TNF-α for instance, can activate NF-κB. This cytokine depletes GSH. Addition of N-acetylcysteine, a precursor of GSH, to TNF-α-treated cells inhibits activation of NF-κB (324, 326). In line with redox implications for NF-κB, thioredoxin increases binding more so than GSH. Low levels of GSSG are required for activation, whereas high levels of GSSG and oxidized forms of thioredoxin inhibit NF-κB DNA-binding activity (327). These results indicate this transcription factor requires a certain thiol content for transactivation and is sensitive to GSSG/GSH levels and the intracellular thiol-disulfide content.

In contrast to thioredoxin's effects on NF-κB, this antioxidant activates AP-1. N-acetylcysteine, an antioxidant and dithiothreitol, a reducing agent increase unstimulated and phorbol ester-stimulated binding of AP-1. Increased AP-1 binding is associated with an increase in transcription of *c-fos* and *c-jun* (327). Once AP-1 is formed from the leucine zipper interaction between c-Fos and c-Jun, Ref-1, a nuclear redox protein, participates in reducing AP-1 proteins. Hypoxia increases levels of Ref-1 and can enhance AP-1 activity. If Cys_{154} of AP-1 is mutated to Ser_{154} in c-Fos, the mutated form has increased binding to AP-1 cis elements in DNA, and is resistant to oxidation. Normally, oxidizing conditions decrease binding. Therefore, thiol-disulfide exchange is extremely important in protein binding of DNA. Thiol state affects the ability of transcription factor to transactivate specific genes.

Hypoxia, TNF-α and agents inducing oxidative stress can increase expression of γ-glutamylcysteinyl synthetase. This most likely occurs through effects on AP-1 elements present in the γ-glutamylcysteinyl synthetase promoter (296, 328, 329). Oxidative stress enhances transcription of the regulatory subunit of γ-glutamylcysteinyl synthetase, the inital and rate-limiting enzyme for de novo synthesis of GSH (330). Oxidative stress initiates the production of nerve growth factor, as part of an injury response. This neurotrophic factor mediates the increase in transcription of γ-glutamylcysteinyl synthetase (331). Nerve growth factor acts through Ras to enhance MAPK activity, which results in the activation of transcription factors, c-Fos and AP-1

(332). Oxidants are known to increase MAPK activity, and activate transcription factors subsequently. Thus, oxidants most likely act through signal transduction pathways, as well as affecting thiol state of specific aa residues, to modulate transcription and subsequent gene expression.

I.6.2 Transcriptional elongation

RNA polymerase II processivity can be affected by the nuclear redox state. To date, very few examples have been cited. In relation to this work, oxidative stress has been shown to play a role in transcriptional elongation of a glutathione-related enzyme. Iron and oxidative stress affect nascent RNA chain elongation of GPx. The iron chelator desferrioxamine decreases nascent chain elongation without affecting mRNA stability. Tert-butylhydroperoxide, an inducer of oxidative stress, and iron donors had the opposite effect. Thus, iron and oxidative stress stimulate transcriptional elongation of GPx (333).

I.6.3 mRNA stability

The best characterized system in which redox status alters post-transcriptional regulation, is the iron-dependent regulation of transferrin receptor (which takes iron up into the cell). Iron-response elements (IREs) in the 3'UTR of transferrin receptor (TfR) mRNA regulate mRNA stability. The TfR imports iron into cells. An IRE-binding protein (IRP-1), now identified as a component of aconitase, binds the IRE when iron is low, thus stabilizing transferrin receptor mRNA. This molecular mechanism functions so that iron will be imported into the cell (334, 335). Under conditions where iron is high, aconitase retains IRP-1 activity, such that it does not bind IREs present in the 3'UTR of TfR mRNA. Therefore, the mRNA is not stable with high levels of iron, and iron is not taken up. H_2O_2 and NO activate IRP-1 to bind RNA, by oxidizing one or

more of its cysteine residues (336). These studies demonstrate iron and possibly •OH from the interaction of iron and H_2O_2 can affect mRNA stability via IRP-1.

Oxidative stress can promote the binding of AU-binding factors to the 3'UTR of labile cytokine mRNAs: TNF-α, IL-1β and GM-CSF, as well as ICAM-1. These mRNAs contain AU-rich elements which confer instability under unstimulated condtions (337). However, oxidative stress induced by phorbol ester can enhance transcription of TNF-α and IL-1, as well as ICAM-1. AU-binding factors can bind and protect mRNAs from degradation: AUBF activity can be reversibly blocked by diamide, but is irreversibly inhibited by N-ethylmaleimide, suggesting that AUBF contains a redox switch as described for other RNA-binding proteins (338). AUBF is also phosphorylated, and redox may affect phosphorylation status by modulating signal transduction (338). AUBF is also sensitive to alkylating agent and selective oxidizing agents, indicating that its binding capacity depends on redox state. Overall, AUBF-binding is sensitive to redox state and enhances mRNA stability of cytokines and ICAM-1.

Cytokines can mediate the effects of oxidative stress on other genes also. For instance, in the lung, TGF-β stabilizes elastin mRNA, through phosphatidylcholine-specific phospholipase C and protein kinase C (339). It is thought that TGF- β affects redox state of RNA binding proteins. Hyperoxia and oxidants increase mRNA stability of another gene in the lung, catalase. Oxidative stress enhances the binding of lung protein to catalase mRNA, thereby stabilizing and promoting expression (340). In addition, selenium deficiency, a condition associated with oxidative stress, enhances mRNA stability of GPx (281). Thus, oxidative stress induces antioxidant defense mechanisms in the lung.

Oxidizing conditions most probably achieve their transcriptional and post-transcriptional effects through signal transduction. Redox state also affects intracellular signalling. Oxidants can affect cell cycle proteins and signal transduction pathways, by altering mRNA stability of p21 (waf1/cip1). Diethyl maleate (DEM), a glutathione depletor stabilizes p21 (waf1/cip1) mRNA so

that it accumulates (341). This is a newer observation and not much is known about this area. The functional effects on cell cycling have not been defined, yet p21 does inhibit cyclin-dependent kinases through p53-dependent pathways. DEM treatment represents a p53-independent pathway. It is known that oxidative stress can alter signal transduction. In relation to this work, DEM is shown to affect mRNA stability of another mRNA, that of eNOS. p21 can be altered by a glutathione depletor (inducer of oxidative stress). It is most likely that oxidizing conditions affect both signal transduction and nucleic acid-binding protein interactions affect each other. These, in turn, affect gene expression.

I.6.4 Translation

Iron levels can often affect redox state and in the case of ferritin, both control its translational processing. IREs in the 5' UTR of ferritin mRNA affect translation of this iron storage protein. During periods of iron deprivation, IRP-binding attentuates translation of ferritin and minimizes storing of iron. Formation of the 5'UTR IRE/IRP complex on the mRNA represses translation by blocking the binding of the small ribosomal subunit to the mRNA (342). The RNA-binding activity of IRP-1 is regulated posttranslationally by a mechanism that interconverts apoprotein IRP-1 and an iron sulfur protein form (aconitase). This interconversion is regulated by iron, oxidative stress and NO, suggesting unexpected roles of IRP-1 in the regulation of cell growth and metabolism (343). Redox state affects RNA binding proteins. As mentioned above, cysteine residues play a key part of IRP-1 binding to IREs of TfR mRNA and ferritin mRNA. If mutated, binding is abolished (344). Thus, redox state exerts effects on mRNA stability and translation of an iron regulatory receptor and storage protein, respectively.

The cellular environment determines the thiol state of nucleic acid-binding proteins. Normally thioredoxin maintain a reducing environment within the cell, such that thiols exceed disulfides at equilibrium. In plants, ferredoxin, ferredoxin reductase and protein disulfide

isomerase maintain an RNA-binding protein, RB47 (47kD). UV light enhances translation of psbA genes by dissociating RB47 from the 5'UTR. The effects of UV light on photosystem I chlorophyll genes are mediated by redox state of this RNA-binding protein. When RB47 is in its reduced form, translation is enhanced. Interestingly, in bacteria oxygen sensors control the activation of oxidative stress genes. GSH and GSH reductase control the thiol-disulfide state of a pair of cysteine residues (Cys_{199} and Cys_{208}). When oxidized, the transcription factor is turned on to induce expression of GSH reductase and hydrogen peroxidase (345). This is an interesting example, as the binding proteins discussed, if oxidized, cannot bind nucleic acids anymore. These studies indicate that thiol state of nucleic acid-binding proteins determines their binding to DNA or RNA. These interactions influence gene expression at multiple levels.

I.7 SUMMARY

Oxidative stress is found in a number of disease processes that involve the endothelium. Examples include atherosclerosis and diabetes. The changes in redox physiology lead to phenotypic alterations and functional changes predicting a variety of changes in gene expression. In the vascular wall, oxidative stress leads to endothelial expression of cellular adhesion molecules that enhance leukocyte interaction and adherence, as well as protective genes. Modulation of redox affect cellular adhesion molecule expression. In diabetes, AGEs, in generating oxidative stress directly and in interacting with RAGE, further enhance gene expression and production of RAGE. This promotes interactions between endothelial cells and macrophages. In attempts to maintain cellular homeostasis, antioxidant production by HO-1 degradation of heme is enhanced in response to oxidative stress.

In addition to increased adherence and concurrent decreased barrier function, dysfunctional endothelium exhibits imbalances in vasomediator activity. Endothelium-dependent vascular relaxation is abnormal in hypercholesterolemia, atherosclerosis, diabetes and hypertension (346).

Dysfunctional regulation of vasomotor tone occurs in hypercholesterolemia and atherosclerosis, as oxygen free radicals abolish endothelium-dependent relaxation (347). On the other hand, augmenting glutathione production, enhances endothelium-derived NO in atherosclerotic patients (348). The purpose of this thesis is to explore the relationship between oxidative stress and gene expression of endothelial-derived vasomediators.

I.8. RATIONALE

Oxidative stress is associated with vascular disease in which vasomotor tone is disturbed. For instance, free radicals that can induce oxidative stress appear to diminish endothelium-dependent vasodilation. Given that regulation of eNOS expression determines the capacity of endothelial cells for longer-term production of NO we sought to determine the effects of oxidative stress on vasomediator function. The issue has been focused in two respects. First, in vitro approaches have been used in which depletion of glutathione has been used as a unique model of oxidative stress. Second, we have addressed the arguably more clinically relevant issue of chronic glutathione depletion and the consequent effect on vasomediator production by vascular endothelium. It is of interest that both transcriptional and post-transcriptional processes contribute to the steady-state regulation of eNOS. Indeed, for many of the stimuli known to alter eNOS mRNA levels, such as TNF-α, proliferation/injury, oxLDL and hypoxia, the latter mechanism figures prominently. Our studies will address the mechanism of any observed changes. Because the balance of paracrine control of the circulation invloves both vasodilators and vasoconstrictors, the effects of chronic glutathione depletion on ppET-1 and ECE-1 mRNA expression were also studied.

II. Hypothesis

Glutathione depletion in endothelial cells leads to decreased steady-state endothelial nitric oxide synthase (eNOS) mRNA and increased steady-state preproendothelin-1 mRNA expression. Underlying the effects of GSH depletion on eNOS expression are molecular mechanisms involving alterations in mRNA stability.

II.1 Specific Aims

1. To determine whether glutathione depletion leads to alterations in endothelial nitric oxide synthase mRNA, protein and functional activity in endothelial cells.

2. To determine whether glutathione depletion leads to alterations in steady-state preproendothelin-1 and endothelin converting enzyme-1 mRNA in endothelial cells.

3. To determine the molecular mechanisms underlying changes in vasomediator steady-state mRNA expression, specifically the role of transcriptional versus post-transcriptional processes.

III. EFFECTS OF OXIDATIVE STRESS ON eNOS, ppET-1 and ECE-1

III.1 METHODS

Reagents–Diethyl maleate, hydrogen peroxide, buthionin sulfoximine, glutathione 5,5'-dithiobis-(2-nitrobenzoic acid) and gelatin were purchased from Sigma (St. Louis, MO). Phorone and guanidium isothiocyanate were from Fluka BioChemika (Buchs, Switzerland). [α-^{32}P]-deoxycytidine triphosphate (3 000 Ci / mmol), [α-^{32}P]-uridine triphosphate (3 000 Ci / mmol), [α-^{32}P]-deoxycytidine triphosphate (800 Ci / mmol) were from Dupont-New England Nuclear (Wilmington, DE). Supplemented bovine calf serum, trypsin, antibiotics, cell culture media and balanced salt solutions were purchased from GIBCO BRL (Grand Island, NY); collagenase type I from Worthington (Freedon, New Jersey); and endothelial mitogen from Biomedical Technologies (Stoughton, CA). RNase inhibitor amd DNase RQ1 were purchased from Promega (Madison, WI); ATP, CTP and GTP were from Amersham Pharmacia Biotech (Arlington Heights, IL); and Century Marker DNA template, RNases A and T1 from Ambion (Houston, TX). Murine anti-human IgG1 eNOS antibody was from Transduction Laboratories (Lexington, KY) and horseradish peroxidase-conjugated sheep anti-mouse secondary antibody, the enhanced chemiluminescence detection system and L-[^{14}C]arginine (300 Ci/mmol) were from Amersham (Arlington Heights, IL). BioRad protein assay kit II and Dowex AG 50WX-8 Na+-exchange resins were from BioRad (Hercules, CA). Histamine and ionomycin were from Calbiochem (Cambridge, MA). All other reagents used were of analytical grade.

Cell isolation and culture–Primary cultures of human umbilical veins (HUVEC) were obtained by cannulating umbilical veins, flushing with saline and incubating with 0.1% type I collagenase at 37 °C (Worthington, Freedon, New Jersey). These cells were subcultured on gelatin-coated plates (0.2 g/dL) in the presence of heparin (17 U/mL), endothelial mitogen (50 ug/mL) (Biomedical Technologies, Stoughton, California), 100 U/mL penicillin G sodium and 100 µg/mL streptomycin sulfate in M199 with 20% fetal calf serum (GIBCO BRL, Grand Island, NY) at 37 °C, 5% CO_2, in a humidified incubator (37, 134, 349). Cells were passaged by

trypsinization with 0.05% trypsin-EDTA (GIBCO) every 5 - 7 days and were used for experiments between passages 2 and 4. Bovine aortic endothelial cells (BAEC) were isolated and propagated in RPMI 1640 medium with 15% supplemented bovine calf serum, 100 U/ml penicillin G sodium, and 100 (g/ml streptomycin sulfate as previously described (99, 350). Endothelial cells at confluence were treated with glutathione depletors, buthionine sulfoximine (BSO) , diethyl maleate (DEM) or phorone every 12 h for 48 h. DEM and phorone were diluted in ethanol, where the final concentration of ethanol did not exceed 0.1%. BSO was diluted in 0.9% NaCl (pH=8). Hydrogen peroxide (H_2O_2) was also given either every 12 h for 48 h, or in combination with DEM at staggered time points. Combination studies were carried out by adding threshold DEM at 12, 24, 36 and 48 h, and H_2O_2 at 18 and 42 h.

Assessing glutathione levels–Cells were trypsinized with 0.05% trypsin, centrifuged and resuspended in 250 µl of 5% salicylic acid. Each sample was sonicated for 30 sec and incubated on ice at 4°C. Serial dilutions of a known glutathione standard were used to generate a standard curve Total cellular glutathione was measured using a colorimetric assay based upon the reactions of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) with non-protein sulfhydryls. The assay allows nonprotein sulfhydryls to react with DTNB as such: 2 GSH+DTNB = GSSG + 2 TNB (351). A 1:10 dilution of GSH standard or cell sample was prepared in a buffer containing 0.2 M Na_2PO_4, 0.2 mM EDTA, 0.1% SDS and 0.2 mM of DTNB for a 15 min incubation. The samples were read on a multiwell plate reader at an absorbance of 414 nm (Titertek Multiscan MCC340, EFLAB, Finland).

In vivo studies–All animal studies were performed in accordance with guidelines of the Toronto Hospital Animal Care Committee and the Canadian Council on Animal Care. Animals were fed standard rat chow and water ad libitum and allowed to acclimatize prior to experiments. Female Swiss-Webster mice were divided into three groups; DEM-treated, sham and control. Mice were injected intraperitoneally every 12 h with 3 mmol/kg DEM in corn oil for 48 h prior to

sacrifice (352). Corn oil was given in the sham group, while control animals did not receive any injections. Lungs and kidneys were harvested and frozen immediately in liquid nitrogen.

RNA extraction and northern blot analysis–Total cellular RNA was isolated from HUVEC using the guanidium thiocyanate-phenol-chloroform method (353). RNA was fractionated by electrophoresis through a 1%-agarose-0.66 M formaldehyde gels in 20 mM 3-(N-morpholino)-propanesulfonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA, pH 7.0 and transferred to a nylon membrane (Hybond N+, Amersham) in 10x SSC. The membrane was UV-crosslinked and prehybridized in 50% deionized formamide, 5x Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA), 0.1% SDS and 25 μg/mL boiled sonicated salmon sperm DNA. Hybridisation was carried out at 42°C overnight using cDNA probes random primer-labelled with [α-^{32}P]-dCTP to a specific activity of averaging 10^9 cpm /μg. Membranes were washed under high stringency conditions. Final wash conditions were 0.1x SSC and 0.1% SDS at 65 °C (26). Blots were subjected to Phosphorimage analysis and analysed densitometrically using ImageQuant (Version 1.1, Molecular Dynamics, Sunnyvale, CA). Specific signals were normalized to GAPDH signals. Membranes were also subject to autoradiography at -80°C using Kodak film (Eastman, NY).

cDNA Probes–An 880 bp *Eco RI* DNA fragment complementary for 3'-regions of murine eNOS from a mouse fetal heart cDNA library was used for murine studies (86) . An *Eco RI* fragment spanning 1.1 kB of cDNA of mid-region human eNOS covering exons 9 through 15 was used for in vitro studies with HUVEC (37). An 880 bp fragment of human GAPDH (354), a full-length 1.2 kB *Eco RI* human preproET-1 cDNA fragment (355), a 630 bp fragment of human heme oxygenase-1 (ID 812038, Genome Systems Inc., St. Louis, MO) and an 880 bp fragment of human ECE-1 spanning exons 2 through 8 (publication pending) were used for northern blot analysis and nuclear run-on studies. Human eNOS genomic and cDNA used in nuclear run-on assays included exon-1 and exon 2 - 20 probes (31, 37).

Nuclear run-on assays–Confluent DEM-treated or vehicle-treated HUVEC cells in 100-mm culture plates were washed 3 times with ice-cold phosphate-buffered saline on ice and lysed in situ with 1.5 ml chilled lysis buffer (10 mM Tris HCl (pH 7.9), 0.15 M NaCl, 1mM EDTA, and 0.6% v/v Nonidet P-40) for 10 min. Cell lysates were centrifuged for 5 min at 500 x g at 4 °C. The cytoplasmic RNA contained in the supernatant was extracted by the guanidinium thiocyanate phenol-chloroform method for steady state mRNA assessment (353). The nuclear pellet was resuspended in 100 µl nuclear storage buffer at 4°C, incubated in trans cription buffer (0.3 M $(NH_4)_2SO_4$, 100 mM Tris HCl (pH 7.9), 4 mM $MgCl_2$, 4 mM $MnCl_2$, 0.2 M NaCl, 0.4 mM EDTA, and 0.1 mM PMSF) containing 0.2 mM DTT, 60 U RNase inhibitor (Promega), 0.2 mM each of ATP, CTP and GTP (Pharmacia Biotech) and 150 µCi [α-^{32}P]-UTP (3 000 Ci / mmol) for 30 minutes at 28 °C. The reaction was inhibited by the addition of DNase RQ1 (10-20 U) (Promega) and 125 µg tRNA and incubated at 37 °C for 10 min. Proteinase K (300 µg/ml) in buffer (10 mM Tris HCl (pH 7.9), 10 mM EDTA and 0.5% SDS) was added and incubated at 42 °C for 30 min. RNA was extracted as described (353) and resuspended in 200 µl hybridization buffer containing 50% formamide, 5x SSPE, 5x Denhardt's solution, 100 µg/ml salmon sperm DNA and 0.5% SDS w/v). Equal cpm of ^{32}P-labelled run-on transcripts were hybridized from each cell type to cDNA probes slot blotted onto nitrocellulose filters for 72 h at 42 °C. Following hybridization, the filters were washed for 15 minutes at each condition as follows: 1x SSC, 1.0% SDS at 55 °C; 0.2x SSC, 0.2% SDS at 55 °C; 2x SSC, 10 µg/ml RNase A and 5 U / ml RNase T1 at 37 °C; 2x SSC at 37 °C. Filters were autoradiographed at -80 °C, phosphorimaged and quantitated using ImageQuant software by Molecular Dynamics.

RNase Protection Assay–To provide an independent method to assess eNOS mRNA expression, RNase protection assays were performed. 50 µCi [α-^{32}P]CTP (800 Ci/mmol) were used to generate an internally labelled antisense riboprobe in an in vitro transcription reaction using

T7 RNA polymerase (Promega) and 1 μg of template DNA linearized with *EcoRI* linearized template DNA spanning the 292 terminal nt of the human eNOS cDNA. A labelled RNA ladder was synthesized similarly using the Century Marker DNA template (Ambion Inc, Houston, TX). DNase digestion (Promega) was used to remove template DNA and the riboprobe was gel purified on a 6% acrylamide/8M urea gel. 1.0 - 1.5x 10^5 cpm of labelled, eluted probe was hybridized in solution to 15 μg of total cellular RNA from control or DEM-treated HUVEC and subjected to RNase A and T1 digestion at 25°C for 30 minutes. The digestion was inactivated according to RPAII kit instructions (Ambion, Houston, Texas) and the RNA preciptated. Samples were electrophoresed through a denaturing 6% acrylamide / 8M urea gel, followed by autoradiography and phosphoimager analysis. Undigested anti-sense probe alone is 330 nucleotides in length and the expected length of the protected fragment is 292 nt in length.

Protein extraction and western blot analysis –Total cellular protein was extracted from confluent HUVEC or BAEC monolayers with boiling Laemmli sample buffer [62.5 mM Tris-HCl (pH 7.9), 2% SDS, 10% glycerol, 5% β-mercaptoethanol] (356). Cell lysates were boiled for 5 min and centrifuged at 100 000 x g for 30 min. The cellular supernatant was collected and protein concentration measure using a Bradford method, the Bio-Rad protein assay kit II (Hercules, CA). 20 μg of protein samples were electrophoresed on a 6% polyacrylamide-SDS gel under reducing conditions. Separated proteins were transferred by electroblotting onto a polyvinyldifluoride membrane and blocked overnight with Tris-buffered saline-0.1% Tween 20 (TBS-T) containing 4% BSA. Blots were incubated with a monoclonal antibody (anti-IgG1) human directed towards the C-terminal portion of human eNOS (Transduction Laboratories), (diluted 1/4 000) and washed with TBS-T. Antigen-antibody complexes were identified with horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (diluted 1/5 000) and the membrane was subsequently exposed to the enhanced chemiluminescence detection system (Amersham) according to the manufacturer's instructions. Blots were briefly exposed to radiographic film at room temperature.

Lung and kidney tissue homogenate samples were extracted using 0.2% Triton X Tris-buffered saline and then separated on a 15% SDS-containing polyacrylamide gel.

Measurement of NOS activity–NOS enzymatic activity was assessed as previously described. NOS activity was measured by L-[^{14}C]arginine to L-[^{14}C]citrulline conversion (26, 133). Briefly, HUVECs were cultured on 6-well plates (Costar, Corning, NY) to confluency and treated with either DEM (100 μM) or vehicle every 12 h for a total of 60 h. Cell monolayers were washed with a physiological salt solution (130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM HEPES [pH 7.4], and 10 mM D-glucose) and equilibrated in 1 ml of the same buffer with or without DEM (100 μM) for 30 min at 37°C. After being labeled with L-[^{14}C]arginine (5x 10^5 cpm/ml) (Amersham, 300 mCi/mmol) for 30 min, cells were treated with either histamine (100 μM), or ionomycin (5 μM) (Calbiochem), or vehicle for 20 min. The reaction was stopped by washing the cell monolayers with 4 ml ice-cold PBS containing 5 mM EDTA. The monolayers were then extracted with 1 ml of 0.3 M $HClO_4$ for 20 min at 4°C. After neutralization, the extract was loaded onto a 1 ml wet bed volume of Dowex AG 50WX-8 cation-exchange resin (Bio-Rad, Na^+ form, 100-200 mesh) followed by 4 ml water. L-[^{14}C]citrulline in the 5 ml column effluent was quantitated by scintillation counting.

Statistics–Unless otherwise indicated, data are means + S.E. from at least three separate experiments. ANOVA was used when comparisons involved multiple samples, for example, northern blot analyses. The paired two-tailed t-tests were used for comparisons in L-arginine to L-citrulline assays and the level of statistically significant difference was defined as $P<0.05$.

III.2 RESULTS

DEM decreased steady-state eNOS mRNA transcript levels in a time-dependent manner– DEM treatment caused a decrease in eNOS mRNA transcript levels in a time-dependent manner over 2 - 72 h. Shown in Figure 1A is a representative northern blot of RNA harvested from HUVEC, from three independent isolations, exposed to DEM 100 μM for increasing lengths of time. DEM was added every 12 h for the longer treatment periods. A mid-region probe spanning exons 9 through 15 was used to assess steady-state eNOS mRNA expression. Incubation of cells with DEM caused evident decreases in the eNOS mRNA (4.8 kB) as early as 8 h, with a maximum effect evident by 48 h. No further decrease was observed at 72 h. Without DEM treatment eNOS mRNA is expressed in confluent cells over this time period. To control for the amount of RNA loaded per lane, nylon membranes were reprobed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. Densitometric quantitation was used to obtain a ratio of eNOS relative to GAPDH (1.2 kB). Results were normalized across experiments and are presented in Figure 1B. DEM-induced decreases in steady-state eNOS mRNA averaged 70 +/- 8%. At the same time, HO-1 mRNA (1.8 kB) levels accumulated compared to GAPDH controls. HO-1 mRNA expression peaked at 48 h and fell slightly at 72 h.

DEM decreased steady-state eNOS mRNA transcript levels in a concentration-dependent manner– DEM treatment caused a decrease in eNOS mRNA transcript levels in a concentration-dependent manner over the range 0 - 100 μM DEM at 48 h. A representative experiment is illustrated in Fig. 2. 100 μM DEM was the maximum concentration used for experiments. Chronic exposure at higher concentrations were cytotoxic to HUVEC. Results indicated a threshold effect of DEM on eNOS mRNA expression at 5 - 20 μM (48 h). These results indicate that DEM, a glutathione depletor decreased expression of eNOS steady-state mRNA as assessed by northern blot analysis.

Figure 1A.

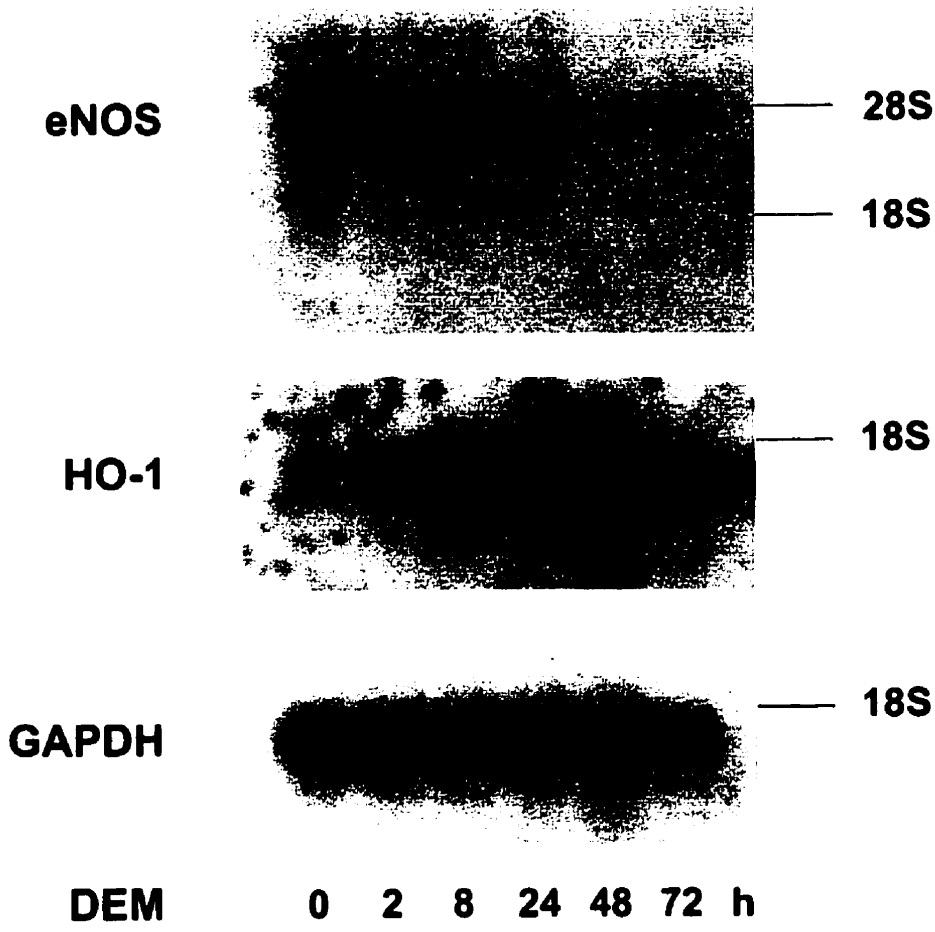


Figure 1B.

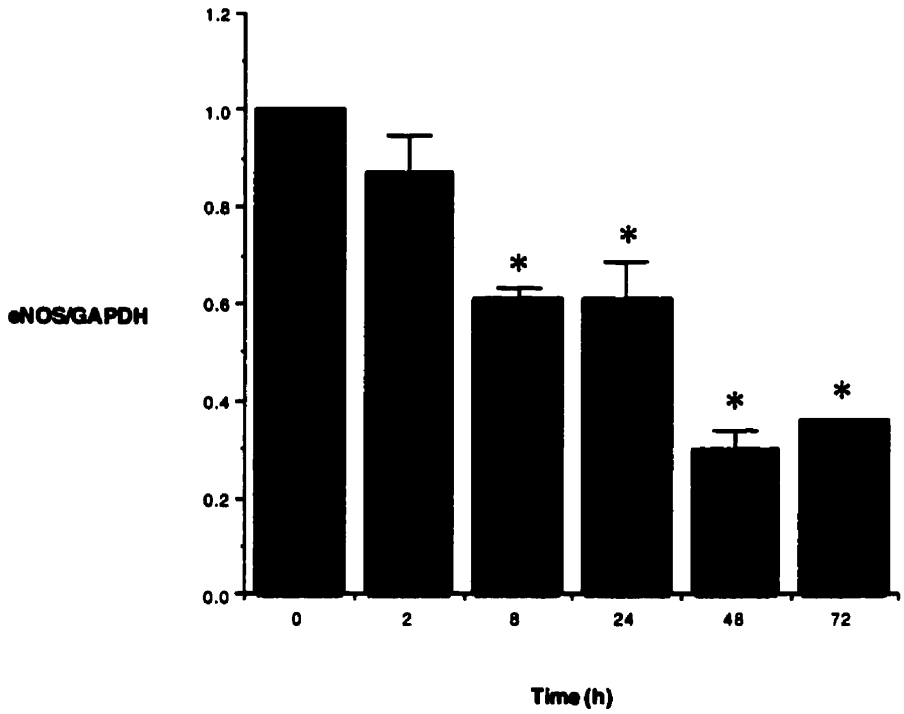


Figure 1. Time-dependent Effects of DEM (48 h) on HUVEC eNOS mRNA expression. A) Northern blot analysis (15 μg/lane) demonstrates the effects of duration of DEM treatment on steady-state eNOS mRNA expression. Cells were treated with DEM (100 μM) for 2, 8, 24, 48 or 72 h prior to harvesting total cellular RNA. For 24, 48 and 72 h timepoints, DEM treatment was repeated every 12 h. Membranes were reprobed with heme oxygenase-1 (HO-1) and glyceraldehyde-3-phosphate (GAPDH) cDNAs. Representative data of three independent experiments are shown. B) Analysis of DEM effect on steady-state eNOS mRNA expression (n=3, mean +/- S. E., $p \leq 0.0001$ ANOVA). Where error bar is not evident, the S. E. M. was insignificant.

Figure 2.

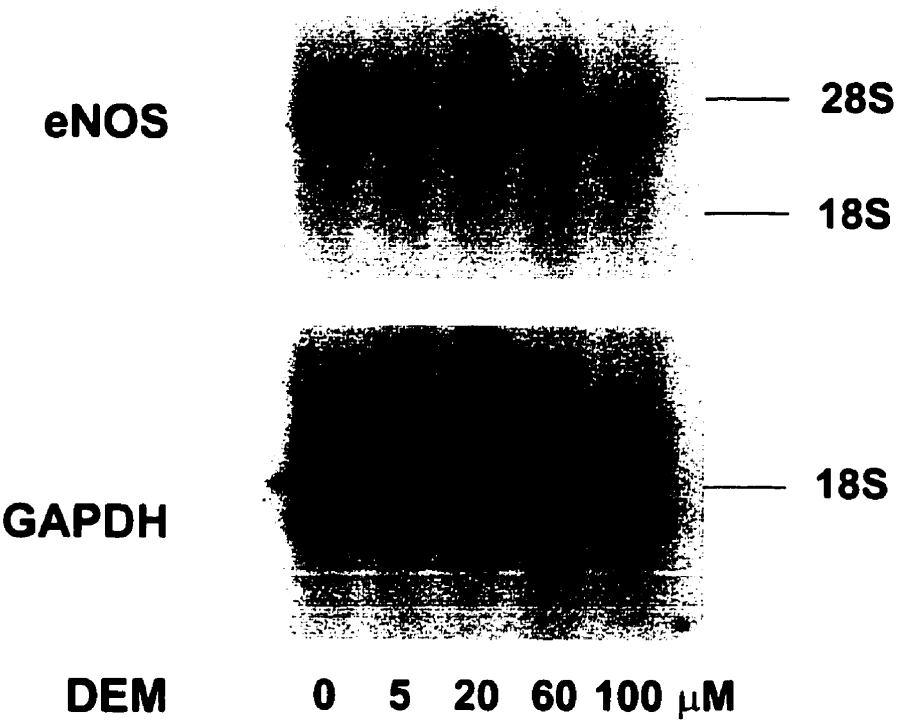


Figure 2. Concentration-dependent Effects of DEM (100 μM) on eNOS mRNA expression. Northern blot analysis (20 μg/lane) demonstrates the effects of varied concentrations of DEM on eNOS mRNA expression in HUVEC. Cells were treated with 0, 5, 20, 60 and 100 μM of DEM every 12 h for 48 h prior to harvesting total cellular RNA. Membranes were reprobed with GAPDH cDNA. Representative data of three independent experiments are shown.

DEM decreased steady-state eNOS mRNA as assessed by an independent method, RNase protection assay–HUVEC incubated with DEM for 48 h at 100 μM exhibited a significant decrease in steady-state expression of eNOS mRNA. This experiment tested whether these effects observed in the northern blot analysis would be confirmed with an independent method. RNase protection analysis was performed, to assess the effects of glutathione depletion on eNOS mRNA. A 3'-end complementary RNA probe encompassing the terminal 292 nt of the eNOS 3'UTR was used in this assay. The data in Figure 3 indicate that DEM decreased eNOS mRNA.

Effects of other pharmacologic depletors of GSH and H_2O_2 on steady-state eNOS mRNA– Glutathione depletion with phorone, also resulted in decreased expression of eNOS mRNA relative to vehicle control (Figure 4A). Phorone (1 mM) consistently decreased eNOS mRNA relative to GAPDH. Compared with vehicle, 1 mM phorone decreased eNOS mRNA expression by 62 +/- 8% compared with control values (n=3). BSO, depletes glutathione by inhibiting GSH synthesis. This compound was designed to interfere with the action of γ-glutamylcysteinyl synthetase. It decreased steady-state eNOS mRNA expression by 48% as shown in Figure 4A); however, results varied (n=3). Shown in Figure 4B) is the addition of H_2O_2. Varying concentrations of H_2O_2, 10, 50 or 100 μM did not decrease eNOS mRNA. In fact, there was a slight increase. However, the combination of H_2O_2 (50 μM) with threshold levels of DEM (5 μM) resulted in decreased expression of eNOS mRNA. Therefore, in the presence of threshold amounts of DEM an effect is evident with H_2O_2.

Figure 3.

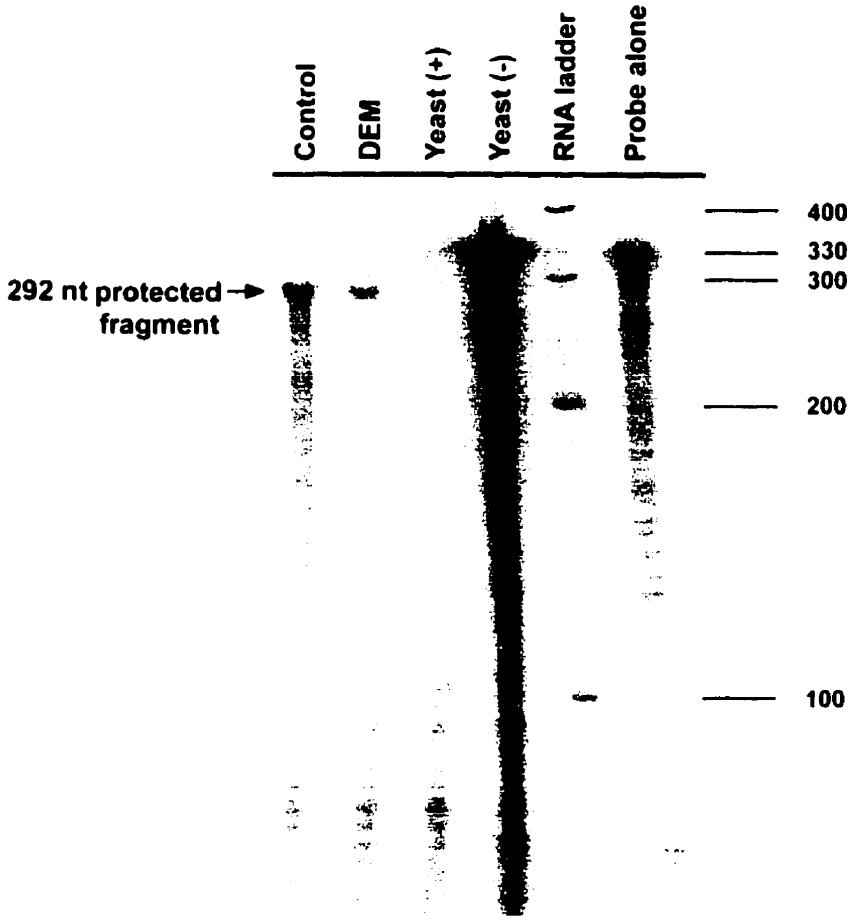

Control
DEM
Yeast (+)
Yeast (-)
RNA ladder
Probe alone
292 nt protected fragment
400
330
300
200
100

Figure 3. Effects of DEM (100 μM) on eNOS mRNA over 48 h of treatment. RNase protection assays were performed on total cellular RNA from HUVEC treated with or without DEM. 15 μg of RNA were analyzed using the α[^{32}P]-CTP labelled riboprobe complementary to the 292 nt 3' of the major site of cleavage and polyadenylation. RNase digestion of yeast RNA (+) was used as an internal control to confirm that RNase digestion was effective and to ensure the specificity of bands. Yeast RNA without digestion allowed to control for RNase contamination. RNA ladder indicates nucleotide size. Undigested probe is 330 nt in length and the expected length of the protected fragment is 292 nt.

Figure 4.

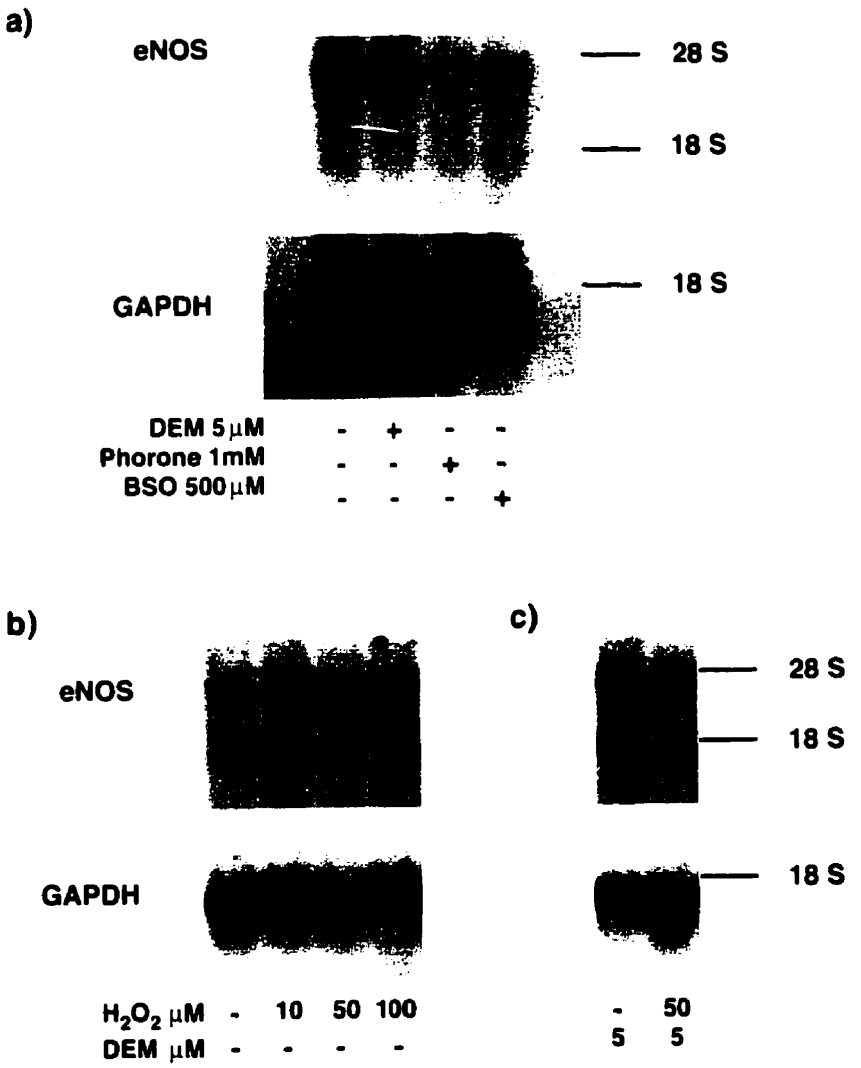

a)
eNOS
28 S
18 S
GAPDH
18 S
DEM 5 μM - + - -
Phorone 1mM - - + -
BSO 500 μM - - - +
b)
eNOS
GAPDH
H2O2 μM - 10 50 100
DEM μM - - - -
c)
28 S
18 S
18 S
- 50
5 5

Figure 4. Effects of various glutathione depletors and reactive oxygen intermediates on eNOS mRNA. a) Confluent HUVEC were treated with DEM (5 μM), phorone (1 mM) or BSO (500 μM) every 12 h for 48 h. b) Varying doses of H_2O_2 (10, 50 and 100 μM) were added every 12 h for 48 h. c) Threshold levels of DEM (5 μM) every 12 h for 48 h. A combination of threshold DEM (5 μM) and H_2O_2 were evaluated.

DEM decreased steady-state eNOS protein–As shown in Figure 5A, HUVEC depleted of intracellular glutathione evidenced decreased eNOS protein expression as evaluated by western blot analysis. Both 100 μM DEM and 1 mM phorone treatment over 48 h caused a decrease in eNOS protein expression relative to vehicle-treated cells. Experiments with another endothelial cell type were used to confirm findings in HUVEC. DEM at 100 μM, given every 12 h for 48 h, demonstrated a slight decrease in levels of steady-state immunoreactive eNOS protein in bovine aortic endothelial cells (BAEC). BAEC mRNA was decreased by 66% in one experiment, but experiments were variable. Taken together, these results show that glutathione depletion by pharmacologic intervention decreased eNOS protein expression in HUVEC and to some degree in BAEC.

DEM, phorone and BSO depleted total cellular glutathione–BSO acts to selectively inhibit γ-glutamylcysteinyl synthetase (357), while DEM and phorone act by conjugating glutathione to form mixed disulfide. Glutathione is the most abundant thiol and readily measurable. It is a marker of thiol reactivity. The standard method to assay for glutathione is by using by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) which reacts with non-protein sulfhydryls. Figure 6 depicts data from five independent experiments each assessing total intracellular glutathione levels as measured by dithiobis-nitrobenzoic (DTNB) acid assays (351). HUVEC were treated with agents every 12 h for 48 h. DEM (100 μM), phorone (1 mM) and BSO (500 μM) depleted intracellular glutathione in HUVEC compared to control. The observed decreases in total cellular glutathione levels reflect both GSSG (oxidized form of glutathione) and GSH (reduced form of glutathione). The ratio is not known from this; however, there is a decrease in available substrate for glutathione peroxidase. Control HUVEC evidenced an average of 6.5 nmol GSH/10^6 cells, with a range of 4.5 - 11.9 nmol/10^6 cells. Treatment with vehicle did not alter intracellular GSH levels. Results for DEM and BSO show a significant difference in GSH from control (Figure 6). DEM decreased GSH to 0.632 +/- 0.172 of control, while BSO decreased GSH to 0.313 +/- 0.079 of control. Phorone at 1 mM decreased GSH across five samples from two independent

experiments, but the decrease was not significantly different from control according to the t-test, because of the small degree of freedom. However, the decrease induced by 1 mM phorone was consistent.

Figure 5.

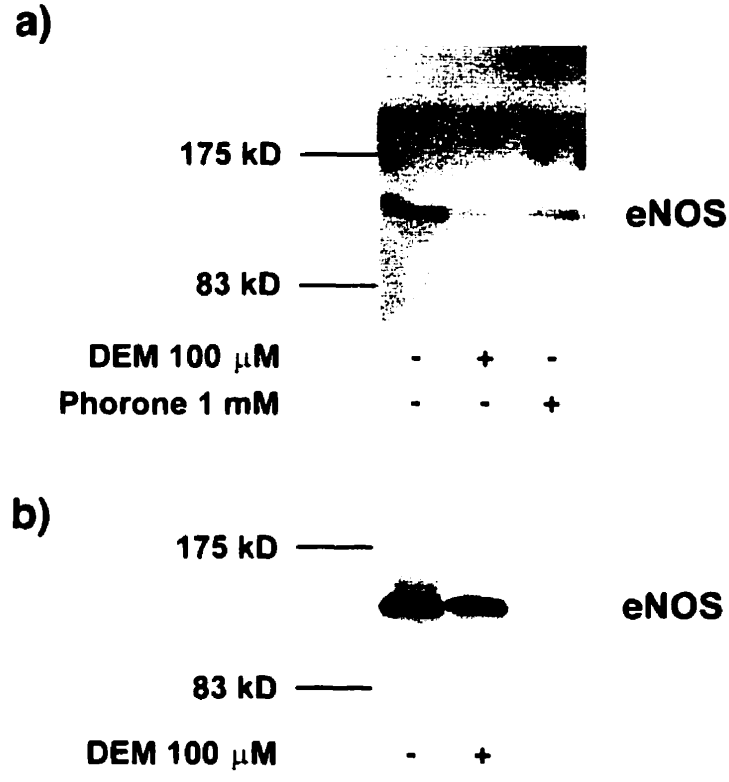

a)
175 kD
eNOS
83 kD
DEM 100 μM - + -
Phorone 1 mM - - +
b)
175 kD
eNOS
83 kD
DEM 100 μM - +

Figure 5. The effects of DEM (100 μM) and phorone (1 mM) on steady-state eNOS protein expression. Shown in panel A) is the effect of DEM and phorone given every 12 h for 48 h to confluent HUVEC. eNOS is 135 kD in size. Panel B) shows the effects of DEM in BAEC.

Figure 6.

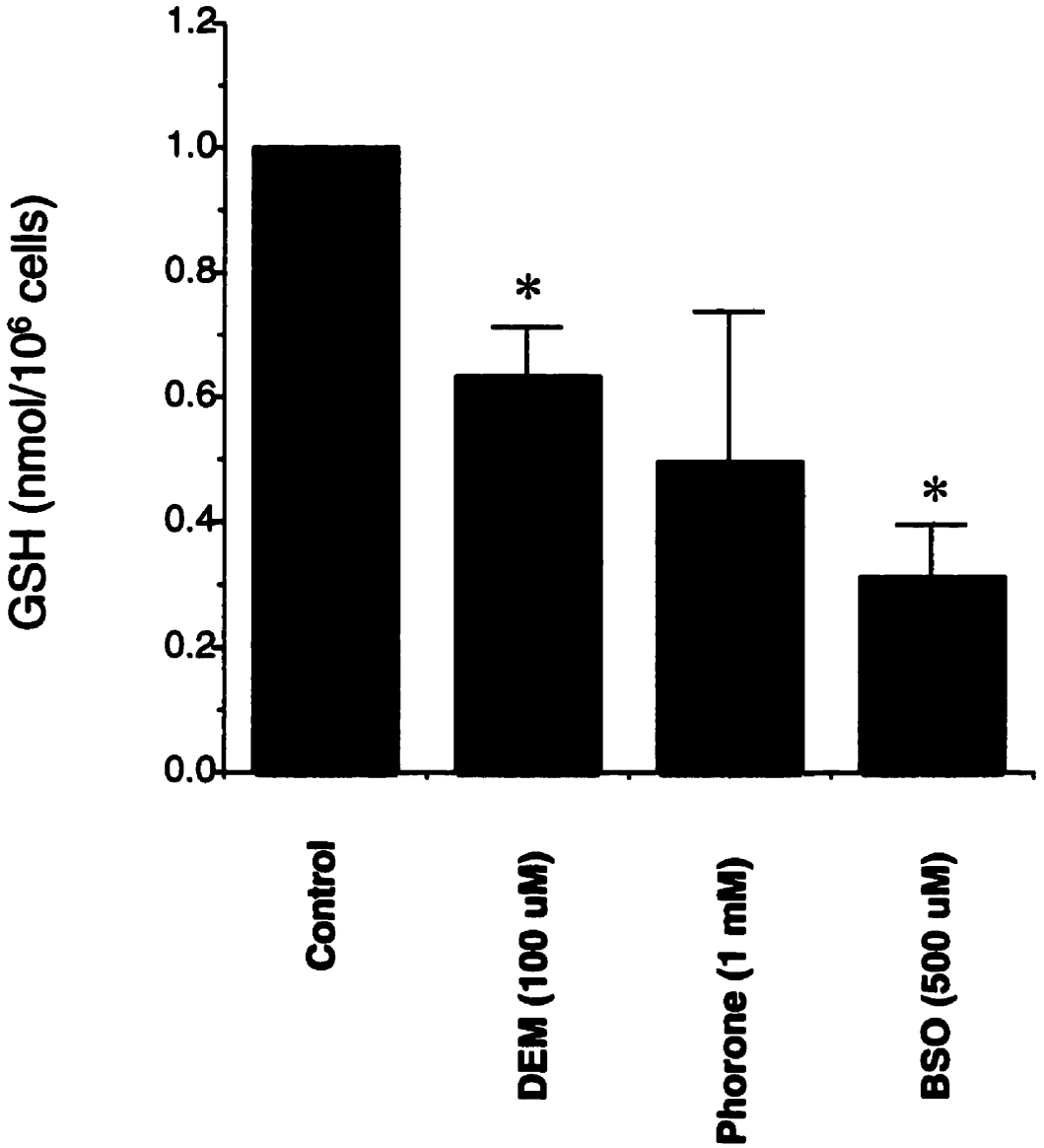

GSH (nmol/10^6 cells)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Control
DEM (100 uM)
Phorone (1 mM)
BSO (500 uM)

Figure 6. Effects of various GSH depletors on total intracellular glutathione content. DEM (100 μM), phorone (1 mM) and BSO (500 μM) (n=3) were given every 12 h for 48 h to confluent HUVEC. Cells were trypsinized, resuspended in 5% salicylic acid and homogenized. Samples were incubated with DTNB for 15 min and read at an aborbance of 414 nm. Results were normalized to cell counts at time of cell harvesting.

DEM decreased NO catalysis–To determine whether DEM-induced decreases in steady-state eNOS mRNA and immunoreactive protein content were important at a functional level, NO synthesis was measured A well-established conversion assay, in which NO is an equimolar byproduct, was used (26, 133). This assay measures [^{14}C]-radiolabelled citrulline. As shown in Figure 7, DEM decreased basal NOS activity as well as histamine- and calcium ionophore-stimulated activity. The effects of DEM on eNOS mRNA and protein are relevant to functional studies, and indicate that the fall in eNOS protein levels resulted in decreased responsiveness to agonist-stimulation or calcium ionophore compared to vehicle control. Although, there is a basal decrease upon DEM treatment, there still is some response to agonists. Compared to control, agonist-stimulated responses were blunted. Histamine targets H_1 subtype-endothelial receptors and activates phospholipase C to produce 1,4,5-inositol trisphosphate and diacylglycerol, which leads to increased intracellular calcium and activation of protein kinase C, respectively. This leads to enhanced eNOS activity and increased vasorelaxation. For this to occur, the molecular chaperone, Hsp90, facilitates translocation of eNOS. To date, no expression of iNOS occurs in endothelial cells, thought it is possible that some of the remaining activity could be due to nNOS (358). In decreasing eNOS mRNA and protein, GSH depletion causes effects at a functional level. Overall, DEM attenuated the histamine- or calcium-ionophore of eNOS (n=3, triplicate determinations).

Glutathione depletion is relevant in vivo to the expression of endothelial vasomediators–Chronic parenteral treatment of Swiss-Webster mice was performed with DEM at 3 mmol /kg intraperitoneally. As shown in Figure 8, total cellular RNA isolated from homogenates of kidney and lung show a decrease in steady-state eNOS mRNA as analyzed by northern blot analysis (n=3). Total cellular protein also exhibited a decrease in eNOS protein as assessed by western blot analysis. eNOS mRNA and protein expression are almost abolished in DEM-treated animals in two different organs. Glutathione levels were not measured as endothelium cannot be easily

separated from these organs. As the eNOS is relatively restricted to endothelium, GSH levels in the endothelium would be indicative of the influence on expression in these organs. In addition, DEM decreases GSH in both the kidney and the lung (359, 360). These results indicate that effects of DEM are relevant in an in vivo setting.

Figure 7.

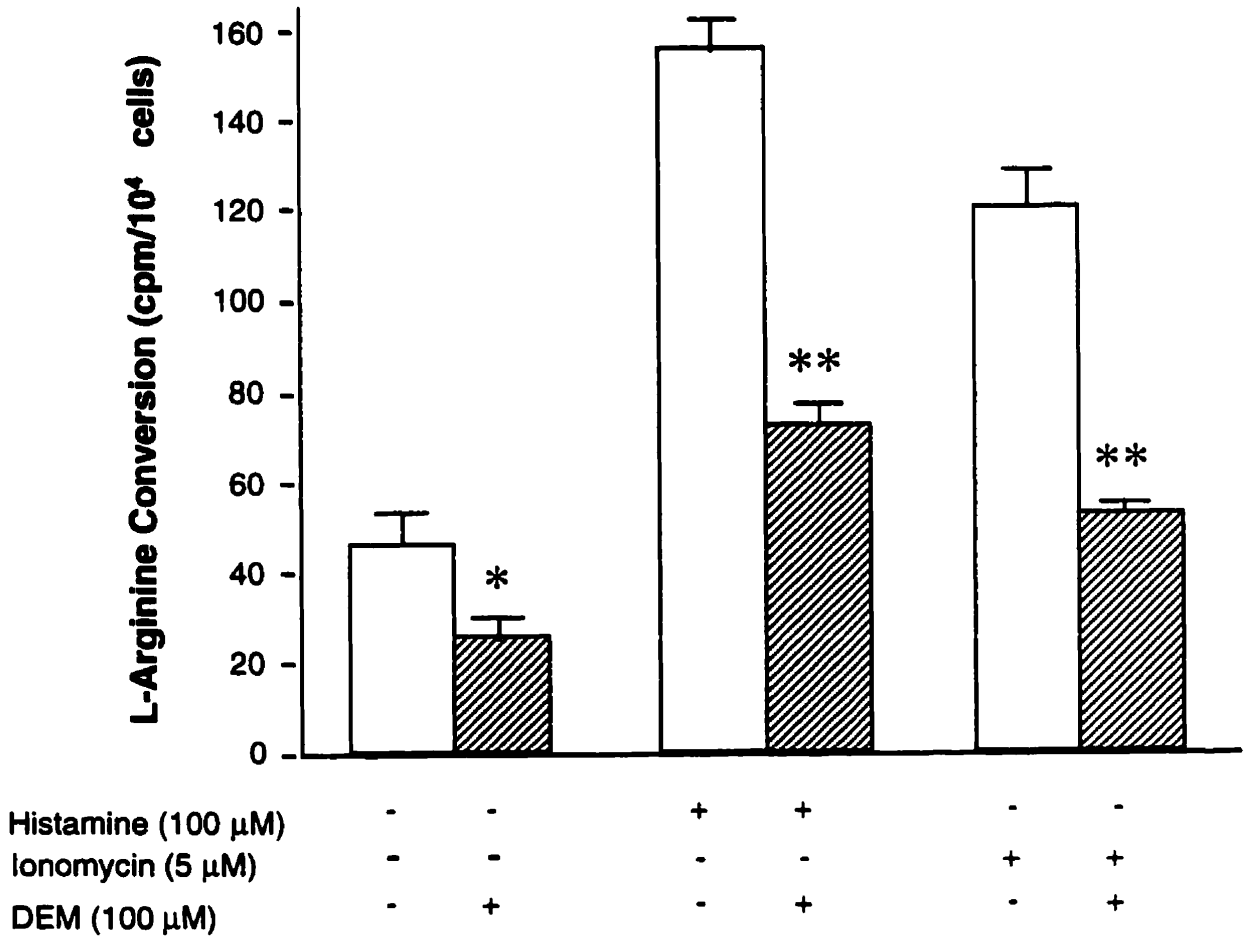

L-Arginine Conversion (cpm/10⁴ cells)
160
140
120
100
80
60
40
20
0
*
**
**
Histamine (100 μM) - - + + - -
Ionomycin (5 μM) - - - - + +
DEM (100 μM) - + - + - +

Figure 7. Functional activity of NOS. Effect of DEM on NOS activity as measured by L-[^{14}C]arginine to L-[^{14}C]citrulline conversion. Confluent HUVECs grown on 6-well plates were treated with either 100 μM DEM (striped bars) or vehicle (open bars) every 12 hours for a total of 60 hours. Cells were then labeled with L-[^{14}C]arginine and stimulated with either vehicle (first set of bars), 100 μM histamine (2nd set of bars), or 5 μM ionomycin (third set of bars). Cell-associated L-[^{14}C]citrulline was measured after cation-exchange chromatography. Results demonstrate significant attenuation of NOS activity by DEM treatment both under basal condition and after stimulation. Bars are mean +/- S. E. M. of three experiments (each in triplicate determinations); * $p<0.01$, **$p<0.0005$ vs. without DEM treatment.

Figure 8.

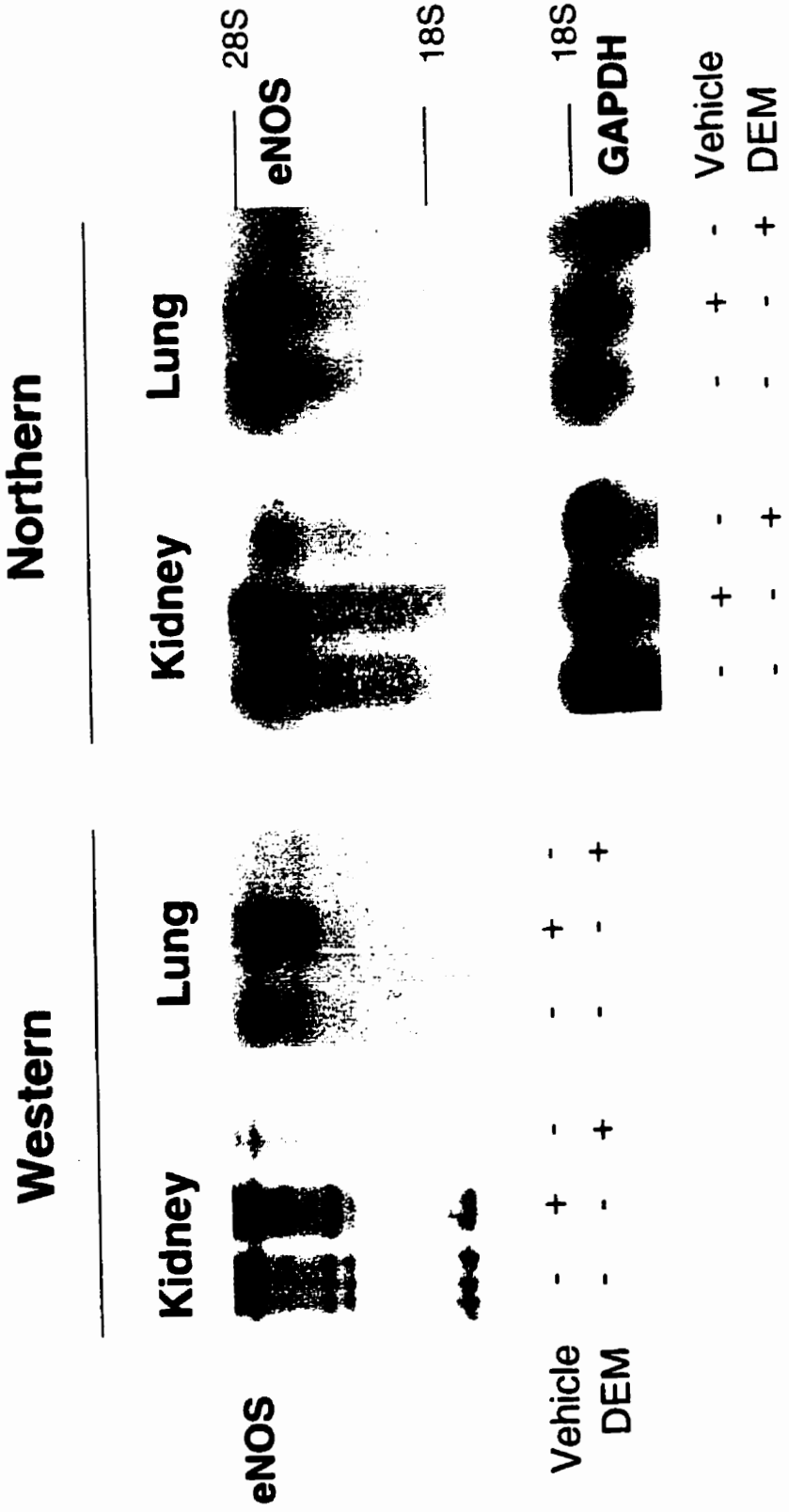

Western
Kidney
Lung
eNOS
Vehicle
DEM
Northern
Kidney
Lung
28S
eNOS
18S
18S
GAPDH
Vehicle
DEM

Figure 8. Effect of glutathione depletion in vivo. Female Swiss-Webster mice were treated with 3mmol/kg DEM every 12 h for 48 h prior to sacrifice. Protein and RNA were extracted from kidney and lung tissue homogenates. Shown in panel A), DEM treatment of mice compared to sham or control decreased eNOS protein expression in the kidney and lung. B) DEM also decreased eNOS mRNA relative to GAPDH in total cellular RNA isolated from kidney and lung. (Sham=Vehicle-treated, where the vehicle is corn oil).

Time-dependent effects of DEM on steady-state ECE-1 and ppET-1mRNA expression – DEM treatment did not alter ECE-1 mRNA transcript levels over 2 - 48 h. Shown in Fig. 9 is a representative northern blot of RNA harvested from HUVEC exposed to DEM 100 μM for increasing lengths of time. DEM was added every 12 h for the longer treatment periods. A mid-region probe spanning exons 2 through 8 was used to assess steady-state ECE-1 mRNA expression. Incubation of cells with DEM caused no evident decrease in the ECE-1 mRNA over a 48 h period. A full-length probe for ppET-1 was used for northern blot analysis. DEM treatment did cause a decrease in ppET-1 mRNA expression by 2 h. This decrease was maintained over a 24 h period. In contrast, DEM treatment failed to consistently modify steady-state ECE-1 mRNA expression. To control for the amount of RNA loaded per lane, nylon membranes were reprobed with a GAPDH cDNA probe.

Effects of DEM on eNOS initiation of transcripts and elongation by RNA polymerase II – To assess the mechanism by which eNOS mRNA expression is decreased, nuclear run-on assays were performed. Nuclear run-on assays assessed loading of RNA polymerase II on the endothelial genes studied in the current work (Fig. 10). These assays were utilized as an indication of the relative contributions of transcriptional and post-transcriptional activity. Nuclear run-on assays were performed with varied cDNA and genomic clones to assess the loading of RNA polymerase II. The probe spanning exons 2 - 20 of eNOS showed no difference between DEM-treated and vehicle-treated dishes, whereas the probe containing only exon-1 detected a decrease with chronic DEM treatment (48 h). Since the GAPDH signal is slightly higher in treated cells, this may even indicate a further decrease in exon 1 signal. The long probe indicated that overall elongation of transcripts is not affected. The decrease in signal of exon 1 probe demonstrates a potential decrease in formation of a pre-initiation complex.

Figure 9

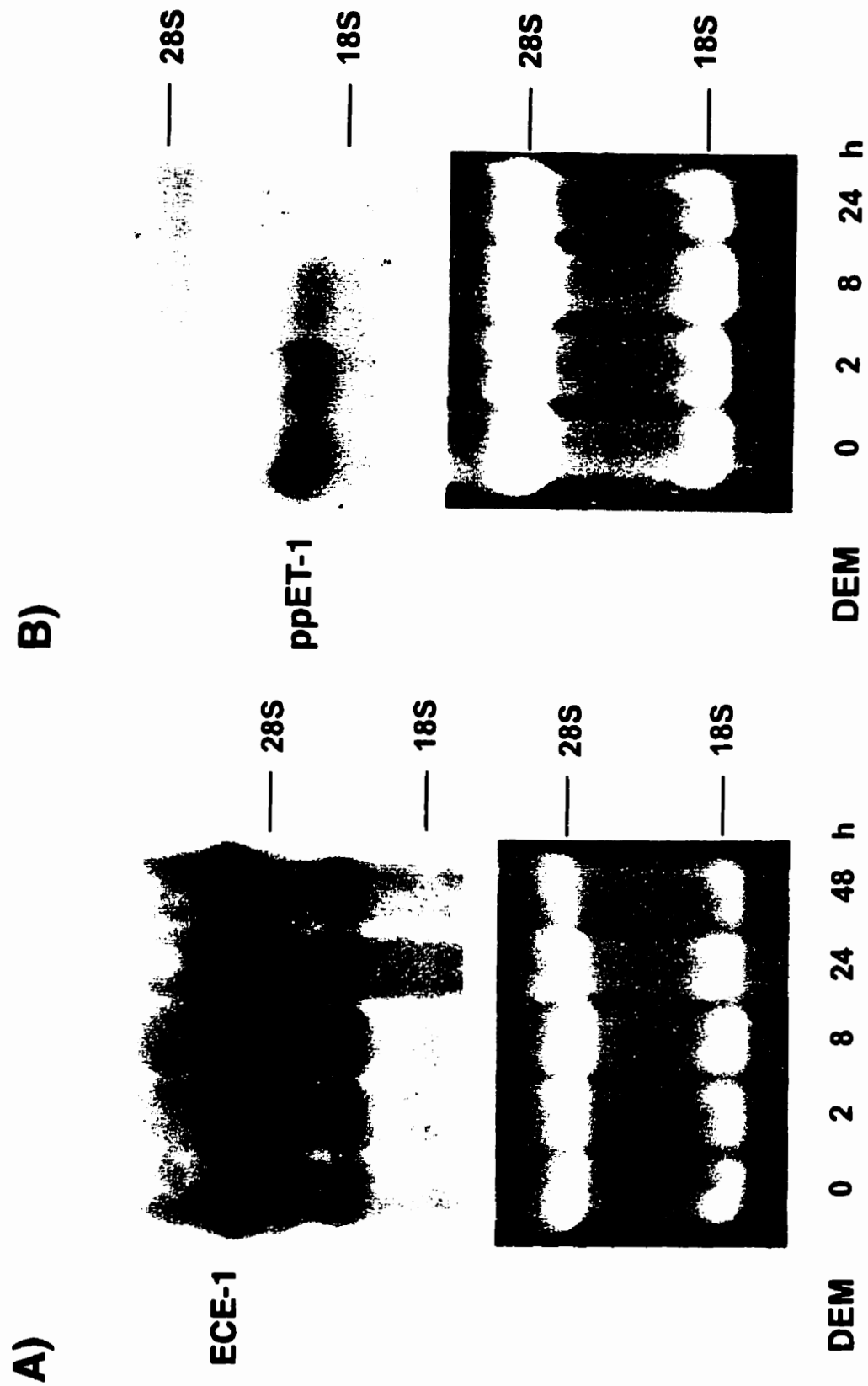

A)
ECE-1
28S
18S
28S
18S
DEM 0 2 8 24 48 h
B)
ppET-1
28S
18S
28S
18S
DEM 0 2 8 24 h

Figure 9. Time-dependent Effects of DEM (100 μM) on ECE-1 and ppET-1 mRNA expression. Northern blot analysis (15 μg/lane) demonstrates the effects of duration of DEM treatment on eNOS mRNA expression in HUVEC. Cells were treated with DEM (100 μM) for 2, 8, 24 or 48 h prior to harvesting total cellular RNA. For 24 and 48 h timepoints, DEM treatment was repeated every 12 h. The lower panel indicates ethidium-bromide-staining. A) Effects on ECE-1. B) Effects on ppET-1.

Figure 10.

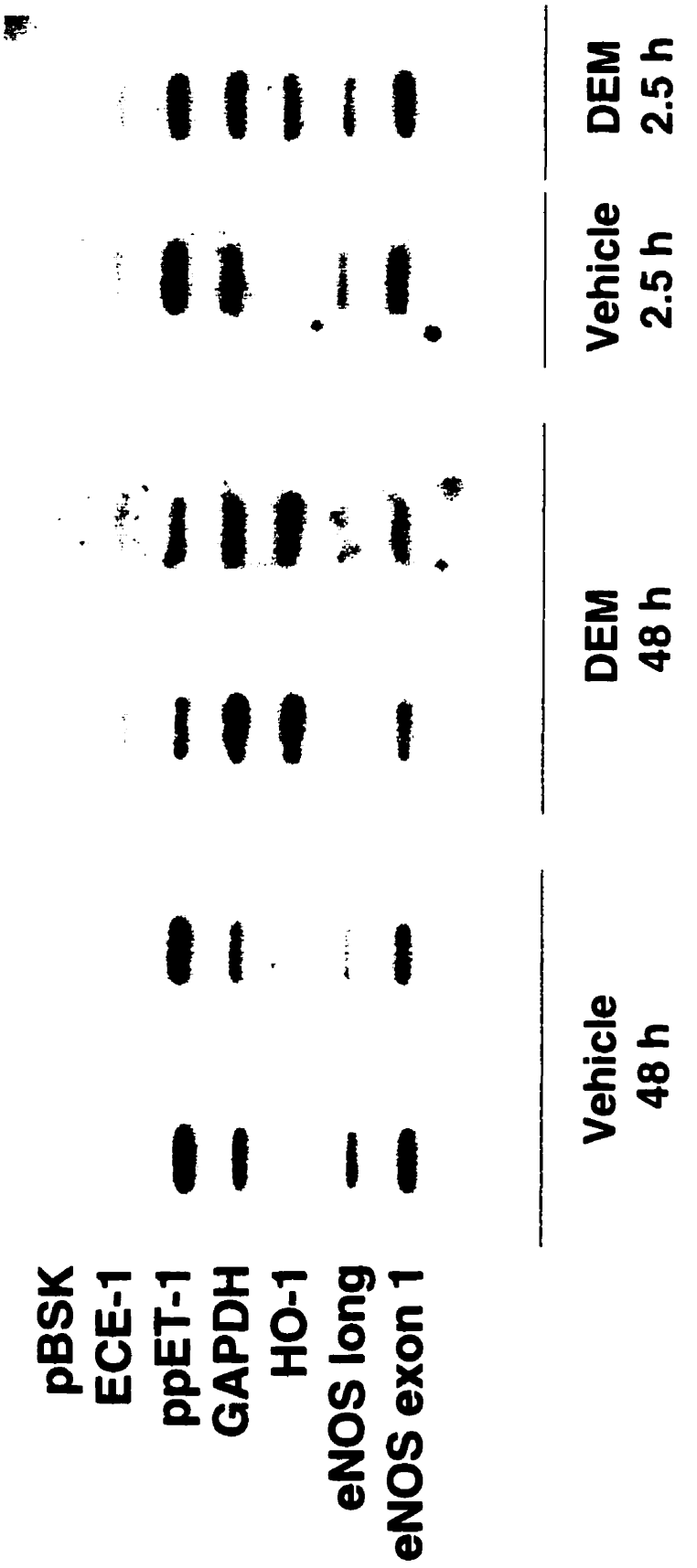

pBSK
ECE-1
ppET-1
GAPDH
HO-1
eNOS long
eNOS exon 1
Vehicle
48 h
DEM
48 h
Vehicle
2.5 h
DEM
2.5 h

Figure 10. Nuclear run-on assays reflect effects of DEM on transcriptional activtity on various transcripts. pBSK=pBluescript or empty plasmid, ECE-1 probe covers exons 2 - 8; ppET-1 represents a full-length probe; GAPDH was used as a control for loading; HO-1 contains 630 bp of the last exon; full-length eNOS spanned exons 2 - 20; and the last probe contained only exon 1 of eNOS. HUVEC were treated with vehicle or DEM (100 μM) for 2.5 h or 48 h. A representative result from 4 independent experiments is depicted.

Overall, these data suggest that the differences seen in northern blots do not seem to be due to a decrease in elongation of eNOS transcripts. HO-1 was used as a positive control, indicating a stress-induced activation of gene transcription in HUVEC following glutathione depletion. ppET-1 displayed a diminished signal in DEM-treated dishes at 48 h of DEM treatment, indicating a decrease in transcriptional activity. ECE-1 evidenced relatively little change consistent with the constant levels of mRNA appearing in northern blots following addition of DEM.

The half-life of eNOS mRNA is between 24 and 48 h (90). Therefore, taken together we conclude that DEM-indeced decreases in steady-state eNOS mRNA transcript levels involve major changes in the stability of eNOS mRNA transcripts.

III.3 DISCUSSION

The major finding of this work is that eNOS mRNA and protein expression fall in the setting of chronic GSH depletion. Depletion of intracellular GSH content results in altered expression of vasomediator genes in endothelial cells. DEM caused a time-dependent and a concentration-dependent decrease in steady-state eNOS mRNA expression. An independent method of assessing eNOS mRNA expression, the RNase protection assay, confirmed our findings with northern blot analysis. In addition, GSH depletion by independent agents caused a decrease in eNOS mRNA expression. The effects of DEM on steady-state eNOS mRNA content were accompanied by a decrease in eNOS protein expression as assessed by western blot analysis, as well as decreases in baseline and calcium-stimulated NOS enzymatic activity. Furthermore, studies performed in mice demonstrate the in vivo relevance of our in vitro findings. This is the first report demonstrating the effects of GSH depletion on eNOS mRNA expression.

Our observations of the effects of glutathione depletion have been divided into two categories; the effects on vasomediator gene expression and the underlying molecular mechanisms. In the time course, DEM addition caused a significant decrease in eNOS mRNA expression by 8 h (Fig. 2). eNOS mRNA normally has a half-life of 24 - 48 h (90). Considering this long half-life, to observe a significant decrease in eNOS mRNA by 8 h indicates that some decrease in mRNA stability must occur. It is plausible that decreased formation of a pre-initiation complex may also contribute. However, rates of RNA polymerase II loading on distal regions of the eNOS gene were not significantly affected by longer-term DEM addition. Overall, the effects of DEM on eNOS steady-state mRNA show that GSH depletion decreased steady-state eNOS mRNA in a time-dependent manner. The relative contributions of transcriptional and post-transcriptional events have been evaluated. Nuclear run-on assays indicate the capacity of RNA polymerase II to elongate initiated transcripts. The long probe for eNOS indicates the capacity of RNA polymerase II to form a full-length intact mRNA transcript. Effectively, no changes were observed upon DEM

treatment indicating that the changes observed in northern blot analysis were not due to decreased transcriptional activity. Exon-1 containing probes did show a slight difference. However, the long probe indicates no difference between the capacity of DEM-treated and vehicle control nuclei to fully transcribe initiated transcripts of eNOS. The differences in exon-1 most likely represent minor transcriptional decreases. This indicates that the differences are due to post-transcriptional changes. Thus, it is inferred that the decrease in eNOS mRNA expression is due to a decrease in mRNA stability of the mature processed cytoplasmic transcript. It is still important to note that the exon-1 containing probe did have an observable decrease. In control nuclei exon 1 signal is strong indicating that RNA polymerase II has transcribed exon 1 more often than the full-length mRNA. In DEM-treated nuclei, RNA polymerase II has the same signal for full-length mRNA, indicating no difference in the overall rate of transcription. Yet, the exon-1 signal is decreased in DEM-treated nuclei. Thus, they may lack this pre-initiation complex that control nuclei have for the eNOS gene. However, this does not reflect the capacity of DEM-treated nuclei to elongate eNOS pre-mRNA transcript fully, as DEM-treated nuclei have the same intensity of signal for full-length pre-mRNA as control nuclei. The enhanced 5'-end signal may be important to the normal course of processing for eNOS mRNA. This has been described for other genes including *c-myc*, Hsp70 and HIV-1 (361). However, this does not appear to be a major operative mechanism here.

mRNA stability is now increasingly recognized as a key regulator in steady-state gene expression. The interactions between cis-RNA elements and trans-RNA binding proteins determine the stability of the mRNA. Certain primary and secondary cis-elements confer stability or instability, and thus, mRNA turnover.

As mentioned in the introduction, AU-rich elements (AREs) are found in unstable mRNAs. In mammalian cells, AREs are the most commonly found cis-elements that determine stability (337). mRNAs of cytokines such as TNF-α (337) and proto-oncogenes, such as *c-fos* (362), *c-myc* (363) and GM-CSF (362) contain AREs. The AU-rich elements have been divided into three

classes. Until recently, it was thought that the pentamer, AUUUA, was the important destabilizing element; however, now it is recognized that the nonamer, UUAUUUA(U/A)(U/A) is the key destabilizing cis-element (364). The 3'UTR of GM-CSF contains a number of nonamers in an AU-rich context. When 51 nt of the 3'UTR of GM-CSF was inserted into the very stable β-globin mRNA, the chimeric fragment induced destabilization (365). This was a key experiment which helped to elucidate the nonamer as an important cis-element.

Generally, the number of nonamers contained within a 3'UTR determines the stability for an mRNA (366). The destabilization effect seems to brought out by a higher number of nonamer copies. However, a single nonamer has been reported to sufficiently destabilize plasminogen activator inhibitor type II mRNA (367). This is in keeping with prior ideas that at least 3 AUUUA motifs are critical for destabilization (368). ARE containing mRNAs have been divided into three classes (362, 369). Class I mRNAs have 1 - 3 copies of scattered AUUUA motifs in a U-rich context. An example of a Class I mRNA is *c-fos*. Class II AREs have multiple AUUU elements that overlap and give rise to nonamers such as in GM-CSF (366). Class III mRNAs do not contain AUUUA motifs, but have U-rich regions. *c-jun* is an example of this class (370). These mRNAs seem to rely on shortening of the poly(A) tail prior to mRNA degradation (362, 363, 371).

Eukaryotic mRNAs are degraded by several different mechanisms. The pathway that a specific mRNA follows sometimes depends on the presence or absence of the poly(A)tail; however, decapping can occur in its absence. Three mechanisms defined to date include: 1) deadenylation-dependent 5'-to-3' exonucleolytic decay, 2) deadenylation-independent decapping followed by 5'-to-3' exonucleolytic decay, and 3) endonucleolytic cleavage (372). Shortening of the poly(A)tail to ~ 25 - 60 A's makes an mRNA more susceptible to be a substrate for decapping reactions. This exposes the mRNA to 5'-to-3' exonucleolytic decay (371, 373). This decapping occurs irrespective of the species of mRNAs and represents a general pathway in mammalian and

yeast cell types (374). Eukaryotic mRNAs can be degraded in a 3'-to-5' direction following deadenylation; however, mRNAs can also exist without a poly(A)tail (373). Histone mRNA exists, for instance, without a poly(A)tail (375). In deadenylation-dependent mRNA decay, differences in destabilization rates result from differences in the rate of shortening and subsequent decapping and exonucleolytic decay. AU-rich elements promote deadenylation, and a fast rate of 5'-to 3' exonucleolytic decay after decapping (371). Decapping can occur regardless of poly(A)tail length in deadenylation-independent pathways. This occurs especially when there are nonsense codons and ensures rapid degradation of incorrectly synthesized mRNAs. This prevents improperly spliced RNAs and RNAs with incorrect early sequences from entering the cytoplasm (376, 377). Another mechanism by which mRNAs can be degraded prior to deadenylation is endonucleolytic cleavage. TfR mRNA fragments are observed (378). Deadenylation is not required as the 3'- fragment remains polyadenylated (378). Although it is clear that iron and oxidative stress control mRNA stability of TfR, it is not clear what stimulates endonucleases to cleave this mRNA. There are AU-rich elements in the 3'-UTR that most likely play a role in conferring instability. The well characterized iron response element (IRE) functions as a stabilizing element within the TfR 3'-UTR upon binding of the IRP-1 protein and responds to depletion of intracellular iron levels (378). It is thought that high iron concentrations enhance hydroxyl radical formation leading to destabilization of this complex. However, short periods of exposure to H_2O_2 or NO actually enhance binding (336, 343). Thus, low physiologic levels of oxidizing conditions can enhance binding; however, glutathione depletors in excess decrease binding (379).

Another set of RNA-binding proteins affected by redox state are trans-factors, known as AU-rich binding proteins. They facilitate AU-rich elements to confer stability or instability upon their cognate mRNAs. AUBFs include AUF-1, also known as heterogeneous nuclear ribonucleoprotein D (hnRNP D). In the case of *c-myc*, binding partially accelerates degradation (380). AUBFs play an important role in the fate of TNF-α, IL-1β, GM-CSF and ICAM-1 (337). Specific cysteine residues are important in binding and stability of these mRNAs upon oxidative

stress (338). In summary, AUBFs have been described to bind AREs in the 3'UTR of a number of mRNAs.

Sometimes degradative elements can exist in the coding region. This occurs in *c-fos*, *c-myc* and β-tubulin and can result in significant alteration in mRNA half-life (381). *c-fos* contains two destabilization signals in the middle of the coding region (382), while *c-myc* also contains destabilization signal in the latter portion of the coding region correlated to the COOH-terminal (383). β-tubulin mRNA stability depends upon the intracellular concentration of tubulin monomers. This destabilization signal occurs in the first 12- to 13-coding nt and is only recognized on a translating ribosome (384).

It is possible with respect to eNOS mRNA that AUBFs may play a role. Human eNOS mRNA contains a sequences in the 3'UTR similar to the nonamer, 5'-GUAUUUAUU-3' (385). Sequence alignments across species, including human, bovine, porcine and murine eNOS cDNA indicate the degenerate nonamer is conserved (86). This indicates this ARE nonamer may play a role in determining the metabolic fate of eNOS. It may be that binding proteins interact with the ARE under basal conditions. When cellular activation leads to decreased eNOS mRNA expression upon treatment with TNF-α (90), oxLDL (100) and AGEs (259), these binding proteins may no longer interact with the degenerate nonamer and stabilize eNOS mRNA. It is plausible that oxidizing conditions may prevent or attenuate binding of stabilizing RNA-binding proteins, as GSH depletion affects thiol state of cysteine residues (326). Often, cysteine residues mediate nucleic-acid binding by proteins. Oxidizing conditions could thereby alter binding, and may have functional effects.

Platelet endothelial cell adhesion molecule (PECAM/CD31) is similar to eNOS with respect to its 3'-UTR and response to cytokine activation (386). Normally, this mRNA transcript has a half-life of 15 h. Following endothelial activation with cytokines, mRNA half-life decreases to 4 h

as is observed for eNOS. PECAM (CD31) has two isolated nonameric sequences within its 3'-UTR, one degenerate and one classical. The presence of an ARE nonamer could predispose this mRNA to be labile. However, PECAM mRNA transcript is stable in endothelial cells under control conditions, just as eNOS is (385). This apparent paradox suggests that the nonamer is suppressed under basal conditions and further indicates that the ARE nonamer may play an important role in determining eNOS mRNA fate. This is particularly important in view of the fact that both eNOS and PECAM are destabilized during cellular activation. It might explain increased decay or instability of eNOS mRNA as well. Elucidation of how their 3'-UTRs function may lead to further insight into how endothelial genes are controlled in response to various stimuli. The effects of oxidative stress on AUBFs influence redox-sensitive genes, such as TfR, TNF-α, IL-1β, GM-CSF and ICAM-1. The cytokine TNF-α, in particular, can affect endothelial genes by decreasing stability of eNOS and PECAM mRNA. It will be of interest to determine the effects of glutathione depletion on steady-state PECAM (CD31) mRNA levels.

In the current study, we examined the effects of glutathione depletors and ROI on endothelial-derived vasomediator mRNA and protein expression. Criteria used to determine that GSH is depleted and that endothelial cells are activated included GSH and non-protein sulfhydryl assays, as well as induction of HO-1 (251) on northern blot analyses and nuclear run-on assays. Nonprotein sulfhydryl assays showed a decrease of total intracellular GSH upon treatment with glutathione depletors. Three reagents were used to deplete endothelial glutathione content. The kinetics of glutathione depletion most likely differ between them, but this was not rigorously evaluated within the context of this thesis (Fig. 6). However, this is an area for future study. DEM and phorone are alkylating agents that conjugate GSH and thus, can act quickly. They are substrates of GSH-S-transferases, and are conjugated to the sulfur atom of cysteine within glutathione (301). BSO blocks γ-glutatmylcysteinyl synthase, the initial rate-limiting step in GSH synthesis.

The rate of change in GSH may be more important than the actual intracellular GSH content. DEM and phorone cause a rapid decrease in eNOS mRNA expression (Figures 1, 2 and 4). BSO is predicted to act more chronically over a 48 h period with repeated dosings. Since these agents act differently, they may induce different kinetics of GSH depletion in this model as they are reported to do so in individual dosings. The rate of GSH depletion may play distinct and separable role from the absolute level of GSH. A faster depletor may induce stress more quickly. Mitochondrial GSH may also be affected differentially by these agents. Although medium is not replaced during the treatment period of 48 h, trace amounts of external glutathione may be recycled. Cells transfected with γ-glutamyl transpeptidase can effectively bypass glutathionedepletion by recycling GSH present in the medium (387). Some endothelial cells express, but can also take up cystine efficiently (388-396). Ischemic injury could be an in vivo example of acute oxidative stress. This type of injury would cause a fast drop in intracellular GSH content. Here, we propose that the kinetics of GSH depletion, such as the quick falls reported to be induced by DEM and phorone, may activate transcriptional processes. On the other hand, an example of chronic oxidative injury, such as those caused by long-term slow release of oxidants, is the AGEs. Slower or longer depletion of GSH may not cause the same "stress" response that acute changes elicit. Chronic oxidative stress may lead to both, changes in transcription, an post-transcriptional control processes. AGEs also cause a decrease in eNOS mRNA and protein expression (259). Lastly, cysteine can accumulate during BSO administration, and glutathione-S-transferases can utilize this amino acid as a substrate. This reasoning could explain differences between agents' effects on eNOS mRNA expression. As a whole, GSH depletion by independent agents decreased eNOS mRNA expression. Rates of change in oxidative stress within the diseased cardiovascular system may well represent a newer and exciting area for study. It remains plausible that acute depletion of intracellular GSH induces a distinct group of mRNAs through changes in transcriptional processes, whereas chronic GSH depletion selectively effects another group of mRNA species. The effects of chronic cellular oxidative stress may involve both transcriptional and post-

transcriptional regulatory processes. Further work in this area is necessary and may be illuminating.

Others have examined the effects of short-term glutathione depletion on NO production. These experiments addressed the hypothesis that thiol and intracellular GSH affect NO production. The first study testing the effects of GSH depletion on NO production were performed in BAEC and indicated no difference in NO production content following administration of BSO (397). A subsequent study was carried out using 20 mM DEM for 30 min. The very short time course precluded effects on eNOS expression, but took into account interactions of NO with thiols. Although there was decreased NO production, the effects were not substantive considering the increased lactate dehydrogenase levels reflecting cytotoxicity (398). Some of the differences observed may be species-related. However, another group studied the effects of GSH depletion in HUVEC and found decreased conversion of L-arginine to L-citrulline under basal and calcium ionophore-stimulated conditions. A small decrease in cGMP levels were observed; however, the addition of sodium nitroprusside, an NO-donating agent, was able to circumvent the decrease. This indicates that the observed decrease in cGMP occurs at the level of NO production. Decreased GSH does not affect cGMP directly, but by decreasing production of NO. This thesis work addresses the mechanism by which NO is decreased following chronic glutathione depletion.

Our work with a chronic model in endothelial GSH depletion also addressed expression of HO-1. HO-1 degrades heme into free iron, carbon monoxide (CO) and biliverdin, an antioxidant. CO plays a role in hepatic sinusoids and can promote dilation and increased blood flow in these areas (399, 400). CO also acts on soluble guanylyl cyclase to increase cGMP levels, just as NO does. Perhaps the decrease in eNOS may be a compensatory mechanism for the increase in HO-1. It has been suggested that there may be interrelationships between NOS and HO, because of the "uncanny similarity" between NO- and CO-generating systems (401). Time course studies showed a time-dependent increase of HO-1 mRNA following DEM addition. This effect was time-

dependent and evidenced maximal induction at 48 h (Fig. 1). HO-1 is known to increase upon GSH depletion (402, 403). Thus, the induction of this stress protein, Hsp32 (HO-1), represents GSH depletion reflecting that cells are in a state of "stress". This work is the first to show that chronic GSH depletion by DEM can induce expression of HO-1 mRNA in vascular endothelium.

Although, there appears to be an inverse relationship between HO-1 and eNOS mRNA in this work, there are instances where HO-1 and eNOS may actually both increase. For instance, recent reports have shown that H_2O_2 generated by oxidase systems, increases eNOS mRNA expression in BAEC with a maximum induction at 8 h and return to control by 24 h (404). Endothelial cells are well-equipped with antioxidant defense mechanisms. Cells produce antioxidants in response to H_2O_2, one of which is biliverdin arising from the induction of HO-1. H_2O_2 does induce expression of HO-1 mRNA. Cells undergo a transiently induction of HO-1 mRNA resulting in the formation of antioxidants biliverdin and bilirubin (305). Cells are also equipped with other defense mechanisms against H_2O_2, such as GPx and catalase, which are also induced by H_2O_2. It is possible that H_2O_2 might affect binding of trans-factors by inducing intracellular antioxidant production. It is not yet clear what leads to the increase in eNOS by H_2O_2. There have been reports that antioxidant response elements exist in the promoter of eNOS (405). Increased binding to a distal antioxidant response element and increased transcription of eNOS is described with antioxidant treatment nordihydroguaiaretic acid (405). Antioxidants, generated as a defense to short-term oxidative stress, could act on elements in the promoter of eNOS to increase transcription (405). Threshold levels of DEM (5 μM) seem to prime the cells for ROI injury induced by H_2O_2. In this case, antioxidant defense mechanisms are utilized by both stresses that may be overwhelming. Threshold DEM may block the increase in antioxidants which H_2O_2 is capable of inducing and result in a longer-term or chronic oxidative stress. This indicates that threshold DEM with H_2O_2 causes a change such that oxidative stress is induced and eNOS mRNA expression is attenuated. Thus, it may not be GSH depletion alone, but ROI injury, in addition, that alters eNOS mRNA expression.

Functional assays were performed to determine whether the effects of oxidant treatment were reflected in differences in enzymatic activity. To test whether NOS activity was affected or not, its catalytic activity was measured by the conversion of radiolabelled substrate to specific product for a fixed period of time. These in vitro enzymatic assays confirmed the functional relevance of DEM treatment. Functional activity over a 48 - 60 h period reflects changes in eNOS mRNA and protein expression. NOS activity was decreased at baseline with DEM-treatment. This most likely reflects a decrease in eNOS steady-state protein. However, upon stimulation with calcium ionophore or histamine, DEM-treated HUVEC were still able to respond. Surprisingly, DEM-treated HUVEC were able to enhance conversion of L-arginine to L-citrulline from basal levels upon stimulation, although clearly this response was attenuated compared to control cells (26). There appears to be a basal decrease of eNOS protein, but responsiveness to stimuli still exists. GSH depletion may increase Hsp90 in this process. This heat shock protein acts as a molecular chaperone for eNOS (57). Thus, induction of Hsp90 by GSH depletion could allow for a continued response to stimulation following a decrease in eNOS steady-state protein. Histamine is known to activate endothelial cells and increase association of Hsp90 with eNOS enzyme (57). Thus, Hsp90 modifies eNOS so that it can either translocate or be more active. Histamine activates eNOS to produce more NO. Histamine can enhance NO production promoting vasodilation in vascular endothelium. In the lung however, histamine induces bronchiole constriction counteracting NO's relaxing effects in the respiratory system (406). As in vitro findings indicated a decrease in histamine-stimulated L-arginine to L-citrulline conversions, in vivo studies were performed.

The effects of DEM were examined in the lung and the kidney. The in vitro effects were confirmed by in vivo data indicating the relevance of these findings to the whole organism. In multiple organs, DEM treatment was observed to decrease steady-state eNOS protein and mRNA expression. In vivo findings indicate broader implications for the in vitro work, where DEM

depleted glutathione, and decreased steady-state eNOS mRNA and protein expression in the lung and the kidney. Functional studies indicated that DEM decreased basal NOS activity as well as calcium-stimulated activity, indicating that decreases in eNOS mRNA and subsequent decreases in eNOS protein, cause a fall in functional NO formation both under basal conditions, and under stimulated conditions. Overall, these results indicate glutathione levels and intracellular oxidation-reduction reactions in endothelial cells alter gene expression of endothelial-derived vasomediators in endothelial cells and in animals.

These results have implications for human disease. For instance, several stimuli known to affect redox state, such as TNF-α, oxLDL and AGEs, also decrease eNOS. Endothelium-dependent vascular relaxation is abnormal in hypercholesterolemia, atherosclerosis, diabetes and hypertension (346). Studies confirm that eNOS mRNA expression is decreased in atherosclerotic patients compared to controls (91). Dysfunctional regulation of vasomotor tone in hypercholesterolemia and atherosclerosis is associated with increased oxidative stress, as oxygen free radicals abolish endothelium-dependent relaxation (347). On the other hand, augmenting glutathione production, enhances endothelium-derived NO in patients (348). Recent studies in animals, have shown that glutathione enhances vasodilation through NO pathways (407). The findings of this thesis work confirm the opposite effect. GSH depletion decreases eNOS mRNA and protein expression in vitro and in vivo resulting in functional effects. Therefore, this work, in conjunction with the above studies, has therapeutic implications.

This thesis work demonstrates that glutathione depletion resulted in decreased eNOS mRNA and protein expression in vascular endothelium. What has not been discussed here are the effects of oxidative stress on other cell types known to express eNOS. Cardiac myocytes express eNOS where eNOS is coupled to muscarinic receptor activation. Thus, eNOS enhances cGMP levels, decreases L-type voltage-sensitive Ca^{2+} channel current and has negative chronotropic effects in vitro (408-412). Oxidative stress is known to have important functional implications for

cardiac myocytes in the setting of varied forms of heart disease (413-415). If eNOS regulation is disrupted by oxidative stress, this may lead to cardiovascular dysfunction. eNOS-derived NO not only plays a role in the vascular system, but also in the nervous system. eNOS is expressed by CA1 neurons (39). The roles of the different NOS isoforms in long-term potentiation have been explored using genetic modelling. eNOS has been shown to play a major role in long-term potentiation (LTP) (38, 416). Oxidative stress has been implicated in Alzheimer's disease, where it is believed LTP is disturbed. β-amyloid that forms a component of senile plaques associated with Alzheimer's enhances cellular oxidative stress (417). Notably, β-amyloid decreases EDRF/NO (418). Thus, oxidative stress may affect eNOS enzymatic activity and possibly, its expression in CA1 neurons. Decreases in NO would have fundamental implications in the nervous system, as NO plays a role in the formation of memory. Finally, eNOS is also expressed in syncytiotrophoblasts. Oxidative stress has been implicated in pre-eclampsia and may contribute to dysregulation of vascular tone in this condition. These effects could occur if oxidative stress decreases expression of eNOS (419-425).

In addition to eNOS, two other endothelial-derived vasomediator genes were evaluated. The effects of GSH depletion on ECE-1 and ppET-1 mRNA expression were tested. Glutathione depletion caused a decrease in steady-state ppET-1 mRNA expression by 2 h that persisted for 24 h. Considering the short half-life of ppET-1 mRNA of approximately 15 - 30 min under basal conditions and that no studies have reported a decrease in ppET-1 mRNA stability, it is likely that this decrease is mediated by changes in transcriptional activity (133). Nuclear run-on analyses demonstrated that ppET-1 mRNA initiated transcripts were fewer in DEM-treated HUVEC compared to vehicle control. It appears that there is an active repression of transcription, considering full-length probes on nuclear run-on assays had decreased signal after 48 h of DEM treatment and some decrease was observed by 2.5 h. This may indicate a dysfunction in endothelial cells in response to DEM exposure for gene expression of vasomediators, and a resulting imbalance between vasoconstrictor and vasorelaxant substances. Recent studies have

demonstrated that H_2O_2, or oxidase systems generating peroxide, diminish ppET-1 mRNA and secreted ET-1 (390, 426, 427). Here, we have defined a potential mechanism for this decrease in transcription. Although our hypothesis predicted that oxidative stress might cause an increase in ppET-1 mRNA expression, this was not observed. It may be that chronic or overwhelming oxidative stress causes a decrease in ppET-1 mRNA through effects on transcription. DEM may modify critical thiols of transcription factors, such as AP-1 that is required for transcription of ppET-1. It is also possible that an early increase in ppET-1 expression was missed. Other stimuli such as phorbol ester can cause a rapid induction and later decrease in ppET-1 mRNA. Since we were interested in studying the long-term and chronic effects of glutathione depletion, earlier time points were not assessed. Future work may need to address this issue.

The decrease in ppET-1 in conjunction with the decrease in eNOS mRNA expression most likely indicates a loss of paracrine control of local blood flow. Interestingly, CO produced via induction of HO-1 is known to decrease ppET-1 mRNA expression (428). As HO-1 mRNA expression was increased in this study, CO derived from HO-1 may play a role in the decrease we observed in ppET-1 mRNA expression in response to DEM treatment. The effects of locally-derived CO on vasomotor tone is an evolving story. In this study, DEM treatment caused an increase in transcriptional activity of the HO-1 gene as early as 2.5 h. This induction is still evident at 48 h.

With regard to ECE-1, there are no observable changes in transcriptional of the human ECE-1 gene following chronic glutathione depletion.

Overall, this study demonstrates that GSH depletion leads to alterations in gene expression of endothelial-derived vasomediators, eNOS and ppET-1. Results demonstrate that the agents used depleted GSH, as confirmed by GSH assays and the sustained induction of HO-1. This causes dysregulation of endothelial-derived vasomediator genes. Considering that TNF-α,

oxidized LDL, and AGEs decrease eNOS mRNA expression, redox reactions may represent the underlying cause of dysregulation of endothelial-derived vasomediator genes. Oxidizing conditions represent a unifying theme for decreased eNOS mRNA expression. eNOS mRNA and protein expression were decreased in response to independent agents that induced GSH depletion. ROI injury by H_2O_2 may play a role, in addition to GSH depletion, in regulating eNOS mRNA expression as demonstrated by experiments using a combination of H_2O_2 and threshold DEM. The observed decrease in eNOS mRNA upon DEM treatment is due primarily to decreases in mRNA stability, with some minor contributions of transcription as assessed by nuclear run-on analyses. Functional assays demonstrated decreases in enzymatic activity corresponding to the fall in eNOS mRNA and protein expression. Finally, eNOS mRNA and protein expression were decreased upon glutathione depletion by DEM in an in vivo setting. The decrease in animals also occurred over the relatively long time period of 48 h.

Thus, chronic oxidative stress may affect post-transcriptional events mainly, and some transcriptional events to regulate gene expression of specific mRNAs. Redox state most likely affects RNA processing at several different stages. Oxidizing condition may pre-empt transcriptional pausing and the formation of complexes essential for proper reading by RNA polymerase II. As of yet, no functional effects have been observed for eNOS mRNA stability as a result of less transcriptional pausing. On the other hand, oxidizing conditions, or sub-optimal GSH levels may also alter thiol state of redox-sensitive RNA-binding proteins at the degenerate nonamer. This is a more established model of how mRNA stability is affected by redox, as examples were discussed on AUBFs' sensitivity to redox state and the functional modulation of mRNA stability. In the cases of TNF-α, IL-1β, GM-CSF and ICAM-1, oxidizing conditions enhance binding and stabilization, but extensive GSH depletion prevent binding (338). In the case of eNOS, basal conditions may favour AUBF-binding and extensive GSH depletion could inhibit AUBF-binding and could lead to decreased mRNA stability. Although the maximal decreases in

eNOS mRNA were observed by 48 h, in contrast, decreases for ppET-1 mRNA were observed at 2 h.

ppET-1 mRNA expression was decreased in response to DEM treatment. This decrease appears to be due to changes in transcriptional activity. Decreasing effects could be attributed to a mRNA general decrease in cellular activity; however, the early and sustained induction of HO-1 mRNA expression affirm the effects are specific. Increases of CO by HO-1 induction have been reported to decrease ppET-1 (428). This may be due to a decrease in transcription. It appears that acute oxidative stress has transcriptional effects, such as the induction of HO-1 mRNA and the repression of ppET-1 mRNA. It is important to note that eNOS and ppET-1 are decreased in by the same condition. The other conditions, such as TNF-α, oxLDL snd AGEs, which cause oxidative stress cause a decrease in eNOS, while increasing ppET-1 mRNA expression. Even stimuli such as proliferation/injury also cause an inverse regulation of eNOS and ppET-1 mRNA expression: eNOS is decreased; whereas, ppET-1 is increased. In this study, both endothelial-derived vasomediator genes had decreased mRNA expression. This indicates *in vivo* other systems, such as the autonomic nervous system and the endocrine systems or myogenic tone may predominate in the control of vascular tone when the endothelium no longer expresses vasomediators. Lastly, ECE-1 mRNA expression and transcriptional activity was not altered by glutathione depletion, confirming the above effects are specific.

Although, the molecular mechanism underlying changes in vasomediator gene expression have not been elucidated, from this work glutathione depletion causes a decrease in eNOS mRNA expression that is likely mediated by a decrease in eNOS mRNA stability, with some minor effects on transcription. Studies on TNF-α and oxLDL, stimuli that have redox as a common factor, cause a similar decrease in eNOS mRNA mediated by changes in mRNA stabiltiy. Thus, a decrease in mRNA stability is predicted to be at least partially responsible for the effect of AGEs on eNOS mRNA expression as well. These effects are observed over a long course of exposure, and

constitute a chronic stress. On the other hand, acute changes in GSH induce expression of HO-1 mRNA, while repressing ppET-1 mRNA expression. DEM exposure stimulated transcriptional processes to induce expression of HO-1 mRNA. This acute oxidative stress also caused an active repression of ppET-1 mRNA expression. It appears that acute oxidative stress may be responsible for changes in transcriptional events, and subsequently affects gene expression. Chronic oxidative stress may affect post-transcriptional processes moreso than transcriptional events, such as is the case for decreased expression of eNOS mRNA. In conclusion, chronic glutathione depletion decreases eNOS and ppET-1 mRNA expression. Glutathione depletion induces an increase in HO-1 mRNA expression and sustains it over a long period of time. This condition does not affect ECE-1 mRNA expression. Glutathione depletion affects transcriptional mechanisms in decreasing ppET-1 and increasing HO-1 mRNA expression. It also affects mRNA stability in decreasing eNOS mRNA expression. Thus, chronic glutathione depletion causes inappropriate expression of endothelial-derived vasomediator genes in vascular endothelium, and may represent an underlying and unifying factor in vascular disease induced by a broad range of initiating insults.

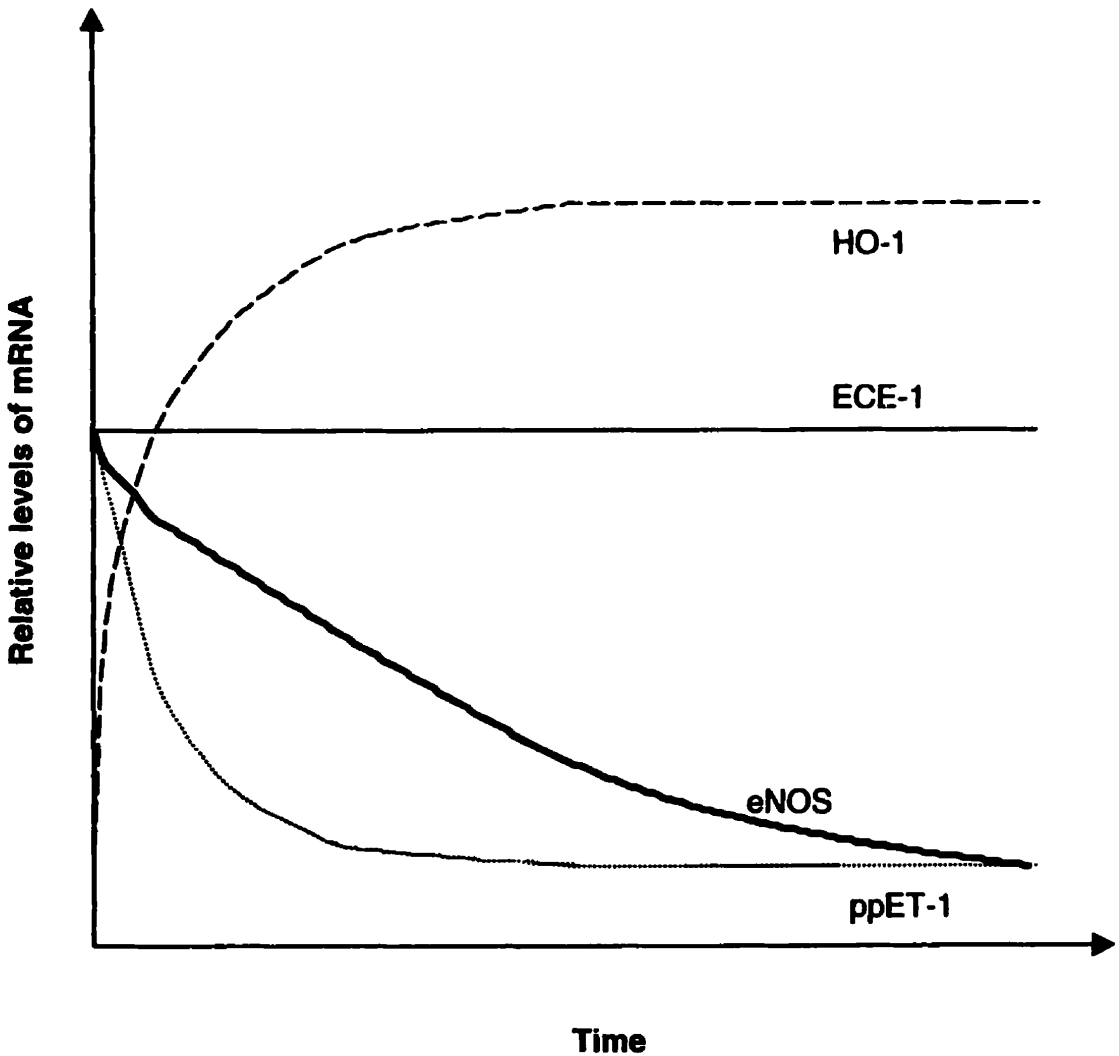


Figure 11. Schematic summary of changes in eNOS, ppET-1, ECE-1 and HO-1 steady-state mRNA expression following chronic glutathione depletion.

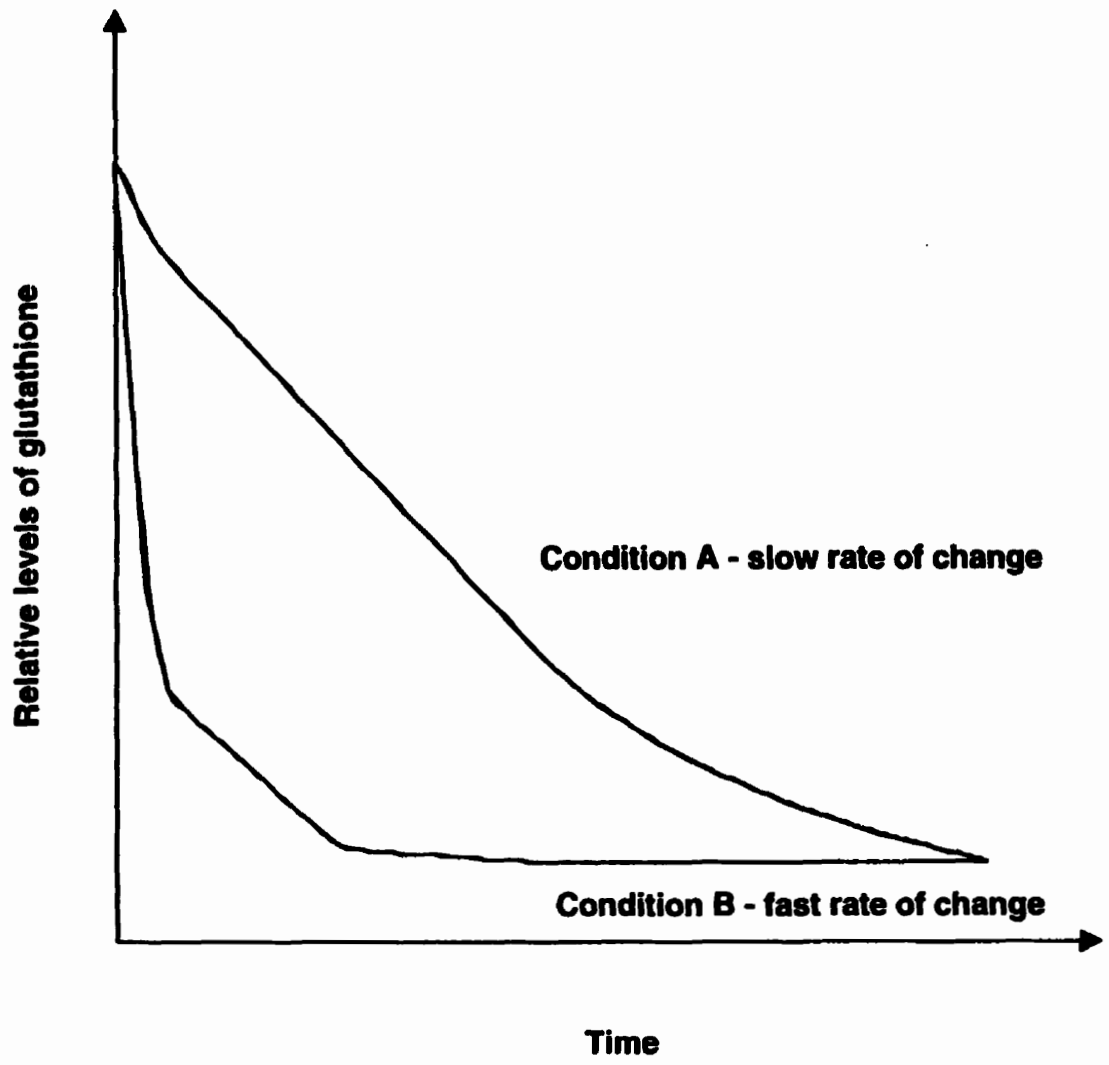


Figure 12. Different kinetic models for depletion of intracellular glutathione to a common endpoint.

IV. FUTURE STUDIES

Several stimuli can decrease eNOS mRNA expression in vascular endothelium. To determine the importance of oxidative stress in relation to other stimuli (e.g. TNF-α), and its possible role in mediating effects of downregulating stimuli, the hypothesis that oxidizing conditions may underlie the effects of various agents on eNOS needs to be tested. A series of studies in endothelial cells can be performed to assess this question. Cells can be treated with stimuli known to decrease eNOS mRNA expression. Thus, TNF-α, oxLDL or AGEs can be applied. In a parallel set of cells, the treatments can be performed in combination with antioxidant defense mechanisms. SOD, catalase, GSH esters or GSH precursor, N-acetylcysteine can be added. In this manner, specific ROI or levels of GSH can be distinguished as to their importance in the regulation of eNOS mRNA expression. If the effects of antioxidants outweigh those of the downregulating stimuli, redox chemistry would have an influential contribution to the steady-state levels of eNOS mRNA. From this point, it would be important to determine at which level the antioxidants acted. They may increase transcription through specific cis-regulatory elements in the promoter. Antioxidants could also affect specific RNA-binding proteins.

This thesis work has reconfirmed the importance of understanding eNOS mRNA expression. Understanding the determinants of eNOS mRNA stability is an evolving field. By constructing RNA chimeras that contain a reporter open-reading frame and different deletional and mutant constructs of eNOS 3'UTR could lead to dissecting out the elements that enhance mRNA stability. Transfecting these constructs into endothelial cells and non-endothelial cells, assessing steady-state mRNA and protein expression from transfections and comparing observations may elucidate the cis-element and potential trans-factors essential for RNA processing. These transfected cells can then be exposed to a series of treatments to determine the effects of oxidative stress on various RNA constructs. These studies would help to elucidate what components of RNA are essential for mRNA stability and which factors in endothelial cells are critical for

producing highly stable mRNAs. RNA-protein interactions could be monitored using gel electrophoresis assays using protein extracts and determining whether different cellular treatments affect the interactions. In addition, the RNA-protein interactions may be modified outside of a cellular system by redox-induced changes in thiol state of binding proteins.

Since this thesis work demonstrated a decrease in ppET-mRNA expression in concert with decreased eNOS mRNA expression, the physiological relevance of cellular and mechanistic findings would need to be addressed. Monitoring blood pressure during DEM-treatment of animals would indicate whether glutathione depletion had an overall effect on cardiovascular hemodynamics in the animals. Aortas could be isolated for determining EDRF bioactivity.

The kinetics of glutathione depletion is an unexplored field. The kinetics by which glutathione depletion occurs may have effects on the cellular expression of various genes, in disparate ways. Since DEM and phorone act by conjugating GSH, these agents can act quickly to deplete glutathione. BSO, on the other hand, specifically inhibits the rate-limiting enzyme involved in synthesizing GSH. Therefore, BSO may lead to a slower rate of glutathione depletion. Thus, it is plausible that the rate of glutathione depletion may play an important role in addition to the absolute levels of glutathione in mediating effects on gene expression. Studies determining the effects of the rate of glutathione depletion on endothelial gene expression would lead to a better understanding of the molecular mechanisms implicated in how oxidative stress induces endothelial dysfunction.

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