University of Alberta

Oxidative Stress as a Modulator of Vascular Function: Implications for Vascular Adaptations to Pregnancy and Preeclampsia

by

Christy-Lynn Marie Cooke 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

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We the undersigned hereby grant permission to **Christy-Lynn M. Cooke** to include our accepted publication "The Receptor for Advanced Glycation End Products (RAGE) is Elevated in Women with Preeclampsia" in her thesis, entitled "Oxidative Stress as a Modulator of Vascular Function: Implications for Vascular Adaptations to Pregnancy and Preeclampsia". This article has been published in "Hypertension in Pregnancy".

Sincerely,

Jerry C. Brockelsby Date: July 23/05

Philip N. Baker Date: July 20, 2003

Sandra T. Davidge Date: July 28/03

University of Alberta

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Oxidative Stress as a Modulator of Vascular Function: Implications for Vascular Adaptations to Pregnancy and Preeclampsia** submitted by **Christy-Lynn Marie Cooke** in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Prof. L. Poston (external examiner)

Dr. B. F. Mitchell

Dr. S. L. Archer

Dr. S. Jacobs

Dr. L. J. Guilbert

Dr. S. T. Davidge

Date: May 26, 2003

ABSTRACT

Pregnancy encompasses dramatic changes to the maternal cardiovascular system which are vital to a healthy pregnancy. In order to accommodate the large increase in blood volume and cardiac output, the maternal vascular resistance decreases, likely due to increased endothelial-derived vasodilators. Preeclampsia, characterized by maternal hypertension and proteinuria, is a severe obstetrical complication affecting approximately 5% of all pregnancies. Although the causes of preeclampsia are unknown, the pathophysiology involves a central endothelial cell dysfunction, possibly mediated by pro-oxidant molecules. Indeed, our laboratory previously found that peroxynitrite is elevated in maternal vessels from women with preeclampsia. However, the role of peroxynitrite in mediating endothelial dysfunction is not fully understood. Furthermore, the mechanisms behind normal vascular adaptations (increased endothelial-dependent relaxation) in pregnancy are still controversial. The central hypotheses of this thesis are: 1. peroxynitrite alters endothelial cell (NOS and PGHS) pathways, leading to reduced vasodilation, thus contributing to vascular dysfunction and 2. mouse pregnancy is associated with enhanced endothelial-dependent vascular relaxation, mediated in part by nitric oxide synthase (NOS), prostaglandin H synthase (PGHS) and cytochrome P450-derived epoxyeicosatreinoic acids (EETs). Our results suggest that peroxynitrite inhibits prostacyclin synthase levels in endothelial cells and increases iNOS through NFκB. This creates a possible positive feed-back loop, consistent with the progression of

preeclampsia. We also found that the novel pro-oxidant pathway, RAGE, is elevated in preeclampsia, which may be a potential source of peroxynitrite in vessels from these women. In an animal model of oxidative stress, the SOD knockout mouse, endothelial-dependent relaxation was significantly impaired in systemic vessels, due to reduced NOS and PGHS mediated relaxation. Finally, due to a paucity of knowledge about vessel function in normal mouse pregnancy, we elucidated that endothelial-dependent relaxation is enhanced in both mesenteric and uterine arteries in pregnant mice, largely due to increased PGHS-mediated vasodilation. The findings presented in this thesis expand our understanding of how pro-oxidants alter the endothelium, and whether these alterations are relevant in mediating abnormal vascular adaptations to pregnancy in women with preeclampsia.

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LIST OF ABBREVIATIONS AND UNITS

The following abbreviations, definitions and units have been used throughout this thesis.

°C ..... degrees Celsius

-/- ..... homozygous knockout mice

-/+ ..... heterozygous knockout mice

+/+ ..... wild type (control) mice

AA ..... arachidonic acid

ad libitum ..... (Latin, 'without preparation')

AGE ..... advanced glycation end products

ANOVA ..... analysis of variance

AT_1 ..... angiotensin 1 receptor

ATP ..... adenosine triphosphate

B_2 ..... bradykinin receptor

BH_4 ..... tetrahydrobiopterin

bp ..... base pair

BP ..... blood pressure

BPH/5 ..... mildly hypertensive mouse strain

BSA ..... bovine serum albumin

Ca^{2+} .... calcium ion

$CaCl_2$ .... calcium chloride

cAMP .... cyclic adenosine monophosphate

cGMP .... cyclic guanidine monophosphate

CGRP .... calcitonin gene related peptide

cDNA .... complementary deoxyribonucleic acid

CO_2 .... carbon dioxide

C_T .... threshold cycle

CuZn SOD .... copper zinc superoxide dismutase

CYP450 .... cytochrome P 450 enzyme family

DAB .... diaminobenzidine

DAG .... diacylglycerol

5β-DHP .... 5β dihydroprogesterone

DMEM .... Delbecco's modified eagles medium

DMSO .... dimethyl sulphoxide

DNA .... deoxyribonucleic acid

dNTP .... deoxy nucleic acid triphosphate

DTT .... dithio-DL-threitol

EC_{50} .... effective dose eliciting 50% response

EC_{80} .... effective dose eliciting 80% response

ECL .... enhanced chemiluminescence

EDHF ..... endothelial-derived hyperpolarizing factor

EDTA ..... ethylenediaminetetraacetic acid di-sodium salt

EET..... epoxyeicosatrienoic acid

EEZE ..... 14,15 epoxyeicosa-5(Z)-enoic acid

EGTA ..... ethylene glycol bis (β-aminoethyl ether) N,N,N, tetra acetic acid

eNOS ..... endothelial nitric oxide synthase

et al...... *et alii* (Latin, 'and others')

EtBr ..... ethidium bromide

EtOH..... ethanol

FAD+..... flavin adenine nucleotide

g..... acceleration due to Earth's gravity (9.8 $m \cdot s^{-2}$)

g..... gram(s)

GPX-1..... glutathione peroxidase 1 gene

h..... hour(s)

H_2O..... water

H_2O_2 ..... hydrogen peroxide

HBSS..... Hank's Balanced Salt Solution

HEPES..... hydroxyethyl-1-piperazineethanesulfonic acid

i.e...... *id est* (Latin, 'that is')

IgG..... Non-specific mouse immunoglobulin G

IHC..... immunohistochemistry

iNOS ..... inducible nitric oxide synthase

IP ..... prostacyclin receptor

IP_3 ..... inositol triphosphate

kb ..... kilobase(s)

KCl ..... potassium chloride

kD ..... kilodalton

l ..... litre(s)

LDH ..... lactose dehydrogenase

L-NAME ..... N^G-nitro-L-arginine methyl ester

LSD ..... least significant difference

m ..... meter(s)

M ..... moles·l^{-1}

MECLO ..... meclofenamate

MEM ..... minimum essential medium

METH ..... methacholine

$MgCl_2$ ..... magnesium chloride

min ..... minute(s)

MLC ..... myosin light chain

MLCK ..... myosin light chain kinase

MLCP ..... myosin light chain phosphatase

mRNA ..... messenger ribonucleic acid

MS-PPOH .... N-methylsulphonyl-6-(2-proparglyloxyphenyl) hexanamide

n..... ..... number of animals or experiments

N/A..... ..... not applicable

NaAc..... ..... sodium acetate

NaCl .... sodium chloride

NAD(P)H .... coenzyme nicotinamide adenine dinucleotide (phosphate)

NaN_3..... ..... sodium azide

NCH .... Nottingham city hospital

N/D..... ..... non-determinate

NFκB..... ..... nuclear factor kappa B

N-terminal..... ..... amino-terminal

NO..... ..... nitric oxide

NOS..... ..... nitric oxide synthase

O_2..... ..... molecular oxygen

O_2^-..... ..... superoxide anion

OD..... ..... optical density

$OH^{\cdot}$ .... hydroxyl radical

p value .... probability (of incorrectly rejecting the null hypothesis)

PBS..... ..... phosphate buffered saline

PCR .... polymerase chain reaction

PDTC..... ..... pyrroline dithiocarbamate

PE .... phenylephrine

PEG-SOD .... polyethylene glycolated superoxide dismutase

pH .... logarithmic unit measuring acidity

PGI_2 .... prostacyclin

PKA .... protein kinase A

PKC .... protein kinase C

PKG .... protein kinase G

PLA_2 .... cytosolic phospholipase A_2

PXR .... pregnane X nuclear receptor

RAGE .... receptor for advanced glycation end products

RAH .... Royal Alexandra hospital

RNA .... ribonucleic acid

ROS .... reactive oxygen species

RT .... reverse transcription

s .... second(s)

SD .... standard deviation of the mean

SDS .... sodium dodecylsulphate

SEM .... standard error of the mean

SERCA .... sarco(endo)plasmic reticulum calcium pump

SIN-1 .... 3-morpholinosydnonimine N-ethylcarbamide

SNP .... sodium nitroprusside

SOD ..... superoxide dismutase

SOD-/- ..... superoxide dismutase knockout mouse

TBARS ..... thiobarbituric acid reactive substances

TBS ..... tris-buffered saline

TNFα ..... tumor necrosis factor-alpha

Tris-Cl ..... tris[hydroxymethyl]-amino methane hydrochloride

tRNA ..... transfer ribonucleic acid

V ..... volts

VCAM ..... vascular cellular adhesion molecule

VEGF ..... vascular endothelial growth factor

WT ..... wild-type

Mathematical prefixes

k..... ..... kilo (10^3)

c..... ..... centi (10^{-1})

m..... ..... milli (10^{-3})

μ..... ..... micro (10^{-6})

n..... ..... nano (10^{-9})

p..... ..... pico (10^{-12})

CHAPTER 1. LITERATURE REVIEW

1.1 PREGNANCY AND THE CARDIOVASCULAR SYSTEM: AN OVERVIEW

The cardiovascular system is the lifeline of the human body, supplying nutrients and oxygen to all tissues. Pregnancy is associated with adaptations to all aspects of the cardiovascular system; it undergoes dramatic alterations to fulfill the needs of the fetus. In order to supply adequate oxygen and nutrients to the developing conceptus, the maternal blood volume greatly increases, along with the cardiac output, which serves to drive the increased blood flow to the feto-placental unit. One fundamental adaptation that must occur concomitant with the other changes (in order to prevent an increase in blood pressure) is a reduction in the maternal peripheral vascular resistance. The mechanisms responsible for these changes include vascular remodeling and a blunted pressor response to vasoconstrictors, possibly mediated by a hormonal-induced increase in endothelial-dependent vasodilation. Maladaptations to this decrease in maternal vascular tone may contribute to conditions such as preeclampsia. Therefore, the primary subject of this thesis is the regulation of peripheral vascular resistance, with a special focus on vascular adaptations to pregnancy.

1.2 ARTERIAL STRUCTURE AND FUNCTION

Peripheral vascular resistance is primarily controlled by small resistance-sized arterioles. These vessels contribute substantially to overall blood pressure and are

responsive to endocrine, autocrine and nervous stimuli. The structure of these vessels is divided into three layers. The outer adventitia, a protective layer consisting mostly of connective tissue; the central media, which is subdivided into many layers of smooth muscle cells. The inner layer comprises the endothelium, which is one cell layer thick and is continuous, although not homogeneous, throughout the entire vascular system. The endothelium plays a crucial role in many different aspects of vascular homeostasis, including regulation of platelet and leukocyte activity, vascular permeability and vasoreactivity (76).

1.2.1 Mechanism of Smooth Muscle Contraction

Regulation of the contractile activity in vascular smooth muscle cells relies on the complex interplay between stimuli that promote constriction (vasoconstrictors) and those that inhibit constriction (vasodilators), such as circulating hormones, neurotransmitters, endothelium-derived vasoactive factors. Excitation-contraction coupling refers to the chain of processes that link a stimulus to the contractile response by a muscle, in which the increase in calcium plays a predominant role (53). The two major types of coupling in vascular smooth muscle cells are electrochemical and pharmacomechanical (59).

Electrochemical coupling involves membrane potential and ion channels, both of which play a large role in regulating calcium influx. Vascular smooth muscle cells express many different types of potassium channels, which are the dominant ion conductive pathway in these cells (59). As such, their activity contributes to membrane potential, due to electrochemical gradient inside versus outside the cell, thus opening potassium channels results in a diffusion of the cation out of the cell, inducing

hyperpolarization. In turn, this hyperpolarization closes voltage-gated calcium channels resulting in vasodilation, where as depolarization of the cell opens the calcium channels, causing vasoconstriction. Vascular smooth muscle cells contain different types of voltage gated calcium channels depending on the location and size of the blood vessel. For example, L-type calcium channels appear to play a particularly important role in the microcirculation, regulating myogenic reactivity and vasomotion (53).

Pharmacomechanical coupling occurs without a necessity for changes in membrane potential. Intracellular calcium stores are the major source of calcium that are liberated upon external stimuli. Upon stimulation by vasoconstrictors, activation of phospholipase C cleaves the phospholipid component of the plasma membrane phosphatidyl 1,5 inositol diphosphate, forming two messenger molecules inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to specific receptors on the sarcoplasmic reticulum and releases calcium. DAG is an activator of specific protein kinases C isoforms (PKC) which are necessary for the phosphorylation and activation of contractile machinery.

Another mechanism by which calcium levels increase in the cytosol is via an influx of extracellular calcium. This can occur through the interaction of ligands directly with receptor-activated calcium channels. Alternatively, plasma membrane calcium channels can become activated by a increase in intracellular calcium derived from cellular stores, termed store operated calcium channels. This secondary rise in calcium levels can serve to prolong the activity of cellular signaling pathways (53).

The changes in cytoskeletal proteins (actin and myosin) occur after the rise in intracellular calcium levels, which cause the activation of calcium-calmodulin sensitive enzyme, myosin light chain kinase (MLCK). This kinase requires binding of the calcium-

calmodulin complex for its activity, which will subsequently phosphorylate the myosin light chain (MLC). Phosphrolyation of the MLC allows for the myosin ATPase to be activated by actin (due to the liberation of the actin-binding site for the myosin heavy chain). Subsequently, the actin-mediated ATP hydrolysis causes the formation of actin-myosin crossbridges, the prerequisite for contraction. The phosphorylated light chains are then de-phosphorylated by myosin light chain phosphatase (MLCP), myosin returns to its inactive state and relaxation results (100).

1.2.2 Regulation of Smooth Muscle Contraction

Vasoactive agonists can modulate the extent of vascular smooth muscle contraction by altering the sensitivity of the contractile machinery to calcium, thus fine tuning vascular smooth muscle responses (Figure 1.1). Indeed, a major mechanism of agonist-induced calcium sensitization is the inhibition of MLCP (100). Phosphorylation by the Rho-Rho kinase and/ or PKC pathways of either the regulatory subunit of MLCP or a smooth muscle specific inhibitory phosphopeptide, will reduce the activity of MLCP. Thus, a decrease in MLC dephosphorylation enhances smooth muscle contraction (100).

Vasodilator signals also regulate the extent of myosin phosphorylation by reducing calcium sensitivity, via stimulation of one of two second messenger pathways, cAMP or cGMP. For example, cAMP-dependent protein kinase (PKA) can phosphorylate MLCK at two sites on the calmodulin-binding domain, leading to a decreased affinity of MLCK for calcium-calmodulin complex. On the other hand, phosphorylation of MLCK by cGMP-dependent protein kinase (PKG) has no effect on the activity of MLCK. Rather, cGMP activates MLCP directly, and/or via an inhibition of the Rho kinase pathway

(100). Therefore, vasoactive molecules regulate smooth muscle contraction by activating second messenger pathways which alter calcium sensitivity, providing another level at which vascular tone can be regulated.

Calcium extrusion from the cytoplasm is as important in the maintenance of vascular homeostasis as is the increase in calcium levels, and is another dimension of smooth muscle function that is modulated by vasodilators. There are two major mechanisms of calcium removal in vascular smooth muscle cells. The plasma membrane calcium ATPase pump has an increased affinity for calcium in the presence of calmodulin. Relaxation can be invoked through the stimulation of the plasma membrane calcium ATPase by PKA as well as PKG. The sarcoendoplasmic reticulum calcium ATPase pump (SERCA) is responsible to returning calcium to intracellular stores in the endoplasmic reticulum. The regulation of its activity converge on a common mediator, phospholamban. In a dephosphorylated state, phospholamban inhibits SERCA. However, phosphorylation by PKA, PKG, or PKC prevents this inhibition of the SERCA, thus removing calcium from the cytosol and allowing for relaxation (53). The stimuli responsible for these alterations in calcium sensitivity, and ultimately vascular relaxation, originate largely in the endothelial cell. The endothelium produces vasodilators that cause the activation of the cAMP or cGMP pathway, thus initiating the aforementioned changes in vascular smooth muscle tone.

1.3 ENDOTHELIAL-DERIVED VASOACTIVE MEDIATORS

The endothelium had originally been regarded as an inert membrane that lined the circulatory system and functioned solely to protect the vessel and maintain vessel wall

permeability. However, a variety of microscopic and physiologic studies led to the current view that the endothelium is a dynamic, heterogeneous organ that encompasses vital vasoactive, thrombostatic and immunologic functions (23). The endothelium covers a surface area of approximately 5 m^2 and lines every vessel in the body, regulating the flow of nutrients and other biologically active molecules. Endothelial cells are strategically located between circulating blood and vascular smooth muscle to respond to a variety of different stimuli. For example, an increase in blood flow activates shear stress-sensitive genes in the endothelium, which are involved in modulating vessel tone. Vessel tone is also regulated through the interactions of circulating, neuronal and paracrine factors with endothelial cell surface receptors, the activation of which cause the production and/or release of vasoactive substances.

Vital to the control of vascular homeostasis are the endothelial-derived vasodilators. Molecules that, when interacting with endothelial cell receptors, cause relaxation include acetylcholine, adrenomedullin, atrial natiuretic peptide, bradykinin, substance P, histamine, calcitonin gene-related peptide (CGRP) and endothelin. Activation of specific receptors on endothelial cells results in the production and/ or release of vasodilator molecules. The endothelium produces three major relaxation factors, prostacyclin, nitric oxide and the group of molecules termed endothelial derived hyperpolarizing factors (EDHF), that all contribute to tonic inhibition of vascular tone and thus oppose the constant sympathetic drive. These molecules are released in varying amounts depending on the vascular bed, species, endothelial agonist and the physiological state of the organism (for example, pregnancy, aging). These three major endothelial-derived relaxing factors [nitric oxide, prostacyclin and epoxyeicosatreinoic acid (EET) as one

possible EDHF] and the factors that regulate their activity are a central focus of this thesis (Figure 1.2).

1.3.1 Nitric Oxide

In 1980, Furchgott and Zawadzki discovered quite serendipitously that relaxation to acetylcholine became impaired if the endothelial layer was accidentally removed. This led to the concept that the endothelium released a diffusible factor that caused vasodilation, which was termed the endothelial-derived relaxing factor (44). Its chemical properties were consistent with a highly volatile, short half-life molecule that reacted with molecular oxygen and hemoglobin, thus becoming inactive. In 1987, the elucidation that the endothelial-derived relaxing factor was indeed nitric oxide sparked the rapid expansion of research into the roles of nitric oxide in physiology. It is now understood that nitric oxide is a ubiquitous cell-signaling molecule. Along with being a very potent vasodilator, nitric oxide counteracts leukocyte adhesion, inhibits smooth muscle proliferation and platelet aggregation (11). These biological actions make nitric oxide a central component in the endogenous defenses against altered vascular reactivity, inflammation and thrombosis (12), key features of many cardiovascular diseases including preeclampsia.

Nitric oxide reduces vascular tone by rapidly diffusing through cell membranes (due to its nature as a small hydrophobic gas) into nearby vascular smooth muscle cells. Here it interacts with the heme moiety of guanylate cyclase, causing enzyme activation by the removal of a histidine residue from its iron center. Guanylate cyclase catalyzes the formation of cGMP, which initiates the activation of PKG (54). Ultimately the processes

that results in vascular smooth muscle relaxation involves PKG-mediated inhibition of calcium release, the stimulation of calcium re-uptake, and membrane hyperpolarization through the opening of potassium channels (143) as well as calcium desensitization of the contractile machinery. However, nitric oxide has also been shown to alter gene expression (113), as well as RNA translation (140) and thus is involved in many aspects of cellular regulation in addition to vasorelaxation.

Nitric oxide is synthesized from L-arginine through a complex oxidation reaction catalyzed by the flavo-hemoprotein nitric oxide synthase (NOS), producing nitric oxide and L-citrulline as a byproduct. NOS exists as three isoforms: neuronal NOS (nNOS) and endothelial NOS (eNOS) are calcium-dependent enzymes. Inducible NOS (iNOS) is independent of calcium for its activity, but its expression is regulated by factors such as pro-inflammatory cytokines (70). The reaction of NOS forming nitric oxide requires at least six co-factors; NADPH, FAD, FMN, tetrahydrobiopterin, heme and calmodulin (57). There are many deleterious effects if one or more of these requirements are not met. For example, low levels of the substrate l-arginine (51) or the co-factor tetrahydrobiopterin (134) can induce NOS-dependent increase in superoxide anion.

eNOS is a constitutively expressed enzyme and its activity is the major point at which regulation of endothelial-derived nitric oxide production occurs. ENOS, a calcium-calmodulin dependent enzyme, requires the association of calcium with calmodulin to stimulate its activity. eNOS is dramatically targeted to special cell surface plasmalemmal caveolae by an integral membrane cell surface protein called caveolin. Indeed, the mechanism behind calcium-calmodulin activation of eNOS may be through an inhibitory

action on caveolin. The binding of calcium-calmodulin to eNOS disrupts the heteromeric complex formed between caveolin and eNOS, thus freeing and activating eNOS (85).

Nevertheless, the expression of eNOS is also altered by external stimuli. For example, increased fluid flow causes shear stress, which upregulates the expression of eNOS and the gene promoter region of eNOS contains shear-stress response elements (28). In addition, the eNOS gene contains a partial response element to estrogen, which may upregulate eNOS gene expression and play a role in vascular adaptations to pregnancy (20). The eNOS gene can also be negatively regulated by cytokines such as TNFα (36).

Experimental evidence suggests that the basal state of vasodilation in the vasculature is due to continuous release of nitric oxide from the endothelium, which opposes the tonic adrenergic stimulation. For example, in isolated rat mesenteric arteries incubation with an arginine analogue inhibitor of NOS (L-NAME) induces vasoconstriction (76). Furthermore, administration of NOS inhibitors elevates blood pressure in both rats (105) and humans (131) illustrating the integral homeostatic role of nitric oxide in modulating vascular resistance.

1.3.2 Prostacyclin

Prostacyclin was discovered to be an endothelial-derived relaxing factor after it was determined to be locally produced in blood vessels, and when perfused through vessel preparations, it caused vasodilation and altered regional blood flow *in vivo* (23). Although the receptors for prostacyclin (IP receptors) are constitutively expressed in

vascular smooth muscle cells, prostacyclin is not basally produced in great quantities and does not appear to regulate basal systemic vascular tone (23).

Unlike nitric oxide, the vasodilation of prostacyclin depends on expression of specific receptors on target tissue. IP receptors are coupled to adenylate cyclase, thus activation will increase levels of cAMP, which can directly stimulate potassium channels causing hyperpolarization and relaxation (97). In addition, through the activation of PKA, prostacyclin can increase extrusion of calcium from vascular smooth muscle cells, thus inhibiting contractile machinery. Interestingly, the actions of prostacyclin may be potentiated by nitric oxide. For example, cGMP can inhibit a phosphodiesterase that breaks down cAMP, thus prolonging the half-life of the second messenger for prostacyclin (88).

Prostacyclin, the major prostaglandin produced by endothelial cells, is formed as a result of arachidonic acid metabolism. Arachidonic acid is liberated from the plasma membrane upon activation of phospholipase A_2 (PLA_2), which is subjected to many different fates. Important to endothelial cell function, arachidonic acid is converted by prostaglandin H synthase (PGHS) into PGH_2 via a two step mechanism. The initial step in the process involves a cyclo-oxygenase activity that incorporates two oxygen into the arachidonic acid molecule, forming the intermediate PGG_2, after which the peroxidase activity of PGHS catalyzes a two electron reduction of PGG_2 into PGH_2. Specific isomerization enzymes catalyze the conversion of PGH_2 into either prostaglandins or thromboxane (120).

The regulation of prostacyclin production occurs at the levels of two enzymes responsible for its production, PGHS and prostacyclin synthase. PGHS exists as two

isoforms; the expression of PGHS-1, although termed the constitutive isoform, is upregulated by factors such as shear stress in endothelial cells. PGHS-2, on the other hand, is highly inducible by many cardiovascular risk factors such as pro-inflammatory cytokines and pro-oxidants (via NFκB activation), cholesterol and lipoproteins (31). The function of the PGHS enzymes can also be regulated at the level of activity. Indeed, an interesting link exists between nitric oxide and the PGHS pathway. It has been reported that nitric oxide can activate PGHS in endothelial cells (120). However, the direct mechanism of nitric oxide activation in a purified enzyme preparation has not been confirmed. On the other hand, peroxynitrite (the pro-oxidant formed by the reaction of nitric oxide with superoxide anion) has been shown to activate PGHS, suggesting that the interplay between NO and PGHS may be mediated by peroxynitrite. Indeed, this creates a potential positive feedback cycle, since there the catalytic functions of PGHS create a significant amount of superoxide anions (17, 144). The role of oxidants in the regulation of PGHS and prostacyclin synthase will be discussed in greater detail in section 1.7.3.

1.3.3 Epoxyeicosatreinoic Acids

Aside from the well characterized vasodilator autocoids, nitric oxide and prostacyclin, there remains a substantial component of the vasodilator response that is insensitive to inhibitors of the NOS and PGHS pathways. This residual dilation, prominent in mesenteric, coronary and renal arteries, coincides with vascular smooth muscle hyperpolarization and is abolished by depolarizing concentrations of potassium. Thus, the nitric oxide/prostacyclin-independent relaxation was determined to be mediated

by an endothelial-derived hyperpolarizing factor (EDHF)(133). The link between cytochrome P450-derived (CYP450) epoxyeicosatrienoic acids (EETs) and EDHF activity has been intensely investigated in recent years.

CYP450 are a family of membrane bound enzymes, with specific vascular isoforms that metabolize arachidonic acid into biologically vasoactive products. For example, epoxygenases of the 2 gene family include CYP450 2B, 2C8, 2C9, 2J2 in humans and 2C28 and 2C29 in mice, which generate a series of stereospecific epoxides including 5,6 EET, 8,9 EET, 11,12 EET and 14,15 EET, all of which are potent dilators of blood vessels depending on the vascular bed studies (4, 110). Another CYP450 isoform, ω-hydroxylase is predominantly in the vascular smooth muscle cell and catalyzes the formation of hydroxyeicosatetraenoic acids (HETEs) which are potent vasoconstrictors. Interestingly, the effect of inhibiting the CYP450 enzymes on vascular function may vary depending on the state of the blood vessel (i.e. stretch, pressure) because this may lead to the vasoconstrictor CYP450 pathway to predominate over the endothelial-derived vasodilators (18).

There is convincing evidence to suggest that EETs are one potential EDHF, since these substances elicit hyperpolarization in endothelial and vascular smooth muscle cells by activation of calcium sensitive potassium channels as well as sodium-potassium ATPase pump (16). Furthermore, CYP450 inhibitors have been shown to eliminate EDHF dependent relaxation (40). However, the traditional pharmacological agents that were utilized in these studies were not specific to any one CYP450 isoform and could interfere with potassium channel function. Therefore, the recent discovery of specific

inhibitors of EET-mediated dilation has lead to the conclusion that these molecules do contribute substantially to EDHF-dependent relaxation (47, 58). Although the identity of EDHF will continue to be intensely researched, it is likely that many different endothelial-derived hyperpolarizing factors exist, which vary depending on the species and vascular bed being investigated.

Blood vessel reactivity is tightly regulated, involving an intricate interplay between many different molecules. Evidence for overlapping vasodilator mechanisms suggests that together the systems are evolutionarily favorable in case one pathway is defective, another may compensate. In addition, different vascular beds rely more heavily on certain vasoregulatory pathways than others. For example, EDHF-dependent relaxation has a large role in modulating vascular function in small resistance arteries compared to reactivity in conduit arteries, which may be more nitric oxide-dependent. Furthermore, it is hypothesized that EDHF mediated relaxation is upregulated in conditions where nitric oxide mediated relaxation is impaired (21, 63). It is now clear that impaired endothelial cell function contributes to cardiovascular disorders such as atherosclerosis, hypertension, and preeclampsia. Thus, it is crucial to have a firm understanding of the mechanisms involved in maintaining normal vascular homeostasis in order to interpret changes in vascular function associated with pathological conditions.

1.4 PREGNANCY ADAPTATIONS

Pregnancy encompasses significant physiological adaptations, which must be integrated in order to ensure a successful outcome. The maternal circulation provides the developing fetus with sufficient oxygen and nutrients, along with the removal fetal

metabolic waste products. To satisfy these necessities, the maternal cardiovascular system undergoes changes that would be considered pathological in non-pregnant women. These changes occur in response to the environment created by the developing conceptus, beginning very early in gestation. An inappropriate maternal response to these fetal signals may contribute to pathological conditions of pregnancy such as preeclampsia. This section will summarize the major maternal cardiovascular changes occurring in a normal pregnancy, with a focus on the mechanisms behind the decrease in peripheral vascular resistance.

1.4.1 Blood Volume Expansion

Plasma volume expansion begins by week seven of human gestation, and reaches its peak by 32 weeks (22). There are several mechanisms that are likely responsible for this increase in blood volume. Aldosterone levels show a two-fold increase, as well as a 15 to 25 time increase in deoxycorticosterone activity. These hormonal changes result in an increase in water retention, by increasing tubule re-uptake of sodium (94). Furthermore, pregnant women have elevated levels of renin and angiotensin, also promoting sodium reabsorption. In order to balance these adaptations, glomerular filtration rate is elevated, along with progesterone levels, which together promote sodium excretion (94). Overall, these physiological adaptations promote a net retention of fluid, resulting in maternal plasma volume reaching levels approximately 40% above those of the non-pregnant state.

Red blood cell mass also increases throughout gestation, reaching levels about 20% above those of non-pregnant women. The increase in hematocrit reflects an increase in production of red blood cells, erythropoiesis, and not a prolonged lifespan of the red cells

(29). The increase in erythropoiesis is stimulated by elevated prolactin, progesterone, and erythropoeitin levels. Interestingly, serum erythropoeitin levels only begin to rise appreciably by mid-pregnancy in humans. Because of the difference in time courses for plasma volume expansion and the increase in hematocrit, pregnancy is associated with a 'physiological' or 'hemodilutional' anemia (84). The expanded blood volume of pregnancy, along with providing an increase in blood flow through the uterus, can also be considered a safeguard against blood loss during delivery of the infant, when the mother might loose a substantial volume of blood (94).

The expanded blood volume creates a remarkable problem for maternal homeostasis; where does the extra blood get distributed? There is a large increase in blood going to the uterus and the feto-placental unit, and thus a disproportionate amount of blood contained in the veins and the pelvis of the lower extremities. During gestation, problems may arise if women are in the supine position for extended time periods, due to compression of the inferior vena cave. This causes blood flow returning to the heart to be diminished, resulting in hypotension, bradykardia and syncope (94). Heart function is very sensitive to changes in body position for this reason, and it is an important determinant in the measurement of cardiac output and blood pressure during pregnancy.

1.4.2 Cardiac Output

Heart function also undergoes dramatic alterations during pregnancy. There is a 30-50% increase in cardiac output compared to non-pregnant women, most of which occurs during the first trimester. This reflects an increase in both stroke volume and heart rate (117). The mechanisms behind the increase in cardiac stroke volume are not entirely

understood. It has been hypothesized that expansion of venous and arterial capacity, secondary to an increase of blood volume, leads to an increase in stroke volume, based on the Frank-Starling relationship (i.e. greater blood volume return to the ventricle increases myocardial fiber length, which in turn will determine the force at which the ventricle contracts and thus the stroke volume) (74). Nevertheless, there does not seem to be evidence that the maternal system is in a state of hypervolemia. For example, pregnant women do not show evidence of an increase in venous pressure. Furthermore, pregnant women respond to salt restriction, or infusion with salt or water loads as through they were normovolemic; that is as though fluid and mineral retention was adequate to fill the increased vascular capacity (84). These observations suggest that volume and osmolality sensors are reset during pregnancy such that the expanded blood volume is perceived as normal. Other hypothesized mechanisms responsible for the increase in cardiac output include an increase in contractility and /or left ventricular mass (108).

1.4.3 Systemic Vascular Resistance

Systemic vascular resistance (total peripheral resistance) is the ratio of cardiac output and mean arterial pressure, which represents (clinically) the amount of impedance to cardiac output (94). The total vascular resistance decreases significantly during pregnancy, an adaptation that must coincide with the elevated cardiac output and blood volume. The decline in peripheral vascular resistance characterizes a normal pregnancy and is required for the maintenance of arterial blood pressure. A decrease in total peripheral resistance has been observed in the late luteal phase of women who are destined to become pregnant, representing one of the earliest manifestations of a maternal

cardiovascular adaptation of pregnancy. This observation supports the theory that vascular changes are an initial stimulus that actually induces the other adaptive cardiovascular changes (94).

1.5 ALTERED VASCULAR REACTIVITY IN PREGNANCY

The regulation of vascular resistance is altered during pregnancy (reviewed in (103)). Indeed, infusion of angiotensin II into a normal pregnant women result in a blunted pressor response compared to non-pregnant women (45) an adaptation that is also common to rat pregnancy (96). This adaptation is consistent with the necessary elevation in renin and angiotensin activity of pregnancy. However, there is also evidence that this blunted pressor effect is not unique to angiotensin. In pregnant rats, the vasoconstrictor effects of phenylephrine are reduced compared to non-pregnant rats (33) as are the responses to vasopressin (87). Overall, there are inherent alterations to the regulation of vascular reactivity, resulting in a reduced constriction and a decrease in peripheral vascular resistance. This modulated constrictor response may be due to an increase in endothelial-derived vasodilators.

1.5.1 Role of Prostacyclin in Vascular Relaxation in Pregnancy

There is much experimental evidence suggesting that pregnancy is characterized by increased endothelial-dependent relaxation. Prostacyclin, the most abundant vasodilator metabolite of arachidonic acid produced by the endothelium, was traditionally considered the major mediator of enhanced relaxation during pregnancy. Prostacyclin is elevated during pregnancy in women, as indicated by measurement of its stable metabolite in the

urine (78, 93). This evidence has been confirmed in the sheep, whereby local production of prostacyclin is increased in the uterine and renal artery during gestation (77) which may actually be stimulated by elevated angiotensin levels (79).

In contrast with these observations, infusion with a PGHS inhibitor in pregnant rats does not induce hypertension, as would be expected if prostacyclin was indeed responsible for enhanced relaxation during gestation (26). Furthermore, the blunted pressor response to phenylephrine was not altered by inhibition of PGHS (33, 122). These data suggest that although prostacyclin may be elevated in pregnancy, it is not playing a major role in vascular homeostasis.

1.5.2 Role of Nitric Oxide in Vessel Function in Pregnancy

There is a significant amount of literature suggesting that nitric oxide-mediated relaxation is enhanced in pregnancy. Indeed, cGMP, the second messenger for nitric oxide, is elevated in the plasma and urine of pregnant rats, along with a rise in urinary excretion of nitrite and nitrate, metabolites of nitric oxide (118). Data on whether nitric oxide is elevated in human pregnancy are conflicting (95, 119). Due to confounding variable of maternal intake of nitrites, Conrad *et al.* performed a longitudinal study in women who were on a nitrite-restricted diet. The results from this study illustrate that neither serum nor urine nitric oxide metabolites were elevated in pregnant women. However, cGMP production was increased in pregnant women (27) suggesting that either other non-nitric oxide mediators are activating the cGMP pathway in pregnancy or it is possible that local production of nitric oxide is elevated, but not measurable systemically, in human pregnancy.

Studies of vessel function *in vitro* also suggest that nitric oxide is involved during the vascular adaptations to pregnancy. In pregnant rats, enhanced sensitivity to the endothelial-dependent vasodilator acetylcholine was observed in mesenteric arteries (33, 98) as well as uterine arteries (91) compared to non-pregnant animals, and in both studies the relaxation was significantly reduced in the presence of a NOS inhibitor. These data coincide with evidence that infusion of NOS inhibitors into pregnant rats eliminates the characteristic blunted pressor response to both angiotensin (86) as well as to phenylephrine (2, 65), such that the pressor response between pregnant and non-pregnant rats was equalized. Furthermore, NOS inhibitors administered in mid-gestation to pregnant rats alters regional hemodynamics and induces hypertension (60), indicating that nitric oxide plays an important role in the cardiovascular adaptations to rat pregnancy.

The investigation of human vessels during gestation indicates that although vascular reactivity is altered, the data involving the role of nitric oxide are conflicting. Nitric oxide mediated forearm blood flow is enhanced in pregnant versus non-pregnant women (3). This is consistent with data in human uterine artery, illustrating that acetylcholine induced relaxation is enhanced in pregnancy due to an increase in nitric oxide-dependent dilation (90). Furthermore, endothelium-dependent dilation to bradykinin induces a greater relaxation response in subcutaneous resistance arteries from pregnant compared to non-pregnant women. Evidence suggests that the nitric oxide component of this enhanced relaxation is upregulated in pregnant women, while the effect of a PGHS inhibitor was similar between groups (67).

1.5.3 Non-Nitric Oxide and Non-Prostaglandin Relaxation in Pregnancy

There is a variety of studies illustrating that pregnancy is associated with enhanced relaxation that is not dependent on nitric oxide or prostacyclin. For example, in pregnant rats, Gerber *et al.* (48) found that residual relaxation to acetylcholine in the presence of both NOS and PGHS inhibitors was enhanced in mesenteric arteries from pregnant rats. This EDHF-like relaxation was inhibited in the presence of potassium chloride and an inhibitor of CYP450, suggesting the involvement of potassium channels and/ or EETs in the relaxation response (48). Furthermore, reduced uterine perfusion pressure in pregnant rats is mediated by an EDHF, since potassium channel blockers, but not a combination of NOS inhibitors, affected the reduced perfusion pressure (43).

Consistent with data in the rat, Pascoal *et al.* (99) found that although overall endothelial-dependent relaxation was not enhanced in omental arteries from pregnant women, there was a pregnancy specific increase in an EDHF-like (potassium channel sensitive) component in bradykinin-induced relaxation. These data were confirmed by Kenny *et al.* who observed that the enhanced bradykinin-induced relaxation in omental arteries from pregnant women was insensitive to NOS and PGHS inhibition (63). Furthermore, in human subcutaneous vessels, relaxation to acetylcholine was not different between non-pregnant and pregnant women and the contribution by nitric oxide to this relaxation was minimal. However, there was a large proportion of this relaxation that persisted after inhibition of nitric oxide and prostacyclin that was attributed to EDHF (82). These data suggest that there may be a specific upregulation of a non-nitric oxide non-prostacyclin dependent molecule in pregnant women.

1.5.4 Transgenic and Knockout Mice in Pregnancy Studies

Recently, much research has focussed on transgenic mice with the hopes of elucidating further mechanistic details about the cardiovascular adaptations to pregnancy. For example, transgenic mice that over-express renin and angiotensinogen become hypertensive during pregnancy (126), and a spontaneously mildly hypertensive mouse model shows manifestations of preeclampsia when pregnant (35). Perhaps most interesting is the observation that mice lacking eNOS do not become hypertensive during gestation; it is hypothesized that other endothelial-dependent pathways are compensating in this mouse model (115). Although, mice undergo many of the same cardiovascular changes in pregnancy, such as an increased cardiac output and a mild reduction in blood pressure (145), the mechanisms associated with the reduced peripheral vascular resistance are poorly understood in this species, which is one focus of this thesis.

1.6 PREECLAMPSIA

Preeclampsia, a pregnancy specific syndrome, is characterized by the *de novo* onset of maternal hypertension and proteinuria in the latter half of gestation. It is also associated with generalized edema and renal dysfunction, which if left untreated can progress to seizures, blindness, or even death (107). Currently, the causes of preeclampsia are unknown and the only cure is the immediate delivery of the fetus and the placenta, which alleviates maternal symptoms, often at the expense of fetal well-being. Consequently, preeclampsia is the major source of idiopathic pre-term delivery and thus perinatal and maternal morbidity and mortality (107).

1.6.1 Pathogenesis: Diversity of Etiology

The distinct maternal pathophysiology of preeclampsia suggest that the symptoms might be caused by abnormal maternal adaptations to the fetal stimulus, in that many of the normal adaptations to pregnancy are compromised in women with preeclampsia. For example, blood volume and cardiac output are reduced, while vascular resistance is elevated (62). Whether, there is a definable defect that initiates the multitude of other maternal problems remains to be elucidated, and might result in a Nobel prize for those who discover the 'cause' of preeclampsia. Until then, it remains a disease of theories, which will be summarized in the following section.

The diverse etiology of preeclampsia has lead to the theory that there are two-steps in the progression of this syndrome. The first step begins at the time of implantation. In a normal pregnancy, fetal extravillous trophoblasts invade the maternal decidua and into spiral arteries. These fetal cells begin to take on an endothelial cell like phenotype, thus enabling the complete remodeling of the spiral arteries from a muscular structure to thin walled, very distensible vessels (102). This adaptation is crucial to allow for adequate perfusion of the placenta with blood. On the other hand, it has been well documented that although spiral arteries in preeclampsia do undergo minimal remodeling, these changes do not extend any further than the mucosal layer (not into the decidua)(101). Furthermore, relatively few trophoblasts are found in the vessels and those that are present do not express an endothelial phenotype (151). This adaptive failure causes a severe reduction in placental perfusion in women with preeclampsia. In normal pregnancy, the spiral artery remodeling is completed by the 21 week of pregnancy, thus

although preeclampsia is not typically diagnosed until the third trimester of pregnancy, its roots are likely early in gestation (107).

A critical analysis of the clinical features and phenotype of women who suffer from preeclampsia led to greater understanding of the pathogenesis of the disease. First, it was noted that reduced placental perfusion is not sufficient to explain preeclampsia. For example, abnormal implantation is associated with both intrauterine growth restriction and pre-term births and occurs in many pregnancies without the systemic manifestations of preeclampsia (107). Interestingly, only one third of births to preeclamptic women are growth restricted (38) while a recent epidemiological study concluded that there is an association of large infants in preeclamptic pregnancies (147). This theory is supported by evidence that conditions associated with larger infants (gestational diabetes, obesity) are also risk factors for developing preeclampsia.

Although reduced placental perfusion may be important in the development of preeclampsia, it is not sufficient. It is proposed that the abnormal placentation must interact with maternal composition (including genetic, behavioral and environmental factors) in order to cause preeclampsia, suggesting that the second hit in the theory is a maternal pre-disposition to cardiovascular disease. Indeed, women who are obese, have atherosclerosis, diabetes, hyperlipidemia or high cholesterol levels are more likely to develop preeclampsia during pregnancy (37). The commonality among these risk factors is that they all place a strain on endothelial cell function, which is crucial for a healthy pregnancy. Thus, in preeclampsia, it is hypothesized that the poorly perfused placenta releases factor(s) that circulate into the mother, and systemically alters endothelial cell

function, which is especially detrimental in women who are susceptible to vascular complications (Figure 1.3).

1.6.2 Markers of Endothelial Cell Dysfunction

The endothelium is considered to be the underlying target of preeclampsia, since endothelial cell dysfunction can be linked to all the clinical manifestations of the syndrome. These include increased thrombogenesis and platelet activation (123) proteinuria due to glomerular endotheliosis (106), edema due to increased capillary permeability (14) and hypertension, possibly secondary to an impaired blunted pressor response to angiotensin II (46). Women with preeclampsia also possess many markers of endothelial cell activation including elevated cellular fibronectin and endothelin (127), factor VIII (104) as well as adhesion molecules such as vascular cell adhesion molecule (VCAM) (7). Furthermore, evidence that urinary metabolites of prostacyclin are reduced, while thromboxane is elevated, suggests an imbalance between PGHS-dependent vasodilation to vasoconstriction in women with preeclampsia (138). Nevertheless, previous clinical trials using low dose Asprin® attempted to equate this imbalance by specifically targeting PGHS-derived thromboxane in platelets, with the hopes of shifting the balance in favour of endothelial produced prostacyclin. However the efficacy in preventing the development of preeclampsia was not significant (116), illustrating the complexity and diversity of preeclampsia.

1.6.3 Altered Maternal Vascular Function

Although an imbalance between endothelial-derived vasoactive factors might partially explain the elevated vascular resistance evident in these women, much remains to be discovered. The mechanisms behind the altered endothelial-dependent vasodilation have been partially elucidated by studying blood vessels from women with preeclampsia *in vitro*, although the results are variable depending on the vascular bed studied or the agonist used. Acetylcholine-dependent relaxation is impaired in omental (99) and subcutaneous (83) resistance arteries from women with preeclampsia, compared to normal pregnant women. However, overall bradykinin-induced relaxation is not impaired in omental (99) nor myometrial (63) (6) vessels from women with preeclampsia, but the pathways mediating its relaxation may be altered. For example, in myometrial arteries, NOS inhibition greatly reduced the bradykinin-induced relaxation in vessels from women with preeclampsia, while in normal pregnant women, a significant proportion of relaxation was attributed to an EDHF-like molecules (63). These data obtained from isolated vessel studies from women with preeclampsia allow for analysis of altered endothelial cell pathways, yet they do not shed any light about whether these alterations are the cause of or result of preeclampsia. An interesting study by Veille *et al.* (135) found that women who were destined to develop preeclampsia had increased vascular resistance and reduced endothelial-dependent blood flow, suggesting that abnormal endothelial function antedates the clinical appearance of preeclampsia.

To summarize, while normal pregnancy is a state of generalized vasodilation, preeclampsia is a state of relative vasoconstriction, leading to hypoperfusion, thus exacerbating organ damage. Indeed, the clinical symptoms are such that once a woman is

diagnosed with preeclampsia, her condition rapidly deteriorates, as though the disease manifests as a feed forward cycle, not subsiding until after delivery of the placenta.

1.6.4 Mediators of Endothelial Cell Dysfunction

Many hypotheses exist about what circulating factor(s) is mediating the systemic maternal endothelial cell dysfunction. For example, changes in certain vasoconstrictor pathways might explain the altered maternal vascular reactivity. Evidence suggests that the levels of angiotensin 1 – bradykinin type 2 receptor heterodimers (AT1-B2) are increased in vessels from preeclamptic versus normal pregnant women. This may enhance sensitivity to angiotensin II via an increase in G-protein coupled activation and thus stimulation of constriction (1). Along the same lines, others have shown that antibodies against the AT-1 receptors might also be mediating vascular dysfunction in women with preeclampsia (136). In addition, Baker *et al.* have hypothesized that vascular endothelial growth factor (VEGF, which is elevated in women with preeclampsia) is mediating the reduced endothelial-dependent relaxation in women with preeclampsia. Indeed, plasma from women with preeclampsia impaired vasodilation in normal blood vessels. However, after incubating the plasma with an antibody that inactivates VEGF, it was much less effective at altering vessel function (52).

There are also circulating lipid factors that could modulate maternal vascular function. Women with preeclampsia also have more small dense LDL particles that are more easily oxidized by pro-oxidants (112). Oxidized LDL molecules may play a role in the pathogenesis of preeclampsia via altering endothelial cell pathways (34) and vascular function, leading to symptoms similar to those observed in preeclampsia (55).

In the circulation of women with preeclampsia, there are also more pro-inflammatory mediators. Circulating levels of TNFα are elevated and may contribute to endothelial cell dysfunction (13). Furthermore, syncytiotrophoblast membrane fragments (released through increased apoptosis of the placenta) are elevated in women with preeclampsia (66). Infusing these subcellular particles into normal pregnant blood vessels can induce changes in vascular reactivity similar to those observed in women with preeclampsia (24). Thus, whether or not any of these circulating factors are involved in the pathogenesis of preeclampsia and how they alter endothelial cell function remains is still an intense area of research.

1.6.5 Oxidative Stress and Preeclampsia

One theory that has gained much support recently is the concept that oxidative stress is enhanced in women with preeclampsia, above levels considered normal in pregnant women (55). In the two-step model of preeclampsia, the oxidative stress theory is as follows: the poorly perfused placenta releases one or more factors that act on the endothelium and result in the generation of reactive oxygen species, which mediate the clinical manifestations of preeclampsia (Figure 3). In support of this theory, stable metabolites of pro-oxidants are elevated in the placentas of women with preeclampsia (138). Furthermore, placental cytotrophoblasts from preeclamptic placentas manifest evidence of oxidative stress, such as active xanthane oxidase and nitrotyrosine, an indicator of peroxynitrite formation (80). Many other investigators have concluded that markers of oxidative stress are elevated systemically in women with preeclampsia. This

includes elevated nitrotyrosine in maternal vessels (109), elevated isoprostanes and lipid peroxides in plasma from women with preeclampsia, and thiobarbituic acid reactive substances (TBARS) in red blood cell lysates (8, 10, 125, 75). Furthermore, women with preeclampsia also show evidence of reduced sera anti-oxidant capacity compared to normal pregnant women (32). For example, CuZn SOD activity and mRNA is reduced in placentas, catalase and glutathione peroxidase activity is reduced in red blood cells and ascorbate depletion is elevated in plasma from women with preeclampsia (56, 75, 137, 141). Nevertheless, the mechanism by which pro-oxidants are inducing endothelial cell dysfunction is not fully understood.

1.7 OXIDATIVE STRESS

There is a vast amount of evidence suggesting that oxidative stress is involved in the vascular dysfunction of many diseases, including atherosclerosis, diabetes and preeclampsia. The following section will summarize some of the known mechanisms by which oxidant stress affects vascular, specifically endothelial function, thus contributing to vascular disease.

1.7.1 Pro-Oxidants versus Anti-Oxidants

The evolution of aerobic organisms that survive in oxygen rich environments required an effective defense system against reactive oxygen species (ROS). Under conditions that ROS (such as superoxide anion, hydroxyl radical, hydrogen peroxide, lipid peroxides, and peroxynitrite) are in excess of the endogenous anti-oxidant defense mechanisms, they potentially oxidize DNA, and can alter the function of proteins and

lipids. This condition, commonly termed oxidative stress, would be an unavoidable consequence of cellular metabolism if it were not for the anti-oxidants that cells have developed to ensure tolerance to the production of reactive oxygen species (Table 1)(149). While superoxide anion and hydrogen peroxide, the primary products of oxygen metabolism, are neutralized via the enzymes superoxide dismutase (SOD) and catalase respectively, defenses against nitrogen derived free radicals such as peroxynitrite are less well developed, possibly contributing to a redox imbalance (149).

The primary defense against the damage caused by superoxide anion and its reactive progeny (peroxynitrite) are the family of enzymes called superoxide dismutase (SOD) (42). At present three distinct isoforms of SOD have been identified in mammals, their genes cloned and protein structure and function described. Two isoforms of SOD have Cu and Zn in their catalytic centre; CuZn SOD (or SOD-1) is localized to cytoplasmic compartments, while extracellular SOD (or SOD-3) is isolated to extracellular spaces (150). EC-SOD was first isolated from human plasma, however, it mainly exists bound onto the extracellular matrix (42). Manganese SOD (Mn-SOD or SOD-2) is localized to the mitochondria and is largely responsible for preventing the excessive accumulation of superoxide anions from cellular respiration (electron transport chain).

The important physiological roles of the three SOD isoforms can be illustrated by the phenotypic abnormalities in mice lacking specific genes. For example, mice lacking Mn SOD die within the first ten days of life due to severe mitochondrial dysfunction, especially in the heart, which exhibits signs of hypertrophy (150). Knockout mice lacking EC-SOD develop normally but were found to be more susceptible to atmospheric oxygen

(42). Furthermore, EC-SOD seems to play an integral role in protecting the neonatal lung from the high oxygen exposure after birth (129).

Mice lacking CuZn SOD also appear normal during development, but do develop distinct pathologic phenotypes when exposed to external perturbations. For example, CuZn SOD knockout mice were less able to recover from axonal injury (114). They also exhibited a shortened lifespan as well as reproductive abnormalities (81). Furthermore, ischemia-reperfusion resulted in reduced coronary blood flow in CuZn SOD knockout mice compared to controls (148), while stroke induced cerebral ischemia caused a larger infarct size in these mice (61).

The role in SOD in regulating vascular function was introduced when it was discovered that the potency of the nitric oxide was enhanced in the presence of exogenous SOD (111). It is now understood that SOD effectively prolongs the half-life of biologically active nitric oxide by preventing the scavenging of nitric oxide by superoxide anion. Furthermore, recent advances in the study of peroxynitrite implicate this potent pro-oxidant is capable of altering vascular function via mechanisms in addition to the depletion of nitric oxide (11). Therefore, because of the key role of intracellular CuZn SOD in preventing the formation and deleterious effects of peroxynitrite, one goal of this thesis was to investigate vascular function in mice lacking CuZn SOD.

1.7.2 Sources of Pro-Oxidants

Although there are a large number of biological sources of pro-oxidants and free radical molecules, this discussion will be limited to cytosolic sources that have been linked to endothelial cell function.

1.7.2.1 NAD(P)H Oxidase

In the endothelium, one major source of free radicals is the activity of NAD(P)H oxidase (17). This enzyme is upregulated in response to many factors that are thought to mediate vascular dysfunction, such as angiotensin and cytokines (49). Indeed, accumulating evidence suggests that NAD(P)H oxidase may be responsible for excessive generation of superoxide anion in conditions of cardiovascular disease. In rats with acute heart failure, NAD(P)H-stimulated superoxide production is elevated in the aorta (9). Further, in saphenous veins taken from patients undergoing routine coronary bypass surgery, both diabetes and hypercholesterolemia were associated with increased NAD(P)H-dependent superoxide. Furthermore, this study showed that the elevated superoxide levels were associated with reduced nitric oxide-dependent relaxation (50). Thus, the association of increased vascular NAD(P)H oxidase activity with endothelial dysfunction and with clinical risk factors suggests an important role for NAD(P)H oxidase-mediated superoxide production in mediating cardiovascular disease.

1.7.2.2 Xanthane Oxidase

Xanthane oxidoreductase, an enzyme involved in the process of purine metabolism, exists as one of two isoforms; xanthane dehydrogenase which prefers reducing NAD+ into NADH or xanthane oxidase, which prefers molecular oxygen, leading to the production of both superoxide anion and hydrogen peroxide (17). Because of its localization within vascular endothelial cells, xanthane oxidase has been hypothesized to mediate altered vessel function in conditions of elevated oxidative stress. For example, in human patients with hypercholersterolemia administration of a xanthane oxidase inhibitor (oxypurinol) improved endothelial-dependent relaxation (19). Furthermore, oxypurinol

was able to reduce blood pressure in SHR rats as well as reduce evidence of microvascular superoxide anion formation (89). These data suggest that xanthane oxidase may, under pathological conditions, be a mediator of oxidative stress induced vascular dysfunction.

1.7.2.3 Nitric Oxide Synthase

In addition to the above major sources of superoxide, in endothelial cells, vasoactive enzymes can also under some circumstances produce free radicals. For example, eNOS, in the absence of either L-arginine or tetrahydrobiopterin, can produce superoxide anion (134). This phenomenon, referred to as NOS uncoupling, has been implicated in a variety of pathophysiological conditions (17). For example, in aortas from stroke prone SHR rats, elevated superoxide production is normalized by administration of NOS inhibitors or removal of the endothelium (64). Furthermore, in aortas from deoxycorticosterone acetate – salt induced hypertensive mice, superoxide anion production is eliminated by NOS inhibitors and does not occur in eNOS knockout mice (17). These data indicate that eNOS under certain circumstances be a significant source of superoxide anion *in vivo.*

1.7.2.4 Prostaglandin H Synthase

The catalytic activity of the PGHS enzyme is another possible source of superoxide anion in vascular cells. Indeed, it has been shown that in endothelial cells in culture, hypoxia/re-oxygenation induced increases in 8-epi isoprostanes can be prevented with specific inhibitors to PGHS (139). Furthermore, piglet cerebral cortex exposed to ischemia/reperfusion, have an increase in superoxide anions due to the activation of PGHS (5). One possible mechanism behind the PGHS-dependent increases in oxidative stress is based on its ability to co-oxidize NAD(P)H (142).

1.7.2.5 Cytochrome P450 Enzymes

Specific CYP450 epoxygenase isoforms (CYP450 2C9) have been shown to also increase levels of superoxide anion. In both native porcine coronary arteries as well as coronary derived endothelial cells, overexpression of the CYP450 2C9 enzyme causes an increase in superoxide generation, as well as an impairment of nitric oxide-dependent vasodilation. Furthermore, evidence indicates that other redox-sensitive biological functions can be altered by the CYP450-induced oxidative stress. For example, CYP450 is thought to mediate activation of the oxidant sensitive transcription factor NFκB, as well as expression of VCAM-1 (41).

Therefore, it is clear that many vasoactive enzymatic pathways produce superoxide anion. Nevertheless, free radicals themselves can interfere with the function and/or expression of these enzymes, and thus may perpetuate vascular dysfunction by creating a deleterious positive feed forward cycle.

1.7.3 Oxidative Stress as a Mediator of Vascular Dysfunction

The endothelium is the integral mediator of vessel tone, because of the production and release of many vasoactive molecules. The effects of two well-characterized vasodilators, nitric oxide and prostacyclin, can be interfered with by the action of pro-oxidant molecules. The actions of EDHF may also modulated by reactive oxygen species through interactions with vascular smooth muscle potassium channels.

Arguably the most important mechanism behind the oxidant-induced decrease in nitric oxide is the scavenging by reactive oxygen species, specifically superoxide anion.

Even before the identity of the endothelial-derived relaxing factor was elucidated, it was known that its efficacy could be reduced by superoxide and restored with SOD (25). Indeed, the interaction of nitric oxide with superoxide occurs at a rate of 6.7 x 10-9 M/s, three-fold faster than the reaction of superoxide with SOD (111). Although there is likely a small amount of peroxynitrite produced at all times in a cell, under physiological conditions, endogenous anti-oxidants minimize this reaction, thus maintaining the delicate balance between nitric oxide, superoxide anion and peroxynitrite.

1.7.3.1 Alteration in NOS Expression and/or Activity

Bioavailability of nitric oxide may be diminished by pro-oxidants by a variety of other mechanisms. For example, the pro-inflammatory cytokine TNFα (which is also known to induce oxidative stress in endothelial cells) decreases protein levels of eNOS by a mechanism that involved mRNA destabilization. Interestingly, this effect can be reversed by treatment with estradiol (124). Furthermore, oxidized LDL particles can negatively regulate eNOS expression through a mechanism that involves both inhibition of transcription as well as RNA destabilization (72). Conversely, Davidge *et al.* showed that plasma from women with preeclampsia increased eNOS protein levels in endothelial cells, due to a factor that had the biochemical profile of LDL (34). Therefore, it is evident that eNOS expression can be both positively and negatively modulated.

The activity of eNOS is also affected by pro-oxidant molecules. For example, evidence indicates that peroxynitrite decreases the activity of NOS via one of two mechanisms. The first involves a direct oxidation-induced uncoupling of the zinc cluster of eNOS (153). In addition, Kohen *et al.* (68) illustrated that peroxynitrite can cause the

destabilization of the important co-factor tetrahydrobiopterin, which not only alters eNOS expression, but also leads to the formation of more superoxide anions. eNOS function is also tightly regulated by its association with caveolae, thus interference with this protein complex will alter eNOS activity. For example, the activation complex of eNOS with caveolin is stabilized by cholesterol, which can be depleted by oxidized LDL molecules. It is hypothesized that in vascular disease, reduced nitric oxide dependent relaxation is, in part, mediated by this reduction in eNOS activity (39).

1.7.3.2 Alteration in the Balance of PGHS-Derived Vasoactive Molecules

The activity of the PGHS pathway is also affected by increased level of pro-oxidant molecules. For example, PGHS activity can be increased by peroxynitrite and lipid peroxides (71, 130). Finally, PGHS is a self-inactivating enzyme that, through the production of hydrogen peroxides, has significantly reduced activity (146).

Zou *et al.* (1994) have extensively characterized that peroxynitrite, via a tyrosine nitrosylation process, inhibits the activity of prostacyclin synthase. Together, these alterations could favour the production of PGHS-mediated vasoconstrictor molecules such as PGH_2 and thromboxane, instead of prostacyclin. Indeed, hypoxia/ re-oxygenation injury induces vasospasm in bovine coronary arteries, due to impaired prostacyclin mediated relaxation (152). Furthermore, placental blood vessels perfused with authentic peroxynitrite show enhanced sensitivity to vasoconstrictors and reduced vasodilation (69). Therefore, these experiments suggest that peroxynitrite is indeed altering endothelial cell pathways, and may be a mediator of altered vascular function.

Interestingly, alterations in the PGHS pathway are becoming more prominent in pathological conditions associated with oxidative stress. In a rat model of aging, a state

that is associated with increased oxidative stress (30, 132) there is evidence of enhanced PGHS-2 dependent vasoconstriction, possibly mediated through the thromboxane/ PGH_2 receptor (121). In renin over-expressing hypertensive rats, impaired endothelial-mediated relaxation is dependent on enhanced vasoconstrictor prostanoid production (92).

1.7.3.3 Alteration in the Activity of Potassium Channels

Although the identity of EDHF is not fully characterized, there is evidence that this pathway can also be modified by peroxynitrite, mainly by its actions on different subtypes of potassium channels. For example, peroxynitrite directly inhibits calcium sensitive potassium channel currents in vascular smooth muscle cells isolated from rat cerebral arteries (15). Furthermore, oxidative stress secondary to hyperglycemia inhibits in voltage gated potassium channels in smooth muscle cells (73) as well as acetylcholine-mediated relaxation in rat aortic rings (128). Interestingly, the effect of pro-oxidant molecules on the CYP450-EET pathway has not been investigated in vascular tissues.

1.8 SUMMARY AND RATIONALE

The regulation of peripheral vascular resistance is a complex process, and plays an integral role in the progression of a healthy pregnancy. The activity of the endothelium is vital, because these cell receive stimuli from circulating blood (physical and humoral) and respond by producing vasodilators. Oxidative stress is hypothesized to be a key feature of many cardiovascular diseases, including preeclampsia. Previous studies from our laboratory indicated that peroxynitrite formation is significantly elevated in resistance-sized vessels from women with preeclampsia, while SOD expression is reduced (109). However, the pathophysiological consequences of peroxynitrite formation

and oxidative stress on endothelial cell function and vasodilation is not fully understood. As discussed in the literature review, pro-oxidants can have negative modulate endothelial-derived vasodilator pathways, including nitric oxide and prostacyclin. Therefore, the unifying theme of this thesis is the alteration of vascular reactivity by oxidants, specifically peroxynitrite, and how these mechanisms may play a role in the vascular adaptations to pregnancy. Elucidating the role of oxidants in vascular dysfunction and pregnancy may improve our understanding of the pathophysiology of preeclampsia and lead to novel and effective treatments for this debilitating pregnancy-specific syndrome.

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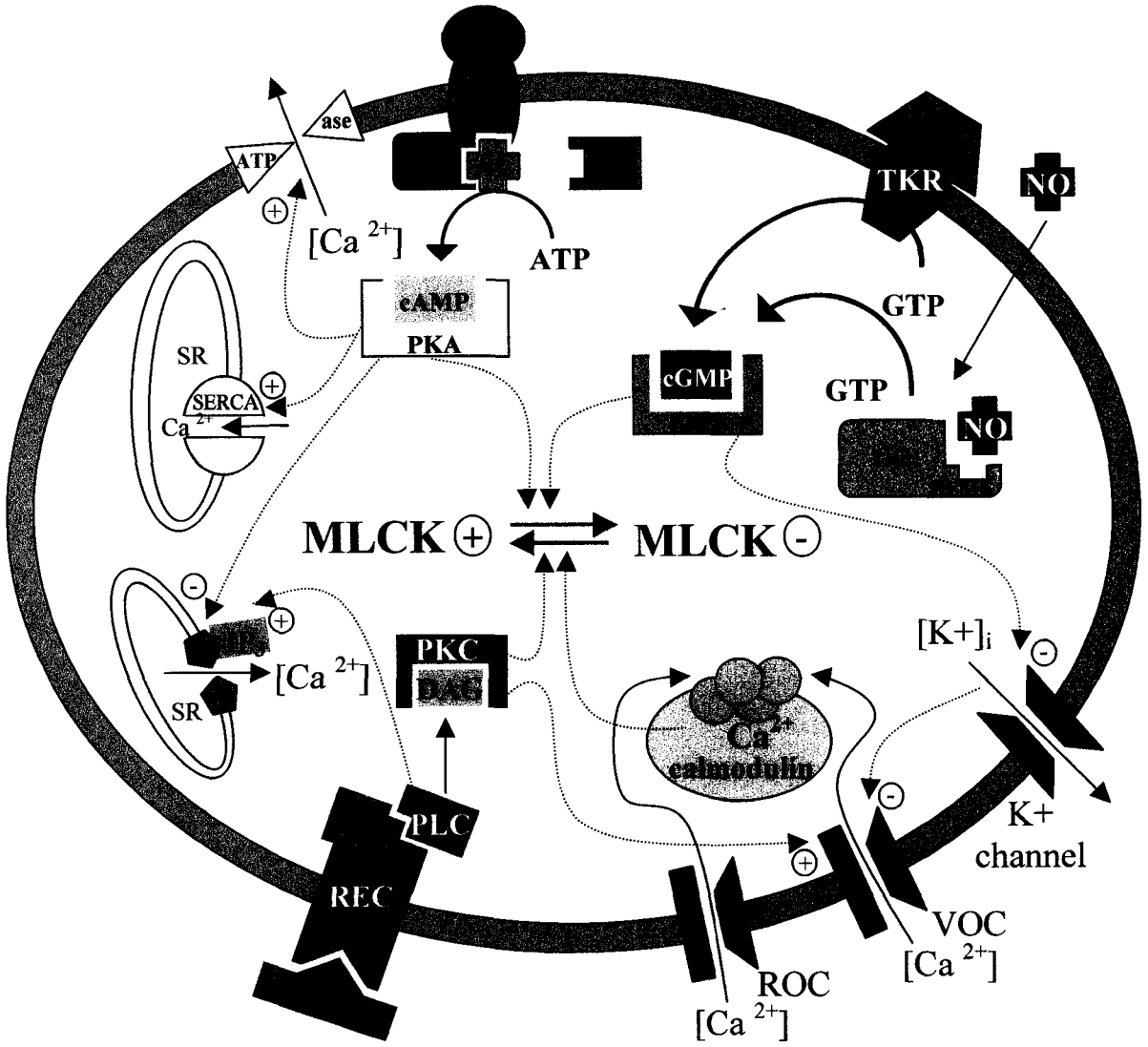

Figure 1.1 Regulation of Vascular Smooth Muscle Calcium and Myosin Light Chain Kinase Activity

Vascular smooth muscle tone can be modified by altering calcium sensitivity of the contractile machinery, especially myosin light chain kinase (MLCK). This enzyme can be positively and negatively modulated directly, or may be regulated indirectly by altering intracellular calcium levels ([Ca2+]) which changes the activity of calcium-calmodulin. Abbreviations: REC – agonist-stimulated receptor; TKR – tyrosine kinase receptor; NO – nitric oxide; AC – adenylate cyclase; GC – guanylate cyclase; PKA – cAMP activated protein kinase; PKG – cGMP activated protein kinase; SERCA – smooth endoplasmic reticulum calcium ATPase pump; MLCK – myosin light chain kinase; ROC – receptor operated calcium channel; VOC – voltage operated calcium channel. Adapted from: Shaw, A.M. and McGrath, J.C. *Pharmacology of Vascular Smooth Muscle.* 103-135, 1997.

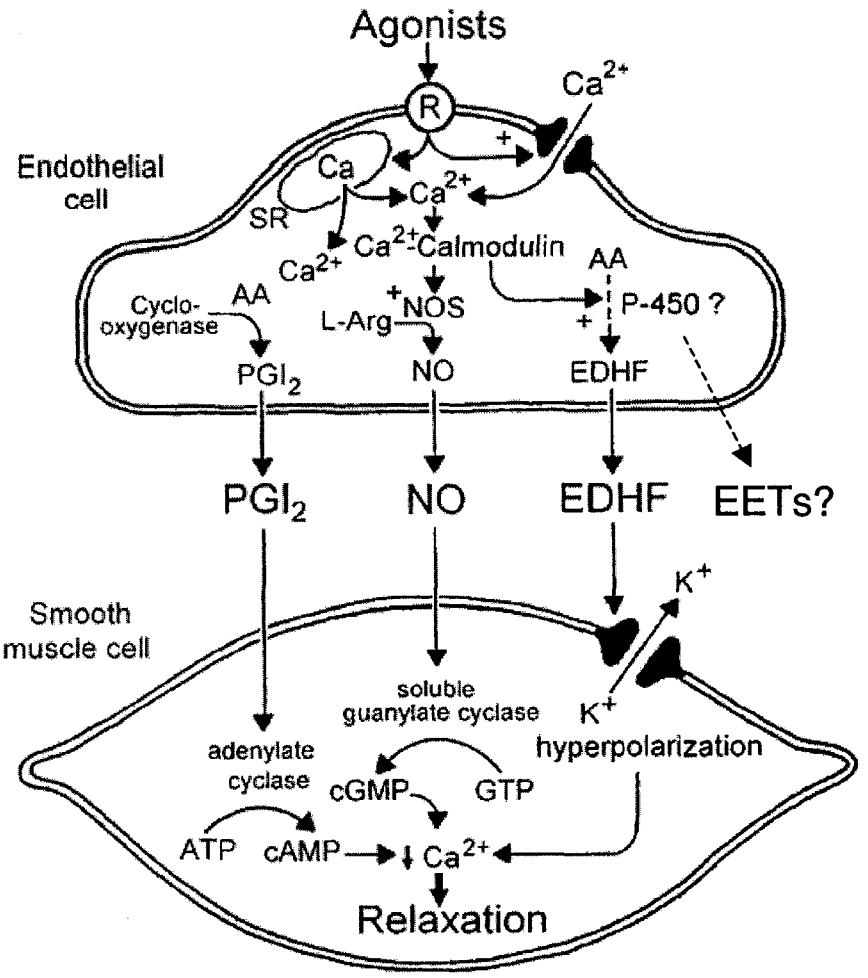

Figure 1.2 Endothelial-Derived Vasodilators Modulate Smooth Muscle Tone

The endothelial cell releases three major vasodilators, prostacyclin (PGI_2), nitric oxide (NO) and the endothelial-derived hyperpolarizing factors (EDHF), including epoxyeicosatreinoic acids (EETs). These molecules act via second messengers and/or potassium channels in the vascular smooth muscle to reduced vascular tone. Adapted from: Vanhoutte, P. M., and J. V. Mombouli.. *Prog Cardiovasc Dis* 39: 229-38., 1996.

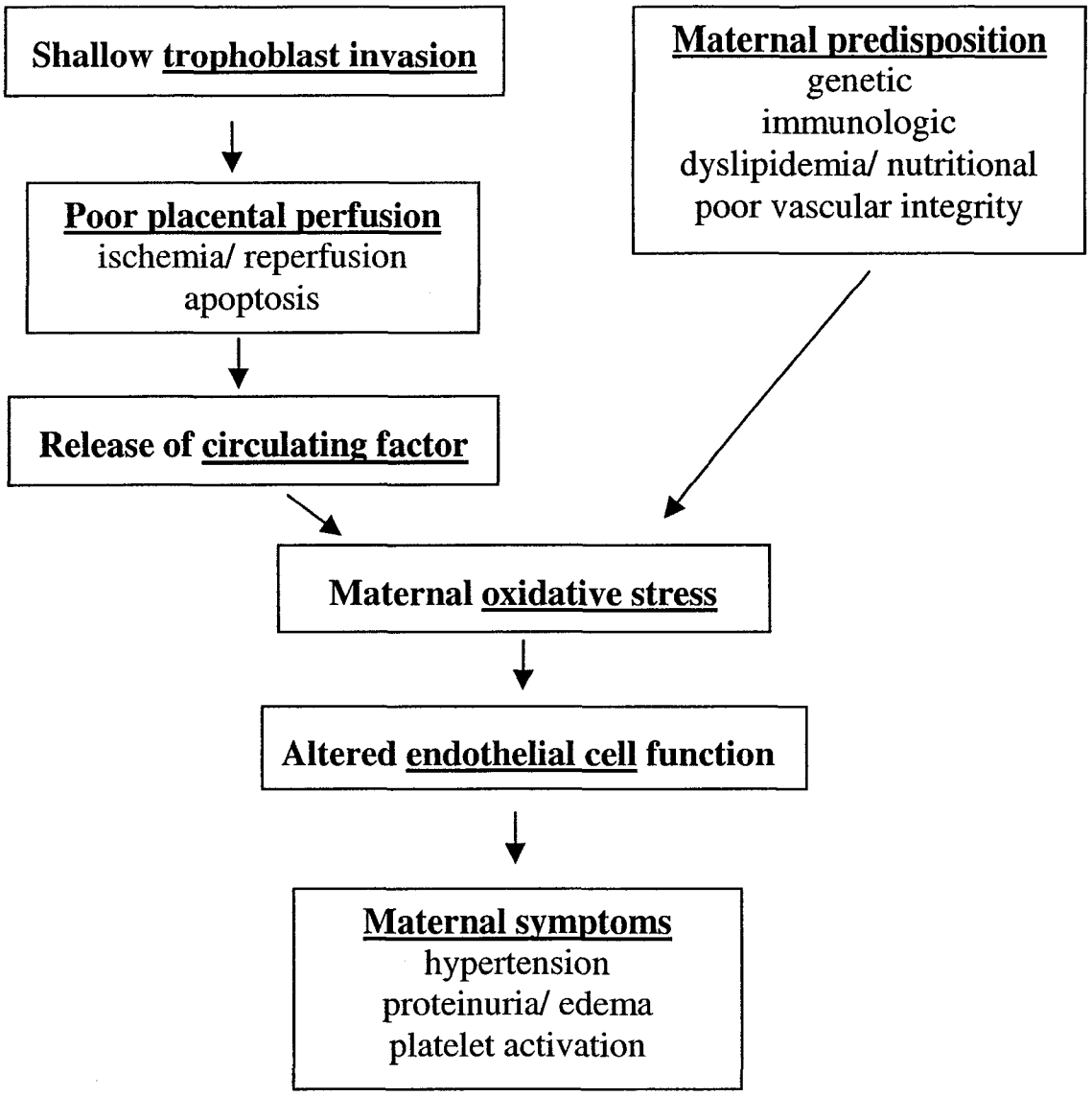

Figure 1.3 The Two-Step Model of Preeclampsia

The etiology of preeclampsia suggests that poor placentation may interact with a Mother's predisposition to vascular abnormalities, and the combination of these factors contributes to this syndrome. The maternal systemic manifestations of preeclampsia center around endothelial cell dysfunction, possible mediated by pro-oxidant molecules. Adapted from: Roberts, J. M., and K. Y. Lain. *Placenta* 23: 359-72.,2002.

Pro-Oxidant Radicals	Pro-Oxidant Non-Radicals
Reactive Oxygen Species	
Superoxide - $^{\bullet}O_2^-$	Hydrogen peroxide - H_2O_2
Hydroxyl - $^{\bullet}OH$	Hydrochlorous acid - HOCl
Peroxyle - $LOO^{\bullet}$	Ozone - O_3
Alkoxyl - $LO^{\bullet}$	Singlet oxygen - $^1O_2$
Hydroperoxyl - $HOO^{\bullet}$	
Reactive Nitrogen Species	Peroxynitrite - $ONOO^-$
Nitric oxide - $NO^{\bullet}$	Nitrous acid - HNO_2
Nitrogen dioxide - $NO_2^{\bullet}$	Dinitrogen trioxide - N_2O_3
	Alkyl peroxynitrite - LOONO

Anti-Oxidant Systems		
Intracellular compartments	Cellular membranes	Extracellular space
Superoxide dismutase	Vitamin E	Ascorbic acid
Catalase	β-carotene	Vitamin E
Glutathione peroxidase		Albumin
Ascorbic acid		EC-superoxide dismutase

Table 1.1 Summary of Reactive Oxygen and Nitrogen Species and Anti-Oxidant Defenses

Table summarizes the major reactive species, both oxygen and nitrogen derived as well as compartmentalization of the various anti-oxidants. Adapted from: Yu, B. P., and H. Y. Chung. *Diabetes Res Clin Pract* 54 Suppl 2: S73-80., 2001.

CHAPTER 2. PEROXYNITRITE INCREASES iNOS THROUGH NFκB AND DECREASES PROSTACYCLIN SYNTHASE IN ENDOTHELIAL CELLS

A version of this chapter has been published. Cooke and Davidge 2002. Am J Physiol. 282: C395-402.

2.1 INTRODUCTION

Peroxynitrite formation has been observed in the maternal vasculature of women with preeclampsia (26), in the placental vasculature of women with preeclampsia and women with diabetes (20), in patients with atherosclerosis (2) and in animal models of aging (34). The localization of peroxynitrite to these tissues suggests that it may be involved in altering vessel reactivity, since all of these conditions are associated with vascular dysfunction. The endothelium has an important function in maintaining vascular tone, which is mediated in part by the enzymes nitric oxide synthase (NOS) and prostaglandin H-synthase (PGHS). Both NOS and PGHS have inducible isoforms (iNOS and PGHS) (9, 27). As such, NOS and PGHS have been implicated in altering vascular function in conditions characterized by oxidative stress (3, 33).

For example, enhanced NOS activity in an environment of oxidative stress would result in scavenging of NO by superoxide anion, forming the potent pro-oxidant, peroxynitrite, thus reducing nitric oxide bioavailability as a vasodilator (3). PGHS-2 may also mediate vascular dysfunction in conditions characterized by oxidative stress. For example, in carotid arteries and macrophages from patients with atherosclerosis, PGHS-2

expression is elevated (2, 33). Furthermore, the enzymatic activity of prostacyclin synthase is inhibited by the pro-oxidant, peroxynitrite, which could result in reduced prostacyclin-mediated vasodilation (38). Thus, endothelial cells maintain a balance of vasodilators and vasoconstrictors, in part through NOS and PGHS-dependent mechanisms, which may be disrupted by oxidative stress. However, the involvement of peroxynitrite in regulating these pathways has not been well elucidated.

The aim of this study was to determine the effect of peroxynitrite on endothelial cell function, focussing on enzymatic pathways that contain oxidant sensitive isoforms. We hypothesized that peroxynitrite would increase the levels of iNOS and PGHS-2, through the activation of NFκB and decrease protein levels of prostacyclin synthase. The results from this study will help determine the mechanisms by which peroxynitrite may alter endothelial cell function, leading to the vascular abnormalities that are characteristic of patients with hypertension, atherosclerosis, and preeclampsia.

2.2 METHODS

2.2.1 Reagents

α-Minimum essential medium (α-MEM) with or without phenol red, horse serum, L-glutamine, trypsin, phosphate buffered saline and penicillin/ streptomycin were purchased from GIBCO (Gaithersburg, MD, U.S). Nystatin, SIN-1, PDTC, gentamycin, kanamycin and bovine serum albumin (BSA) were purchased from SIGMA (Oakville, ON, Canada).

2.2.2 *Endothelial Cell Culture*

Bovine microvascular endothelial cells (B88 cell line) were selected for our study because they originate from a resistance sized vascular bed and therefore intrinsically function to regulate systemic vascular resistance. When cultured, these cells show typical endothelial cell characteristics: growth in a monolayer, cobblestone morphology and positive detection of von Willebrand factor. Cells were grown at 37° C in an atmosphere of 5% CO_2 and 95% air. Growth media (α–MEM) were supplemented with 1% L-glutamine, 10% horse serum, 5 μg/ ml gentamycin, 20 μg/ ml kanamycin, penicillin-streptomycin and nystatin. At confluence, cells were plated into six well plates (10^6 cells per well) in a volume of 1 ml. After 24 hours, cells were quiesced overnight with phenol red free media (also supplemented as above). Prior to stimulation, plates were replaced with fresh media. After experimentation, the total protein content of the cells was determined using the Bradford Method (4), with BSA used as a standard.

2.2.3 *Experimental Protocol*

Our experiments involved using 3-morpholinosydnonimine N-ethylcarbamide (SIN-1), a peroxynitrite donor that breaks down at physiological pH to form nitric oxide and superoxide anion simultaneously (10). SIN-1 is commonly used as an effective pharmacological agent for administering peroxynitrite at the level of the cell in a relatively stable form, as opposed to using authentic peroxynitrite, which is highly volatile and may decompose into inactive metabolites before reaching the cell. We determined the formation of peroxynitrite in our cells by detection of nitrotyrosine, using

both Western immunoblots and immunocytochemistry (Figure 2.1). Furthermore, in order to confirm that the effects of SIN-1 were mediated by peroxynitrite, we studied the effect of exogenous peroxynitrite (Cayman Chemicals, Ann Arbor, MI) as well as the effect of the NO donor sodium nitroprusside (SIGMA), on endothelial cell function.

SIN-1 was prepared immediately before use and was tested initially in a range of doses (0.1-1.0 mM) and for various lengths of stimulation (2, 6, 18 h) based on the literature (21, 22, 37). From these experiments, 0.5 mM and 6 h were selected as the most effective dose and length of stimulation and thus used for all subsequent experiments. Higher doses and longer incubation times resulted in an increased incidence of cell damage, as assessed by the lactate dehydrogenase (LDH) assay. After 24 hours of quiescence, cells were stimulated for 6 hours with 0.5 mM SIN-1 in the presence or absence of 0.5 mM pyrroline dithiocarbamate (PDTC), a NFκB inhibitor (19). Treatments were performed in triplicate, and at least six separate experiments were conducted. At the end of the stimulation period, the supernatant was collected and stored at -80° C. Cells were rinsed with PBS, scraped and collected into 200μl of homogenizing buffer (25 mM Tris-Cl with 0.1% Triton X-100). Cells were then sonicated for approximately 5 seconds and stored at -80° C.

2.2.4 Nuclear Protein Preparation

At confluence, cells were quiesced for 24 hours in a T-75 flask (10 ml volume). Afterwards, cells were stimulated with 0.5 mM SIN-1 for 4 hours. This time frame was chosen based on previous accounts of NFκB activation (7). Nuclear protein was

extracted from the cells following the method described by Schreiber *et al.* (28). Cells were washed with 10 ml tris buffered saline (TBS) and pelleted by centrifugation at 1500×g for 5 minutes. The pellet was resuspended in 1 ml TBS and pelleted again by spinning in a microfuge for 12 000×g for 15 seconds. The pellet was then resuspended in cold Buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1.0 mM DTT, 0.5 mM PMSF). The cells were allowed to swell on ice for 15 minutes, then 25 µl of 10% Nonidet NP-40 (Boehringer Mannheim, Germany) was added. The tube was vortexed for 10 seconds and centrifuged for 30 seconds at 12 000×g. The nuclear pellet was resuspended in 50 µl of ice-cold Buffer B (20 mM HEPES, 0.4 M NaCl, 1.0 mM EDTA, 1.0 EGTA, 1.0 mM DTT, 1.0 mM PMSF). The tube was agitated for 15 minutes at 4° C, then centrifuged for 5 minutes and the supernatant containing the nuclear protein was stored at –80° C for future use.

2.2.5 Western Immunoblotting

Western immunoblots were performed to measure levels of eNOS, iNOS, PGHS-1, PGHS-2, prostacyclin synthase and nitrotyrosine in endothelial cells and NFκB in endothelial cell nuclei. Samples were diluted 1:4 with sample buffer (1.0 M Tris-Cl, glycine, 2% SDS, 2% Bromophenol Blue, beta-mercaptoethanol) and boiled for 3 minutes. 8 µg of protein from each sample was loaded per well and 10 µl of Kaleidoscope molecular weight marker (BIORAD, Hercules, CA) was loaded in a separate well to allow for accurate determination of molecular weight. eNOS and iNOS protein was run on an 8% acrylamide gel while PGHS-1, PGHS-2 , prostacyclin synthase,

NFκB and nitrotyrosine were run on a 10% gel. Protein was separated by electrophoresis at 120 volts for 1.5 hours in a mini gel apparatus after the procedure of Laemmi (16).

Following separation, protein was transferred onto a nitrocellulose membrane (Osmonics, Westborough, MA). Membranes were incubated for 2 hours at room temperature with primary antibody [rabbit polyclonal anti-PGHS-2, mouse monoclonal anti-PGHS-1 or anti-prostacyclin synthase (Cayman Chemicals, Ann Arbor, MI) or mouse monoclonal anti-eNOS, anti-iNOS or anti-nitrotyrosine (Transduction Laboratories, San Diego, CA)] or overnight at 4° C [rabbit polyclonal anti-NFκB p65 (Santa Cruz Biotechnology Inc., Santa Cruz, CA)]. Secondary antibody was incubated for 1 hour at room temperature (polyclonal anti-rabbit or anti-mouse horseradish peroxidase conjugated, Jackson Immunoresearch, West Grove, PA). Afterwards, protein bands were visualized using the ECL detection system (Amersham LIFE Sciences, Buckinghamshire, UK) and quantified using the Fluor-S MultiImager (BIORAD).

2.2.6 Immunocytochemistry

Cells were plated onto 22x22 mm glass cover slips, and treated with 1.0 mM SIN-1 for 6 hours. Afterwards, the cover slips were fixed with 10% formalin-phosphate and stored overnight at 4° C. SIN-1 treated cells were incubated with 1:100 rabbit polyclonal anti-NFκB (Santa Cruz) or 1:100 monoclonal anti- nitrotyrosine (Transduction Laboratories) overnight at 4° C. A standard immunostaining protocol was followed using the Vectastain ABC kit from Vector Laboratories (Burlingame, CA).

2.2.7 *Total RNA Isolation*

After stimulation, cells were lysed directly in a 6-well plate by addition of 1 ml of TRizol reagent (GIBCO, Burlington, ON), passing the cell lysate through a pipette several times and allowing the cells to sit for 5 minutes at room temperature. The lysate was transferred into eppendorf tubes, 0.2 ml chloroform was added and each tube was vigorously shaken. Tubes were centrifuged for 3 minutes at 12 000×g at 4° C. The aqueous phase was transferred into a fresh tube, 0.5 ml isopropanol was added and tubes were incubated at room temperature for 10 minutes. Afterwards the tubes were centrifuged at 4° C at 12 000×g for 10 minutes. The RNA pellet was washed with 75% ethanol and resuspended in 50 µl TE buffer (10 mM Tris-Cl, pH 7.5 and 1.0 mM EDTA, pH 8.0). The RNA concentration was determined by measuring the absorbency at 260 nm and calculating the optical density.

2.2.8 *Real Time-Polymerase Chain Reaction*

First-strand cDNAs were synthesized by incubating 1.0 µg of total RNA from endothelial cells with 1.0 µM random primers (Stratagene, La Jolla, CA), in a 20 µl reaction volume containing cDNA buffer (50 mM Tris-Cl, 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT), 2.5 mM deoxynucleotide triphosphates and 1 U reverse transcriptase (Superscript II, GIBCO). The mixtures were incubated for 50 minutes at 48 °C, followed by 5 minutes at 85 °C. The PCR reaction contained 25 µl of the SYBR green Master Mix Kit (Applied Biosystems, CA, U.S. containing DNA polymerase, deoxynucleotide triphosphates (dNTPs) and $MgCl_2$) 100 nM sense and anti-sense primers

and 2 μl of the reverse transcriptase reaction. The temperature profile was 3 minutes at 95° C followed by 38 cycles of 30 seconds at 95° C and 30 seconds at 60° C (or 30 seconds at 53° C for cyclophilin, a housekeeping gene). The following primers were used: Bovine prostacyclin synthase: Forward 5′- AGGATGAAGGAGAAGCATGG - 3′; Reverse 5′- GGGCTCCTCGAGTTCTCCTA - 3′; Cyclophilin: Forward 5′- CACCGTGTTCTTCGACATCAC - 3′; Reverse 5′- CCAGTGCTCAGAGCTCGAAAG - 3′.

2.2.9 Data Analysis

Western immunoblot density was quantified using the Fluor-S Max Quantity One software (BIORAD). Protein values are expressed as percent of control ± SEM. RNA content was measured and quantified using the I-Cycler software (BIORAD). The starting quantity of RNA was determined based on the number of cycles required to amplify the cDNA above a set threshold (C_T). Starting quantity values (for both cyclophilin and prostacyclin synthase) were obtained from a standard curve, created from control RNA (ranging from 1.0 ng to 1000 ng RNA) then normalized to cyclophilin RNA for each experimental group. Data are expressed as the standardized amount of RNA/ cyclophilin RNA ± SEM. Either Student's t-test (for comparison between two groups) or One-way ANOVA followed by a Fisher LSD post hoc test were used to determine statistical significance between groups ($P<0.05$). Immunocytochemistry of NFκB and nitrotyrosine immunostaining was qualitatively described.

2.3 RESULTS

2.3.1 *Effect of a Peroxynitrite Donor on iNOS Protein Levels and NFκB Activation in Endothelial Cells*

We investigated the effect of peroxynitrite on endothelial cell enzymes that are important for vascular function and are known to be oxidant sensitive. In this study SIN-1 was used as an endogenous peroxynitrite donor (21, 22, 37). Cell damage was assessed using a LDH assay and was negligible at all doses.

Untreated (control) endothelial cells show low levels of constitutive iNOS expression, which has also been shown in a variety of other cell types (15, 23). SIN-1 treatment significantly increased iNOS protein levels when compared to untreated endothelial cells (167±24.2%, $P<0.05$, Figure 2.2A), whereas eNOS protein mass was not altered by SIN-1 treatment (Figure 2.2B). Since we observed a SIN-1 induced increase in iNOS protein mass, we examined whether the NFκB inhibitor, PDTC could block this effect. Indeed, co-incubation of endothelial cells with SIN-1 in combination with PDTC, prevented the increase in iNOS protein levels (78±18.3%, $P<0.05$).

In order to further substantiate the hypothesis that peroxynitrite is activating NFκB, we investigated the effect of SIN-1 on endothelial cell nuclei. Since NFκB activation requires protein translocation into the nucleus, we assessed nuclear NFκB levels in the presence or absence of SIN-1 treatment. Untreated cells show low levels of NFκB in the nucleus, which is consistent with the constitutive expression of iNOS we reported above. However, we found that treatment of cells with 0.5 mM SIN-1 significantly increased NFκB protein mass in the nucleus (135±10.0%, $P<0.05$, Figure 2.3). Furthermore, using

immunocytochemistry as a qualitative tool, we found that NFκB immunostaining was diffuse and localized mainly to the cytosol in untreated cells (Figure 2.4A) where as, in SIN-1 treated cells, NFκB immunostaining was prominently localized to the nucleus (Figure 2.4B).

2.3.2 Effect of a Peroxynitrite Donor on PGHS-2 and Prostacyclin Synthase Expression

We also studied the effect of peroxynitrite on the enzymes PGHS-1, PGHS-2 and prostacyclin synthase. Contrary to our hypothesis, treating endothelial cells with a peroxynitrite donor did not increase PGHS-2 protein levels (104±8.7%, $P>0.05$, Figure 2.5A). The constitutively expressed PGHS-1 also was not altered by SIN-1 treatment (Figure 2.5B). On the other hand, prostacyclin synthase, the enzyme downstream of PGHS which forms the vasodilator prostacyclin, was significantly inhibited by SIN-1 treatment of the endothelial cells (78±8.9%, $P<0.05$, Figure 2.6).

We further investigated whether this down-regulation of prostacyclin synthase was at the level of transcription, using real time PCR to measure changes in mRNA expression in endothelial cells after SIN-1 treatment. The results (expressed as a ratio of standardized prostacyclin synthase to cyclophilin RNA) indicate that there was no difference between the amount of prostacyclin synthase RNA in control vs. SIN-1 treated endothelial cells (0.66±0.2 vs. 0.59±0.1, Figure 2.7). Therefore, peroxynitrite does not seem to be altering prostacyclin synthase at the level of gene expression.

2.3.3 Effect of Authentic Peroxynitrite on iNOS and Prostacyclin Synthase

In order to confirm the cellular effects of peroxynitrite formed by SIN-1, we treated endothelial cells with 100 μM authentic peroxynitrite for 6 hours. Our data show that the same changes in protein levels occur with peroxynitrite treatment as with SIN-1. For example, iNOS protein levels increased (133±9%, P=0.07, Figure 2.8A) while prostacyclin synthase was significantly reduced (73±5.5%, P<0.05, Figure 2.8B). Therefore, these observations support the results obtained with the peroxynitrite donor SIN-1 and strongly suggest that peroxynitrite is capable of mediating changes in protein levels in the endothelium.

2.4 DISCUSSION

This study shows that peroxynitrite alters endothelial cell pathways, which are important for vascular function. The results illustrate that the peroxynitrite donor SIN-1 significantly upregulates iNOS protein mass in endothelial cells. This novel finding has important implications for the development of vascular dysfunction, especially in diseases characterized by oxidative stress. By increasing iNOS levels in the vasculature, peroxynitrite is capable of stimulating large quantities of nitric oxide, which in an environment of oxidative stress, will rapidly be scavenged, forming more peroxynitrite and culminating in a positive feedback cycle (Figure 2.9). Thus, the detrimental effect(s) of peroxynitrite may be exacerbated by this feed forward mechanism, in addition to reducing the bioavailability of nitric oxide as a potent vasodilator.

A role for iNOS in vascular pathophysiology is supported by data in the SHR rat, showing a reduction in the development in hypertension by a specific iNOS inhibitor (11). Furthermore, patients with atherosclerosis show elevated iNOS in coronary plaques, which co-localizes with nitrotyrosine, a marker for peroxynitrite (6). Our data using endothelial cells suggests that peroxynitrite induces iNOS protein. However, it has also been reported that peroxynitrite inhibits iNOS activity in lung epithelial cells (24). This is one potential mechanism by which peroxynitrite may counterbalance the positive feedback cycle, as discussed above. Moreover, the increased expression of iNOS by peroxynitrite may also help explain paradoxical observations in women with preeclampsia; while nitric oxide-mediated vasorelaxation is reduced in maternal blood vessels (1) there is reportedly either no change (5) or elevated (29) levels of nitric oxide metabolites. Thus, the impaired endothelium-dependent relaxation, with no concomitant decrease in nitric oxide *per se* suggests that there is increased nitric oxide scavenging by oxygen free radicals. Indeed, our laboratory reported evidence of peroxynitrite formation in the vasculature of women with preeclampsia (26).

Currently, little is known about the effect(s) of peroxynitrite on cellular signaling pathways, therefore we studied the mechanism by which peroxynitrite is altering the expression of iNOS. Since NFκB is an oxidant-sensitive transcription factor, we investigated whether the pro-oxidant peroxynitrite could potentially activate NFκB. Using Western blot analysis of cell nuclei, a technique used by others (31), we showed that SIN-1 treatment was capable of increasing NFκB levels in the endothelial cell nuclei. These data provides strong correlative evidence that peroxynitrite can induce nuclear

translocation of NFκB. Our results are further supported by immunocytochemical data, illustrating diffuse cytosolic immunostaining in control cells, but intense nuclear staining in SIN-1 treated cells. Furthermore, the peroxynitrite-induced increase in iNOS protein levels was diminished in the presence of a pharmacological NFκB inhibitor, PDTC. Taken together, our data support the hypothesis that peroxynitrite is a potential activator of NFκB in endothelial cells.

The activation of NFκB by peroxynitrite has been implicated in other cell types. In LPS-stimulated human whole blood, peroxynitrite can induce interleukin-8 gene expression, which is blocked by the NFκB inhibitor, PDTC (8). Additionally, during hepatocyte isolation, NFκB activation can be inhibited by both nitric oxide synthase blockade (L-NAME) or through administration of the anti-oxidant/ peroxynitrite scavenger TROLOX^{TM} (25). In rat lung epithelial cells, SIN-1 activated a NFκB-dependent luciferase reporter vector after 8 hours of stimulation (12). Therefore, the pro-oxidant peroxynitrite is likely able to activate NFκB and our data extend this mechanism to include endothelial cells.

Peroxynitrite has previously been shown to activate PGHS (17). In our study, we investigated whether peroxynitrite could increase protein expression. We were unable to detect a statistically significant increase in PGHS-2 levels in endothelial cells treated with SIN-1. Since our data suggests that peroxynitrite activated NFκB in the endothelial cells, it is possible that other nuclear factors are necessary for full activation of PGHS-2 expression, including high-mobility-group protein I (Y), which is required for complete upregulation of PGHS-2 under hypoxic conditions (13). Therefore, the formation of

peroxynitrite *in vivo* may act in concert with other physiological events that were not present in our experiments, to increase PGHS-2 production in endothelial cells.

Prostacyclin synthase, an enzyme downstream of PGHS, was significantly reduced in endothelial cells treated with SIN-1. However, this effect of peroxynitrite on prostacyclin synthase does not seem to be at the level of transcription, since we did not observe any effect of SIN-1 on prostacyclin synthase mRNA expression. One possible mechanism by which peroxynitrite may decrease prostacyclin synthase protein mass is via increasing proteolytic degradation of the enzyme. Zou *et al.* (38) have illustrated that peroxynitrite inhibits the activity of prostacyclin synthase through a tyrosine nitration-dependent mechanism. Furthermore, recent findings show that nitration of tyrosine residues in certain proteins can increase proteolytic degradation, by enhanced targeting to the proteosome (30). Thus, prostacyclin synthase is a potential candidate for enhanced proteolytic degradation by peroxynitrite-induced tyrosine nitration.

The effects of peroxynitrite on the PGHS pathway illustrate another important mechanism by which peroxynitrite can alter endothelial cell function. We have shown, for the first time, that peroxynitrite is capable of inhibiting prostacyclin synthase at the level of protein, in addition to the well-documented effects of peroxynitrite on prostacyclin synthase activity (38) (Fig. 9). Furthermore, peroxynitrite has been shown to increase PGHS activity (17). Therefore, in endothelial cells, peroxynitrite formation shifts the balance away from the vasodilator prostacyclin and towards the vasoconstrictors, PGH_2 and thromboxane. Again, this theory correlates well with data from women with preeclampsia, who show reduced levels of prostacyclin and elevated levels of thromboxane metabolites (36). Furthermore, in atherosclerosis, PGHS-2 is

highly expressed and co-localizes with iNOS and nitrotyrosine (2). Finally, PGHS-2-mediated vasoconstriction is more pronounced in aging (34), which is also a state of oxidative stress in which peroxynitrite levels have been shown to be elevated (35).

Although it has been proposed that peroxynitrite may have similar physiological effects as nitric oxide in certain biological systems (18) our results are likely specific to peroxynitrite. There are reports illustrating NO-dependent modulation of NFκB, however most of these papers show an inhibitory effect of NO on NFκB activity (14, 32). Furthermore, for comparative purposes, we observed that a NO donor did not increase iNOS in our endothelial cells (data not shown) whereas authentic peroxynitrite induced similar changes in protein levels as SIN-1. Therefore, in an endothelial cell culture model, we find that SIN-1 is an effective, endogenous peroxynitrite donor, which can alter intracellular enzyme expression.

In conclusion, it is well documented that peroxynitrite is elevated in the vasculature of many conditions characterized by oxidative stress. Although peroxynitrite is hypothesized to be involved in the vascular pathophysiology of these conditions, few studies have focused on the effects of peroxynitrite on endothelial cell pathways that regulate vessel function. This study illustrates that peroxynitrite is a novel mediator of endothelial cell function. By activating NFκB, thus increasing the expression of iNOS and inhibiting prostacyclin synthase, peroxynitrite can contribute to the altered vascular reactivity in a variety of conditions in which the clinical manifestations are mediated by oxidative stress.

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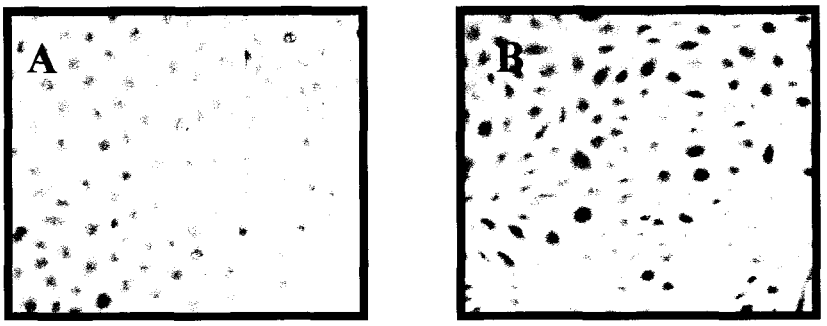

Figure 2.1 SIN-1 Treatment of Endothelial Cells Induces Peroxynitrite Formation

Immunocytochemistry of endothelial cells stained for nitrotyrosine. Cells were plated onto glass cover slips and treated with 0.5 mM SIN-1 for 6 h. Cells were incubated overnight at 4° C with 1:100 anti-nitrotyrosine monoclonal antibody. A) Representative slide of unstimulated endothelial cells. B) Representative slide of endothelial cells stimulated with 0.5 mM SIN-1.

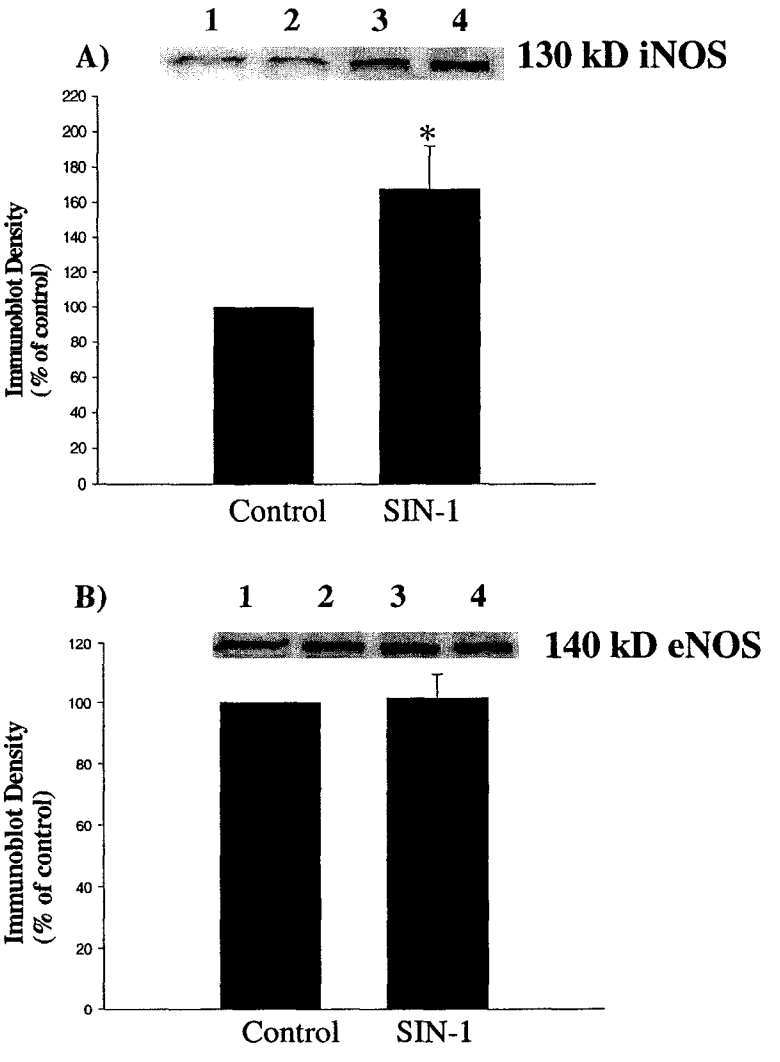

Figure 2.2 Treatment of Endothelial Cells with SIN-1 Increases iNOS Protein Levels

Western immunoblot analysis of iNOS and eNOS protein mass. Equal amounts of protein extracts (8 μg) were separated on a 8 % SDS-PAGE gel. A) Representative immunoblot of iNOS: lanes 1-2: unstimulated cells; lanes 3-4: endothelial cells treated with 0.5 mM SIN-1 for 6 h. B) Representative immunoblot of eNOS. Lanes 1-2: unstimulated cells; lanes 3-4: cells treated with 0.5 mM SIN-1 for 6 h. Summary data are expressed as percent of control ± SEM of six separate experiments; *P < 0.05.

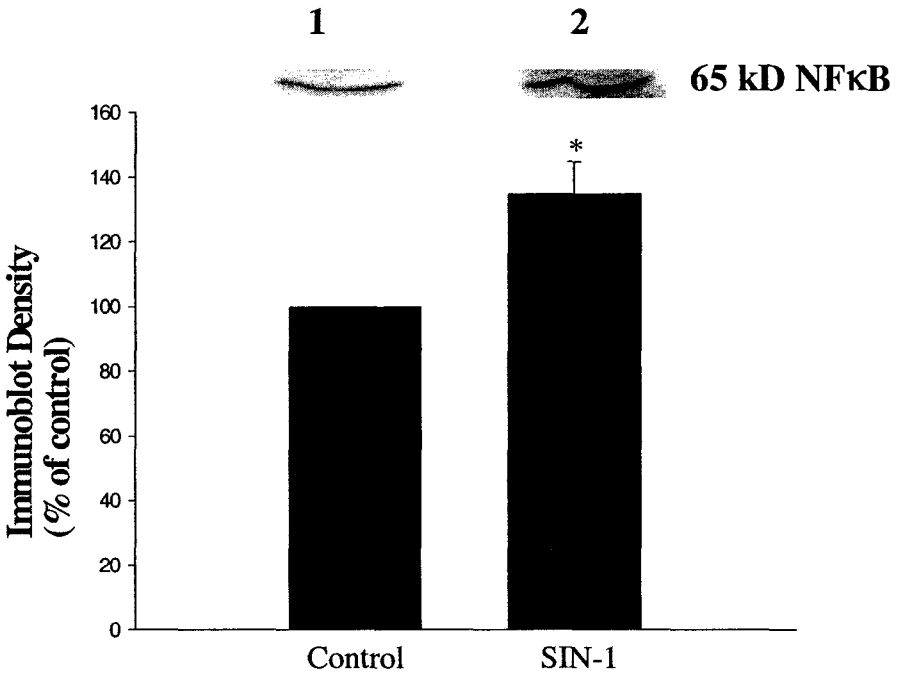

Figure 2.3 SIN-1 Treatment of Endothelial Cells Increases NFκB Protein Levels in the Nucleus

Western immunoblot analysis of NFκB protein mass in endothelial cell nuclei. Equal amounts of protein (10 μg) was separated on a 10 % SDS-PAGE. Representative immunoblot of one experiment: lane 1: unstimulated endothelial cell nuclei; lane 2: endothelial cell nuclei treated with 0.5 mM SIN-1 for 4 h. Summary data are expressed as percent of control ± SEM of four separate experiments, *$P < 0.05$.

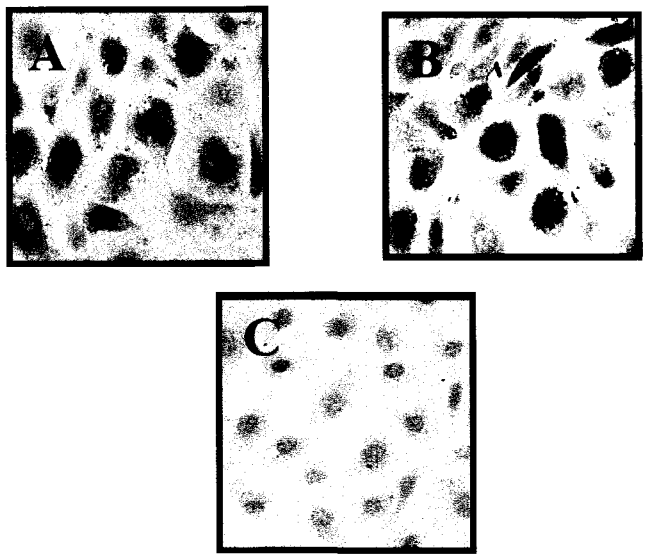

Figure 2.4 SIN-1 Stimulates Nuclear Translocation of NFκB in Cultured Endothelial Cells.

Immunocytochemistry of endothelial cells stained for NFκB. Cells were plated onto glass cover slips, and treated with 0.5 mM SIN-1 for 4 h. Cells were then incubated overnight at 4° C with 1:100 anti-NFκB p65 polyclonal antibody. A) Representative slide of unstimulated endothelial cells. B) Representative slide of endothelial cells stimulated with 0.5 mM SIN-1. C) Immunostaining for non-specific IgG, negative control.

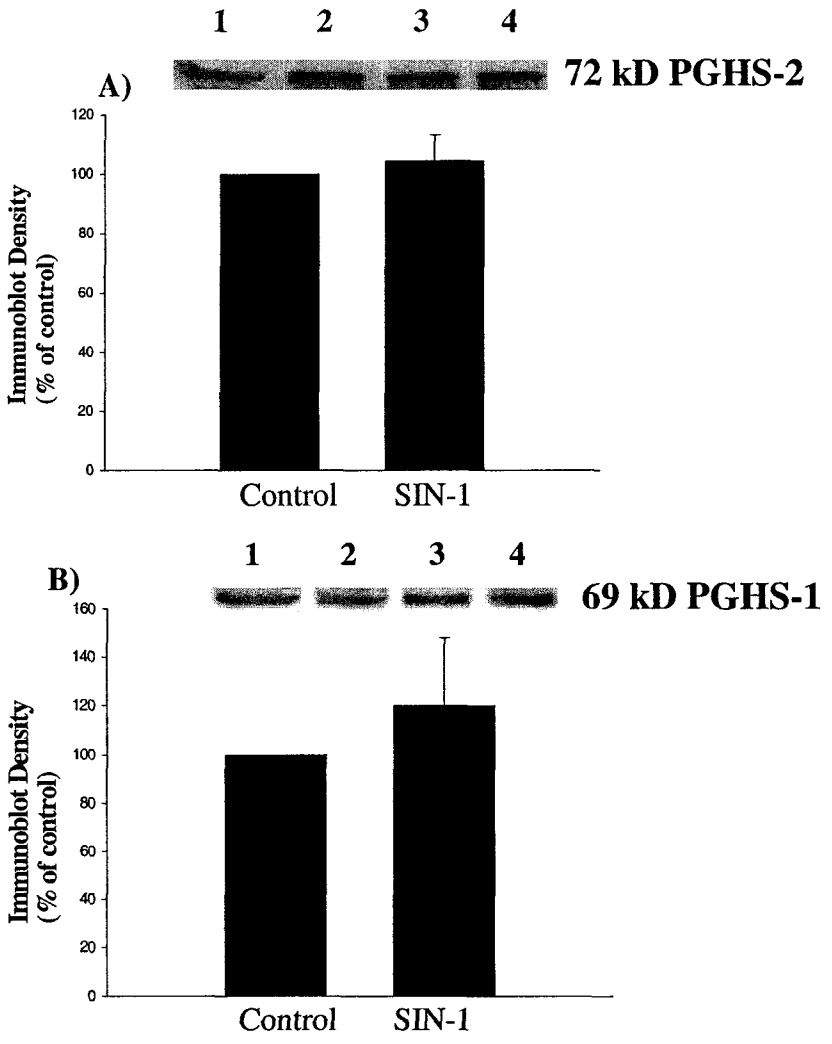

Figure 2.5 SIN-1 Does Not Affect Protein Levels of Either PGHS-1 or PGHS-2.

Western immunoblot analysis of PGHS-2 and PGHS-1 protein mass. Equal amounts of protein extracts (8 μg) were separated on a 10 % SDS-PAGE. A) Representative immunoblot of PGHS-2. Lanes 1-2: unstimulated endothelial cells; lanes 3-4: endothelial cells treated with 0.5 mM SIN-1 for 6 h. B) Representative immunoblot of PGHS-1. Lanes 1-2: unstimulated endothelial cells; lanes 3-4: endothelial cells treated with 0.5 mM SIN-1 for 6 h. Summary data are expressed as percent of control ± SEM of six separate experiments.

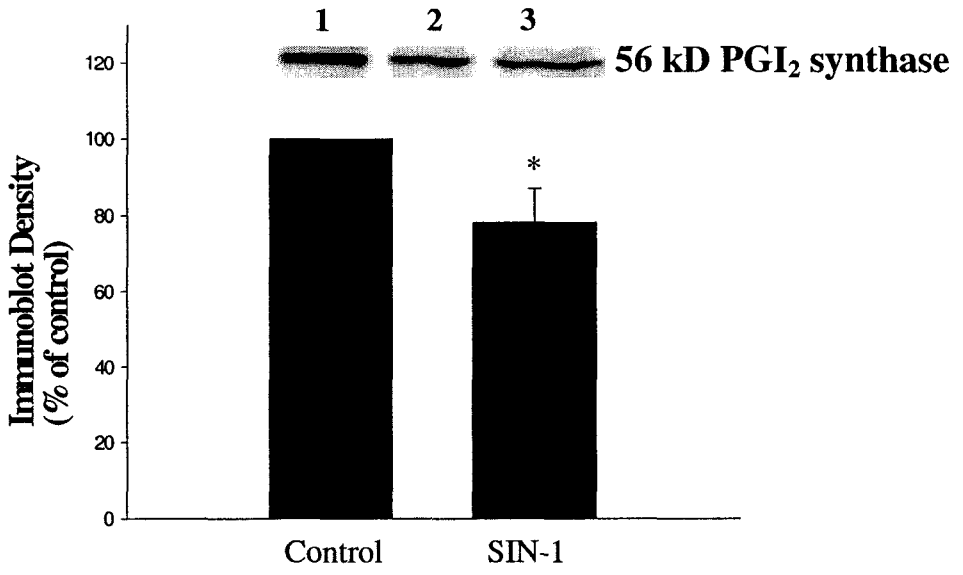

Figure 2.6 SIN-1 Reduces Protein Levels of Prostacyclin Synthase

Western immunoblot analysis of prostacyclin synthase protein mass. Equal amounts of protein extracts (8 μg) were separated on a 10 % SDS-PAGE gel. Representative immunoblot of one experiment: lane 1: unstimulated endothelial cells; lanes 2-3: endothelial cells treated with 0.5 mM SIN-1 for 6 h. Summary data are expressed as percent of control ± SEM of six separate experiments.

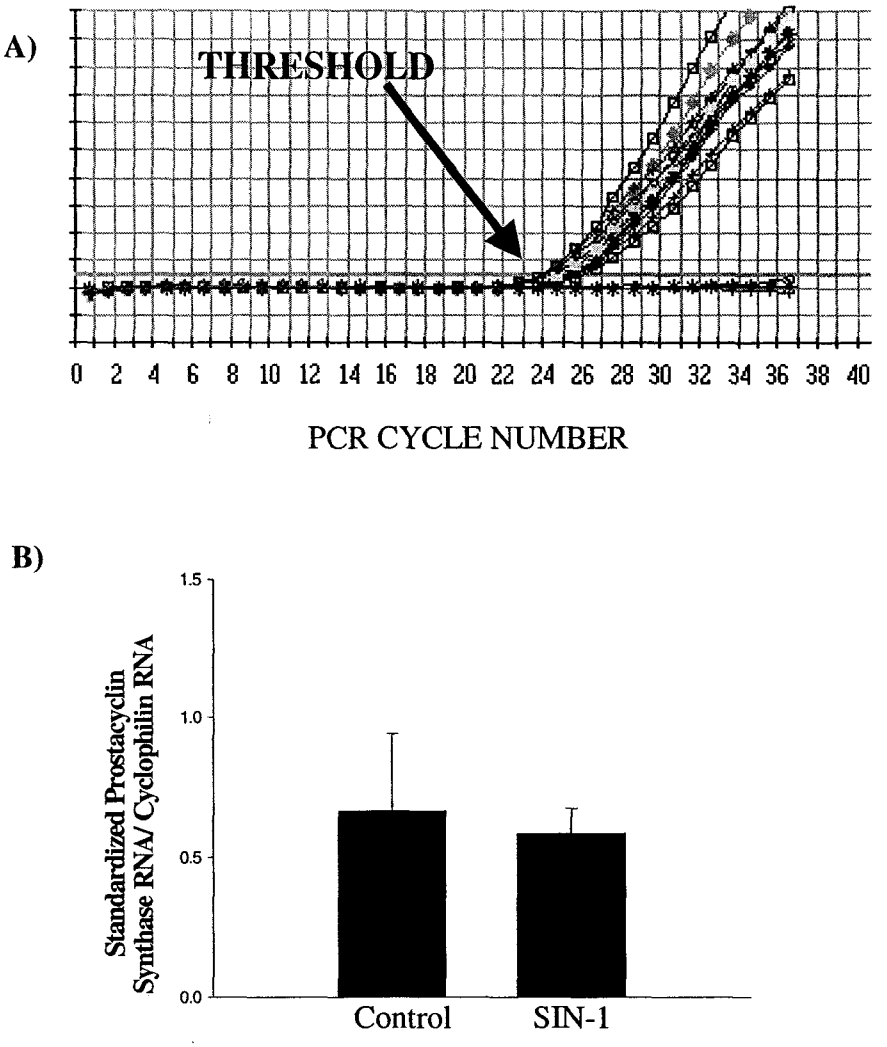

Figure 2.7 The Effect of SIN-1 on Prostacyclin Synthase mRNA Expression

Real time PCR measuring prostacyclin synthase RNA in endothelial cells. PCR was performed using 2 μl of the reverse transcriptase reaction in a 50 μl reaction containing SYBER green Master Mix (Applied Biosystems, CA) and 100 nM of the sense and anti-sense prostacyclin synthase primers. Relative amounts of RNA were calculated based on the number of cycles required to cross a set threshold. A standard curve was constructed and RNA values were normalized to cyclophilin. A) Representative trace of real time PCR, illustrating threshold cycle. B) Summary of PCR data for prostacyclin synthase. Data are expressed as standardized amount of RNA/ amount of cyclophilin RNA. There were no significant differences in relative amounts of RNA between control and SIN-1 treated endothelial cells.

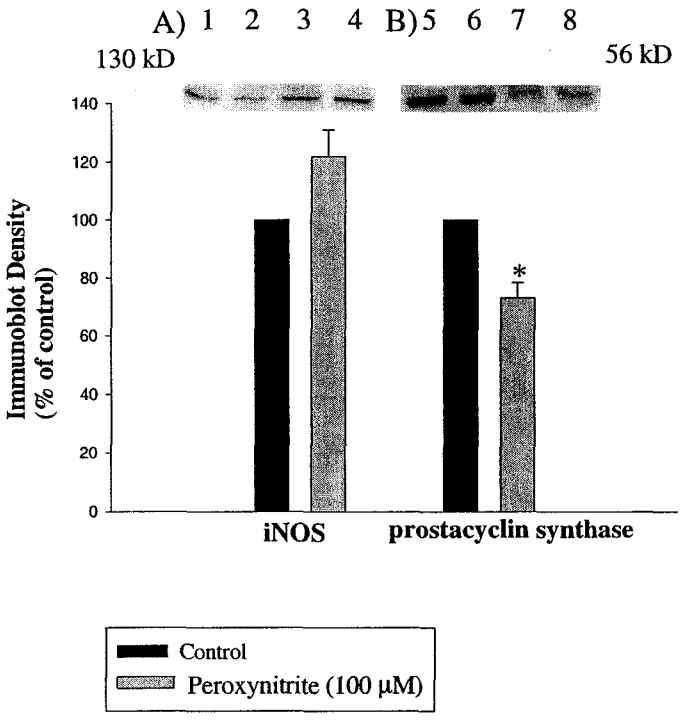

Figure 2.8 The Effect of Authentic Peroxynitrite on iNOS and Prostacyclin Synthase Protein Levels

Western immunoblot analysis of iNOS and prostacyclin synthase protein mass. Equal amounts of protein extracts (8 μg) were separated on either a 8 or a 10 % SDS-PAGE gel for iNOS or prostacyclin synthase respectively. A) Representative immunoblot of iNOS. Lanes 1-2: unstimulated endothelial cells; lanes 3-4: endothelial cells treated with 100 μM peroxynitrite for 6 h. B) Representative immunoblot of prostacyclin synthase. Lanes 5-6: unstimulated endothelial cells; lanes 7-8: endothelial cells treated with 100 μM peroxynitrite for 6 h. Summary data are expressed as percent of control ± SEM of three separate experiments.

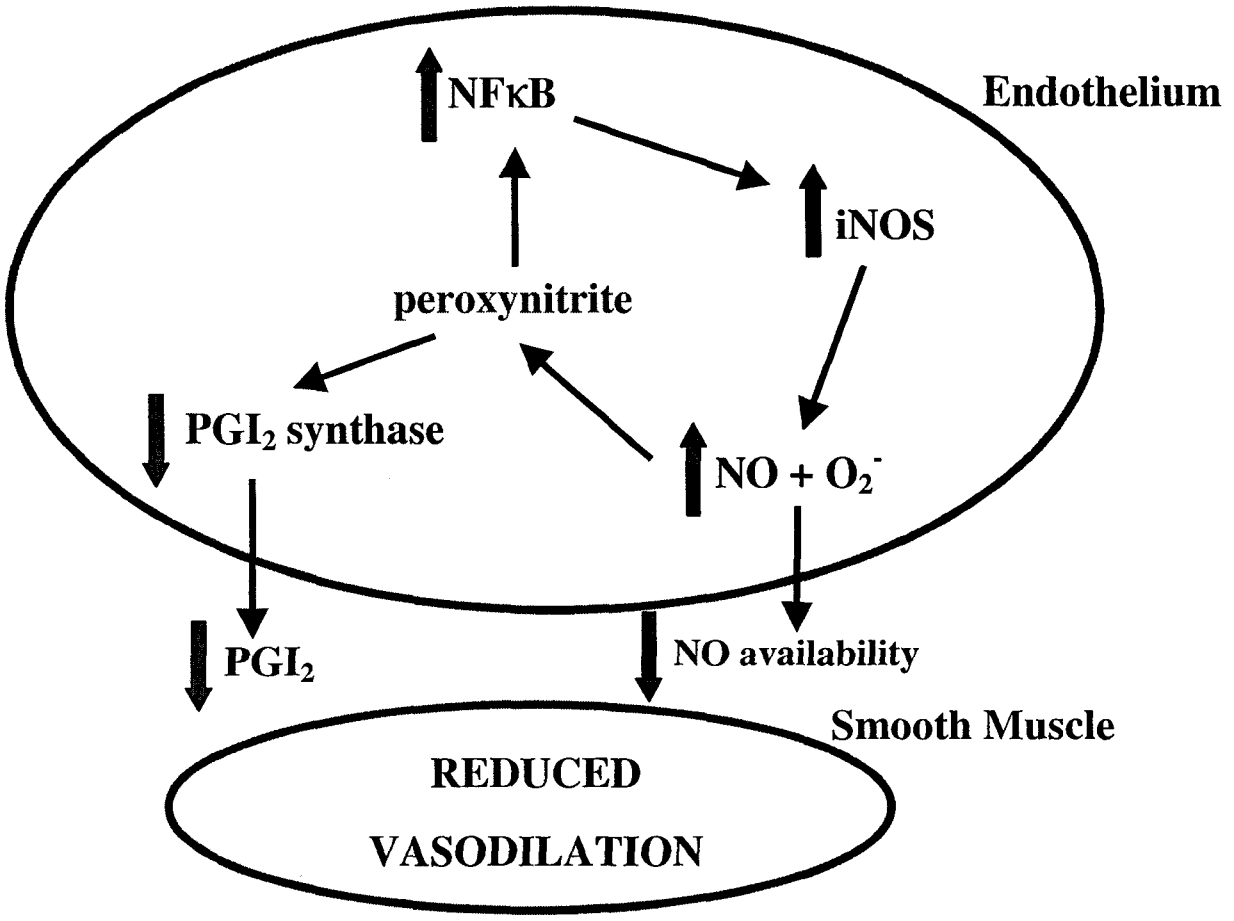

Figure 2.9 Proposed Effects of Peroxynitrite on the Endothelium

Our results show that the peroxynitrite donor, SIN-1 can increase iNOS protein levels in endothelial cells, through the activation of NFκB. In an environment of oxidative stress, this would enhance peroxynitrite formation, causing a positive feedback loop, and reduce the bioavailability of nitric oxide as a vasodilator. Furthermore, peroxynitrite reduces the protein mass of prostacyclin synthase, thus inhibiting prostacyclin - mediated vasodilation.

CHAPTER 3. ENDOTHELIAL-DEPENDENT RELAXATION IS REDUCED IN MESENTERIC ARTERIES FROM THE CuZn SOD KNOCKOUT MOUSE

A version of this chapter has been submitted to British Journal of Pharmacology, Cooke and Davidge, 2003.

3.1 INTRODUCTION

Superoxide anion, although a deleterious via the initiation of the harmful Fenton-Weiss reaction (8, 9) is also a precursor to many reactive oxygen/ nitrogen species. For example, superoxide anion is the precursor to hydrogen peroxide, which can be hydrolyzed into the potent hydroxyl radical, an initiator of lipid peroxidation. Further, superoxide anion scavenges nitric oxide, forming peroxynitrite, which occurs at a rate significantly more rapid than the reaction of superoxide with the enzyme superoxide dismutase (SOD) (1, 29).

The reaction of superoxide with nitric oxide reduces the bioavailability of nitric oxide as a vasodilator. Additionally, peroxynitrite itself substantially impacts the vascular system. We previously found that peroxynitrite affects endothelial cells in culture by reducing protein levels of prostacyclin synthase, as well as by inducing nuclear translocation of the oxidant-sensitive transcription factor, NFκ-B (4). Other laboratories have found that peroxynitrite activates the enzyme upstream of prostacyclin synthase, prostaglandin H synthase (PGHS) which also produces the vasoconstrictor thromboxane,

in addition to prostacyclin (17). Overall, these alterations may shift the balance between dilators and constrictors, leading to a reduced capacity for vasorelaxation.

Pro-oxidants are typically maintained at low intracellular levels by both enzymatic and non-enzymatic mechanisms. Pertinent to our study, superoxide dismutase (SOD) is the enzyme responsible for converting the free radical superoxide anion into hydrogen peroxide, which is then neutralized by catalase into water (12). SOD exists as three isoforms; SOD-1 is copper-zinc dependent and localized to the cytosol (CuZn SOD), SOD-2 is manganese dependent and found in the mitochondria (Mn SOD) and there is also an extracellular SOD (EC-SOD)(8). The Mn SOD isoform plays a critical role in the protection from oxidants inside the mitochondria, which produces copious amounts during cellular respiration. Although homozygous knockout Mn SOD mice die within the first ten days of life, (19), the phenotype of the heterozygotes includes increased susceptibility to oxygen toxicity (33) as well as altered cardiac mitochondrial function and increased oxidative injury (35). On the other hand, the cytosolic isoform, CuZn SOD scavenges superoxide that has been produced from enzymatic processes, such as NADPH oxidase (39). Thus, the CuZn SOD-/- mouse constitutes a proficient model to assess the influence of elevated superoxide anion, *in vivo*, on vascular function.

Based on the importance of oxidative stress in the pathogenesis of vascular dysfunction, we sought to determine the effect of increased cytosolic superoxide radicals on blood vessel reactivity, focussing on endothelial cell vasoactive pathways that are altered by pro-oxidant molecules. We hypothesized that resistance-sized blood vessels from SOD-/- mice will have reduced endothelial-mediated relaxation and enhanced vasoconstriction, due to impaired PGHS and nitric oxide-dependent mechanisms.

3.2 MATERIALS AND METHODS

3.2.1 Animal Housing and Protocols

Heterozygous pair of B6;129S-SOD1 mice were purchased from Jackson Laboratories (Bar Harbour, ME), and were subsequently bred at our facility. Mice were housed in a virus-antigen free environment, kept at 20% humidity, under 12 hour light: dark cycles and fed standard lab chow and water *ad libitum*. Genotype was determined at the University of Alberta Transgenic Facility using specific primers for the mutated gene. Experimental groups consisted of wild type (control; females n = 6 and males n = 8) versus homozygous knockout littermates (SOD-/-; females n = 5 and males n = 7). Initial experiments considered males and females as separate groups, but after data analysis, we found that there was not a statistical difference, thus data from both groups were pooled. Mice were used between 5-7 months of age. All protocols used for this study were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee and were in accordance with the Canadian Council on Animal Care.

3.2.2 Vessel Function

This study focuses on the characterization of vascular reactivity in mesenteric arteries, since they are small resistance sized arteries that contribute substantially to overall peripheral vascular resistance (3). Mesenteric arcades were removed from the animal after cervical dislocation and placed immediately into ice-cold Delbecco Modified Essential Medium (DMEM) buffer (1 mmol/L sodium pyruvate, 25 mmol/L sodium bicarbonate, 5 mmol/L Hepes, 5 mmol/L D+ glucose; pH 7.4) which was used to maintain vessel viability (20). Second-order mesenteric arteries (~150 μm) were cleaned

free of fat and connective tissue under a light microscope. After threading with two smooth 20 μm tungsten wires, vessels were mounted in an isometric wire myograph system (Kent Scientific, Litchfield CT), warmed to 37°C (bathed in 5 ml of DMEM buffer) and equilibrated for 30 minutes.

3.2.3 Myograph Protocols

At the start of each experiment, vessel length was measured using a micrometer and a passive circumference-tension curve was performed for each vessel to determine the optimum resting tension (see Appendix for expanded methods). Cumulative dose response curves were initially performed for phenylephrine (1 – 50 μmol/L). Vessels were used for subsequent relaxation curves if they attained a minimum of 1.0 mN/mm of tension in response to phenylephrine. Phenylephrine dose response curves were repeated after a 20 minute pre-incubation with either N^G-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L; nitric oxide synthase (NOS) inhibitor) or meclofenamate (1.0 μmol/L, PGHS inhibitor). The concentration of phenylephrine required to produce an 80 % response (EC_{80}) was used to pre-constrict the vessels for subsequent vasodilation curves because this dose provides a greater range over which relaxation could be assessed. Methacholine (muscarinic-agonist; endothelial-dependent vasodilator) dose response curves (10 nmol/L – 10 μmol/L) were performed alone or after a 20 minute incubation with L-NAME alone, meclofenamate (MECLO) alone, L-NAME and MECLO together, or L-NAME, MECLO plus inhibitors of calcium-sensitive potassium channels (apamine; 10 μmol/L and charybdotoxin; 0.1 μmol/L).

In a separate set of experiments, methacholine curves were also assessed after a one-hour pre-incubation with the soluble polyethylene glycol-SOD (PEG-SOD; 50 units per ml) either alone or in combination L-NAME (20 minutes, 100 μM). Relaxation responses were also measured over a range of doses (1.0 nmol/L – 10 μmol/L) to an exogenous nitric oxide donor, sodium nitroprusside (SNP), as well as a stable prostacyclin analogue, epoprostenol. All drugs, unless otherwise stated, were purchased from Sigma Chemicals (St. Louis, MO).

3.2.4 Immunohistochemistry for peroxynitrite formation and vasoactive enzymes

At the time of dissection, mesenteric arteries that were not used for myograph experiments were fixed with 4% formaldehyde, and dehydrated overnight in 30 % sucrose at 4 °C. Arteries were embedded in tissue freezing compound (O.C.T. Tissue Tek™, Fisher Scientific) and sectioned using a cryostat (-25° C) at a thickness of 10 μm. The immunostaining protocol is described in brief. Slides were fixed with acetone, and blocked for 30 minutes at room temperature with 2 % bovine serum albumin (BSA) in PBS (pH 7.4). Sections were then incubated with rabbit polyclonal anti-nitrotyrosine (1:100; Transduction Laboratories, San Diego, CA), prostacyclin synthase, PGHS-1 (1:200; Cayman Chemicals, Ann Arbor MI) or eNOS (1:200; Santa Cruz, CA) overnight at 4°C. The anti-rabbit secondary antibody, containing a rhodamine-TRITC fluorescent tag, was incubated for 40 minutes at room temperature. Sections were mounted with Vectashield H-1200 solution (containing DAPI, Burlingame, CA) and slides were sealed. For detection of nitrotyrosine fluorescence in mesenteric arteries, images were captured

using an Olympus Microscope System (Model BX40) with Reflected Light Fluorescence Attachment (Model BX-FLA) and analyzed with Image Pro-Plus software (Media Cybernetics, Silver Spring, MD). Negative controls were performed by incubating slides either without primary or secondary antibody, or incubating with excess nitrotyrosine (10 μM in PBS, SIGMA) before the addition of primary antibody (32). All negative controls showed minimal immunofluorescence.

3.2.5 Oxidative Fluorescent Microtopography

Superoxide anion production was detected as previously described (23) using the fluorescent dye hydroethidium (Molecular Probes; Eugene Oregon), which is oxidized into ethidium bromide in the presence of superoxide (28). Ethidium bromide is excited at 488 nm and has an emission spectrum of 610 nm. Aortas were snap frozen immediately following dissection of the animal, then were embedded in tissue compound (Fisher Scientific) and sliced using a cryostat while still frozen. Care was taken to not allow samples to thaw until the assay was performed in order to minimize any additional stress to the tissue. Slides of unfixed aorta sections (30 μm thick) were removed from –80 °C, and the hydroethidium dye was topically applied (2.0 μmol/L stabilized in DMSO). The slides were incubated at 37 °C for 30 minutes in the dark and then images were captured in the dark using a 510 nm long-pass filter and analyzed with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Due to the nature of the fluorescent dye (which fades over time), the images were captured as quickly as possible, with the identity of the sample concealed.

3.2.6 Data Analysis and Statistics

Dose response curves are graphically depicted as either percent relaxation or constriction and each point represents mean ± standard error of the mean. EC_{50} concentrations were calculated and compared between groups for the phenylephrine, SNP and epoprostenol responses using either a Student's t-test or a One Way Analysis of Variance (ANOVA), where appropriate. However, since methacholine-induced responses were non-sigmoidal in shape and because of the variability in the slopes of the curves, we were not able to use EC_{50} doses for comparison. Thus, statistical significance between wild type and SOD-/- mice, as well as the effect of inhibitors for methacholine relaxation was determined using a Two-Way ANOVA for repeated measures, followed by a Tukey post hoc test. Statistical significance was considered at $P< 0.05$. Immunofluorescence for nitrotyrosine was quantified using Sigma Scan 5.0 software, which measured mean optical intensity of the red fluorescence in mesenteric vessels. These data were for normalized for vessel size by dividing the optical intensity by area and data are expressed using this ratio. Statistical analysis was performed using a Student's t-test, $P < 0.05$. Ethidium bromide fluorescence (detecting presence of superoxide anion) was qualitatively described.

3.3 RESULTS

3.3.1 Vascular reactivity to phenylephrine in SOD-/- and wild type mice

There were no significant differences in overall sensitivity to phenylephrine between wild type and SOD-/- mice (EC_{50} values: 4.53 ± 0.45 μmol/L vs. 5.03 ± 0.51 μmol/L;

Figure 1A). Interestingly, the effect of PGHS inhibition on vasoconstriction was different between groups. In wild type mice, pre-incubation with MECLO (PGHS inhibitor) significantly enhanced the mesenteric sensitivity to phenylephrine, as indicated by a lower EC_{50} concentration (EC_{50}: 3.18 ± 0.20 μmol/L vs. 4.53 ± 0.45 μmol/L; $P<0.05$; Figure 1B) suggesting modulation by a PGHS-dependent vasodilator. In contrast, MECLO had no effect on the phenylephrine dose response curve in SOD-/- mice (EC_{50}: 4.48 ± 0.41 μmol/L vs. 5.03 ± 0.51 μmol/L; Figure 1C). There was a trend towards enhanced sensitivity to phenylephrine in wild type mice after L-NAME treatment, however this effect did not reach statistical significance (EC_{50}: 3.55 ± 0.32 μmol/L vs. 4.53 ± 0.45 μmol/L; $P = 0.09$; Figure 3.1B). Pre-incubation with the NOS inhibitor did not shift the constrictor response in SOD-/- mice (EC_{50}: 4.85 ± 0.32 μmol/L vs. 5.03 ± 0.51 μmol/L; Figure 3.1C).

3.3.2 Endothelial-dependent relaxation in SOD-/- and wild type mice; Role of NOS and PGHS

Methacholine-induced relaxation was significantly impaired in SOD-/- mice compared to wild type mice (Figure 3.2A). In wild type mice, methacholine relaxation was significantly blunted after incubation with either L-NAME or MECLO (Figure 2B). Conversely, in mesenteric arteries from SOD-/- mice, neither L-NAME nor MECLO had a significant effect on methacholine induced relaxation (Figure 3.2C). These data suggest that NOS and PGHS-mediated vasorelaxation in SOD-/- mice is compromised.

By incubating vessels with a combination of both L-NAME and MECLO, we assessed the remaining component of endothelial-dependent relaxation. In mesenteric arteries from both wild type and SOD-/- mice, blocking NOS and PGHS significantly reduced methacholine-induced relaxation (Figures 3.2B and 3.2C). However, this residual relaxation was not statistically different between groups (maximum relaxation: 26 ± 4.3% versus 29 ± 5.0%, P = 0.6). We also incubated mesenteric vessels from both wild type and SOD-/- mice with a combination of apamine and charybdotoxin (in the presence of L-NAME and MECLO) and these inhibitors completely blocked relaxation (data not shown), indicating that the remaining relaxation was indeed mediated by a factor(s) that acts on potassium channels (EDHF-like).

In order to determine whether the impaired endothelial-dependent relaxation in SOD-/- mice was due to acute superoxide production leading to scavenging of nitric oxide, we treated mesenteric vessels with an exogenous cell permeable SOD (PEG-SOD) alone or in combination with L-NAME. Our data illustrate that pre-incubation with PEG-SOD was able to significantly enhance methacholine-induced relaxation in vessels from SOD-/- mice. Furthermore, administering L-NAME to vessels treated with PEG-SOD blunted the enhanced relaxation, such that the relaxation was not different from control curves (Figure 3.3). Interestingly, administering PEG-SOD to vessels from wild type mice caused a reduction in methacholine induced relaxation (maximum relaxation: 56.2 ± 4.0% vs. 22.9 ± 3.2%; $P < 0.05$, data not shown). These data suggest that physiological, low levels of superoxide anion are important for mediating endothelial-dependent vascular functions (22, 25, 30).

3.3.3 Effect of exogenous nitric oxide and prostacyclin on relaxation

Mesenteric arteries from wild type mice were significantly more sensitive to the nitric oxide donor, SNP, compared to arteries from SOD-/-mice (EC_{50}: 0.24 ± 0.03 μmol/L vs. 0.65 ± 0.04 μmol/L; $P < 0.05$; Figure 3.4A). In order to determine whether this effect was specific to nitric oxide, we also used the prostacyclin agonist, epoprostenol. Our data show that the responses to the prostacyclin analogue were not significantly different between groups (EC_{50}: 0.24 ± 0.02 nmol/L vs. 0.27 ± 0.06 nmol/L; Figure 3.4B), suggesting that nitric oxide specifically is being interfered with in SOD-/- mice.

3.3.4 Detection of superoxide anion and peroxynitrite in vessels from SOD -/- mice

Using hydroethidium as a fluorescent dye that is excited by the presence of superoxide anion, we found that as expected, aortas from SOD-/- mice had significantly more fluorescence of ethidium bromide than the wild type mice (Figure 3.5A and 3.5B). We also performed immunohistochemistry for nitrotyrosine, which is a marker of peroxynitrite formation, in mesenteric arteries (31). Our data illustrate that peroxynitrite formation was elevated in mesenteric vessels of SOD-/- mice (Figure 3.6B) when compared to wild type mice (Figure 3.6A). The mean intensity of immunofluorescence in wild type mice was 0.28 ± 0.03 compared with 0.78 ± 0.14 in vessels from SOD-/- mice ($P < 0.05$; $n = 5$). In SOD-/- mice, nitrotyrosine immunostaining was evident mainly in the endothelium, but was also present in vascular smooth muscle. Incubation of sections without primary antibody result in a very low signal (Figure 3.6C) and was consistent for

all other negative controls performed. We also measured expression of the enzymes PGHS-1, prostacyclin synthase and eNOS in the mesenteric arteries from wild type and SOD-/- mice and no differences between groups were detected (data not shown).

3.4 DISCUSSION

This study utilized the CuZn SOD knockout mouse to highlight mechanisms behind oxidant-induced vascular dysfunction. We demonstrated that in this model, in which peroxynitrite levels are elevated, vascular relaxation is significantly impaired, due to reduced PGHS and nitric oxide-dependent vasodilation. Furthermore, although we did not observe an overall difference in vasoconstrictor responses between the two groups, PGHS inhibition increased phenylephrine sensitivity only in wild type mice, which corroborates our conclusion that SOD-/- mice have an impaired PGHS modulation of vascular reactivity, in addition to the reduced nitric oxide-dependent vasorelaxation.

Our hypothesis that oxidative stress is mediating the vascular dysfunction in SOD-/- mice is supported by evidence that after pre-incubation with PEG-SOD, methacholine-induced relaxation in SOD-/- mice was enhanced. The use of PEG-SOD to restore oxidant-induced vascular dysfunction has been used in a mouse model of diabetes, where it was also effective at reversing the deleterious effects of superoxide anion (11). In our study, we speculate that the acute removal of superoxide anions reduces scavenging of nitric oxide, thus increasing its bioactivity as a vasodilator. Supporting this theory, relaxation in PEG-SOD-treated vessels was significantly reduced after incubation with L-NAME, whereas in untreated vessels from SOD-/- mice, L-NAME did not affect methacholine-induced relaxation. Thus, exogenous SOD enhanced NOS-mediated

relaxation in knockout mice. However, it is likely that chronic elevated levels of pro-oxidants also alter enzymes important to vascular function (such as prostacyclin synthase and/ or eNOS), hence acute PEG-SOD could not fully compensate for these changes. Indeed, relaxation in vessels from SOD-/- mice was only partially restored after PEG-SOD treatment and did not vasodilate to the levels observed in wild type mice.

Our data illustrates highly impaired endothelial-dependent relaxation, and is in agreement with a recent study that observed a significantly reduced response to a bolus administration of acetylcholine in the carotid artery of CuZn SOD-/- mice (5). Further, in other animal models of oxidative stress (glutathione peroxidase gene (GPx-1) knockout mouse and apolipoprotein E knockout mouse) endothelial-dependent relaxation was found to be impaired, which also supports the findings of the current study (7, 10). However, the contribution of PGHS and nitric oxide in modulating the vascular reactivity was not demonstrated in these studies.

Our results illustrate that PGHS-mediated relaxation is compromised in mesenteric vessels from SOD-/- mice and thus, is partially responsible for the altered vascular reactivity. We speculate that the mechanism behind this reduced PGHS-mediated dilation is a decrease in prostacyclin (the predominant vasodilator produced by PGHS in mesenteric arteries) (18). In the current study, we observed no difference in relaxation responses to exogenous prostacyclin, supporting the conclusion that production of a PGHS-dependent vasodilator is indeed reduced in SOD-/- mice, rather than the vessels being less sensitive to prostacyclin. In vessels from SOD-/- mice, we found increased evidence of nitrotyrosine, a marker of peroxynitrite. Evidence suggests that this reactive nitrogen species can alter the production of prostacyclin through a variety of

mechanisms. For example, our laboratory found that peroxynitrite reduces protein levels of prostacyclin synthase in endothelial cells (4). Additionally, Zou et al. have extensively described how peroxynitrite inhibits the activity of prostacyclin synthase through a tyrosine nitrosylation-dependent reaction (40, 41). In mesenteric arteries from SOD-/- mice, we did not detect reduced levels of prostacyclin synthase, suggesting that in this model, inhibition of prostacyclin synthase activity by peroxynitrite may be responsible for the reduced PGHS-dependent vasodilation.

Nitric oxide mediated vasodilation is also compromised in SOD-/- mice, as evidenced by reduced NOS-dependent endothelial-mediated relaxation and reduced sensitivity to the nitric oxide donor sodium nitroprusside. It is possible that nitric oxide is simply unavailable since it is being scavenged, forming peroxynitrite. However, reactive nitrogen species can also alter other aspects of the NOS pathway. For example, peroxynitrite can decrease the activity of eNOS via either directly uncoupling (oxidation) the zinc cluster of eNOS (42) or by the destabilization of tetrahydrobiopterin, an important co-factor for NOS activity (16). These effects on eNOS activity may be involved in the impaired endothelial-dependent relaxation in mesenteric arteries from SOD-/- mice, since eNOS protein levels were not different between SOD-/- and wild type mice. Our conclusion is in agreement with other mouse models in which NOS-dependent relaxation was impaired (6). Therefore, the mechanism(s) by which reactive nitrogen species impair vascular function are likely complex, affecting many different pathways involved in vascular relaxation.

EDHF-mediated relaxation has been described as endothelial-dependent relaxation that is not inhibited by NOS and PGHS antagonists, but is inhibited by potassium channel

blockers (2). In the SOD-/- mice, the magnitude of relaxation that is dependent on an EDHF-like factor was not different from arteries from wild type mice. This is in contrast to other models of vascular dysfunction, in which it is suggested that an EDHF-like factor is compensating, including in vessels from eNOS knockout mice (36), women with preeclampsia (15) and hypercholesterolemic rabbits (24).

In conclusion, our data illustrate that CuZn SOD knockout mice have reduced NOS and PGHS-dependent modulation of vascular reactivity. These abnormalities may be due to increased peroxynitrite-induced endothelial cell dysfunction, which is also indicative of a reduced capacity of nitric oxide to induced vasodilation. The findings of this study are relevant to clinical conditions of vascular dysfunction, because reduced SOD expression and activity, (and increased peroxynitrite formation) are associated with preeclampsia (26, 37), aging (14, 34), atherosclerosis (21, 38) and diabetes (13, 27). Using the CuZn SOD knockout mouse as a tool to better understand the mechanisms of oxidant-induced endothelial cell dysfunction provides new opportunities to investigate novel therapeutic targets for the treatment and prevention of vascular disease.

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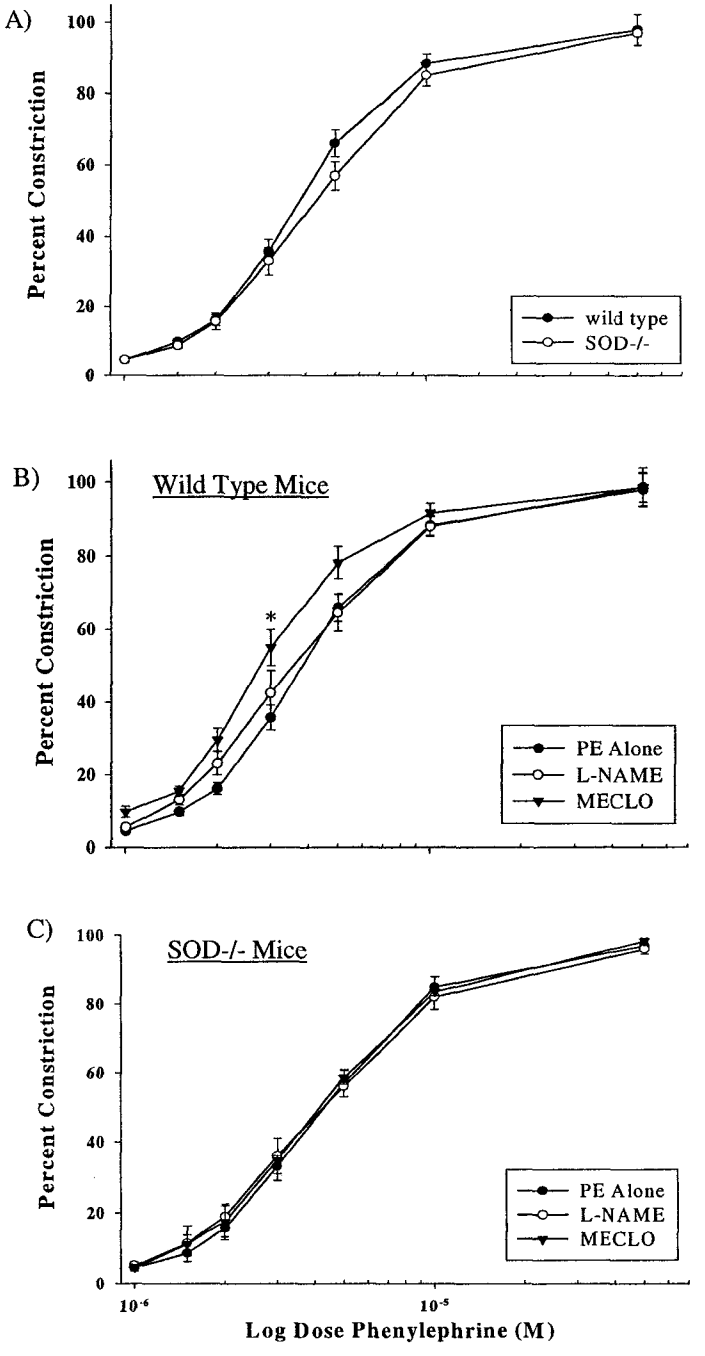

Figure 3.1 Overall Sensitivity to Phenylephrine is Not Different in Mesenteric Arteries from Wild Type and SOD-/- Mice.

Panel A) Concentration response curves to phenylephrine in wild type (•; n = 12) and SOD-/- (○; n = 12) mice. Phenylephrine-induced vasoconstriction in wild type mice (Panel B) and SOD-/- mice (Panel C) in the presence or absence of L-NAME (○; 100 μM) or meclofenamate (MECLO; ▼; 10 μM). Responses are expressed as percent constriction, each value represents mean ± SEM, *P<0.05.

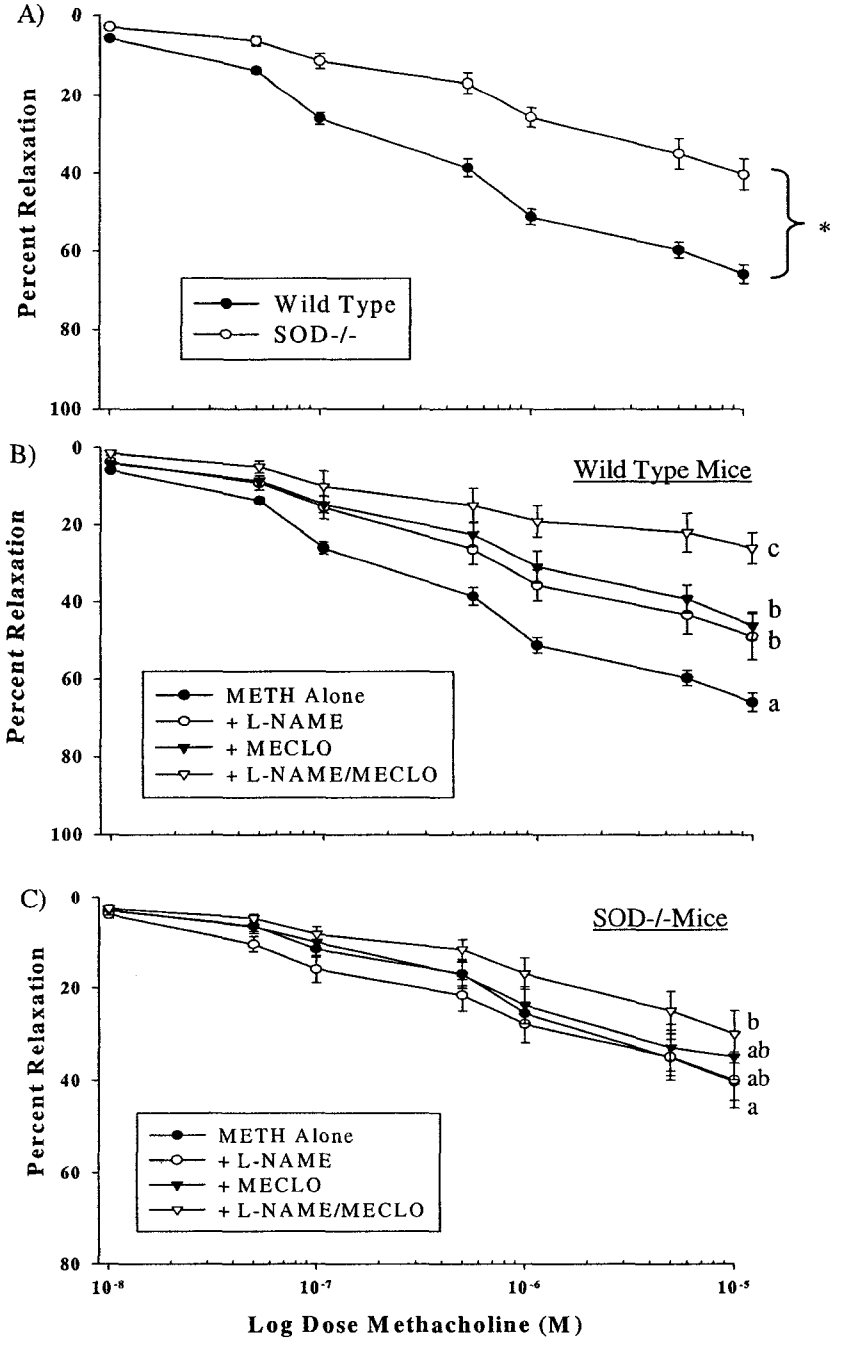

Figure 3.2 Endothelial-Dependent Relaxation is Reduced in SOD-/- Mice Due to Impaired NOS and PGHS Pathways

Panel A) Methacholine dose response curves in wild type (•; n=14) and SOD-/- (○; n=12) mice. Mesenteric artery relaxation in wild type mice (Panel B) and SOD-/- mice (Panel C) in the presence or absence of L-NAME (○; 100 μmol/L) or meclofenamate (MECLO; ▼; 1.0 μmol/L) or both L-NAME and MECLO (▽). *P < 0.05 vs. wild type. Different letters indicate statistically different dose response curves at.

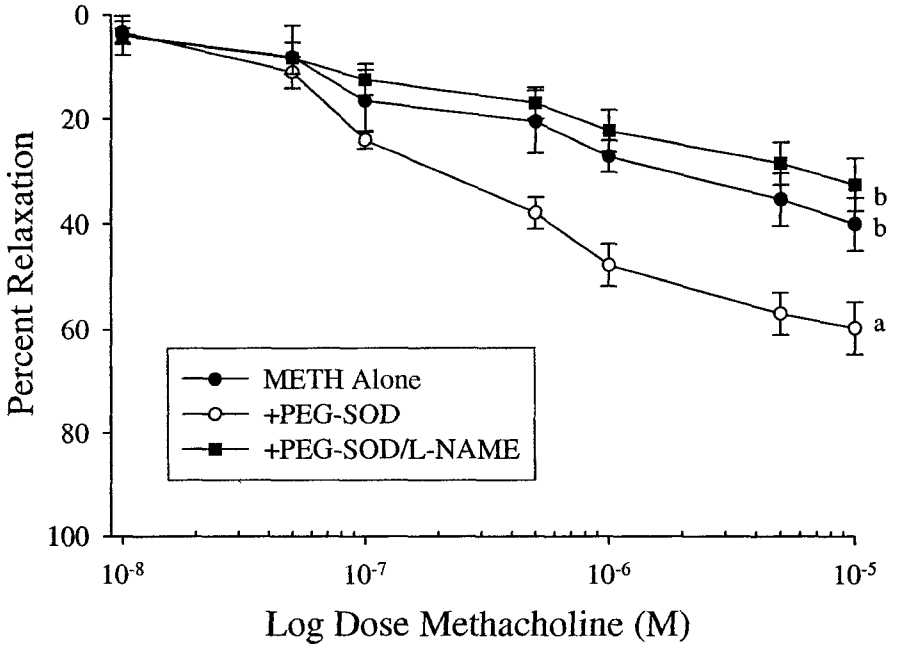

Figure 3.3 Exogenous SOD Partially Restores Endothelial-Dependent Relaxation in Vessels from SOD-/- Mice.

Endothelial-dependent relaxation in mesenteric arteries from SOD-/- mice after one hour incubation with PEG-SOD. Methacholine-induced relaxation in vessels from SOD-/- mice *before* PEG-SOD (•; n = 6) *after* PEG-SOD alone (○; n = 6) and after both PEG-SOD and L-NAME (■; 20 minutes, 100 μM; n = 5). Responses are expressed as percent relaxation, each value represents mean ± SEM. Different letters indicate statistically different curves at $P < 0.05$.

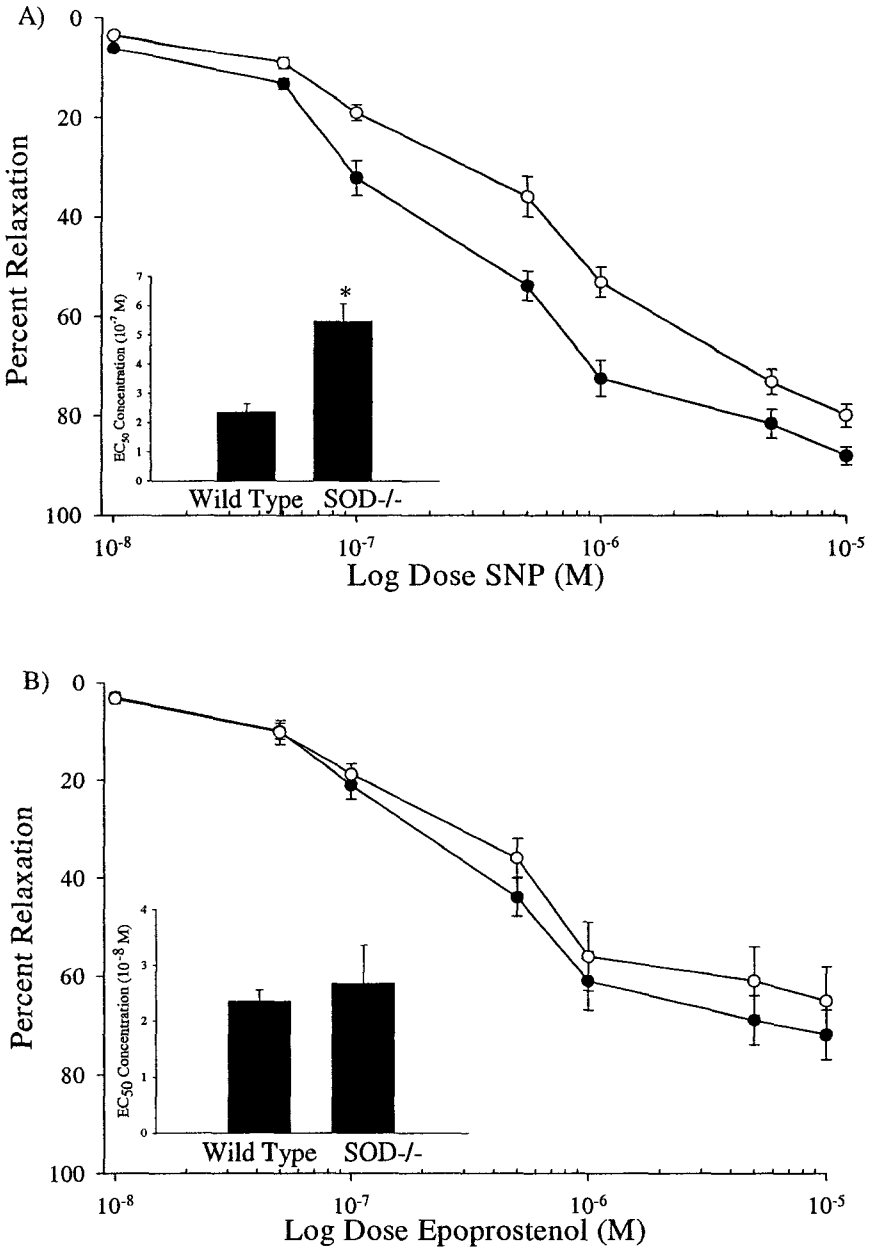

Figure 3.4 Endothelial-Independent Relaxation is Impaired to a Nitric Oxide Donor but not a Prostacyclin Analogue in SOD-/- Mice.

Concentration response curves to endothelial-independent agonists. Panel A) Sodium nitroprusside (SNP) induced relaxation in wild type (•; n = 14), SOD-/- (○; n = 12) mice. Panel B) Epoprostenol-induced relaxation in wild type (•; n = 6) and SOD-/- (○; n = 6) mice. Responses are expressed as percent relaxation and each value represents the mean ± SEM. *Inset:* Bars indicate average EC_{50} doses for wild type and SOD-/- mice. *$P < 0.05$ vs. wild type.

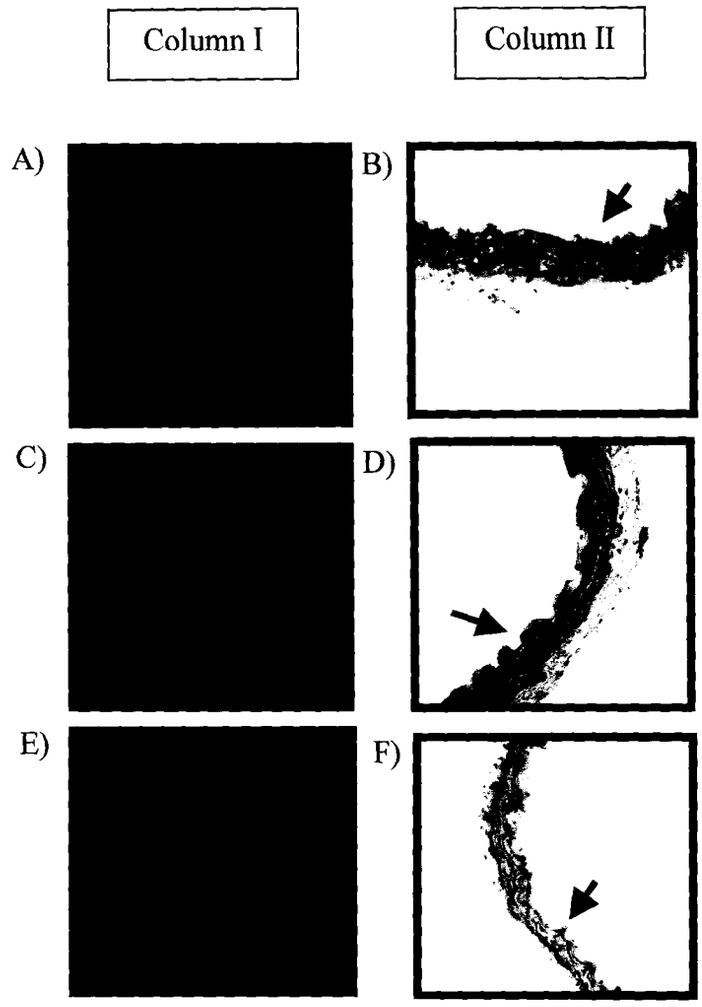

Figure 3.5 Peroxynitrite Formation and Superoxide Anions are Elevated in the Aortas of SOD-/- Mice.

Detection of superoxide anion (column I) and peroxynitrite (column II) in aortas from wild type and SOD-/- mice. Panels A and B are representative aortic sections from wild type mice (n = 5). Panels C and D are representative aortic sections from SOD-/- mice (n = 5). Panel E is a negative control for hydroethidium dye (aortic section incubated with vehicle (DMSO) alone). Panel F is a negative control for nitrotyrosine immunohistochemistry (section incubated without primary antibody). All images were taken at 100x magnification; arrow depicts luminal surface of the aorta.

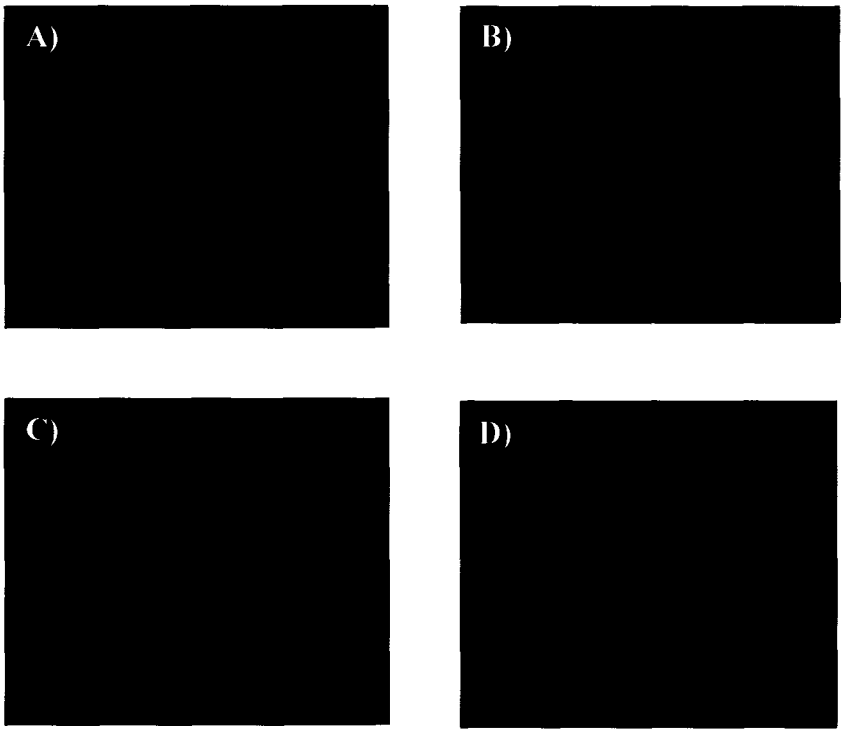

Figure 3.6 Peroxynitrite Formation is Increased in Mesenteric Arteries from SOD-/- Mice

Immunohistochemistry for nitrotyrosine, a marker of peroxynitrite formation. Representative sections of mesenteric arteries from A) wild type mice (n = 5) and B) SOD-/- mice (n=5) incubated overnight with rabbit polyclonal anti-nitrotyrosine antibody. Panel C is a negative control, which is a section incubated without primary antibody and panel D is the corresponding DAPI stained nuclei, in order to illustrate presence of the blood vessel. All images were taken at 100x magnification.

CHAPTER 4. THE RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE) IS ELEVATED IN WOMEN WITH PREECLAMPSIA

A version of this chapter has been accepted for publication. Cooke, Brockelsby, Baker and Davidge, Hypertension in Pregnancy, 2003.

4.1 INTRODUCTION

Our previous experiments illustrated that peroxynitrite is elevated in vessels from women with preeclampsia and can activate NFκB in endothelial cells. In this study, we speculate that activation of the RAGE pathway (receptor for advanced glycation end products) may be one potential mechanism that stimulates the production of pro-oxidants, thus leading to exacerbation of the vascular disturbances in women with preeclampsia. RAGE, a member of the immunoglobin receptor-type superfamily (14), is a multi-ligand receptor which recognizes families of ligands, including advanced glycation end products (AGEs), amphoterins and S100/calgranulins, rather than one individual molecule (19). AGEs (the best characterized of these molecules) are generated by the reaction of glucose with protein amino groups, which undergo further processing with reactive oxygen species forming these irreversible compounds (4).

The RAGE promoter consists of binding sites for the oxidative-sensitive transcription factor NFκB, which suggested a link between RAGE activation and oxidative stress (10). Indeed, activation of RAGE by its ligand(s) stimulates the

formation of pro-oxidants, and activates NFκB, which can upregulate RAGE expression, initiating a positive feedback cycle which favours cellular perturbation rather than homeostasis (27). This feed-forward pathway of RAGE-induced cellular dysfunction is consistent with women with preeclampsia, whose condition continually deteriorates, which often mandates the pre-mature delivery of the fetus (17).

The role of RAGE as a key mediator in the progression of vascular diseases characterized by oxidative stress and inflammation is becoming increasingly recognized (19). For example in endothelial cells, RAGE activation can i) increase TNFα release via NFκB activation (12), ii) reduce release of the vasodilator prostacyclin and iii) increase release of the potent vasoconstrictor endothelin (15). Of interest, these alterations are commonly observed in women with preeclampsia (24, 29, 31).

Therefore, since normal pregnancy is a state of insulin resistance (5), we hypothesized that RAGE protein is elevated in normal pregnant women compared to non-pregnant women. Furthermore, since preeclampsia is considered to be a state oxidative stress, combined with insulin resistance, we hypothesize that RAGE protein is further elevated in women with preeclampsia.

4.2 MATERIALS AND METHODS

4.2.1 Patient Demographics

Women were recruited from the Royal Alexandra Hospital (RAH; Edmonton, Canada; n=11) and from Nottingham City Hospital (NCH; Nottingham, U.K; n=10) at the time of admittance to labour and delivery, using protocols approved by the University

of Alberta and the University of Nottingham Ethics Committees. Six women from the RAH and 5 women from the NCH had preeclampsia using the criteria of hypertension and proteinuria. Hypertension was defined as an absolute blood pressure >140/90 mm Hg on 2 occasions at least 6 hours apart and occurring after the 20th week of gestation. Proteinuria was defined as >500 mg /24 hour urine collection or a 2+ on a voided urine Dipstick test. Five women from the RAH and five women from NCH had uncomplicated pregnancies. No subject was known to have a history of chronic hypertension, diabetes or liver, renal or metabolic disease. Non-pregnant samples were obtained at the time of hysterectomy at both the RAH and the NCH (n=5 and n=4 respectively).

4.2.2 Tissue Collection and Immunohistochemistry

Biopsies of myometrium (NCH) or omental fat (RAH) were obtained, snap frozen in liquid nitrogen and stored at –80 °C. Frozen tissue was sliced into consecutive 12-16 μm thick sections (approximately 30 sections per sample) using a cryostat and mounted onto glass slides at –25 °C. The presence of blood vessels, as well as an intact endothelium, was determined for each sample using monoclonal antibody against α-actin (1:400; Oncogene Research Products; Boston, MA) or Von Willebrand factor (1:100; Sigma, St. Louis, MO) respectively. Sections were immunostained using polyclonal antibody against RAGE (1:75 dilution; Santa Cruz; Santa Cruz, CA). Negative controls were stained without primary antibody. The Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) protocol was followed for detection of RAGE protein. The counterstain used was a 1:1 mixture of alcian blue and methyl green.

4.2.3 Data Collection and Analysis

Sections were evaluated with the identity of the subject concealed. The intensity of RAGE immunostaining was qualitatively described as well as semi-quantified using a scale of zero (absent) to four (intense) for each patient. A one-way analysis of variance (ANOVA) followed by a Fisher LSD post hoc test or a Student's t-test (where appropriate) was used to determine statistical significance between groups (*P< 0.05). Summary data are expressed as mean plus or minus standard error of the mean. The arbitrary intensity of staining scores were verified using image analysis software (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). After image acquisition (SPOT Advanced, Diagnostic Instruments, USA) the optical intensity of the blood vessel sections was measured and data are expressed in mean optical density units. Optical density data were pooled for omental and myometrial vessels from normal pregnant women and women with preeclampsia due to small sample size and variability. Non-pregnant samples were not quantified since the optical intensity readings were artificially high due to the intense dark blue counterstain.

4.3 RESULTS

Patient demographics are summarized in Table 4.1. Women with preeclampsia had significantly elevated blood pressure and proteinuria compared to normal pregnant women (by definition). Women with preeclampsia (from both Edmonton and Nottingham) also gave birth to smaller babies (2200 ± 300 vs. 3400 ± 200 grams; P<

0.05) and had earlier deliveries (Range: 29-38 weeks). However, the intensity of RAGE expression in samples from women with preeclampsia did not vary with gestational age.

In myometrial tissue, the endothelial cell specific antibody Von Willebrand factor was used to detect blood vessels (Figure 4.1A), since α-actin would have also detected uterine smooth muscle. The presence of blood vessels in omental tissue was determined using a vascular smooth muscle cell specific antibody, α-actin. Blood vessels were present in all omental tissue sections (Figure 4.1B). Negative controls were performed for all antibodies in all groups and no immunoreactivity was detected (one representative sample is illustrated in Figure 1C). Vessel sizes (from both omental and myometrial tissue) were measured using the Image-Pro Plus software. Vessels had a median diameter of 230 μm (range: 130 μm – 465 μm). Interestingly, vessels from women with preeclampsia tended to be smaller than those from normal pregnant women (199±12 μm vs. 330±25 μm, P=0.07).

In the vascular tissues, RAGE staining was located primarily in the vascular smooth muscle, but was also present in the endothelium, which is consistent with previous reports in conditions such as diabetes and atherosclerosis (21). RAGE intensity was consistently low in connective tissue. Vessels from non-pregnant women showed very low levels of RAGE immunostaining and in some women it was below detection (Figure 4.2A and 4.3A). As hypothesized, normal pregnant women had significantly elevated levels of RAGE expression in both omental and myometrial blood vessels compared to non-pregnant women (Figure 4.2B and 4.3B). The most striking result was observed in women with preeclampsia, who consistently showed intense RAGE immunostaining in

the vasculature (Figure 4.2C and 4.3C). In omental vessels, mean RAGE immunostaining in non-pregnant, normal pregnant and preeclampsia was 1.2 ± 0.3 vs. 2.2 ± 0.3 vs. 3.0 ± 0.3 arbitrary units respectively (Figure 4.2D). In myometrial blood vessels, RAGE intensity was 1.0 ± 0.3 vs. 2.8 ± 0.2 vs. 3.8 ± 0.1 arbitrary units respectively (Figure 4.3D). Image analysis software was also utilized to quantitate the optical intensity of the staining in the blood vessels. These data corroborate that obtained by the investigator, showing that both omental and myometrial vessels from women with preeclampsia have a higher optical density than vessels from normal pregnant women (402 ± 80 vs. 187 ± 16 optical density units, $P< 0.05$).

In addition to the RAGE expression in the vasculature, it was noted that RAGE was also evident in myometrial smooth muscle. The intensity of RAGE immunostaining followed a similar pattern as described above for the blood vessels. Non-pregnant women had very low levels of RAGE protein (Figure 4.4A), while RAGE was significantly elevated in normal pregnant women (Figure 4.4B). The most striking upregulation of RAGE was observed in women with preeclampsia, who showed intense RAGE immunostaining in the myometrium (Figure 4.4C).

4.4 DISCUSSION

In this study we characterized levels of RAGE protein expression in myometrial and omental vascular beds during pregnancy and preeclampsia. Interestingly, normal pregnant women have significant increased levels of RAGE protein in both myometrial and omental vascular beds. Pregnancy is a state of insulin resistance (5) in which glucose

levels are elevated in order to supply adequate nutrients to the developing fetus; this may explain the increased staining for RAGE in pregnancy. In preeclampsia (pregnancy associated with oxidative stress) immunoreactivity of RAGE was consistently observed to be highly elevated in the vasculature. Thus, although RAGE protein is elevated in normal pregnancy, preeclampsia may be at the far end of a pregnancy continuum, such that moderate levels of RAGE activity are tolerated, but excessive RAGE may contribute to vascular dysfunction in women with preeclampsia.

Previous experiments from our laboratory illustrated that peroxynitrite levels are elevated in maternal vessels from women with preeclampsia (18). We also determined that peroxynitrite can alter endothelial cell vasoactive pathways through the activation of NFκB and inhibition of prostacyclin synthase protein expression (6). However, the link between preeclampsia, peroxynitrite and endothelial dysfunction is unknown. In endothelial cells, the primary signal transduction cascade initiated by RAGE activation includes an increase in superoxide anion, which stimulates NFκB-mediated changes in gene transcription (20). Thus, we speculate that elevated RAGE in vessels from women with preeclampsia could be one factor responsible for the increased formation of peroxynitrite.

RAGE immunoreactivity is also elevated in patients with atherosclerosis and diabetes (2, 23). Although the origins of the diseases are quite distinct, the vascular dysfunction evident in atherosclerosis and diabetes is remarkably similar to that seen in women with preeclampsia. For example, hypertension is a common complication in atherosclerosis and diabetes, similar to preeclampsia. Furthermore, endothelial-dependent

vasodilation is reduced in atherosclerosis and diabetes, which is thought to be mediated by oxidative stress (13) again consistent with observation in women with preeclampsia (1).

The mechanisms by which RAGE induces vascular dysfunction are currently an intense area of research. RAGE activation in microvascular endothelial cells reduces prostacyclin secretion, while also increasing plasminogen activator inhibitor-1, a potent platelet aggregator, together promoting vasoconstriction and thrombogenesis (32). Furthermore, in cultured endothelial cells, AGE administration (a RAGE ligand) increases endothelin expression through an activation of NFκB (15). In retinal epithelial cells and vascular smooth muscle cells, the RAGE pathway has been linked to an increase in vascular endothelial growth factor (VEGF), which is implicated in the retinal vasculopathy in diabetes (11). Finally, in a rat model of diabetes, soluble RAGE, which binds to ligands, thus blocking activation of cell-surface RAGE, prevents vascular hyperpermeability and edema (30). These effects of RAGE activation are also observed in women with preeclampsia (3, 25, 26, 29).

In addition to its effects on vascular reactivity, RAGE activation may also mediate inflammatory processes (9). For example, stimulation of the RAGE pathway increases TNFα secretion from endothelial cells (27) as well as increases the expression of VCAM-1 (22). The pathogenesis of preeclampsia has also been linked to a pro-inflammatory response, such that inflammatory molecules may be altering vascular function (7, 16). Indeed, preeclampsia is associated with an increase in a variety of immunological factors compared with normal pregnancy. For example, TNFα and its soluble receptor are

elevated in women with preeclampsia (31) as are activated neutrophils (28) and vascular adhesion molecules, important in leukocyte adhesion with endothelial cells (8).

In conclusion, many consequences of RAGE activation (including vascular dysfunction and inflammation) are characteristic of women with preeclampsia. We have shown for the first time that RAGE is elevated in resistance-sized systemic and myometrial blood vessels from women with preeclampsia, providing a new and intriguing insight into the pathophysiology of preeclampsia, which warrants further research. Interestingly, there are novel mouse models of diabetes, atherosclerosis and oxidative stress that provide potential systems in which to investigate the effects of RAGE activation on vessel function in pregnancy. However, little research has examined the mechanisms behind vascular adaptations to pregnancy in normal mice, which is the subject of Chapter 5.

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Measure	Non-Pregnant		Normal Pregnant		Preeclampsia	
	NCH (n=4)	RAH (n=5)	NCH (n=5)	RAH (n=5)	NCH (n=5)	RAH (n=6)
maternal age (year)	36 ± 2.2	32 ± 3.0	29 ± 2.3	33 ± 1.4	31 ± 1.2	25 ± 2.1
pre-preg mass (kg)	61 ± 3.0	67 ± 10	65 ± 2.0	63 ± 3.1	67 ± 2.2	74 ± 11
pre-preg BP mmHg	111/70 ± 4/2	106/72 ± 8/6	117/63 ± 4/2	119/71 ± 9/1	116/67 ± 7/7	115/66 ± 15/3
Term BP mmHg	N/A	N/A	127/70 ± 7/1	117/70 ± 4/5	149/104 ± 3/2 *	146/101 ± 3/2 *
Protein-uria	N/A	N/A	N/D	N/D	730 ± 10 * (mg/24 hr)	2.5+ ± 0.3* (dipstick)
Gestation (weeks)	N/A	N/A	38 ± 0.5	39 ± 0.2	33 ± 1.8 *	36 ± 0.9 *
Infant mass (g)	N/A	N/A	3200 ± 200	3540 ± 90	2100 ± 400*	2442 ± 300*

Table 4.1 Non-Pregnant, Normal Pregnant and Preeclamptic Patient Demographics

Patient Demographics for women from Royal Alexandra Hospital (RAH) and Nottingham City Hospital (NCH) from whom omental or myometrial biopsies were obtained. N/A = not applicable; N/D = not detectable; BP = blood pressure; * $P < 0.05$ (vs. normal pregnant women from the same hospital).

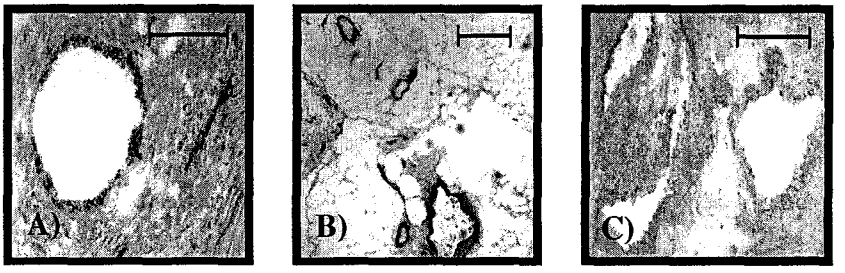


Figure 4.1 Endothelial and Smooth Muscle Immunostaining: Positive Controls

Representative sections of immunohistochemical staining. A) endothelial cell specific Von Willebrand factor in myometrial vessels from a non-pregnant women (1:100 dilution, polyclonal antibody B) α-actin in omental vessels from a woman with preeclampsia (1:400 dilution, monoclonal antibody) and C) negative control (section incubated without primary antibody) from a woman with preeclampsia. All images were taken at 100x magnification. Vessel diameter was measured using the Image Pro-Plus software; the black bar depicts 200 μm in each figure.

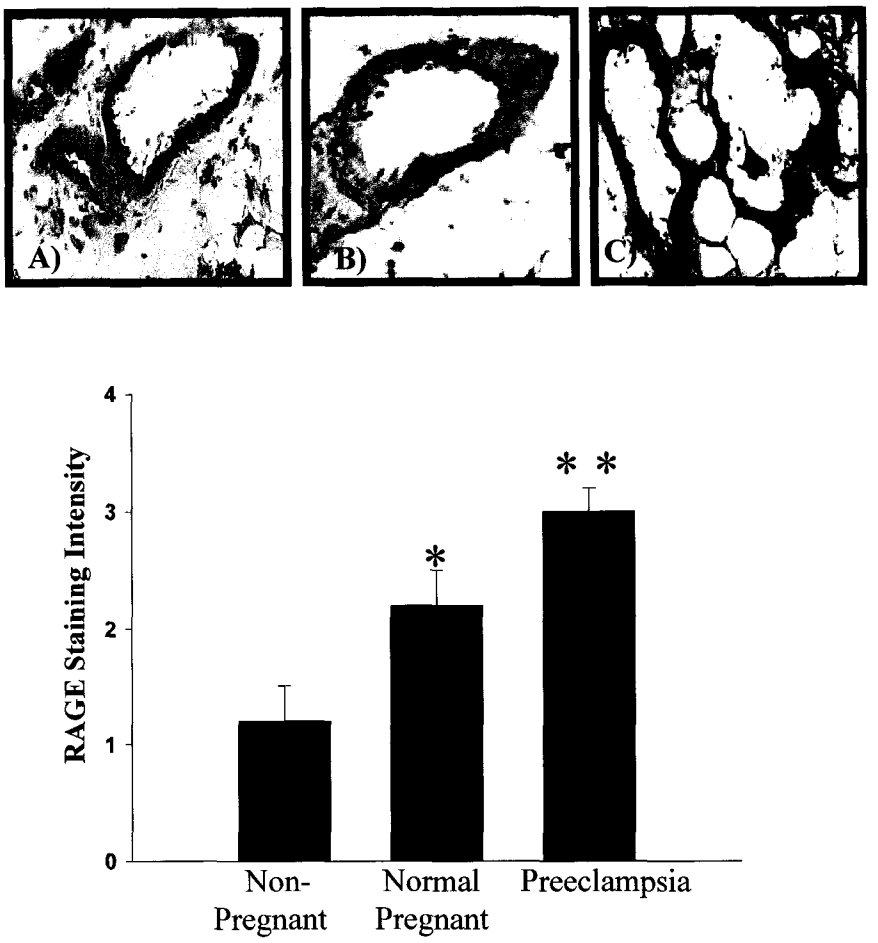
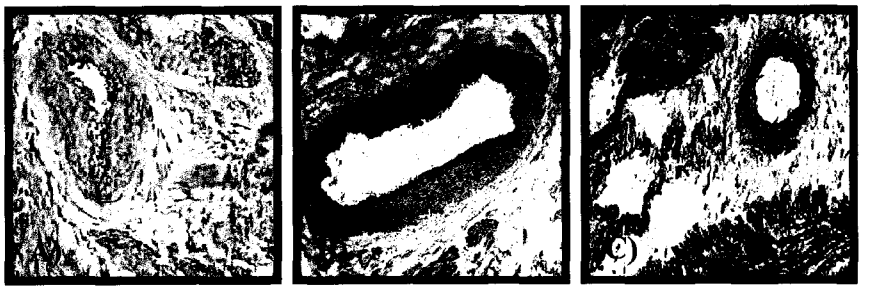


Figure 4.2 RAGE Protein Expression is Elevated in Omental Vessels from Women with Preeclampsia

Representative sections of omental biopsies immunostained for RAGE protein (1:75 dilution, polyclonal antibody). A) RAGE protein expression in non-pregnant subject B) normal pregnant subject and C) a patient with preeclampsia. All images were taken at 100x magnification. D) Summary histogram of the mean staining intensity ± SEM, n = 5 for non-pregnant and normal pregnant and n = 6 for preeclampsia. * P< 0.05.



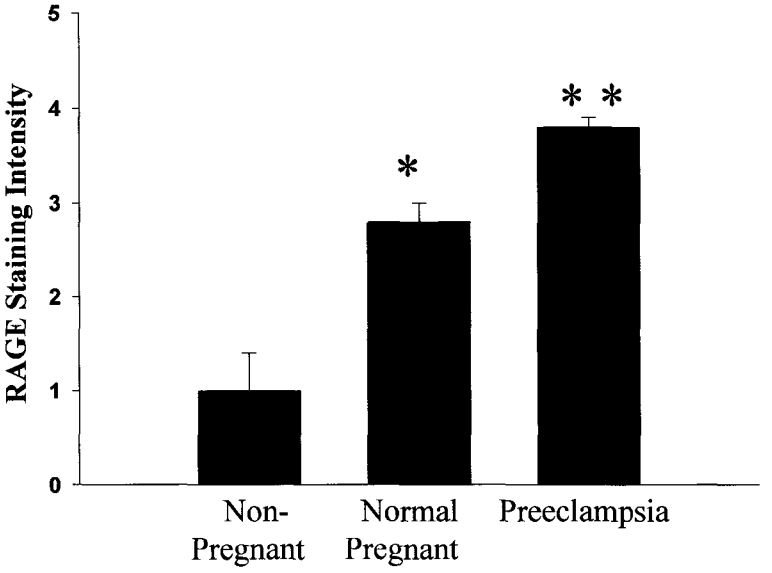


Figure 4.3 RAGE Levels are Elevated in Myometrial Vessels from Women with Preeclampsia

Representative sections of myometrial blood vessels immunostained for RAGE protein. A) RAGE protein expression in non-pregnant subject B) normal pregnant subject and C) a patient with preeclampsia. All images were taken at 100x magnification D) Summary histogram of the mean staining intensity ± SEM, n = 5 for non-pregnant, normal pregnant and for preeclampsia. * $P < 0.05$.

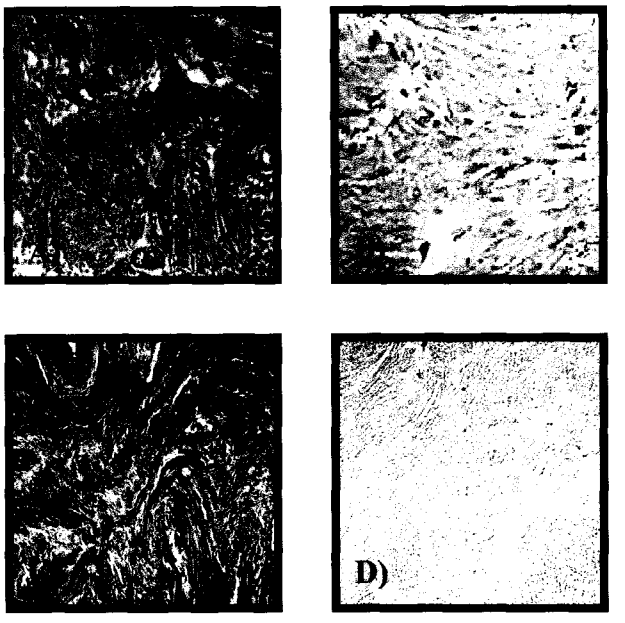


Figure 4.4 Myometrial Smooth Muscle from Women with Preeclampsia has Elevated RAGE Protein Levels.

Representative sections of myometrial smooth muscle immunostained for RAGE protein in A) non-pregnant subject B) normal pregnant subject and C) a patient with preeclampsia D) Representative negative control showing no immunostaining. All images were taken at 100x magnification.

CHAPTER 5. PREGNANCY- INDUCED ALTERATIONS OF VASCULAR FUNCTION IN MOUSE MESENTERIC AND UTERINE ARTERIES

A version of this chapter has been published. Cooke and Davidge. Biol Reprod. 2003; 68(3): 1072-1077.

5.1 INTRODUCTION

Recent advances in genetic manipulations have lead to the development of transgenic mouse models, many of which focus on elucidating the mechanisms involved in pregnancy adaptations. For instance, cardiovascular abnormalities have been documented during pregnancy in renin-angiotensin overexpressing mice (26) as well as an inbred mildly hypertensive mouse strain (BPH/5) (7). Yet these studies did not investigate the specific pathways mediating cardiovascular changes. Based on the importance of the nitric oxide pathway in rat gestation, many thought that nitric oxide synthase knockout (NOS-/-) mice would provide a good model to study mechanisms of abnormal vascular adaptation to pregnancy. However, these mice do not become hypertensive and in fact appear to have a normal pregnancy (24), suggesting that the mechanisms mediating vessel function in pregnant mice may be unique. We proposed to investigate vascular adaptations to pregnancy in a mouse model of oxidative stress (SOD-/- mice). However, few studies have been specifically designed to investigate the mechanisms mediating

changes in vascular function during normal mouse pregnancy, which is the focus of this Chapter.

Indeed, animal models (particularly small mesenteric arteries from pregnant rats) have been examined to elucidate changes in vessel function during gestation. Similar to changes in vascular function observed in humans (10), rat pregnancy is associated with a blunted systemic vasoconstrictor response to adrenergic agonists (6) and angiotensin (18) as well as an enhanced endothelial-dependent vasodilation (11, 19). The increase in endothelial-dependent vasodilation systemically in the rat seems to involve enhanced nitric oxide modulation of vascular tone (19) and possibly EETs (11) rather than prostacyclin (6, 5).

The uterine circulation is also known to undergo dramatic alterations during pregnancy, leading to increased endothelial-dependent vasodilation. In the rat uterine artery, enhanced nitric oxide-mediated relaxation is an important adaptation during pregnancy (17), which is similar to mechanisms involved in sheep pregnancy (21). However, studies that measure in vivo perfusion pressure of the rat uterine circulation suggest that endothelial-derived hyperpolarizing factors is also involved, which interestingly, does not seem to be CYP450-dependent (8).

The dramatic hormonal changes that occur early on in gestation (and are necessary for pregnancy maintenance) are thought to be one initiator of the reduced peripheral vascular resistance (10, 20). Progesterone, the hormone of pregnancy, is metabolized into many biologically active molecules. One major metabolite of progesterone is 5β-dihydroprogesterone (5β-DHP). Recently, 5β-DHP was discovered to be a potent ligand

for the pregnane X receptor (PXR), an orphan nuclear receptor. In the liver, PXR activation increases the expression of specific isoforms of CYP450 (including the proposed EDHF synthase 2C9)(1), although the possible role of 5β-DHP in stimulating PXR induced EET-dependent vasorelaxation has not been investigated.

Therefore, this study addresses two hypotheses. First, we hypothesized that mouse pregnancy will involve enhanced endothelial-dependent vasodilation in both the mesenteric and uterine circulation. The relative contribution of the major endothelial-derived vasodilator pathways (NOS, PGHS and CYP450) will be assessed. Second we propose that 5β-DHP is a novel stimulator of enhanced vascular relaxation, via the activation of PXR and increase in CYP450 and EET-mediated vasodilation.

5.2 MATERIALS AND METHODS

5.2.1 Animals and Breeding

Female C57BL/6J mice (12 weeks of age) were purchased from Jackson Laboratories, Bar Harbour, ME) and housed in a temperature and humidity controlled environment, under 12 hour light-dark cycles. Water and standard laboratory rodent chow were available *ad libitum* to the mice. Females were bred for two hours (1:2 male to female ratio) and the presence on a vaginal plug signified successful mating (day 0 of gestation, n = 8). Experiments were performed on day 17-18 of gestation; delivery occurs on day 19 of gestation. Non-pregnant mice (n = 10) were used at various times throughout their estrous cycle. However, there was not a significant variation in vessel function among groups and therefore, data were pooled.

In order to assess the role of 5β-DHP is vascular relaxation, 20 mg time-release pellets of 5β-DHP were implanted subcutaneously into non-pregnant wild type (control; n = 5) and PXR knockout (PXR-/-) female mice (n = 5), for seven days. Placebo-treated wild type (n = 4) and PXR-/- mice (n = 4) were used as controls for these experiments. On the day of experiment, all mice were sacrificed by cervical dislocation. These protocols were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee and was in accordance with the Canadian Council on Animal Care.

5.2.2 Wire Myography Experimental Protocol

The uterine and mesenteric arteries were assessed for vascular reactivity using the wire myograph system as previously described in Chapter 3. We chose to study these two vessel types for distinct reasons. The uterine artery is known to undergo dramatic physiological changes and restructuring during pregnancy, due to the large increase in blood flow to the placenta. On the other hand, small mesenteric blood vessels are important in regulating overall peripheral vascular resistance (4). Since pregnancy is a state of generalized vasodilation and mesenteric blood flow is known to increase during gestation (14), it is important to understand how this vasculature has adapted to account for these changes. Furthermore, by comparing two vascular beds, we were able to elucidate whether different mechanisms are involved in the vascular changes. Our initial studies investigated vascular function in the aorta, and there were no differences in vascular function between pregnant and non-pregnant mice (data not shown).

After pre-constriction of mesenteric and uterine arteries with an EC_{80} dose of phenylephrine, methacholine (muscarinic-agonist; endothelial-dependent vasodilator) dose response curves were performed alone, or after a 20 minute pre-incubation with: L-NAME alone (NOS inhibitor; 100 μM); meclofenamate alone (MECLO; PGHS inhibitor; 1.0 μM); L-NAME and MECLO together; L-NAME, MECLO plus inhibitors of calcium-sensitive potassium channels (apamine; 10 μM and charybdotoxin; 0.1 μM). Endothelial-independent relaxation was also assessed in response to sodium nitroprusside (SNP).

In a separate set of experiments, the role of CYP-EET dependent relaxation was assessed in pregnant mice (versus non-pregnant) and in non-pregnant wild type and PXR-/- mice treated with 5β-DHP or a placebo pellet. Methacholine dose response curves were performed alone or after a 20 minute pre-incubation with MS-PPOH (1.0 μM; CYP450 epoxygenase inhibitor) or 14,15 EEZE (10 μM; antagonist to EET-dependent relaxation). All vasorelaxation dose response curves were performed in the range of 10^{-8} to 10^{-5} M.

5.2.3 Data Analysis and Statistics

Dose response curves are graphically depicted as percent relaxation and each point represents mean ± standard error of the mean. Curves were summarized using either the EC_{50} dose or maximal relaxation to methacholine and these values were used to compare vascular function between groups as previously described (Chapter 3). $P< 0.05$ was considered significant.

5.3 RESULTS

Initially, we assessed vascular sensitivity to α_1-mediated vasoconstriction using phenylephrine. Uterine and mesenteric vessels from pregnant mice had a blunted sensitivity to phenylephrine compared to non-pregnant mice, but only at the low range of doses (data not shown). However, EC_{80} concentrations were not different between groups (Uterine EC_{80}: 5.6 ± 0.96 μM vs. 7.7 ± 0.9 μM; P = 0.2; Mesenteric EC_{80}: 8.3 ± 2.2 μM vs. 11 ± 1.2 μM, P = 0.09). For this reason, we used the EC_{80} dose to pre-constrict vessels for subsequent relaxation curves. Furthermore, pre-constricting vessels with the EC_{80} concentration provided a greater range of tensions over which relaxation could be measured.

5.3.1 Endothelial-Dependent Relaxation in Uterine and Mesenteric Arteries: Role of NOS and PGHS

As hypothesized, methacholine-induced relaxation was significantly enhanced during pregnancy in both uterine (Figure 5.1A) and mesenteric blood vessels (Figure 5.1B), however, the effect was more pronounced in the uterine vasculature. To address whether NOS and/ or PGHS were involved in the enhanced vasodilation of pregnancy, methacholine-induced relaxation was assessed in presence or absence of specific inhibitors to these pathways. In the non-pregnant uterine vasculature, pre-incubation with either L-NAME or MECLO did not alter relaxation to methacholine (Figure 5.2A). However, in uterine arteries from pregnant mice, blocking either NOS or PGHS shifted the dose response curve to the right, indicating an impaired relaxation capacity (Figure

5.2B). By comparing the maximum relaxation to methacholine before and after inhibitors, it is evident that both L-NAME and MECLO significantly reduced relaxation in uterine arteries from pregnant mice (Figure 5.2B). These results suggest that both NOS and PGHS-dependent relaxation is enhanced in the uterine artery from pregnant mice.

Unlike the uterine vasculature, pre-incubation with L-NAME in non-pregnant mesenteric arteries impaired methacholine-induced relaxation, although the effect of MECLO treatment was minimal (Figure 5.3A). In mesenteric arteries from pregnant mice, both L-NAME and MECLO alone inhibited the relaxation to methacholine to a similar extent. Indeed, the maximum relaxation to methacholine was significantly reduced after blocking either NOS or PGHS (Figure 5.3B).

5.3.2 Non-Nitric Oxide and Non-Prostaglandin Mediated Relaxation in Uterine and Mesenteric Arteries from Pregnant Mice

Endothelial-dependent relaxation that is insensitive to NOS and PGHS antagonists is thought to be due to EDHF-like molecule(s) (27). By pre-incubating the vessels with the combination of L-NAME and MECLO, we were able to examine the remaining EDHF-like relaxation to methacholine. Our data illustrate that both uterine and mesenteric arteries have non-NO, non-prostacyclin mediated relaxation from both non-pregnant and pregnant mice. A comparison of the percent maximal relaxation to methacholine (in the presence of L-NAME and MECLO) shows that in the uterine artery, the remaining methacholine-induced relaxation is reduced in pregnant mice (Figure 5.4A). However, in mesenteric arteries, there is no difference in the relaxation between pregnant and non-pregnant vessels (Figure 5.4B). Because, hyperpolarizing factors act via potassium

channels to induced relaxation, we pre-incubated vessels with a combination of potassium channel blockers (apamine and charybdotoxin) together with the NOS and PGHS inhibitors. In all vessel types from both non-pregnant and pregnant mice, relaxation to methacholine was completely abolished (data not shown) suggesting that this 'EDHF-like' molecule is characteristically working through potassium channels to induce relaxation.

5.3.3 Endothelial-Independent Relaxation in Uterine and Mesenteric Arteries from Pregnant Mice

We also assessed endothelial-independent vasorelaxation using SNP. Uterine vessels from pregnant mice were significantly more sensitive to SNP than uterine vessels from non-pregnant mice indicated by a left shift in the dose response curve and a reduced EC_{50} concentration (Figure 5.5A). However, there was no difference in SNP-induced relaxation between non-pregnant and pregnant mesenteric vessels (Figure 5.5B).

5.3.4 The Role of CYP450 and EETs in Mesenteric Relaxation in Mouse Pregnancy

In a separate study, we assessed the role of the cytochrome P450 epoxygenase in the enhanced endothelial-dependent relaxation of pregnancy, by pre-incubating mesenteric vessels from pregnant and non-pregnant mice with a CYP450 inhibitor. Based on the observation that a very small component of uterine relaxation was non-nitric oxide and non-prostacyclin dependent, the role of EETs in the uterine vasculature was not investigated. Interestingly, in non-pregnant mice, MS-PPOH enhanced mesenteric dilation compared to methacholine alone (maximum relaxation: 50 ± 2.3% vs. 61 ± 3.9%;

P<0.05; Figure 5.6A), suggesting an arachidonic acid-derived vasoconstrictor predominates in mesenteric vessels from non-pregnant mice. Conversely, in pregnant mice, inhibiting CYP450 significantly blunted endothelial-dependent relaxation, suggesting an increase in CYP450-dependent dilators in pregnancy (maximum relaxation: 81.5 ± 4.5% vs. 59 ± 4.1%; P < 0.05; Figure 5.6B). In accordance with these results, blocking EET-mediated dilation in mesenteric vessels from non-pregnant mice had no effect (Figure 5.6A), whereas in pregnancy mice, the antagonist 14,15 EEZE significantly reduced methacholine induced dilation (Figure 5.6B).

5.3.5 Role 5β-DHP in CYP-EET Mediated Relaxation in Non-Pregnant Control and PXR-/- Mice

We also investigated whether the progesterone metabolite 5β-DHP could stimulate CYP450-EET dependent vasorelaxation, by activating PXR. After implanting time-release pellets of 5β-DHP into wild type or PXR-/- mice for one week, endothelial-dependent relaxation was assessed in mesenteric vessels. As hypothesized, methacholine-induced relaxation was significantly enhanced in 5β–DHP treated wild type mice compared to placebo treated animals (Figure 5.7A). In order to determine a role for CYP450-EET mediated relaxation in this enhanced relaxation, we pre-incubated vessels for 20 minutes with either MS-PPOH or 14,15 EEZE. Incubation of vessels from placebo wild type mice with MS-PPOH or 14,15 EEZE had little effect on relaxation (Figure 5.7B). On the other hand, both MS-PPOH and 14,15 EEZE significantly reduced

relaxation in vessels from 5β-DHP treated mice (Figure 5.7C), suggesting an increase in CYP450-EET mediated relaxation is elevated in 5β-DHP treated non-pregnant mice.

In contrast to wild type mice, in PXR-/- mice 5β-DHP had no effect on endothelial-dependent relaxation, such that the methacholine dose response curves in placebo and 5β-DHP treated mice were identical (Figure 5.8A). Furthermore, we observed no change in methacholine-induced relaxation after incubation of vessels from PXR-/- with either MS-PPOH or 14,15 EEZE (Figure 5.8B), suggesting that 5β-DHP is acting via PXR.

5.4 DISCUSSION

Mouse pregnancy may be a suitable model for the study of human pregnancy, considering the similarities in trophoblast invasion and placental development (22). A recent study investigating cardiovascular changes in early and late mouse gestation found that mean arterial pressure is reduced in early pregnancy, while by late gestation, the cardiac output was increased and the pressor response angiotensin was reduced (29). These data are in accordance with cardiovascular changes in human pregnancy (25). However, the mechanisms responsible for these cardiovascular changes during mouse pregnancy are not fully understood.

The first part of this study defines the role of NOS and PGHS-mediated relaxation in vascular adaptations during pregnancy in the mouse. These data illustrate that in both uterine and mesenteric vessels from pregnant mice, endothelial-dependent relaxation was greatly enhanced due to an increase in NOS and PGHS-dependent vasodilation. The effect of PGHS inhibitors was minimal in both uterine and mesenteric arteries from non-

pregnant mice, while in pregnant mice, blocking PGHS greatly reduced endothelial-dependent relaxation, suggesting a specific increase in prostacyclin mediated vasodilation during mouse pregnancy. Our results also show that non-NO, non-prostacyclin mediated relaxation is not different between mesenteric arteries from non-pregnant and pregnant mice, while in the uterine vasculature from pregnant mice, the EDHF-like relaxation is reduced compared to non-pregnant mice.

The study of vessel function in mice is currently an emerging field. Many studies conducted on mouse vessel function have compared transgenic mice to their controls (2, 3, 12, 28). In our study using mesenteric arteries, the non-pregnant control mice exhibited both NO and EDHF-like relaxation. In agreement with our data, mesenteric artery relaxation to acetylcholine was mainly NO-dependent, in female control animals for transgenic mice overexpressing the growth hormone gene. However, the PGHS pathway was not investigated in this study (2). In eNOS+/+ (wild-type) mice, blocking NOS, but not PGHS, partially inhibited acetylcholine induced relaxation in mesenteric vessels (3) which is also in accordance with our results. However, the combination of NOS and PGHS inhibitors completely eliminated endothelial-dependent relaxation in eNOS+/+ mice (3), suggesting that EDHF does not play a significant role in mesenteric relaxation. In contrast, our data illustrates that after inhibiting both NOS and PGHS in non-pregnant mice, approximately 35% of the methacholine-induced relaxation persists. The discrepancy between our study and that of Chataigneau *et al.* may be due to strain variability, which can alter vascular responses (23).

Interestingly, our results demonstrate that in mesenteric arteries from pregnant mice, the effect of blocking NOS or PGHS alone was almost identical. This suggests that

during pregnancy, the NOS and PGHS pathways are redundant, such that when one is inhibited, the other provides adequate relaxation. Indeed, we also found that non-NO, non-prostacyclin dependent relaxation is not significantly different between mesenteric arteries from non-pregnant and pregnant mice. Although this data suggests that hyperpolarizing factors are not involved in the mesenteric adaptations to normal mouse pregnancy, during pathological conditions, EDHF-like pathway(s) may be upregulated, thus compensating for a vasodilator that is lacking. In non-pregnant mice that lack eNOS, there is enhanced sensitivity to PGHS inhibitors compared to wild type mice (3), as well as increased EDHF-mediated relaxation (3, 28). These observations are in accordance with the above theory and may also explain why NOS-/- mice are not hypertensive during pregnancy, although this hypothesis has not been specifically investigated (24). It would be interesting to investigate the contribution of CYP450-EET dependent relaxation during pregnancy in models such as the eNOS-/- mouse. Indeed, our data suggests that a relatively small component of endothelial-dependent relaxation is mediated by EETs in normal pregnant mice, possibly allowing for an upregulation of this pathway in pathological situations.

Uterine artery reactivity has not been studied in the mouse; however, pregnancy adaptations in the uterine artery of the rat and sheep are well characterized. Previous to the discovery of EDHF, studies in the rat showed that NO was responsible for the increase in vasodilation and reduced pressor response during pregnancy (17). However, recent evidence suggests that an EDHF-like molecule plays a role in both rat uterine artery (8) and human myometrial vessels (13) during pregnancy. In sheep, enhanced uterine blood flow likely involves both NOS and PGHS pathways, since NO and

prostacyclin metabolites are elevated during ovine pregnancy (15, 16). In the uterine artery from pregnant mice, we found that in the presence of NOS or PGHS inhibitors, there was a significant reduction in relaxation, suggesting that these pathways have a role in the enhanced sensitivity to methacholine. We also showed that the uterine artery was more sensitive to endothelial-independent vasodilators during pregnancy, suggesting increased smooth muscle sensitivity, possibly to NO. Finally, our data suggests that EDHF-like relaxation is reduced in uterine artery from pregnant mice. We believe that during gestation, the component of relaxation that is mediated by NOS and PGHS is so great that it minimizes contributions of hyperpolarizing factors to vascular function in uterine arteries from pregnant mice.

The factors responsible for altering maternal vascular reactivity in pregnancy are not fully understood. Progesterone is likely involved, although the effects of this steroid hormone may in fact be mediated by one of its metabolites. Indeed, early studies into the blunted pressor response in normotensive pregnant women showed that the progesterone metabolite, 5α-dihydorprogesterone, reversed the indomethacin-induced increase in the pressor response in normal pregnant women (9). Nevertheless, the mechanism behind these effects and additional roles of progesterone metabolites in pregnancy adaptations are not known. Our data illustrate that treating non-pregnant mice for one week with 5β-DHP enhances endothelial-dependent relaxation to a similar extent as that observed in pregnant mice. This effect was likely through the activation of the nuclear receptor PXR, since treatment with 5β-DHP in PXR-/- was not able to increase endothelial-dependent relaxation. Since PXR increases the expression of the CYP450 family of enzymes (in

liver tissue, (1)), we speculate that 5β-DHP is causing enhanced endothelial-dependent relaxation via this pathway. Indeed, inhibiting either CYP450 or EETs significantly blunted relaxation in 5β-DHP treated wild type mice, yet these pharmacological agents had no effect in vessels from 5β-DHP treated PXR-/- mice. Additional experiments are required to confirm an increase in CYP450 expression in vascular tissue from 5β-DHP treated mice, as well as confirm if the 5β-DHP is elevated in pregnant mice. Nevertheless, we speculate that 5β-DHP may be an important stimulus to the vascular adaptations to pregnancy.

In conclusion, these studies highlight pathways involved in vascular adaptations during mouse pregnancy in both the mesenteric and uterine vascular beds. We found that although nitric oxide is involved in the vasodilation of mouse pregnancy, the PGHS pathway is also an important mediator of endothelial-dependent relaxation in both the uterine and mesenteric vasculature. Furthermore, CYP450 derived EET-induced relaxation is enhanced in mesenteric arteries from pregnant mice, possibly due to the progesterone metabolite 5β-DHP. Our data illustrate that vascular reactivity in mouse pregnancy is distinct from the rat, and provides information that will improve our understanding of altered vascular function in transgenic mouse models of pregnancy.

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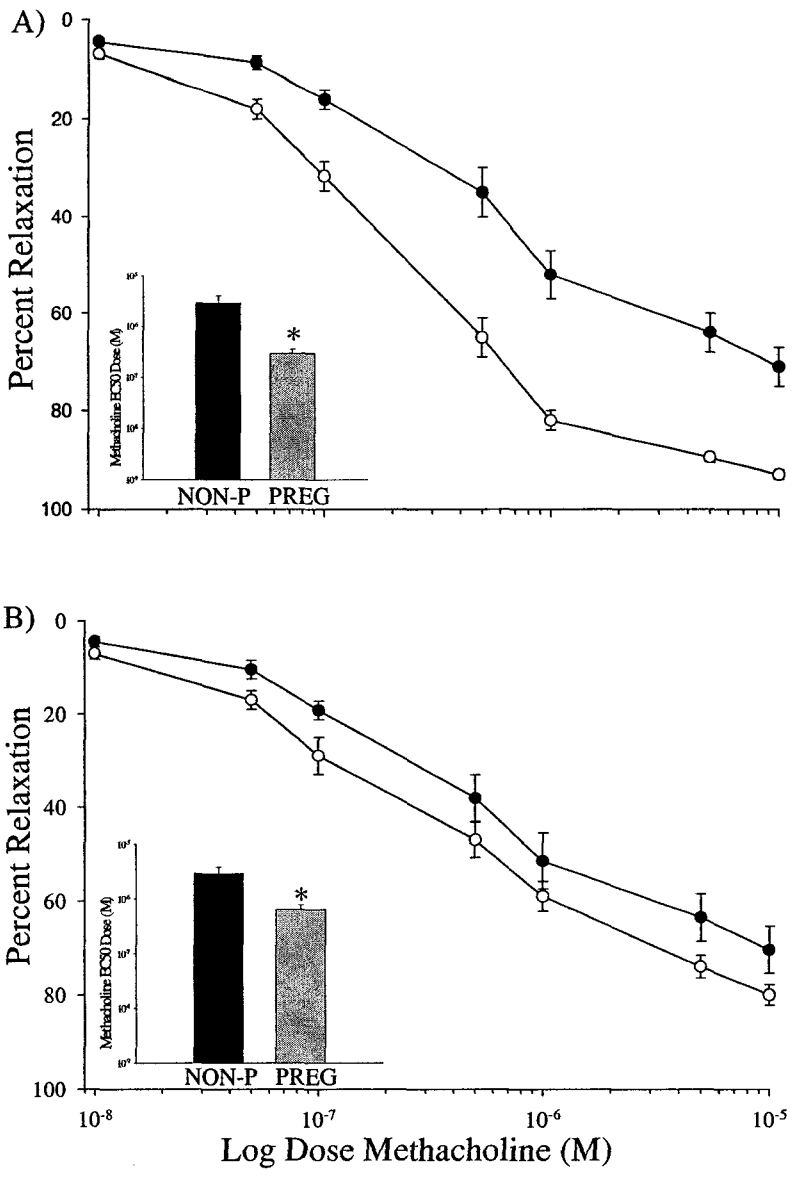

Figure 5.1 Endothelial-Dependent Relaxation is Enhanced in Uterine and Mesenteric vessels from Pregnant Mice.

Concentration response curves to methacholine in non-pregnant (•; n = 7) and pregnant (○; n = 10) mice. A) Methacholine-induced vasodilation in uterine arteries and B) methacholine-induced dilation in mesenteric arteries. Responses are expressed as percent relaxation and each value represents the mean ± SEM. *Inset:* Average EC_{50} doses for non-pregnant (NON-P) and pregnant (PREG) mice. *P<0.05 vs. non-pregnant EC_{50}.

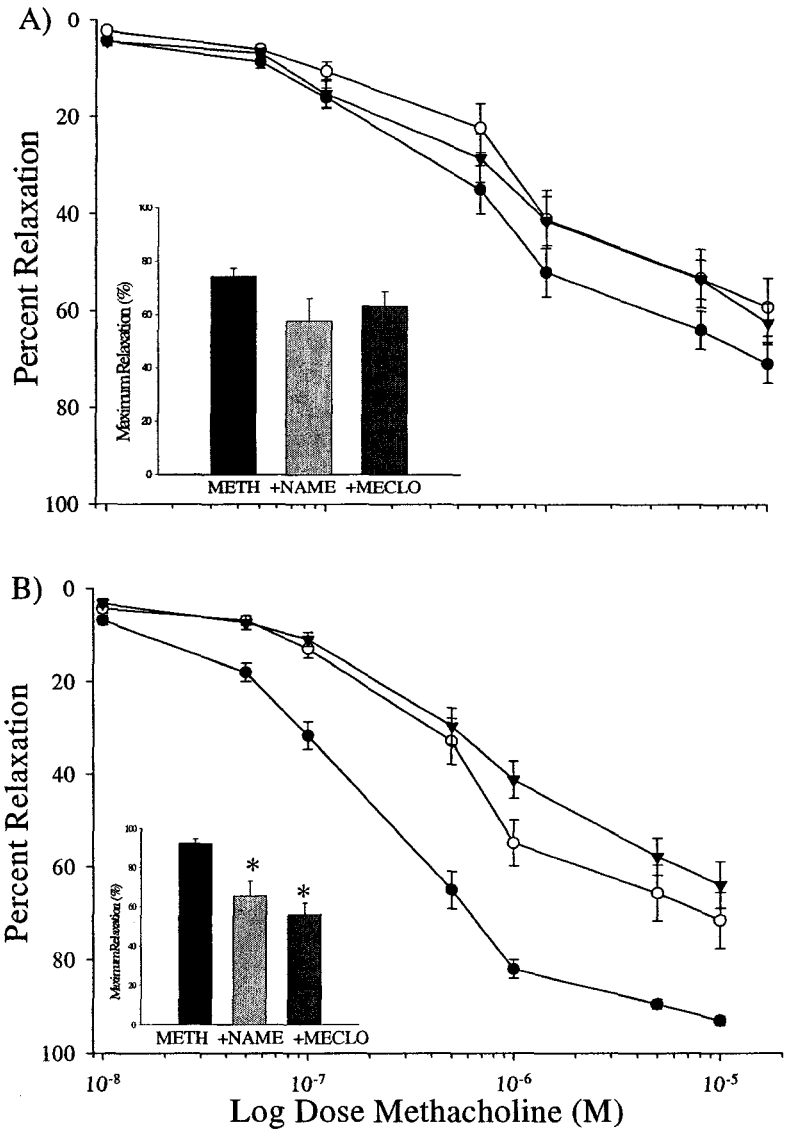

Figure 5.2 Enhanced Endothelial-Dependent Relaxation in the Uterine Artery is Due to Increased NOS and PGHS Mediated Relaxation.

Methacholine concentration response curves in mouse uterine artery. Relaxation was assessed to methacholine alone (METH; •) and in the presence of L-NAME (○; 100 μM) or meclofenamate (MECLO; ▼; 10 μM). Methacholine-induced dilation in A) non-pregnant uterine arteries (n = 7) and B) pregnant uterine arteries (n = 10). Responses are expressed as percent relaxation and each value represents the mean ± SEM. *Inset:* Bars represent mean maximum relaxation to methacholine. *P < 0.05 vs. maximum relaxation to methacholine alone.

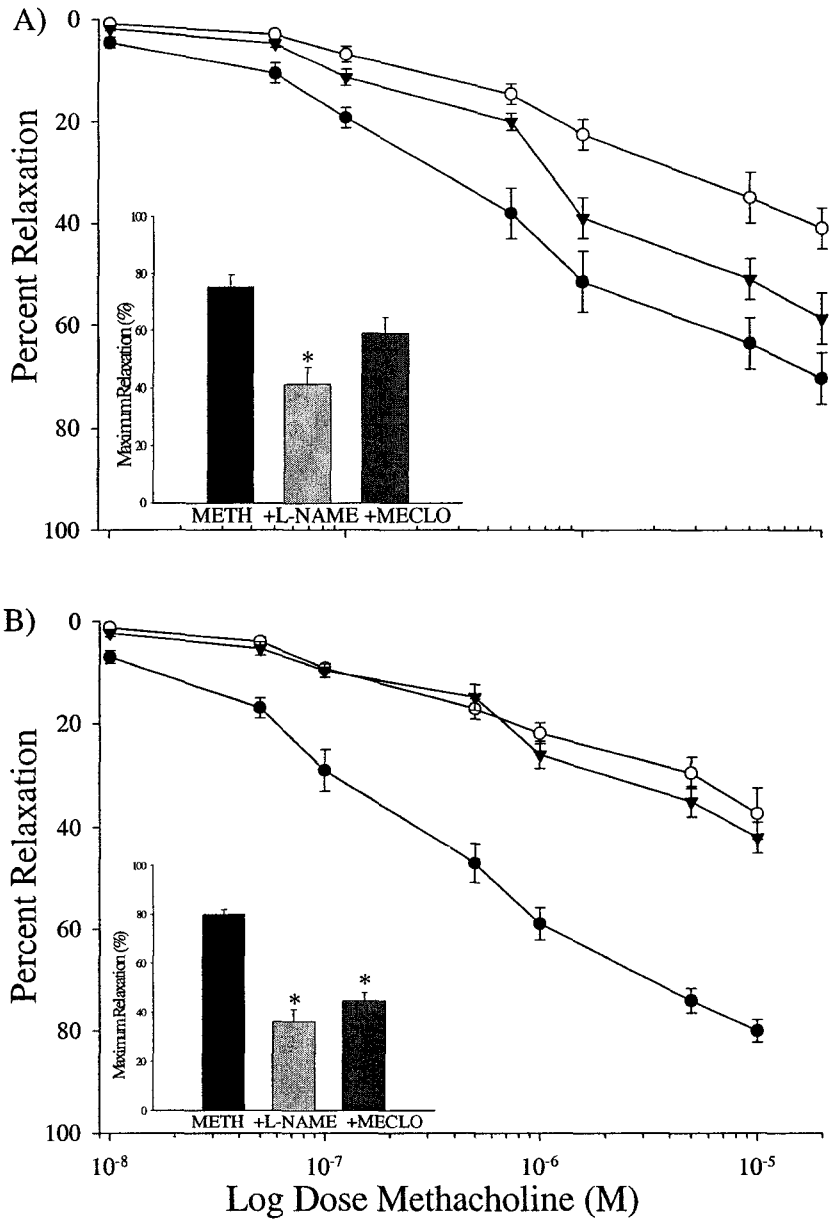

Figure 5.3 Mesenteric Arteries from Pregnant Mice have Enhanced PGHS-Dependent Vasodilation.

Methacholine concentration response curves in mouse mesenteric artery. Relaxation was assessed to methacholine alone (METH; •) and in the presence of L-NAME (○; 100 μM) or meclofenamate (MECLO; ▼; 10 μM). Methacholine-induced dilation in A) non-pregnant mesenteric arteries (n = 7) and B) pregnant mesenteric arteries (n=10). Responses are expressed as percent relaxation and each value represents the mean ± SEM. *Inset:* Bars represent mean maximum relaxation to methacholine. *P<0.05 vs. methacholine alone.

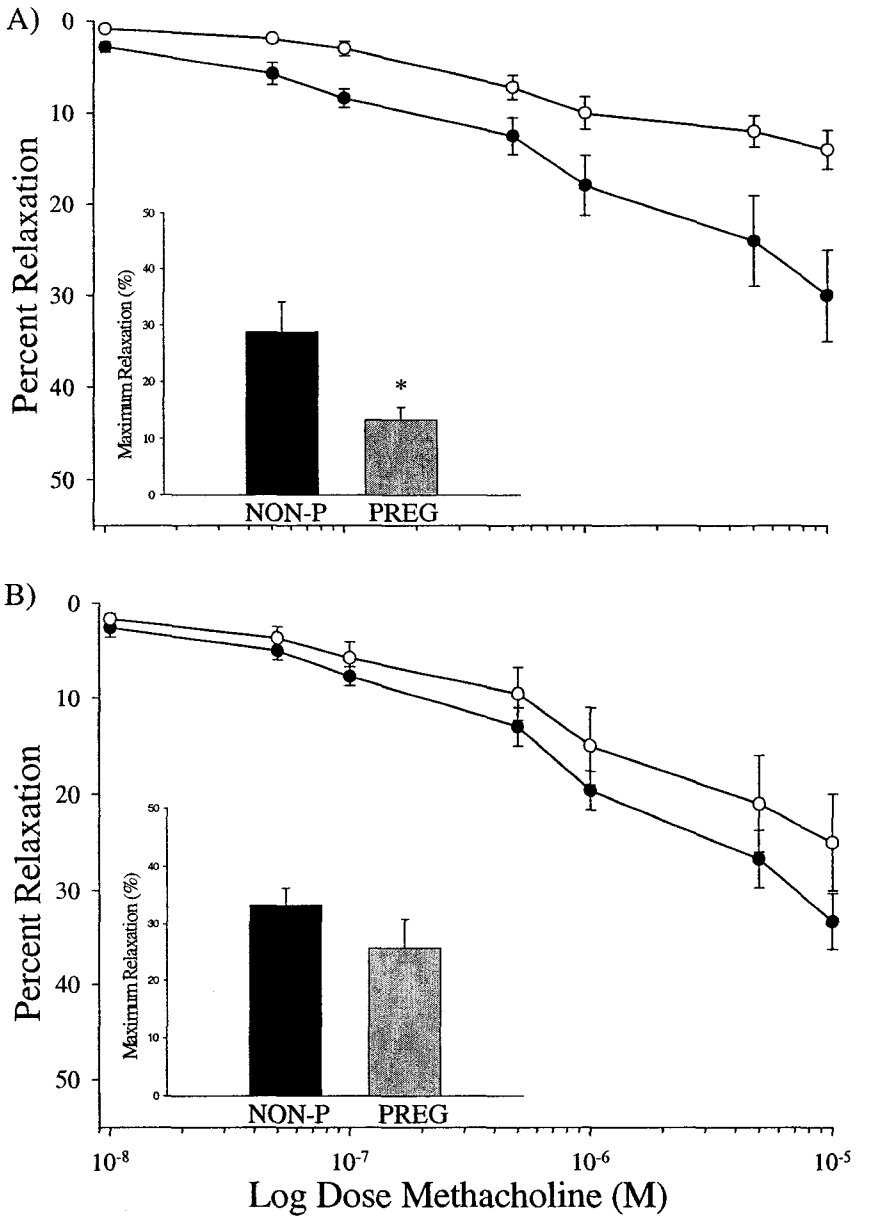

Figure 5.4 Non- Nitric Oxide, Non-Prostacyclin Mediated Relaxation is Reduced in Uterine Vessels from Pregnant Mice.

Methacholine concentration response curves in the presence of both L-NAME (100 μM) and meclofenamate (10 μM) in non-pregnant (•; n=7) and pregnant (○; n=10) mice. Methacholine-induced dilation in A) uterine arteries and B) mesenteric arteries. Responses are expressed as percent relaxation and each value represents the mean ± SEM. *Inset:* Bars represent mean maximum relaxation to methacholine. *P<0.05 vs. maximum relaxation in non-pregnant mice

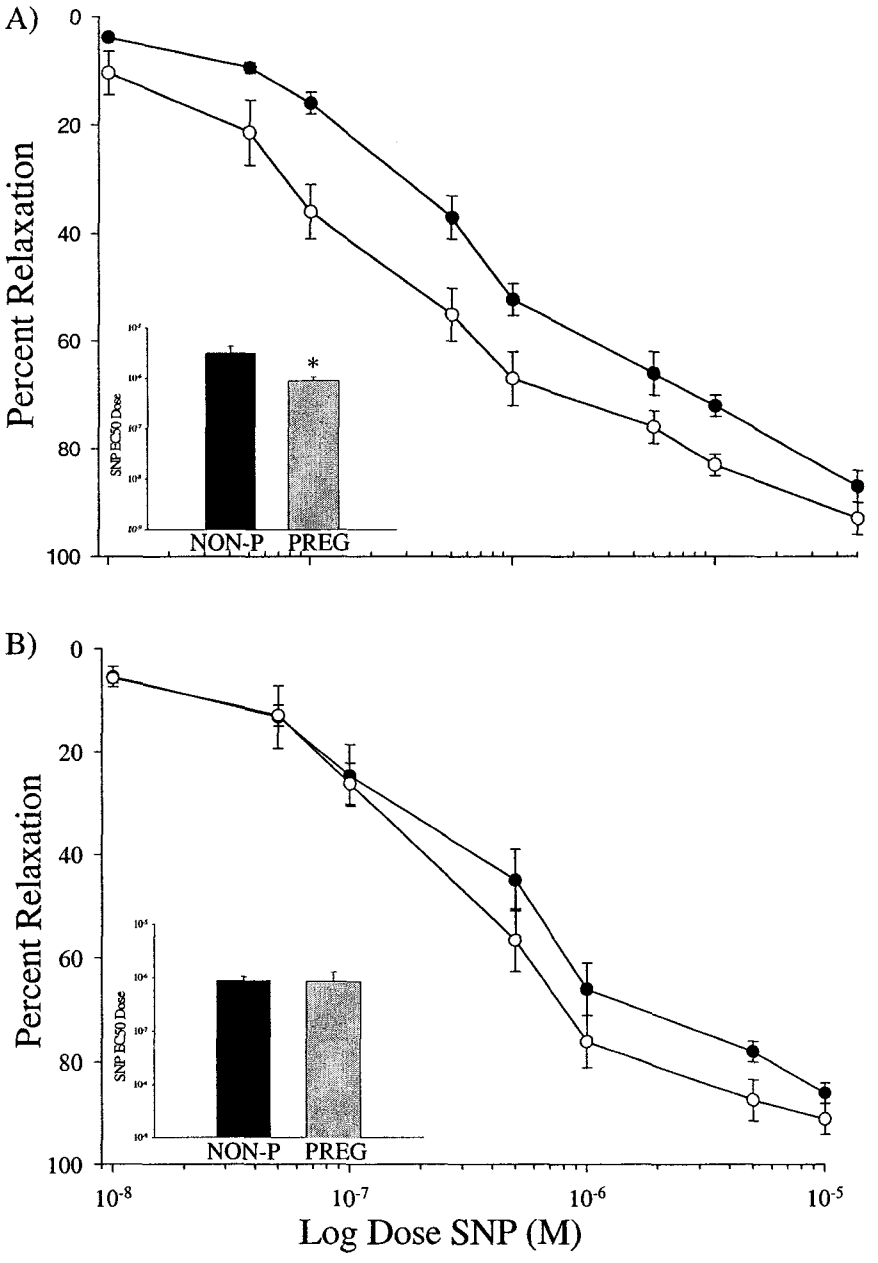

Figure 5.5 Endothelial-Independent Relaxation is Enhanced in Uterine but not Mesenteric Arteries from Pregnant Mice

Dose response curves to sodium nitroprusside (SNP) in non-pregnant (•; n = 7) and pregnant (○; n = 10) mice. SNP-induced dilation in A) uterine arteries and B) mesenteric arteries. Responses are expressed as percent relaxation and each value represents the mean ± SEM. *Inset:* Bars indicate average EC_{50} doses for non-pregnant (NON-P) and pregnant (PREG) mice. *$P < 0.05$ vs. non-pregnant EC_{50}.

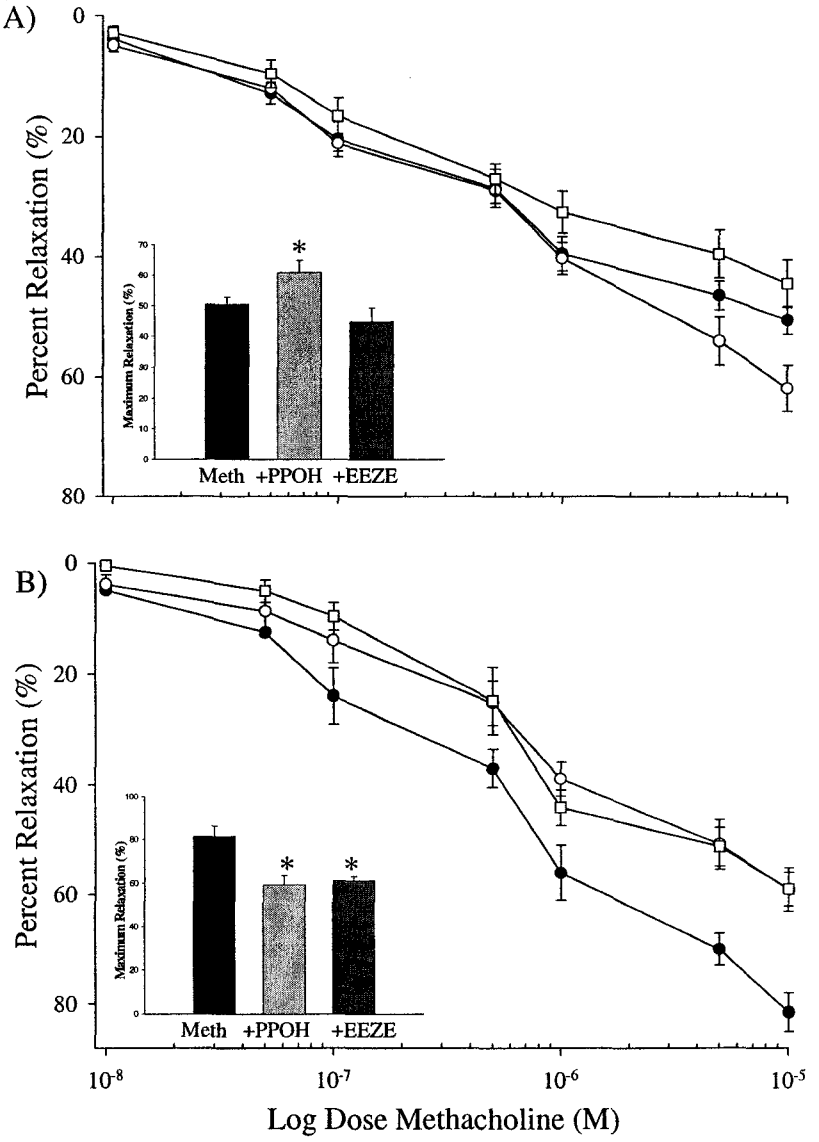

Figure 5.6 CYP450-Derived EET-Mediated Relaxation is Enhanced in Mesenteric Arteries from Pregnant Mice

Methacholine concentration response curves in mesenteric vessel from A) non-pregnant mice (n = 5) and B) pregnant mice (n = 6). Relaxation was assessed to methacholine alone (METH; •) and in the presence of MS-PPOH (○; 1.0 μM) or 14,15 EEZE (□; 10 μM). Responses are expressed as percent relaxation and each value represents the mean ± SEM. *Inset:* Bars represent maximum relaxation. *P<0.05 vs. methacholine alone.

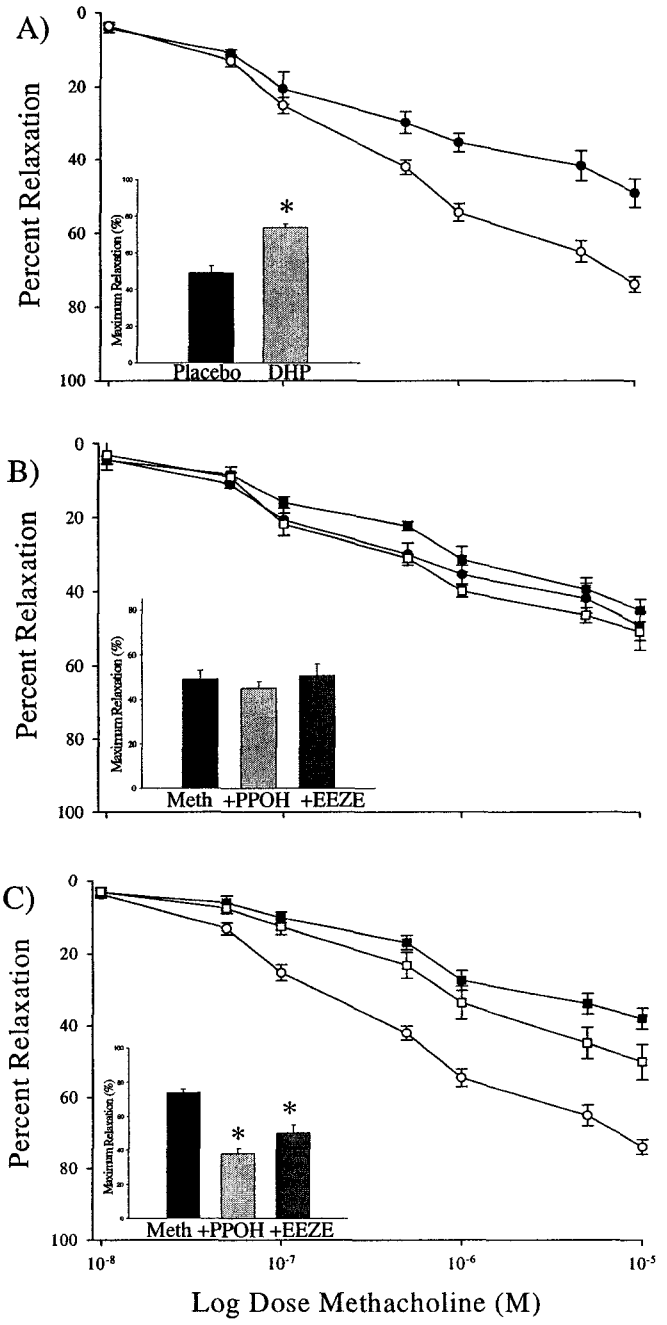

Figure 5.7 5β-DHP Increases EET Induced Relaxation in Wild Type Mice

A) Methacholine relaxation curves in mesenteric arteries from wild type mice after chronic treatment with 5β-DHP (○; n = 6) or placebo (●; n = 6). Methacholine-induced relaxation in B) placebo mice or C) 5β-DHP treated mice in the presence of MS-PPOH (■; 1.0 μM) or 14,15 EEZE (□; 10 μM). *Inset:* Bars represent maximum relaxation. *P< 0.05 vs. methacholine alone or placebo treated mice.

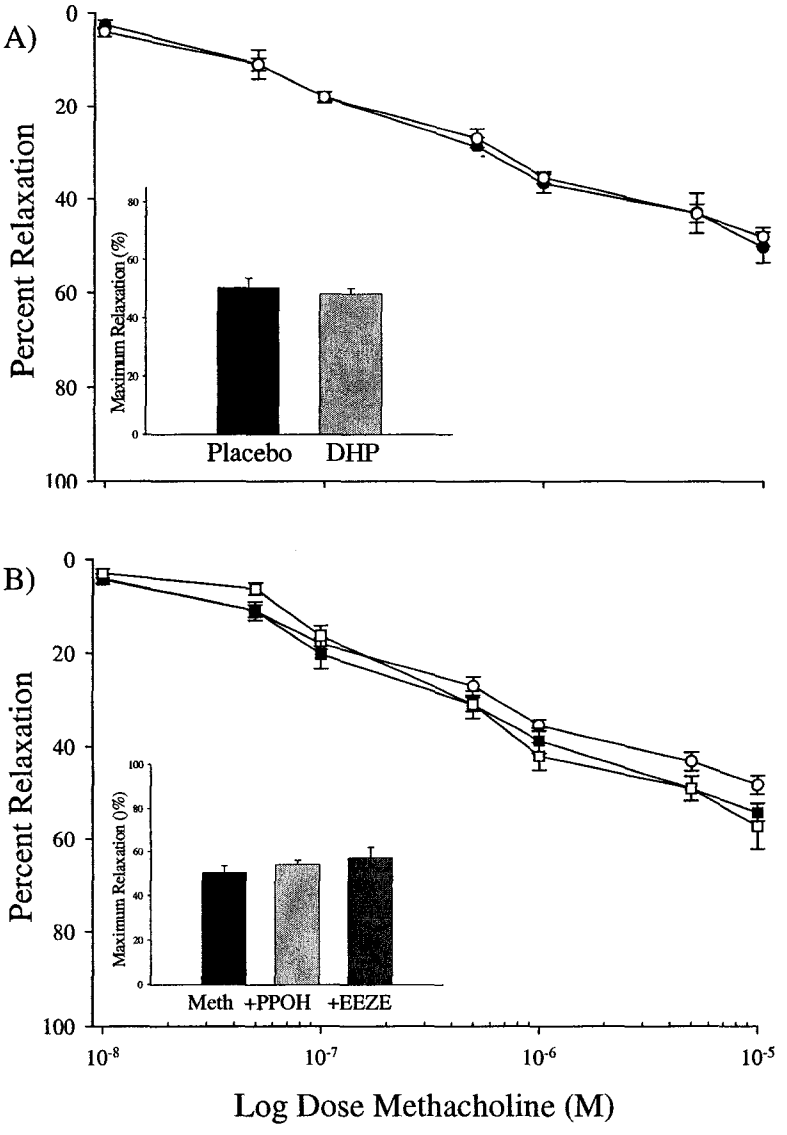

Figure 5.8 5β-DHP Does Not Enhance Endothelial-Dependent Relaxation in Mesenteric Arteries from PXR-/- Mice

A) Methacholine relaxation curves in mesenteric arteries from PXR-/- mice after chronic treatment with 5β-DHP (○; n = 6) or placebo (●; n = 6). B) Methacholine-induced relaxation in 5β-DHP treated mice in the presence of MS-PPOH (■; 1.0 μM) or 14,15 EEZE (□; 10 μM). *Inset:* Bars represent maximum relaxation. *P< 0.05 vs. methacholine alone or placebo treated mice.

CHAPTER 6. GENERAL DISCUSSION

6.1 SUMMARY

The major focus of this thesis is the study of mechanism(s) behind altered vascular reactivity in response to oxidative stress and/ or pregnancy. The endothelial cell is central to regulating vascular tone and the modulation of its vasoactive pathways were the central focus of the experiments. Using endothelial cells in culture, we demonstrated that peroxynitrite can reduce prostacyclin synthase expression and increase iNOS via NFκB, potentially producing more peroxynitrite. To test whether these alterations could translate into altered vascular function *in vivo*, we used SOD-/- mice (which show increased levels of peroxynitrite) and found that indeed, the NOS and PGHS pathways are compromised. In vessels from women with preeclampsia, we found that RAGE (a potent stimulus of oxidative stress that is induced by NFκB activation) is elevated and may be one potential mediator of peroxynitrite formation in this pregnancy-specific syndrome. In order to establish the mouse as a model for vessel function in pregnancy, we elucidated mechanisms behind vascular adaptations in normal mouse pregnancy. We found that pregnancy adaptations in the mouse involve enhanced prostacyclin-mediated vascular relaxation in both the uterine and mesenteric circulation, with a systemic component mediated by EETs. Finally, the novel role of 5β-DHP in stimulating EET-induced vascular relaxation via PXR was determined. These findings are summarized in Figure 6.1.

6.2 PREGNANCY ADAPTATIONS IN SOD-/- MICE

Our initial hypothesis was that an animal model of oxidative stress would demonstrate abnormal vascular adaptations to pregnancy due to altered endothelial cell pathways. Over the course of experiments, we found that SOD-/- mice were incapable of maintaining pregnancy, since over an intensive course of breeding, we achieved pregnancies in all of the wild type mice but in none of the SOD-/- animals. The reproductive capacity of the SOD-/- has been partially characterized. One study reported that fertility was dramatically reduced due to increased fetal loss (8). Another study found that ovarian function was impaired due to altered hormonal levels (12).

The abnormal reproductive capacity in the SOD-/- mice is a fascinating observation, especially considering future research into the possible effects of pro-oxidants on implantation and early placental development (3). Additionally, this animal model may provide an excellent opportunity for dietary interventions in the maternal system (vitamin E and vitamin C) to determine whether *in vivo* scavenging of pro-oxidants could improve reproductive function in the SOD-/- mice. These experiments could further elucidate the effects of vitamin supplementation on vascular function during pregnancy in comparison to the non-pregnant state. This subject warrants further research, since recent evidence suggests that vitamin supplementation was detrimental to vascular function in normal non-pregnant mice (6), suggesting that vitamin treatment may only be beneficial under conditions of oxidative stress.

6.3 ROLE OF OXIDANTS IN NORMAL PHYSIOLOGY

The role of oxidative stress in the progression of many pathological conditions is increasingly becoming apparent. However, moderate levels of pro-oxidants are physiological, and indeed likely play an important role as cellular messengers. Examples of this concept are evident in this thesis. We found that in mice lacking CuZn SOD, endothelial-dependent relaxation was highly impaired. This altered vascular reactivity could be partially restored using a cell permeable SOD added exogenously to the vessel bath. On the other hand, vessel relaxation in wild type mice was significantly reduced after treatment with the exogenous SOD. These data suggest that in mice with normal levels of SOD, adding an excess of the enzyme interferes with normal vascular function be depleting the vessel of physiological levels of superoxide anion. Indeed, there are many mechanisms that might be responsible for these observations. For example, low levels of peroxynitrite have been shown to have vasorelaxant properties (19). Furthermore, an excess of SOD may alter the cellular redox balance, which is known to affect vascular smooth muscle hyperpolarization, ion channel function and ultimately vascular relaxation. Additionally, an excess of hydrogen peroxide, the product of the SOD enzyme, can be hydrolyzed into the hydroxyl radical, a vasoconstrictor, and thus impair vasorelaxation. These findings also illustrate the concept that in moderation, oxidative stress is a healthy part of vascular function, yet when encountered in excess, it may lead to deterioration in vessel function.

This thesis also presented data which supports the theory that normal pregnancy is a state of mild oxidative stress. We illustrated that although RAGE levels were the highest in women with preeclampsia, normal pregnant women had significantly elevated

levels of this novel pro-oxidant, compared to non-pregnant women. This reiterates that low levels of pro-oxidants may serve an important physiological role in pregnancy.

The observation that RAGE is elevated in women with preeclampsia has interesting ramifications. First, it exemplifies another similarity between the vascular pathophysiology of atherosclerosis and preeclampsia. In animal models of atherosclerosis (and diabetes) specific inhibitors of RAGE are able to significantly improve vascular function (17). It would be interesting to elucidate the effect of inhibiting the RAGE pathway in an animal model of oxidative stress, such as the SOD-/- mouse, or investigating the vascular effects of blocking RAGE in vessels from women with preeclampsia. Indeed, a soluble RAGE inhibitor (that is effective at reversing some symptoms of atherosclerosis in mice (13)) may provide a possible therapeutic target for women with preeclampsia, especially those who have co-incident metabolic abnormalities.

The fact that the RAGE pathway in inherently feed-forward suggests that it might be capable of not only initiating, but also perpetuating the impaired vascular reactivity in women with preeclampsia. This theory fits into the two-step model of preeclampsia. Women who are not pre-disposed to abnormal vascular function can tolerate increases in RAGE expression during pregnancy by eliminating pro-oxidants produced by this pathway. However, women destined to develop preeclampsia likely have compromised anti-oxidant capacity, thus allowing for the deleterious feed-forward pathways to commence.

6.4 THE STUDY OF VASCULAR FUNCTION IN TRANSGENIC MICE

The increasing usage of mice to study not only pathological conditions, but also pregnancy, holds great possibilities due to advances in genetic manipulations of this species. However, understanding normal mouse physiology is still greatly lacking in certain areas. Because the rat has been traditionally used as the classical laboratory animal, the intricacies of pregnancy adaptations in this species have been well characterized. Our data suggests that mouse physiology during pregnancy is distinct from the rat, which must be kept in mind when interpreting physiological changes in pregnancy and/or transgenic strains. Another important consideration is that different mouse strains have dramatically different cardiovascular profiles. Indeed, a recent study found that in 5 normotensive mouse strains, the maximal endothelial-dependent relaxation was significantly different among groups and was inversely correlated to blood pressure (15). The implications of these differences on mouse pregnancy adaptations is not fully understood, although a recent study determined that a mildly hypertensive inbred mouse strain develops vascular abnormalities similar to women with preeclampsia when pregnant (4).

In the SOD-/- mouse model that we have employed, the mice are born in and develop in a state of reduced CuZn SOD capacity. Thus, it is conceivable that these mice may have upregulated other physiological anti-oxidant systems to help maintain viability. For example, one study found that metallothioneine expression is increased in the livers of CuZn SOD-/- mice, which may serve as an alternative superoxide anion scavenger (7). An alternative method of investigation could incorporate genetic techniques that

selectively turn on or off genes for a pre-determined length of time (tet-on tet-off mice), thus eliminating the developmental (compensatory) aspects of utilizing knockout mice.

On the other hand, one could perceive that these mice have a genetic predisposition to vascular abnormalities. Indeed, in contrast to other models of oxidative stress, the SOD-/- mice only exhibit pathophysiological responses when faced with perturbations to their systems. For example, heart function is normal in SOD-/- mice until after ischemia-reperfusion injury which leads to reduced coronary blood flow and a larger infarct size compared to control mice (18), and neurological defects do not present except in aged mice (16). Furthermore, although SOD-/- mice are not hypertensive (5), when we stimulate endothelial-dependent relaxation *in vitro*, we observe impaired vascular dilation. Therefore, these observations suggest that SOD-/- mice function normally until faced with a perturbation.

Pregnancy can be considered a perturbation to normal physiological processes. Indeed, most women who develop preeclampsia have normal vascular function and are normotensive before pregnancy. It is the 'insult' of the pregnancy that triggers their susceptibility to vascular dysfunction, and may continue to have ramifications that last once the pregnancy is completed and the symptoms have subsided. Indeed, women who have previously suffered from preeclampsia are more likely to develop abnormal vascular function later in life (11). Although in our studies, homozygous SOD-/- mice were not able to maintain a viable pregnancy, current experiments are underway to investigate vascular adaptations in heterozygous SOD mice.

6.5 COMPENSATION FOR IMPAIRED NITRIC OXIDE-MEDIATED RELAXATION?

In conditions where nitric oxide and/or prostacyclin are reduced, a compensatory upregulation of alternative (EDHF) endothelial-dependent pathways is an intriguing concept. In pregnant women, there seems to be a switch from nitric oxide-prostacyclin dependent relaxation in normal pregnancy to EDHF-like relaxation in women with preeclampsia (10). In our animal model of oxidative stress, we observed very little effect on vasorelaxation in the presence of both NOS and PGHS inhibitors, but in the presence of potassium channel blockers, endothelial-dependent relaxation was eliminated. This suggests that molecule(s) acting via potassium channels is allowing for methacholine-induced relaxation, since the NOS and PGHS pathways are compromised. The role of EET-mediated relaxation in the SOD-/- mice has not been investigated, but would provide interesting information on the interaction of CYP450 and EETs with pro-oxidants. This hypothesis has been addressed in a study by Huang *et al.* in which they found that flow-induced dilation in eNOS-/- mice is mediated by a CYP450-derived EDHF as opposed to wild type mice, where nitric oxide and prostacyclin are responsible for dilation (9). Therefore, it is possible that the EET pathway is less affected by pathological situations (or oxidative stress) and may benefit endothelial-dependent relaxation. Future experiments could investigate the regulation of CYP450 expression in conditions of oxidative stress, such as in the SOD-/- knockout mouse.

6.6 NOVEL MECHANISMS OF VASCULAR ADAPTATIONS IN PREGNANCY

This thesis also presents preliminary data on a novel mechanism mediating enhanced vascular relaxation; the 5β-DHP-induced increase in CYP-EET dependent relaxation.

Interestingly, it has long been hypothesized that progesterone is involved in uterine smooth muscle quiescence, which is integral to pregnancy maintenance, especially in rodents. The mechanisms behind these effects are unknown, and may not depend on changes in progesterone, but in its receptor or other progesterone metabolites. It is evident that metabolites of progesterone have specific and important physiological roles that are distinct from progesterone. The potent activation of PXR by 5β-DHP has been investigated in the liver, where it was shown to increase the expression of the CYP2C family of enzymes. In vascular tissue (where we observed PXR to also be expressed), CYP2C enzymes catalyze the formation of EETs. Our experiments illustrate that one-week treatment with 5β-DHP caused an increase in EET-dependent vasorelaxation in mice. The role of this pathway is not fully understood in pregnancy, and represents a new and exciting area of research into the regulation of vascular reactivity. There are many possible future experiments, including determining whether levels of 5β-DHP are different in normal versus complicated pregnancies, examining pregnancy adaptations in PXR knockout mice, and further characterizing the role of the CYP450-EET pathway in pregnancy.

6.7 LIMITATION OF MOUSE VESSEL FUNCTION STUDIES

There are certain limitations to using mice for cardiovascular experiments, due to the limiting factor of their small size. Indeed, one complication encountered in our experiments was the that although small mesenteric vessels were used to assess vascular reactivity (as an indication of peripheral vascular resistance) it is extremely difficult to

characterize amounts of protein, or the metabolized products of enzymes in these vessels. Thus, we have turned to the aorta for analysis of markers of oxidative stress. Our results indicate that it was possible to detect qualitative differences in peroxynitrite and superoxide anion levels. However, in the pregnancy study, we attempted to quantify changes in NOS and/or PGHS protein levels in the aorta, but were unable to observe quantifiable differences between pregnant and non-pregnant mice (data not shown). Currently, there are new methods for the high yield extraction of protein and RNA utilizing RNAlater ™ (14). Future experiments could attempt this method for extracting mouse mesenteric protein and RNA in order to determine changes in the expression of various enzymatic pathways.

6.8 SIGNIFICANCE OF EXPERIMENTS

The application of information from mouse studies to the human/clinical setting is not always possible. Nevertheless, the results presented in this thesis provide novel and important information about oxidative stress-mediated vascular dysfunction, with implications for the pathophysiology of preeclampsia. Indeed, an anti-oxidant trial involving a cohort of women who were at risk for preeclampsia was successful at decreasing the incidence of preeclampsia (1). In a follow up study, it was determined that markers of endothelial dysfunction and oxidative stress were reduced in the anti-oxidant treated group, illustrating that pro-oxidants are likely mediating the development of vascular endothelial-cell dysfunction in preeclampsia (2). Although the root of the problem of preeclampsia lies in the placenta, the harmful effects of that insult are in the maternal response to endothelial cell disruption. This thesis identifies mechanisms by

which pro-oxidants alter endothelial cell and vascular pathways that mediate adaptations to pregnancy, which could allow for targeted development of therapeutics to treat or prevent oxidative stress-induced vascular dysfunction. In addition, we have elucidated factors that may play a novel role in both normal pregnancy (5β-DHP) and preeclampsia (RAGE) of which future research will expand our understanding of vascular adaptations in normal pregnancy as well as the pathophysiology of preeclampsia.

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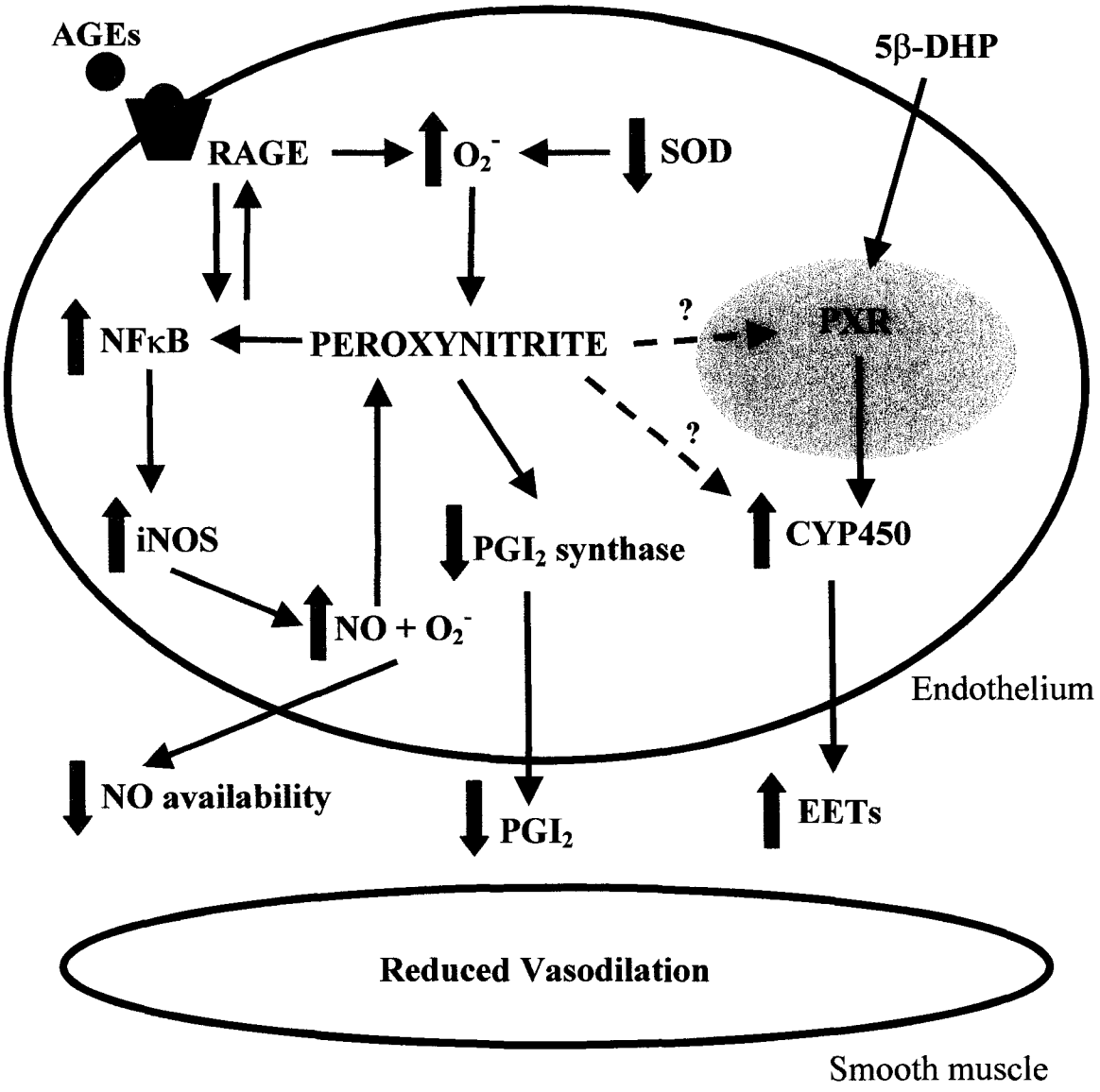


Figure 6.1 Possible Interactions of Peroxynitrite with Endothelial Cell Pathways Involved in Vascular Adaptations to Pregnancy

Vascular reactivity in women with preeclampsia may be impaired due to increased peroxynitrite formation (possibly stimulated by RAGE), inducing a deleterious positive feedback cycle. It is possible that the CYP450-EET pathway is compensating in the vessels such that some vasodilatory capacity remains. The effect of pro-oxidants and/ or peroxynitrite on the CYP450-EET pathway is not known.

APPENDIX I. Wire Myography Expanded Methods

Determination of Optimal Resting Tension for Small Mouse Arteries in a Wire Myograph System

The use of wire myography techniques to investigate vascular reactivity *in vitro* has been employed for many years. Early observations were made that the quantitative outcome of an experiment can depend on the initial passive conditions under which the vessel is placed (resting tension)(2). The amount of active tension developed by a muscle when it contracts varies with the length (or circumference for vascular muscle) of the fiber at the time of contraction (3). Thus, determining the optimal resting tension each blood vessel plays two major roles. First, it allows for a vessel to be set at a point at which it can develop maximal active with a minimal amount of passive tension (which increases as the vessel muscle fiber is stretched). Second, this normalization of blood vessels (which will each have a unique resting tension) takes into account the variability existing in diameter between vessel segments as well as among different animal groups.

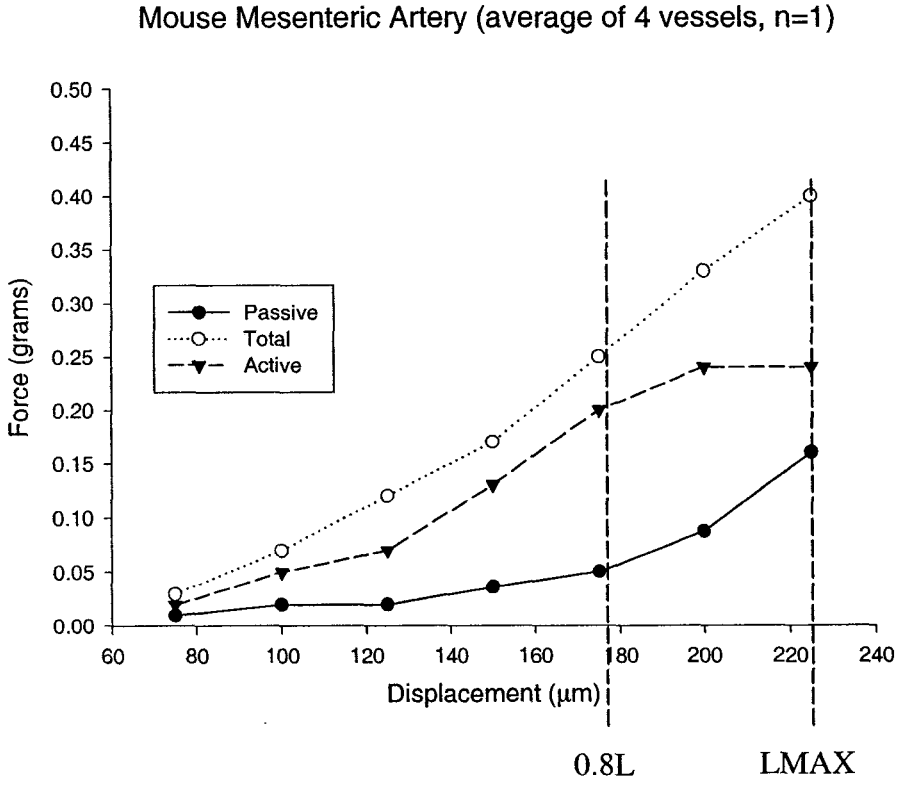
The method used was devised by Mulvany and Halpern (1) who showed that the point of maximal active tension can be calculated empirically from the passive diameter (circumference) tension relationship. This method involves calculating an equivalent radial distending pressure from the measured arterial circumference and the radial force, assuming that the walls of the artery are thin and one can apply the Law of LaPlace

(circumferential wall tension = force X axial raduis x 2). The specific protocol is as follows:

1. Measure the axial length of vessel with a FILAR^{TM} eyepeice.
2. Bring wires together so they are just touching. Note micrometer reading, which represents zero displacement.
3. Separate wires until transducer reads just positive. Note micrometer reading, which can be used to estimate vessel diameter (current displacement – zero displacement). This reading is your initial displacement equal to minimum tension on vessel.
4. Continue to separate wires with micrometer one increment at a time (= 25 μm) until a maximal tension is attained (LMAX = length at which maximal tension results). For mouse mesenteric vessels, this is typically not more than 4 increments or a tension of 0.15 g).
5. These values [displacement (μm) and tension (g)] are entered into a computer program that takes into account vessel axial length, wire diameter and the assumed *in vivo* pressure of the vessel.
6. The program then generates an optimal resting tension based on a value that is sub-maximal, we have determined from preliminary experiments to be L_{80}.
7. The vessel is set at this tension and allowed to equilibrate for 20 minutes before experimentation begins.

Because our laboratory had not previously used mouse vessels for wire myograph studies, we conducted preliminary experiments using the following protocol to determine

if L_{80} was the optimal setting at which the vessel would generate maximal active force with minimal passive tension. First, the above steps were performed in the presence of a high potassium buffer (140 mM KCl) and the subsequent displacement – tension curve was constructed, which represents the total curve (active + passive). Then, the length – tension curve was performed in a calcium-free buffer (including the calcium chelator papaverine; 10^{-4} M), which represents the Passive Curve. In order to generate the active curve, the passive curve was subtracted from the total curve. The following figure represents an example of these curves. Indeed, we found that in mouse mesenteric and uterine vessels, 0.8L was an appropriate setting that allows for maximal active tension with tension generated from passive vessel characteristics.



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