2003

VASCULAR CELL DYSFUNCTION AND TRANSENDOTHELIAL MIGRATION OF NEUTROPHILS IN PREECLAMPSIA

Courtney Elizabeth Leik

Virginia Commonwealth University
School of Medicine

This is to certify that the dissertation prepared by Courtney Elizabeth Leik entitled, "Vascular Cell Dysfunction and Transendothelial Migration of Neutrophils in Preeclampsia" has been approved by her committee as satisfactory completion of the dissertation requirement for the degree of Doctor of Philosophy.

[redacted]
Scott W. Walsh, Ph.D., Director of Dissertation

[redacted]
George D. Ford, Ph.D., School of Medicine

[redacted]
John R. Grider, Ph.D., School of Medicine

[redacted]
Raphael J. Witorsch, Ph.D., School of Medicine

[redacted]
Martin F. Graham, M.D., School of Medicine

[redacted]
Margaret C. Biber, D. Phil., Department Chair

[redacted]
H. H. Newsome, Jr., M.D., Dean, School of Medicine

[redacted]
F. Douglas Boudinot, Ph.D., Dean, School of Graduate Studies

September 25, 2003
Date

VASCULAR CELL DYSFUNCTION AND TRANSENDOTHELIAL MIGRATION OF NEUTROPHILS IN PREECLAMPSIA

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

Courtney Elizabeth Leik
B.S. Villanova University, 1997

Director: Dr. Scott W. Walsh
Professor
Departments of Obstetrics and Gynecology, and Physiology

Virginia Commonwealth University
Richmond, Virginia
August 2003

Dedication

For my parents, Charles and Wilma Leik.
For your commitment to one another and to your family.

Acknowledgments

A dissertation is a collaborative achievement. These pages represent the collected efforts of many individuals within the Virginia Commonwealth University community over the past four years. They also represent encouragement and support from my VCU colleagues, friends and family. I am grateful to all.

My mentor, Dr. Scott Walsh, was a wonderful teacher, supportive guide, and good friend during my journey through graduate school. I am thankful for the experience of working together over the past few years.

The greatest strength of VCU is its faculty. They greatly enhanced my experience by supporting my intellectual, professional, and personal growth. The faculty are not only experts in their fields, they are approachable and proactive toward student development. Faculty members wrote letters on my behalf, attended my poster and oral presentations, and had a genuine interest in my research and development. I am especially grateful to my committee members.

I would like to specifically acknowledge Dr. John T. Povlishock and Dr. Arun J. Sanyal for allowing us to use their lab equipment. Furthermore, their lab technicians, Sue Walker and Farid Mirshahi, were always helpful and willing to provide technical assistance. Our lab technician, Amy Willey, also was vital to this project with her expertise in cell culture, Western blotting, and smooth muscle cell isolation.

These experiments would not have been possible without the assistance of Labor and Delivery staff, and OB/GYN residents and faculty at MCV Hospital. They were invaluable in helping me obtain clinical samples, including fat biopsies, placentas, and blood.

This journey was a "family project". It began a long time ago with moldy bread and intoxicated paramecia. I feel very blessed to have Dad, Mom, Seth, and Monica cheering for me throughout my life, especially these past few years. Thank you! I love you.

Jim you are amazing! Thank you for your support and sacrifice in helping me achieve this goal. I look forward to many more shared successes in our life together. I love you.

Finally, I thank God for providing me with the strength, courage, and determination necessary for this journey.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AA	arachidonic acid
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CO_2	carbon dioxide
COX	cyclooxygenase
Cu	copper
^{51}Cr	chromium 51
ddH_2O	double distilled water
ET	endothelin
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
Fe	iron
$FeSO_4$	ferrous sulfate
FMLP	N-formyl-methionyl-leucyl-phenylalanine
GSH	glutathione
GSSH	glutathione disulfide
HBSS	Hank's balanced salt solution
HETEs	hydroxyeicosatetraenoic acids

HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell
HX	hypoxanthine
H_2O_2	hydrogen peroxide
ICAM-1	intercellular adhesion molecule-1
IL-1	interleukin-1
IL-8	interleukin-8
KCL	potassium chloride
6-ketoPGF$_{1\alpha}$	6-keto prostaglandin F$_{1\alpha}$
$L^{\bullet}$	lipid radial
LA	linoleic acid
LDL	low density lipoprotein
LH	unsaturated lipid
LOH	lipid alcohol
$LO^{\bullet}$	alkoxyl radical
$LOO^{\bullet}$	peroxyl radical
LOOH	lipid hydroperoxide or lipid peroxide
LPO	lipoxygenase
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
MDA	malondialdehyde
Me^{n+}	a transition metal (e.g. Cu^{+}, Cu^{++}, Fe^{++}, Fe^{+++})

MPO	myeloperoxidase
MUFA	monounsaturated fatty acid
M199	Media 199
NADPH	nicotinamide-adenine dinucleotide phosphate
NOS	nitric oxide synthase
O_2	oxygen
OH^-	hydroxyl radical
$ONOO^-$	peroxynitrite anion
Ox	oxidizing solution (hypoxanthine, xanthine oxidase, $FeSO_4$)
OxLA	oxidizing solution enriched with linoleic acid
PAF	platelet activating factor
PASM	placental arterial smooth muscle
PBS	phosphate buffered saline
PECAM	platelet endothelial cell adhesion molecule
PG	prostaglandin
PGI_2	prostacyclin
PLA_2	phospholipase A_2
PMA	phorbol-12-myristate acetate
PSG-1	P-selectin glycoprotein ligand-1
PTA_2	pinane thromboxane A_2
PUFA	polyunsaturated fatty acid
RNS	reactive nitrogen species

ROS	reactive oxygen species
SOD	superoxide dismutase
STBM	syncytiotrophoblast micromembrane fragments
TMP	1,1,3,3-tetramethoxypropane
TNFα	tumor necrosis factor-α
TX	thromboxane
TXA_2	thromboxane A_2
TXB_2	stable metabolite of thromboxane A_2
VE	vitamin E
VC	vitamin C
VCAM-1	vascular cell adhesion molecule-1
XO	xanthine oxidase

List of Units of Measurements

L	liter
mL	milliliter
µL	microliter
m	meter
mm	millimeter
µm	micrometer
nm	nanometer
g	gram
mg	milligram
µg	microgram
ng	nanogram
pg	picogram
M	molar (moles / liter)
mM	millimolar (millimoles / liter)
µM	micromolar (micromoles / liter)
Ci	Curie
µCi	microCurie
cpm	counts per minute

x g	times gravity
°C	degrees Celsius
rpm	revolutions per minute
IU	international units

ABSTRACT

VASCULAR CELL DYSFUNCTION AND TRANSENDOTHELIAL MIGRATION OF NEUTROPHILS IN PREECLAMPSIA

By Courtney E. Leik, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2003

Director: Dr. Scott W. Walsh
Professor
Departments of Obstetrics and Gynecology, and Physiology

Oxidative stress, hyperlipidemia, neutrophil activation and endothelial cell dysfunction are characteristic of women with preeclampsia. We used in vitro experiments to test if a combination of oxidative stress and linoleic acid favors a mechanism for neutrophil transendothelial migration. We used linoleic acid because it is one of the fatty acids elevated in preeclampsia and the precursor for arachidonic acid and its inflammatory metabolites. For these studies, we developed a methodology for isolating and culturing human vascular smooth muscle cells from placental chorionic plate arteries. Treatment of these cells with an oxidizing solution enriched with linoleic acid, but neither

component alone, led to increased production of interleukin-8 (IL-8), a potent neutrophil chemotactic agent. This treatment solution also stimulated arachidonic acid metabolites, including leukotriene B_4, another potent neutrophil chemotactic agent. The same treatment solution rapidly activated neutrophils to produce superoxide. These observations suggested there might be neutrophil transendothelial migration in women with preeclampsia because increased expression of IL-8 by vascular smooth muscle would attract neutrophils to the vasculature, and activation of neutrophils would prime them for transendothelial migration. These predictions were confirmed using immunohistochemical staining of systemic vascular tissue in preeclamptic women, as compared to normal pregnant and normal non-pregnant women, by demonstrating vascular smooth muscle cell expression of IL-8 coincident with neutrophil infiltration into systemic vessels. Endothelial cells and vascular smooth muscle cells also expressed ICAM-1, a cell adhesion molecule necessary for neutrophil infiltration.

This investigation is the first to demonstrate vascular smooth muscle cell expression of IL-8 and ICAM-1 coincident with neutrophil transendothelial migration into systemic vascular tissue in women with preeclampsia. These observations provide evidence for total "vascular cell dysfunction", not only endothelial cell dysfunction, in women with preeclampsia. Together they link vascular cell dysfunction to a single mechanism, transendothelial migration of neutrophils, which could explain the clinical symptoms of hypertension, proteinuria, and pathological edema. These results bolster the use of antioxidants in preventing preeclampsia and suggest novel treatments for preeclampsia based on neutralizing antibodies to IL-8 or cell adhesion molecules.

Chapter 1

GENERAL INTRODUCTION

A. Introduction

Preeclampsia is a significant pregnancy-related disorder that affects 6-8% of all human pregnancies and is the leading cause of intrauterine growth retardation (IUGR), premature delivery, and maternal death [1]. It is a multi-system disorder clinically characterized by maternal hypertension ($\geq$ 140/90 mm Hg), proteinuria (> 300 mg/24 hours), pathological edema, and coagulation abnormalities. There is also evidence of compromised uteroplacental, fetoplacental, and maternal organ perfusion in preeclamptic women. Although preeclampsia does not present clinically until the second or third trimesters of pregnancy, the pathological process is believed to begin at placentation.

A working hypothesis for the pathophysiology of preeclampsia states that the disorder has two stages [2]. The first stage is the initiation of the pathophysiological process caused by abnormal placental implantation, which leads to reduced placental blood perfusion. The second stage is characterized by maternal endothelial cell dysfunction and subsequent clinical symptoms. The link between the first and second stage is not clear, although it is hypothesized that abnormal placentation results in the

release of toxic agents that enter the maternal circulation, causing endothelial cell dysfunction. The clinical manifestation and progression of preeclampsia depends on the mother's pre-disposing circulatory health [3].

Research supports the belief that oxidative stress links the two stages of preeclampsia. Evidence confirms oxidative stress in the placenta and maternal circulation and suggests agents that could transfer oxidative stress between the two compartments. This literature review will summarize the current understanding of the role of oxidative stress in the pathogenesis of preeclampsia, with a focus on maternal vascular smooth muscle, endothelial, and neutrophil dysfunction. First, a general review of free radical and antioxidant biochemistry is essential to understanding the relationship between oxidative stress and preeclampsia.

B. Free Radicals, Antioxidants, and Oxidative Stress

Multicellular organisms require oxygen for the efficient production of energy. Oxygen, however, is a toxic gas. Over the past 50 years it has become evident that oxygen mediates toxic effects through the production of free radical species. A free radical, as defined by Halliwell, is "any species capable of independent existence that contains one or more unpaired electrons" [4]. Free radicals are highly reactive species because they will attack nearby atoms or molecules for electrons to complete their outer electron orbital and, therefore, restore their stability.

Approximately 1-3 % of inhaled oxygen forms free radicals [5]. The body produces free radicals spontaneously and deliberately during chemical reactions. Reactive oxygen species (ROS) are natural byproducts of oxidative metabolism. During oxidative metabolism, electrons flow through a series of electron carriers, eventually to the final electron carrier of cytochrome oxidase, which reduces oxygen to water. A small percentage of electrons leak from the electron transport chain and combine with oxygen to produce the free radical, superoxide ($O_2^{\bullet -}$) [5].

Oxidase enzymes also yield ROS byproducts. For example, xanthine oxidase spontaneously produces $O_2^{\bullet -}$ during purine base catabolism, while cyclooxygenase (COX) and lipoxygenase (LPO) produce ROS during arachidonic acid (AA) metabolism [6]. An oxidase enzyme that deliberately produces ROS is the nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase of neutrophils. Upon neutrophil activation, NADPH oxidase incorporates into the cell membrane and reduces molecular oxygen to $O_2^{\bullet -}$, which leads to the formation of ROS for the destruction of bacteria and other pathogens. NADPH oxidase also was localized recently to endothelial and vascular smooth muscle cells, where ROS may play a role in cell signaling [7].

ROS is a collective term that encompasses oxygen radicals and non-radicals. Many ROS are pro-oxidants, agents that oxidize various targets by removing electrons or hydrogen, or by adding oxygen [4]. The "reactive" in ROS is relative since their reactivity varies depending on environmental conditions [4]. $O_2^{\bullet -}$ is an important oxygen free radical produced when diatomic oxygen accepts an extra electron (Table 1). It is not particularly reactive alone, but in the presence of transition metals and/or other ROS, highly toxic

species are produced. For example, $O_2^{\bullet-}$ reacts with transition metals to form the highly reactive hydroxyl radical (OH•). H_2O_2, a non-radical derivative of oxygen, also produces the OH• by a metal-catalyzed reaction.

Reactive nitrogen species (RNS) are a recent addition to free radical biochemistry. Nitric oxide ($NO^{\bullet}$) is the most commonly known RNS. $NO^{\bullet}$ is a free radical with a single unpaired electron formed in the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) [4]. Similar to $O_2^{\bullet-}$, $NO^{\bullet}$ is not a very reactive free radical and actually has a more important physiological role (i.e. vasodilatation) than pathological role [4]. $NO^{\bullet}$ can, however, react with $O_2^{\bullet-}$ to produce the strong oxidizing agent, peroxynitrite ($ONOO^-$) (Table 1). $ONOO^-$ formation occurs three times faster than the dismutation of $O_2^{\bullet-}$ to H_2O_2 [8].

Since oxidative metabolism and other physiological processes produce free radicals, mechanisms are necessary to inhibit free radical damage and maintain oxidant-antioxidant balance. Endogenous antioxidant defense mechanisms are either enzymatic or nonenzymatic. The three most important cellular antioxidant enzymes are superoxide dismutase (SOD), catalase, and glutathione peroxidase (Table 2). They function together to eliminate ROS generated during oxidative metabolism, which can damage cellular membranes, proteins, and DNA (Figure 1). SOD dismutates $O_2^{\bullet-}$ to H_2O_2 and molecular oxygen. Thus, it is a significant antioxidant enzyme because it quenches $O_2^{\bullet-}$ produced during oxidative metabolism. Cellular SOD exists in two forms; copper zinc SOD is located in the cytosol while manganese SOD is located in the mitochondria. (There is also an extracellular SOD, which functions to minimize oxidative stress in the plasma.)

Catalase is located in the cytosol and functions in conjunction with SOD to catalyze the conversion of H_2O_2 to water and molecular oxygen. Glutathione peroxidase is a selenium-dependent enzyme that is similar to catalase in that it catalyzes the reduction of H_2O_2 to water and molecular oxygen. Selenium-dependent glutathione peroxidase, however, unlike catalase, also reduces free lipid peroxides to nonreactive fatty acid alcohols [9]. Selenium-dependent glutathione peroxidase resides in the cytosol. A second form of glutathione peroxidase, phospholipid hydroperoxide glutathione peroxide, is located in the cell membrane and acts on membrane-bound lipid peroxides [10]. This form is also selenium-dependent.

Nonenzymatic antioxidants can be divided into a fat-soluble group and a water-soluble group [4]. For example, vitamin E is a fat-soluble vitamin composed of tocopherols. Of these, d-α-tocopherol has the greatest biological activity [11]. Vitamin E is found in cell membranes and functions as a chain-breaking antioxidant to inhibit lipid peroxidation [9, 11]. Vitamin E also modulates redox cell signaling pathways to prevent free radical damage [11]. In the process of inhibiting lipid peroxidation, vitamin E becomes a radical and must be recycled (reduced) by vitamin C [5]. Other lipid soluble antioxidants include: carotenoids (i.e. β-carotene and lycopene), flavonoids, and vitamin A [12].

Vitamin C (ascorbic acid) is a water-soluble chain-breaking antioxidant [13]. It is considered important for its role in recycling the vitamin E radical and in scavenging other free radicals. Metal transport and storage proteins are also water-soluble antioxidant defense mechanisms. These proteins bind iron and copper ions to prevent metal-catalyzed free radical damage, lipid peroxidation, and OH• formation [9]. Iron is bound to transferrin

in the plasma and to lactoferrin in body secretions [9]. Ceruloplasmin binds and transports copper [9].

Oxidative stress is a state of imbalance between ROS/RNS and antioxidant defenses that favors ROS/RNS. This imbalance occurs when there is either a decrease in antioxidants (enzymatic and/or dietary) or an increase in the production of free radicals. Increased concentrations of ROS/RNS may result from: 1) excessive oxygen levels, 2) toxins, 3) ischemia-reperfusion, 4) leukocyte activation, etc. [4].

An imbalance toward reactive species leads to unchecked free radical cascades that cause transient or permanent tissue injury. During initial periods of oxidative stress tissues adapt and produce additional antioxidant molecules to maintain cell homeostasis. Severe oxidative stress, however, causes irreversible cell damage that ultimately leads to cell death. For this reason oxidative stress is a pathological mechanism of tissue injury in a wide variety of diseases [9].

Many functionally and structurally important biological molecules are targets of oxidative stress. Consequently, oxidative stress often alters cell behavior. The effects vary according to the cell type, the involved ROS/RNS, and the severity of the stress. Cell functions that are affected by oxidative stress include: altered membrane function, cell division, intercellular communication, and signal transduction [4].

Lipids, especially polyunsaturated fatty acids (PUFAs), are often involved in oxidative stress because they are the major constituents of biological membranes. Since the lipid bilayer serves as a protective barrier, oxidative stress initially alters membrane function to cause: increased fluidity, increased permeability to proteins, and inactivation

of membrane-bound enzymes [4]. PUFAs are especially vulnerable to oxidation at carbon-carbon double bonds. Initiation of lipid peroxidation occurs at fatty acid double bonds, by a reactive species that can remove a hydrogen atom to form a lipid peroxyl radical (Figure 2). OH• and $ONOO^-$ are primary agents of lipid peroxide initiation. Following initiation, the lipid undergoes a molecular rearrangement to form a conjugated diene. Propagation occurs when the lipid peroxyl radical attacks an adjacent fatty acid to form another peroxyl radical. Lipid peroxyls and hydrogen ions then interact to produce lipid peroxides. Another membrane lipid, AA, undergoes free radical, nonenzymatic peroxidation to produce toxic products called isoprostanes. Isoprostanes are prostaglandin-like compounds that mediate a variety of damaging actions, including potent vasoconstriction.

C. Oxidative Stress in Preeclampsia

Oxidative stress occurs during normal pregnancy, but antioxidant defenses simultaneously increase to oppose free radical activity [14]. In preeclampsia, however, there is greater lipid peroxidation and oxidant activity with a net decrease of antioxidants, producing a state of oxidative stress [14].

The dominating hypothesis proposes that the primary pathological event of preeclampsia is insufficient trophoblast invasion of the spiral arteries during placental implantation [15]. Incomplete spiral artery remodeling creates a vasoconstrictive placental bed characterized by ischemia-reperfusion and the production of ROS. In the presence of

ROS, abundant placental PUFAs serve as substrates for lipid peroxidation. Lipid peroxides and ROS attack placental cell membranes to perpetuate oxidative stress and damage. Gradually, as placental mass increases, placental oxidative stress is transferred to the mother.

Oxidative stress appears to be the link between placental pathology and vascular cell dysfunction in the maternal compartment, which results in the clinical manifestation of preeclampsia. The placenta intimately links and mediates exchange between two genetically distinct organisms, the mother and the fetus. Through the placenta, the maternal system maintains the proper functioning and homeostasis of the fetal compartment. Thus, oxidative stress in one compartment may alter homeostasis in another compartment. The distinctive roles and functions of the fetal, placental, and maternal compartment demand that each system be analyzed individually for oxidative stress.

i. Fetal Oxidative Stress

There is little evidence for fetal oxidative stress during preeclamptic pregnancy. The child's health during a preeclamptic pregnancy depends more upon uteroplacental perfusion than oxidative stress within the fetal compartment [15]. The mechanics and innate properties of the placenta, however, may protect the fetus from oxidative stress during normal and preeclamptic pregnancies. This is especially important because oxidative

stress in the placenta is the likely origin of excessive maternal oxidative stress during preeclampsia.

ii. Placental Oxidative Stress

Lipid peroxidation in the placenta during normal pregnancy was first described in 1979 [16]. Approximately ten years later Wang et al. demonstrated elevated lipid peroxides in preeclamptic placentas as compared to normal placentas by measuring malondialdehyde (MDA) and H_2O_2 equivalents [17]. This initial study was followed quickly by research demonstrating secretion of lipid peroxides predominately toward the maternal circulation upon placental perfusion of t-butyl hydroperoxide as compared to Krebs-Ringer bicarbonate buffer [18]. Simultaneous placental perfusion of aspirin blocked the secretion of lipid peroxides, suggesting cyclooxygenase (COX) involvement. A comprehensive follow-up study showed significantly greater lipid peroxide, thromboxane A_2 (TXA_2) and prostacyclin I_2 (PGI_2) production by trophoblast cells isolated from preeclamptic placentas as compared to normal placentas [19]. Furthermore, villous core tissue isolated from preeclamptic placentas produced significantly more lipid peroxides and TXA_2, but not more PGI_2, than villous core tissue of normal placentas. Additional studies demonstrated that there is compartmentalization within the placenta: trophoblast cells produce more lipid peroxides and TXA_2, whereas vascular tissue (chorionic plate arteries) produces more PGI_2.

Lipid peroxides are not only a consequence of oxidative stress, but also influence enzyme activity to further alter the balance between oxidants and antioxidants. Specifically, lipid peroxides stimulate COX to generate prostaglandins and thromboxanes, including the vasoconstrictor TXA_2 and the vasorelaxant PGI_2 [14](Figure 3). Lipid peroxides, however, simultaneously inhibit the PGI_2 synthase enzyme so that PGI_2 is not available to counteract the vasoconstricting actions of TXA_2. Thus, elevated lipid peroxides in preeclamptic placentas would lead to an imbalance of increased TXA_2 and decreased PGI_2. Walsh analyzed TXA_2 and PGI_2 production from term placentas and was the first to describe that preeclamptic placentas produce seven times more TXA_2 than PGI_2 compared to normal placentas. This TXA_2/PGI_2 imbalance could explain many clinical findings of preeclampsia [20].

The generation of ROS from enzymes, cytokines, and biochemical processes are sources of placental oxidative stress during preeclampsia. Preeclamptic placentas have greater COX-2 trophoblast expression and activity, as well as increased COX-1 placental bed protein and mRNA levels [21, 22]. Elevated COX-1 & –2 protein levels may contribute to placental oxidative stress by the spontaneous release of $O_2^{\bullet-}$ during COX activation, while amplifying the TXA_2/PGI_2 imbalance due to COX activation by lipid peroxides.

The NADPH oxidase enzyme, which mediates the phagocytic respiratory burst of leukocytes, was localized recently to endothelial and vascular smooth muscle cells [7]. As an important ROS-generating system, NADPH oxidase activation in the placenta may cause or contribute to oxidative stress during preeclampsia. Only one study has evaluated NADPH oxidase in preeclamptic placentas. Although NADPH oxidase staining pattern

and intensity of synctiotrophoblast cells was equivalent in normal and preeclamptic placentas, enzyme activity may differ and lead to greater placental oxidative stress [23].

Cytokines, specifically TNFα, may also increase ROS by stimulating $O_2^{\bullet-}$ production by coenzyme Q of the electron transport chain in mitochondria of preeclamptic placentas [24]. Wang et al. found elevated TNFα mRNA and protein in preeclamptic placentas, which indicated that TNFα was available to stimulate coenzyme Q and result in $O_2^{\bullet-}$ production [25]. The number of mitochondria, a source of oxygen free radicals, was also elevated in preeclamptic placentas and may act to increase ROS [26].

ROS perpetuate oxidative stress by lipid peroxidation and isoprostane formation. Walsh et al demonstrated both elevated lipid peroxidation and increased 8-isoprostane release from preeclamptic placentas [18, 27]. Superoxide is the primary agent in causing AA peroxidation for the formation of isoprostanes [28]. Significantly elevated protein carbonyls in the placental and decidual tissue of preeclamptic women confirm ROS-mediated protein damage and general placental oxidative stress [29].

In addition to attacking proteins and lipids, $O_2^{\bullet-}$ interacts with $NO^{\bullet}$ to produce $ONOO^-$ in the placenta. Preeclamptic placentas have evidence of this $ONOO^-$ formation. Myatt et al. illustrated the presence of nitrotyrosine residues, the stable product of $ONOO^-$ and protein interaction, in preeclamptic placentas by immunostaining placental villous vascular endothelium and smooth muscle [30]. Preeclamptic placentas, furthermore, released greater concentrations of $ONOO^-$ into the maternal circulation [30]. $ONOO^-$ action further contributes to the feed-forward cycle of oxidative stress present in preeclamptic placentas.

Placental antioxidant mechanisms oppose free radical damage in healthy pregnancy. During normal pregnancy, placental SOD and catalase activity increase while glutathione peroxidase activity remains steady [24]. In preeclampsia, however, placental antioxidant protection is decreased. Preeclamptic placentas have reduced Cu-Zn SOD and glutathione peroxidase activity and mRNA expression [31, 32]. A recent study, however, showed increased glutathione levels and glutathione peroxidase activity in placental and decidual tissue from preeclamptic women [33]. Studies performed by Poranen et al. on preeclamptic placentas confirmed decreased SOD activity, but not decreased glutathione peroxidase activity [34]. Decreased SOD levels imply that $O_2^{\bullet-}$ is available for longer periods in which it can react with AA or $NO^{\bullet}$ to produce isoprostanes or $ONOO^-$, respectively. Normal and preeclamptic placentas showed similar extracellular SOD localization and activity, indicating that extracellular SOD is probably not significant in the pathology of preeclampsia [35]. Another group demonstrated, in severe preeclamptics, a decrease of thioredoxin and glutaredoxin [36]. These antioxidant enzymes are involved in the thiol redox control of enzymes and transcription factors that mediate transcription and translation during oxidative stress.

The role of placental lipid- and water-soluble vitamins in preeclampsia also has been studied. There is evidence of lower vitamin E levels in placental tissue from preeclamptic women [31]. Addition of vitamin E blocked peroxide-induced vasoconstriction in a placental perfusion model [37]. Other studies confirmed the importance of antioxidants by perfusion of preeclamptic placentas with vitamins E and C [38] or β-carotene [39] to reduce lipid peroxide production. These studies suggested that

preeclamptic placentas may have lower dietary antioxidant concentrations and, therefore, be unable to control lipid peroxidation. Decreased antioxidant molecules and enzymes in preeclamptic placentas may not be the cause of oxidative stress but, rather, may be the result of oxidative stress. Whatever the case, decreased antioxidant protection would worsen oxidative stress.

Oxidative stress may be the link between insufficient placental perfusion and maternal endothelial cell dysfunction in preeclampsia. The direct exchange between the placenta and the maternal circulation allows for the transfer of placental oxidative stress to the mother by lipid peroxides, ROS, circulating leukocytes, and oxidized syncytiotrophoblast micromembrane (STBM) fragments. Although oxidative stress originates in the placenta, it is systemic maternal oxidative stress that leads to cellular dysfunction, which causes the clinical presentation of preeclampsia. This is discussed in the next section.

iii. Maternal Oxidative Stress

Maternal plasma lipid peroxide levels are increased above non-pregnant levels during normal pregnancy and further increased during preeclampsia [40-42]. A likely source of elevated circulating lipid peroxides during pregnancy is the placenta [14]. Normally placental growth parallels a progressive rise in antioxidant defenses during pregnancy, which counteracts oxidative stress produced by the placenta [24]. In preeclampsia, however,

significantly elevated lipid peroxides and decreased antioxidant mechanisms support self-propagating lipid peroxidation and widespread cell damage.

Research has demonstrated increased serum lipid peroxides in preeclamptic women. Maternal serum lipid peroxides, measured by the breakdown product, MDA, were significantly elevated in mild preeclampsia and even more elevated in severe preeclampsia compared to normal pregnancy [43]. Hubel et al. demonstrated elevated levels of serum lipid peroxides, as well as elevated levels of serum triglycerides and free fatty acids (FFA) in women with preeclampsia. The levels significantly decreased by 24-48 hours post-partum, implicating the placenta as the source of the elevated levels of lipids [44]. Other studies confirmed maternal oxidative stress with evidence of elevated conjugated dienes, 8-isoprostanes, triglycerides, and iron levels in preeclamptic individuals [24]. A recent study, which measured oxidized low density lipoproteins (LDL), rather than lipid peroxide metabolites, did not find an increase in oxidized LDL in preeclamptic patients [45]. These investigators suggested that secondary lipid peroxidation products, such as aldehydes or lipid peroxide metabolites originating in the placenta, might mediate oxidative damage in the maternal circulation in preeclampsia.

Maternal oxidative stress may be responsible for the TXA_2/PGI_2 imbalance that characterizes not only the placenta, but also the systemic maternal compartment in preeclampsia [43, 46, 47]. Lipid peroxides secreted by the placenta could activate COX, while inhibiting PGI_2 synthase of endothelial cells to increase TXA_2 and decrease PGI_2 plasma levels. Oxidized fatty acids may also explain increased TXA_2 and decreased PGI_2 production by monocytes from women with preeclampsia as compared to women with

normal pregnancy [47]. Finally, ROS from other sources (i.e. activated leukocytes and enzymes) would exacerbate this TXA_2/PGI_2 imbalance by stimulating and propagating lipid peroxidation.

Antioxidants, the opponents of free radical cascades, are reduced in the maternal circulation during preeclampsia. Preeclamptic women have decreased plasma levels of vitamin C, vitamin E, vitamin A, β-carotene, glutathione, and iron-binding proteins [24]. Hubel et al. further demonstrated increased plasma vitamin C depletion rates, indicative of oxidative stress, from preeclamptic women [15]. Moreover, early prophylactic treatment with vitamins E and C decreased the incidence of preeclampsia in women at high risk [48]. This correlated with lower urinary isoprostane levels, indicating a reduction of maternal oxidative stress, and supporting the role of oxidative stress in the pathology of preeclampsia [49]. The activity of the antioxidant enzyme, SOD, also was decreased in erythrocytes and leukocytes [50]. As in the placenta, oxidative stress propagates because low levels of SOD allow excess $O_2^{\bullet-}$ to interact with AA and $NO^{\bullet}$ to form powerful oxidants. Furthermore, $O_2^{\bullet-}$ was shown to increase free iron levels, which leads to accelerated cell membrane lipid peroxidation by the Fenton reaction [51, 52]. Decreased plasma antioxidants are likely a result of widespread maternal oxidative stress and not the primary stimulus for the oxidant imbalance.

These data clearly demonstrate the involvement of oxidative stress in the pathophysiology of preeclampsia. Oxidative stress is the result of excess oxidants in the presence of decreased antioxidants. Preexisting conditions that involve oxidative stress, such as obesity, diabetes and hyperlipidemia, increase the risk of preeclampsia. Since the

balance is already tipped toward oxidative stress in the pre-partum state for these individuals, additional lipid peroxides secreted from a growing placenta can lead to a state of maternal oxidative stress by the second or third trimesters of pregnancy, resulting in the clinical symptoms of preeclampsia [24](Figure 4).

D. Maternal Cell Dysfunction in Preeclampsia

Oxidative stress of the preeclamptic placenta may be transferred to the maternal circulation by secreted lipid peroxides, ROS, cytokines, circulating leukocytes, and/or STBM particles to induce maternal cell dysfunction in many organ systems. These factors may influence one another to propagate oxidative stress and produce cellular damage leading to the clinical syndrome of preeclampsia. Next, this review will focus on the effects of oxidative stress on vascular smooth muscle cells, endothelial cells, and neutrophils and, finally, their linked role in the pathogenesis of preeclampsia.

i. Vascular Smooth Muscle

Vascular smooth muscle is critical in mediating the normal hemodynamic changes associated with pregnancy and the abnormal hemodynamics of preeclampsia [53]. Since the endothelium modulates vascular smooth muscle tension, most preeclampsia research has focused on endothelial cell physiology despite the obvious role of vascular smooth muscle in hypertension. Only recently groups have begun to investigate vascular smooth

muscle functioning during preeclampsia. Bearchell et al. treated vascular smooth muscle with preeclamptic serum and measured changes in vascular smooth muscle oxygen consumption. They found an increase in oxygen consumption, which is indicative of muscle contraction [54]. Removal of preeclamptic serum returned oxygen consumption to control levels. They hypothesized that a toxic factor might be present in preeclamptic serum that directly alters vascular smooth muscle function. Another study demonstrated that placental extracts from women with preeclampsia lead to increased oxygen consumption by porcine carotid arteries as compared to placental extracts from normotensive women. Furthermore, placental extracts from women with preeclampsia produced greater histamine-induced vasoconstriction of these vessels than extracts from normotensive women [55]. Additional experiments indicated lipid peroxides to be the active agents of preeclamptic placental extracts. Another group corroborated that preeclamptic serum altered vascular smooth muscle function. Specifically, preeclamptic serum attenuated whereas non-pregnant and normal pregnant serum augmented the intracellular calcium response of vasoactive agents [56]. Although the implications are unclear, these data demonstrate vascular smooth muscle dysfunction in the presence of preeclamptic as compared to normal pregnant serum factors. The studies completed so far have shown that vascular smooth muscle function is altered in the presence of preeclamptic serum, but they have not shown that there is specific dysfunction of vascular smooth muscle. In Chapter 3, we will show specific and direct evidence for dysfunction of vascular smooth muscle in preeclampsia.

ii. Endothelium

The endothelium is a complex organ that serves as a regulator of vascular smooth muscle tone and as a mechanical barrier to prevent blood and other circulating factors from entering the extravascular space. Therefore, it is a source of important vascular smooth muscle functional mediators, including PGI_2, endothelin (ET), and $NO^{\bullet}$. Endothelial cells also release proteins that inhibit platelet aggregation, prevent intravascular coagulation, and orchestrate inflammatory cell influx [57].

During pregnancy, endothelial cell behavior is modified to satisfy the demand for increased blood volume while maintaining lower blood pressures and reduced vasopressor sensitivity [58]. High blood pressure and increased vasopressor sensitivity during preeclampsia suggest endothelial cell involvement. The original description of "endothelial cell dysfunction" in preeclampsia combined morphological, biochemical, in vivo and in vitro evidence [59]. Since the hypothesis was first presented, research has continued using techniques, such as treating cultured endothelial cells with plasma from non-pregnant, normal pregnant, and preeclamptic women or measuring plasma markers of endothelial dysfunction.

a. Evidence for Endothelial Cell Dysfunction

i.) Histological and Cell Culture

Morphological observations of the vasculature highlight endothelial destruction and dysfunction in preeclampsia. Glomerular endotheliosis is a common pathological finding and direct evidence of endothelial cell involvement. In glomerular endotheliosis, there is swelling of glomerular capillary endothelial cells, often blocking capillary lumens, and deposition of protein between the basal lamina and endothelial cells [1]. Additional histological evidence of endothelial and vessel damage in preeclampsia is "acute atherosis" in decidual vessels, which is similar to atherosclerotic foam cell formation [58].

Initial in vitro experiments tested if preeclamptic serum was cytotoxic to human umbilical vein endothelial cells (HUVECs) using radiolabeled chromium (^{51}Cr) release [60]. Cells treated with preeclamptic serum showed increased levels of ^{51}Cr in the cell supernatant, which illustrated that cell permeability was compromised [60]. Cytotoxic effects quickly disappeared with treatment of post-delivery serum, which parallels the abatement of preeclamptic symptoms following delivery [60]. Further research, however, clarified that preeclamptic serum was not cytotoxic, but rather, caused selective activation of endothelial cells [61]. Using phase contrast microscopy and fluorescence-activated cell sorting, it was determined that membrane integrity remained intact following treatment with preeclamptic serum [61]. The conclusion that preeclamptic serum

was not cytotoxic to endothelial cells was confirmed by Endresen et al. using a broad set of cytotoxicity measurements including ^{51}Cr release, Trypan blue exclusion, plating efficiency, DNA and protein synthesis, and cell growth [62].

ii.) PGI_2 and TXA_2

Prostaglandins and thromboxanes are 20-carbon unsaturated fatty acids derived from AA upon cleavage of membrane phospholipids by phospholipase A_2 (PLA_2), followed by COX activation (Figure 3). Although they are similar in structure, their actions are varied and often opposing. All, however, are characterized by local production, short half-lives, and inhibition by anti-inflammatory agents [63].

PGI_2 is a prostaglandin produced from AA by COX and PGI_2 synthase [64]. PGI_2 is a potent vasodilator and inhibitor of platelet aggregation. The production of PGI_2 by endothelial cells exceeds all other prostaglandins, suggesting a significant physiological role [63]. Thus, PGI_2 production is an excellent gauge for endothelial cell homeostasis and a deficiency in PGI_2 could explain hypertension in preeclampsia. Accordingly, decreased PGI_2 levels are the best evidence for endothelial cell dysfunction in preeclampsia. Measurement of the stable PGI_2 metabolite, 6-keto prostaglandin $F_{1\alpha}$ (6-ketoPGF$_{1\alpha}$), in plasma and urine has demonstrated decreased PGI_2 biosynthesis in preeclamptic compared to normotensive pregnant individuals. Recently, a comprehensive longitudinal study by Chavarria et al. showed that plasma PGI_2 was significantly decreased by the third trimester in preeclamptic as compared to normally pregnant women [46]. This is

consistent with previous measurements [17, 65]. These data correlate with decreased urine PGI_2 levels in women with preeclampsia as compared to women with normal pregnancy. An initial study showed a significant increase of urine PGI_2 levels during normal pregnancy, but less of an increase in preeclamptic pregnancy [66]. Mills et al. confirmed that women with preeclampsia had significantly lower excretion of PGI_2 metabolites throughout pregnancy as compared to normal pregnant women, even prior to clinical symptoms as early as 13 to 16 weeks of gestation [67].

Initial in vitro studies with preeclamptic plasma reported conflicting results concerning endothelial PGI_2 production. Preeclamptic plasma was reported to both decrease [68, 69] and increase PGI_2 production by HUVECs [70-72]. Baker et al. clarified that in vitro PGI_2 production by HUVEC was dependent upon the length of plasma incubation. Acute administration of preeclamptic plasma increased PGI_2 (by stimulating COX), but longer incubations with preeclamptic plasma decreased PGI_2 concentrations (by inhibiting PGI_2 synthase) [73, 74]. HUVECs treated with preeclamptic plasma, as compared to those treated with normal pregnant plasma, also were shown to have increased triglyceride incorporation and reduced PGI_2 production [68]. This study suggested a link between hyperlipidemia and altered endothelial cell function in preeclampsia.

TXA_2 is an AA metabolite produced by COX and thromboxane synthase. It is a potent vasoconstrictor. Chavarria et al. showed that plasma TXA_2 was elevated as early as 9 weeks of gestation in women destined to develop preeclampsia [46]. In women that developed severe preeclampsia, TXA_2 was two times as high as those diagnosed with mild preeclampsia. Similarly, Wang et al. showed significantly elevated plasma TXA_2

levels in women with severe preeclampsia in comparison to women with mild preeclampsia [43]. Urine TXA_2 metabolites were also elevated in women with preeclampsia [67, 75, 76]. Elevation of urinary TXA_2 metabolites was observed as early as 21 weeks gestation [67] and 25 weeks gestation [76], indicating that altered TXA_2 levels precede clinical symptoms. Urinary excretion of TXA_2 metabolites decreased rapidly to normotensive levels by 24 hours post-partum [75].

The TXA_2/PGI_2 imbalance strongly suggests endothelial cell dysfunction in preeclampsia. Although many tissues produce PGI_2 and TXA_2, endothelial cells produce the greatest quantity of PGI_2, so decreased PGI_2 production in preeclampsia indicates impaired endothelial function.

iii.) Coagulation Abnormalities

The procoagulant state of preeclampsia, evidenced as disseminated intravascular coagulation, microthromboses, and uteroplacental circulation restriction may reflect endothelial cell activation. In vivo studies demonstrated decreased anticoagulant protein levels (antithrombin III, protein C, and protein S) and increased procoagulant protein expression. Elevated plasma levels of tissue factor, von Willebrand factor, platelet-activating factor (PAF), β-thromboglobulin, cellular fibronectin, and thrombomodulin strongly suggested endothelial cell dysfunction in preeclampsia [77]. Endothelial cells release ET and von Willebrand factor following endothelial cell damage and/or coagulation system activation. von Willebrand factor initiates platelet activation while

endothelin causes vasoconstriction of subjacent vascular smooth muscle [77]. ETs form a family of vasoactive peptides with the primary ET, ET-1, released from endothelial cells [78]. Clark et al. correlated ET elevation during preeclampsia with a rise in plasma uric acid [79]. Elevated plasma uric acid levels, caused by a decrease in uric acid clearance, indicated glomerular damage that reflected disease severity and altered glomerular filtration rate [79]. Fibronectin is a high-molecular-weight surface glycoprotein of endothelial cells involved in cell adhesion and migration. Fibronectin is constantly shed into the circulation but at increased concentrations during endothelial cell damage, making it a good marker for endothelial cell activation. Longitudinal studies showed significantly elevated plasma levels of fibronectin by the second [80] and third trimesters [81] in preeclamptic women as compared to normal pregnant women.

iv.) Cell Adhesion Molecules

Investigators also have measured plasma levels of soluble cell adhesion molecules to determine endothelial cell dysfunction in preeclampsia [82-87]. Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are constituently expressed on endothelial cells. These proteins are shed into the circulation upon endothelial cell damage or dysfunction. In preeclamptic plasma, Austgulen et al. found elevated concentrations of soluble ICAM-1, VCAM-1, and E-selectin [84]. This was corroborated by Krauss et al. who reported elevation of both ICAM-1 and VCAM-1 [88]. They concluded that elevated second trimester VCAM-1 and ICAM-1 levels were

predicative of developing preeclampsia [88]. Other investigators have observed increased soluble VCAM-1, but not ICAM-1 levels [85, 89, 90]. Finally, a study conducted at 18 weeks of gestation found an increase in ICAM-1, but not VCAM-1, in women destined to develop preeclampsia [83]. The levels did not correlate with endothelial cell dysfunction and instead were suggested to reflect abnormal placentation, which occurs prior to 20 weeks of pregnancy. The reason for discrepancies between some studies is not known, but overall, it appears that VCAM-1, and probably ICAM-1, levels are elevated in women with preeclampsia, which is consistent with endothelial cell dysfunction. The levels, however, apparently vary throughout pregnancy and may reflect pathophysiological processes occurring prior to endothelial dysfunction and the clinical manifestation of preeclampsia.

b. Explanations for Endothelial Cell Dysfunction

i.) Hyperlipidemia and Dyslipidemia

Dyslipidemia could mediate endothelial cell dysfunction in preeclampsia. It is well documented that both hyperlipidemia and lipid profile alter the expression of adhesion molecules and the general function of the endothelium [91-95]. Potter et al. demonstrated increases of cholesterol and triglyceride during pregnancy, which peaked in the second and third trimesters, and declined sharply with delivery [96]. Thus, normal pregnancy is characterized by hyperlipidemia. In preeclampsia, however, triglycerides are

significantly elevated above normal pregnant levels [96, 97]. This difference was present at ten weeks of gestation in women destined to develop mild and severe preeclampsia, and was not observed in pregnant women with other forms of hypertension [97].

In conjunction with hyperlipidemia, there is evidence that preeclamptic women have altered lipid profiles. Serum FFAs were increased in preeclamptic patients, and positively correlated with serum triglycerides and MDA [44]. Elevated FFAs included: palmitic (16:0), oleic (18:1 n-9), and linoleic (18:2 n-6) acids [98, 99]. These alterations were present as early as 17 weeks of pregnancy in women destined to become preeclamptic, and were also present in women with preeclampsia [98]. There was no difference in the level or the composition of esterified fatty acids in early pregnancy between normal pregnant and preeclamptic groups [98]. In later pregnancy, however, women with preeclampsia had increased oleic acid in the phospholipids fraction, and had decreased linoleic acid in the phospholipid and triglyceride factions [98]. These data confirm earlier work that characterized the preeclamptic state by decreased esterified PUFAs, including linoleic acid, linolenic acid, and eicosapentaenoic acid [100]. Decreased PUFAs, later once preeclampsia is established, may indicate maternal oxidative stress, since PUFAs are vulnerable to oxidation.

Further studies demonstrated that fatty acid composition, particularly levels of certain PUFAs, may influence one's risk of developing preeclampsia. Williams et al. showed that women with the lowest levels of omega-3 PUFAs (n-3), a PUFA rich in fish oil, had a 7.6-increased risk of developing preeclampsia [101]. A later study confirmed this, showing that women with increasing levels of n-3 PUFAs had decreased risk for

developing preeclampsia [102]. In contrast, their risk was increased with higher levels of n-6 PUFAs, especially for AA [102]. This was corroborated by studies that have demonstrated elevated AA in maternal serum phospholipids and cholesterol esters [103] and increased total plasma AA concentrations [104] in preeclamptic as compared to normal pregnant women.

These data would suggest that dietary modifications might be beneficial in preventing preeclampsia. Research substantiates this proposal demonstrating that diets of women who develop preeclampsia were characterized by increased consumption of total lipids, particularly of palmitic, stearic, oleic and linoleic acids [105]. A prospective study also showed that a greater intake of PUFAs lead to an increased risk of developing preeclampsia [106]. Since linoleic acid is a precursor to AA and its inflammatory metabolites, dietary restrictions of linoleic acid might be helpful in preventing the development of preeclampsia. In preeclampsia, accumulation of linoleic acid and AA by endothelial cells could alter eicosanoid synthesis [69] and contribute to the TXA_2/PGI_2 imbalance. In contrast, ingestion of omega-3 PUFAs would alter AA metabolism, leading to the production of thromboxane A_3, a much less potent vasoconstrictor than TXA_2, and production of PGI_3, a vasodilator as potent as PGI_2, to maintain TXA_2/PGI_2 levels that favor vasodilatation.

ii.) Neutrophils

One theory of endothelial cell dysfunction proposes that neutrophils are activated as they circulate through the intervillous space by lipid peroxides secreted by the placenta (Figure 5). As they return from the intervillous space to the maternal circulation, they produce systemic vascular cell damage by adhering to endothelial cells [14, 107]. Thus, neutrophils would transfer the oxidative stress of the placenta to the maternal compartment. Neutrophil activation causes enhanced expression of cell adhesion molecules and release of proteolytic enzymes, ROS and cytokines, which can directly damage the endothelium. Endothelial cell adhesion molecules mediate leukocyte binding in preparation for neutrophil transendothelial migration. Greer et al. first demonstrated neutrophil activation in preeclampsia by showing elevated plasma levels of neutrophil elastase [108]. Plasma levels of neutrophil elastase were correlated with plasma levels of von Willebrand factor, suggesting that neutrophil activation could be responsible for endothelial cell dysfunction [108].

iii.) ROS

The vasculature, specifically the endothelium, is both a target for and a source of many oxidant species. Oxidants, such as $ONOO^-$, lipid peroxides, oxidized LDLs, and $O_2^{\bullet -}$, directly damage endothelial cells. All of these species can act as toxic agents and could propagate placental oxidative stress to the maternal compartment.

The presence of ROS in the maternal circulation could affect endothelial vasoactive pathways resulting in endothelial cell dysfunction. An example is impairment of $NO^{\bullet}$ signaling, which is evidenced by inhibition of endothelium-dependent relaxation in coronary ring preparations in the presence of $O_2^{\bullet-}$ [109]. The role of $NO^{\bullet}$ in preeclampsia has yielded conflicting results. Studies have demonstrated unchanged, reduced or elevated circulating $NO^{\bullet}$ metabolites in preeclampsia compared to normal pregnancy [109]. During preeclampsia $NO^{\bullet}$ production may be reduced locally in endothelial cells due to the interaction of $NO^{\bullet}$ and abundant $O_2^{\bullet-}$. Not only does this reaction produce the powerful $ONOO^-$, which acts as an oxidant, it also decreases the $NO^{\bullet}$ available to mediate vasorelaxation. Therefore, increased $O_2^{\bullet-}$ in preeclampsia may not directly cause vasoconstriction, but it may prevent $NO^{\bullet}$ from completing its vasorelaxant role. This speculation was supported by immunohistochemical staining of increased $ONOO^-$ and endothelial nitric oxide synthase (NOS) staining and decreased SOD in endothelial cells of preeclamptic women as compared to normal pregnant women [110]. Additionally, Davidge et al. has demonstrated decreased $NO^{\bullet}$ production from HUVECs upon treatment with preeclamptic plasma. Isolation and purification studies suggested that oxidized LDL was the mediating factor. They speculated that perhaps $NO^{\bullet}$ is increased in preeclampsia, but in the presence of oxidative stress it is rapidly converted to $ONOO^-$ so that local endothelial cell $NO^{\bullet}$ levels are decreased [111]. Finally, $ONOO^-$ is not only a result of oxidative stress, but also a strong oxidant that may directly attack endothelial cell membranes to cause cell necrosis or apoptosis, resulting in increased cell permeability and fluid loss [109].

Treatment of HUVECs with preeclamptic plasma (containing elevated lipid peroxides, as measured by MDA) activated NF-κB and up-regulated ICAM-1 expression, which was inhibited by vitamin E [112]. These data suggested that lipid peroxides in the plasma were the mediators of endothelial cell activation. NF-κB activates the interleukin-8 (IL-8) promoter to increase IL-8 expression. Simultaneous expression of ICAM-1 and IL-8, a potent neutrophil chemokine, suggests a mechanism for neutrophil transendothelial migration.

ROS, generated by activated neutrophils, also could damage endothelial cells to cause dysfunction. For example, $O_2^{\bullet-}$ attacks AA in cell membranes to produce vasoconstrictive isoprostanes, which also could propagate lipid peroxidation within the maternal vasculature.

iv.) Cytokines

Cytokine production by the placenta or leukocytes also could activate endothelial cells as part of the pathophysiological process of preeclampsia. Cytokine levels correlated with VCAM-1 expression in the maternal circulation suggesting interaction between activated leukocytes and endothelium [113]. Cytokines, such as TNFα, IL-1β, and IL-2, affect endothelial cell prostaglandin formation and may enhance the PGI_2 and TXA_2 imbalance.

v.) STBM deportation

STBM deportation into the maternal circulation, which is increased in preeclamptic patients, also has been suggested to be the toxic agent responsible for endothelial cell dysfunction [114]. In vitro research showed that incubation of STBM and HUVECs suppressed cell proliferation and altered cell integrity [115]. Cockrell et al. confirmed STBM-induced endothelial dysfunction by demonstrating that perfusion of subcutaneous arteries with STBM vesicles inhibited endothelium-dependent vasodilation [116]. Finally, ultrastructural endothelial cell disruption of STBM-perfused arteries was validated using transmission electron microscopy.

These studies suggest that oxidative stress is spread within the maternal compartment by placental-derived toxic agents, which produce direct damage of the endothelium while interacting with maternal vascular constituents to generate additional oxidants and to promote further destruction. The condition of the maternal circulatory system prior to pregnancy (e.g., diabetes, hyperlipidemia, obesity, etc.) could exacerbate the propagation of oxidative stress during pregnancy. In preeclamptic pregnancy, maternal oxidative damage leads to a dysfunctional endothelium, unable to regulate smooth muscle tone, resulting in hypertension.

iii. Neutrophils

The primary role of neutrophils or polymorphonuclear leukocytes is the killing and phagocytosis of pathogens for host defense [117]. Neutrophils release destructive agents, such as ROS and proteolytic enzymes, to fulfill this function [117]. Cytokines, including TNFα and IL-6, modulate or "prime" neutrophils for enhanced activation, but do not themselves activate neutrophils [118, 119]. Neutrophils have plasma membrane receptors that interact with specific chemoattractants to initiate activation events, including: chemotaxis, respiratory burst, phagocytosis, degranulation, and transendothelial migration [119]. These end results are mediated by various cell signaling pathways, which are activated by certain stimuli [119]. Recently, neutrophils have been implicated in many diseases because unchecked neutrophil activity causes damaging tissue degradation and can lead to inflammatory conditions [120].

A great deal of evidence suggests that uncontrolled neutrophil activation may play a significant role in the pathophysiology of preeclampsia. Neutrophils are likely mediators in preeclampsia for several reasons: 1) they are the most abundant of the leukocytes, 2) their numbers increase in pregnancy [1], 3) their numbers further increase in preeclampsia [121], and 4) they produce toxic substances (ROS, myeloperoxidase (MPO), TXA_2, and TNFα), which could cause endothelial and vascular smooth muscle cell dysfunction and lead to vasoconstriction and proteinuria.

a. Evidence for Neutrophil Dysfunction

i.) Neutrophil Elastase

Greer et al. first demonstrated neutrophil activation in preeclampsia by measuring significantly elevated plasma levels of the neutrophil protease, elastase, in preeclamptic patients [122]. Elastase is a potent enzyme released as a host-defense mechanism upon neutrophil activation [123]. It is stored pre-packaged in the azurophil granules and is released to degrade bacteria and connective tissue during inflammation [123]. The presence of neutrophil specific elastase in plasma indicated in vivo lysosomal granule release and thus, neutrophil activation. It also suggested a potential mechanism for endothelial cell dysfunction in preeclampsia mediated by neutrophil release of ROS, cytokines, and proteases. Further investigation compared maternal and umbilical vein plasma neutrophil elastase levels and found neutrophil activation was confined to the maternal compartment [124]. Immunocytochemical evidence of increased elastase production and neutrophil infiltration in the decidua, but not in the placenta or subchorionic plate, suggested that the mechanism of neutrophil activation may occur at the maternal-fetal interface [125]. Recently, plasma levels of the proteolytic MPO enzyme, also released by activated neutrophils, were found to be elevated during preeclampsia [126].

ii.) Neutrophil ROS

The respiratory burst is the primary result of neutrophil activation. It is also a useful tool to measure neutrophil activity. Tsukimori et al. measured neutrophil $O_2^{\bullet-}$ production using a SOD-inhibited reduction of ferricytochrome C with a spectrophotometer to first demonstrate that neutrophils from preeclamptic patients had greater spontaneous $O_2^{\bullet-}$ production than those from normal pregnant and non-pregnant patients [127]. Furthermore, neutrophils, primed with N-formyl-methionyl-leucyl-phenylalanine residues (fMLP), and incubated with sera from preeclamptic patients had twice the neutrophil $O_2^{\bullet-}$ production than sera from normal pregnant patients. There was no significant difference between non-pregnant and normal pregnant groups. Since preeclamptic serum enhanced neutrophil $O_2^{\bullet-}$ release, it was suggested that a humoral factor activates neutrophils.

Sacks et al. used whole blood flow cytometry to show that basal intracellular ROS values of granulocytes, monocytes, and lymphocytes were significantly greater for preeclamptic as compared to normal pregnant and non-pregnant women [128]. The oxidative burst showed an increasing trend from non-pregnant, to normal pregnant, to preeclamptic patients. Thus, the evidence clearly supports that neutrophil intracellular ROS and respiratory burst are altered in preeclampsia.

iii.) Neutrophil Adhesion Molecules

Measurements of neutrophil adhesion molecules further confirm neutrophil activation in preeclampsia. Expression of the neutrophil integrins, CD11b and CD64, were increased in both normal pregnant and preeclamptic patients [128]. In addition, there was a decrease in L-selectin expression on neutrophils from preeclamptic women [128]. Quantitative flow cytometry confirmed that there was increased CD11b and decreased L-selectin expression on neutrophils from preeclamptic patients [82, 129, 130]. Furthermore, the enhanced CD11b expression positively correlated with plasma uric acid levels in women with preeclampsia, suggesting that neutrophil activation reflected disease severity [82]. An animal study also demonstrated neutrophil activation by measuring adhesion molecules in an experimental rat model of preeclampsia induced by low-dose endotoxin [131]. While neutrophils from healthy pregnant rats exhibited an up-regulation of CD11a and CD49d, there was even greater CD11b and CD49d expression and a decrease of L-selectin on neutrophils from the endotoxin-induced preeclamptic rats [131]. One study, however, did not find an alteration in the expression of cell adhesion molecules (CD11, CD18, and CD26) on neutrophils isolated from non-pregnant, pregnant, and preeclamptic women [132].

iv.) Neutrophil Lactoferrin

Neutrophil lactoferrin release and locomotion have also been analyzed. Crocker et al. observed no significant difference in lactoferrin production among neutrophils

obtained from non-pregnant, pregnant, and preeclamptic women [132]. Preeclamptic plasma or serum also did not alter neutrophil locomotion [133]. These data suggested that specific rather than general neutrophil activation occurs in preeclampsia.

b. Causes for Neutrophil Dysfunction

These studies support the idea that a certain stimulus, most likely a plasma component, activates specific neutrophil pathways in preeclampsia. Research groups have investigated a variety of potential neutrophil activators including: fatty acids, cytokines, complement molecules, and STBM particles [134-138]. One hypothesized mechanism of neutrophil activation proposes that neutrophils become "activated" from placental-derived lipid peroxides and TNFα in the intervillous space (Figure 5) [107].

i.) Lipids

The altered lipid profile of preeclampsia may lead to neutrophil activation in both the intervillous space and the maternal circulation. Major fatty acid concentrations are normally modified in the pregnant state and further altered in preeclampsia. Specifically, both palmitic and linoleic acids are elevated in women with preeclampsia [98]. Crocker et al. demonstrated that PUFA incorporation lead to altered neutrophil NADPH oxidase function, producing enhanced respiratory bursts [134]. PUFAs also may directly alter NADPH oxidase activation. Gorog et al. showed direct and differential activation of the

respiratory $O_2^{\bullet-}$ burst by oxidized PUFAs [139]. This mechanism could account for excessive $O_2^{\bullet-}$ production in preeclampsia, since elevated plasma FFAs and free radicals would produce oxidized PUFAs.

ii.) Cytokines

Cytokines, from various sources, may be involved in neutrophil activation during preeclampsia. TNFα, TNFα receptor, interleukin-6 (IL-6), and interleukin-12 (IL-12) are all increased in preeclamptic plasma [135-137]. The placenta, activated endothelial cells, or activated neutrophils could secrete these cytokines, and by exerting paracrine or autocrine effects, induce genomic changes to alter cell function. TNFα and IL-6 are known to prime neutrophils, which enhances subsequent activation, perhaps by a preeclampsia-specific agent, such as lipid peroxides [118, 119].

iii.) Complement

Neutrophil activation in preeclampsia also could occur by the complement system. The terminal complement complex, C5a, is increased in the severe preeclamptic state and could activate neutrophils [140]. Furthermore, C5a and neutrophil elastase levels positively correlated in plasma from preeclamptic patients, while in vitro work showed a dose-dependent release of neutrophil elastase upon stimulation with recombinant C5a [140]. Another study, however, did not find evidence that the complement system was activated

in mild preeclampsia [126]. These investigators reported that C4b and levels of other complement mediators in preeclamptic women were comparable to those of non-pregnant women.

iv.) STBM particles

More STBM particles are shed from the placenta in preeclampsia than normal pregnancy [114]. These particles could potentially link placental and endothelial cell dysfunction in preeclampsia. Endothelial cell activation by STBM also could lead to the release of factors that activate neutrophils [115, 141]. The involvement of STBM particles in neutrophil activation was analyzed by changes of intracellular calcium, pH, and ROS in the presence of supernatants from cultured HUVECs previously exposed to STBM [138]. The supernatants increased neutrophil intracellular calcium and ROS concentrations, and decreased intracellular pH, demonstrating a mechanism for neutrophil activation in preeclampsia [138].

The mechanism for neutrophil activation during preeclampsia is not clear. There is, however, a growing understanding of the complex inter-relationship among lipids, cytokines, complement, STBM particles, and neutrophil activation. Since the exact relationship of neutrophil activation and endothelial cell dysfunction remains elusive, it must be determined if neutrophils are the primary stimulus for endothelial activation or if they are activated secondarily to endothelial cell damage. In Chapter 5, we present

evidence for the former by demonstrating massive neutrophil adherence to endothelial cells and infiltration into systemic vasculature in women with preeclampsia.

iv. Vascular Smooth Muscle, Endothelial, and Neutrophil Interaction

The preceding review demonstrates that the vasculature is a dynamic environment. Vascular smooth muscle cells, endothelial cells, and neutrophils interact and respond to various physiological and pathophysiological conditions to maintain homeostasis. Coordinated and appropriate behavior is achieved through cellular communication by mediators, such as cell surface receptors, adhesion molecules, cytokines, chemokines, and ROS. Interaction among dysfunctional vascular smooth muscle cells, endothelial cells, and neutrophils in preeclampsia may lead to uncontrolled neutrophil transendothelial migration, which could explain the clinical symptoms of preeclampsia.

Transendothelial migration or diapedesis is a multi-step process coordinated by expression of chemotactic agents and various adhesion proteins on neutrophils and endothelial cells (Figure 6). The mechanism of neutrophil transendothelial migration consists of margination (rolling and capture), firm adhesion, and extravasation. Each step is controlled to direct specific aspects of neutrophil transendothelial migration [142].

Margination occurs under normal physiological circumstances. Neutrophils roll along the vessel wall and transiently adhere to the endothelium via constitutively expressed leukocyte selectin (L-selectin). Selectins are transmembrane glycoprotein

adhesive molecules that consist of a calcium-dependent lectin domain on the extracellular NH_2-terminus that is joined to an epidermal growth factor-like domain. Short consensus sequences bind extracellular domains to a short intracellular domain, which is coupled to signaling molecules. The L-selectin ligand is CD34, a long protein chain molecule with O-linked sugar and sialyl groups [143].

Inflammatory stimuli such as complement, ROS, and certain cytokines increase platelet selectins (P-selectin) and endothelial selectins (E-selectin) of endothelium to facilitate irreversible neutrophil binding. P-selectin is stored in intracellular Weibel-Palade bodies of endothelial cells. Appropriate inflammatory stimuli induce mobilization of P-selectin to the cell surface. P-selectin interacts with P-selectin glycoprotein ligand-1 (PSG-1), which is constitutively expressed on neutrophils, to slow rolling and establish firm neutrophil-endothelial attachments. PSG-1 is a protein ligand molecule, similar to CD34, modified by O-linked sugar and sialyl groups. It is a homodimer, however, and can simultaneously bind two P-selectin ligands. E-selectin expression, unlike P-selectin, requires gene transcription and translation following endothelial cell stimulation. The time necessary for E-selectin mobilization may coordinate with the down-regulation of P-selectin to maintain neutrophil rolling and adhesion. In vivo and in vitro evidence demonstrate that E-selectin binds protein ligands, similar to those for P- and L-selectin, with sugar and sialyl group modifications [143].

Complementary surface receptors on neutrophils (L-selectin) and endothelium (P- and E-selectins) mediate neutrophil rolling and capture. Neutrophils begin to adhere firmly to the endothelium upon integrin and cell adhesion molecule expression. Integrin

up-regulation occurs in a timely, sequential manner by inflammatory cytokines or by signaling of activated selectins. Since L-selectin has cytoplasmic domains linked to signal transduction machinery, it is a likely candidate for the activation of neutrophil integrins [143]

Integrins are a group of heterodimeric transmembrane glycoproteins involved in neutrophil cell-cell and cell-extracellular matrix adhesion. Each integrin contains an extracellular α- and β-subunit for ligand binding and a cytoplasmic domain with phosphorylation sites and signal transduction proteins. Macrophage antigen-1 (Mac-1; $\alpha_M\beta_2$; CD11b/CD18) is an important neutrophil integrin in transendothelial migration. Neutrophils package Mac-1 in secretory vesicles and granules for rapid mobilization. Potent Mac-1 stimulators, including the bacterial fMLP peptide, cause degranulation. Lipopolysaccharide (LPS) and TNF-α, as weaker stimuli, mobilize Mac-1 secretory vesicles for cell surface expression [143].

ICAM-1 on endothelial cells interacts with the neutrophil Mac-1 integrin to mediate firm binding. ICAM-1 is an immunoglobulin-like molecule that is constitutively expressed on endothelium. It is greatly up-regulated in the presence of inflammatory cytokines or oxidative stress [144]. VCAM-1 is similar to ICAM-1, but functions in the transmigration of monocytes and eosinophils by binding to β-1 integrins. Neutrophil transendothelial migration may use both ICAM-1 and VCAM-1 [143].

Following tight adhesion of neutrophils to the endothelium, neutrophils migrate through intercellular junctions and move toward a chemotactic stimulus within the interstitial tissue [142]. The exact extravasation mechanism, unlike the previously described

steps of transendothelial migration, is not clear. It is understood, however, that platelet-endothelial cell adhesion molecule (PECAM-1) of endothelium is an important homing receptor, strategically positioned at intercellular junctions where neutrophil transendothelial migration occurs. PECAM-1 has an immunoglobulin domain that can form homodimers with PECAMs on other cells, such as neutrophils [143].

Soluble exogenous or endogenous mediators regulate the process of vascular cell activation and neutrophil transendothelial migration. These mediators can be further classified as non-chemoattractant or chemoattractant. Non-chemoattractant cytokines, such as TNF-α and IL-1, increase expression of adhesive proteins, but are not chemoattractant for neutrophils. These inflammatory cytokines up-regulate adhesion molecules on neutrophils and endothelial cells in preparation of transendothelial migration. Directed transendothelial migration, however, requires neutrophil chemoattractants. Chemokines are chemotactic cytokines that mediate inflammation [145]. They form a group of approximately forty proteins that share a common four cysteine residue structure, which is used to classify chemokines and produces varying leukocyte chemoattractant responses [143].

Neutrophils have receptors for chemoattractant agents: IL-8, platelet activating factor (PAF), C5a complement protein, leukotriene B_4 (LTB_4), and fMLP. A variety of cells produce these neutrophil chemoattractants. fMLP is an exogenous mediator, while LTB_4 and PAF are important endogenous mediators. Monocytes and neutrophils produce LTB_4 from AA. LTB_4 is 10 to 1000 times more potent than PAF in causing neutrophil chemotaxis [143].

The chemokine, IL-8, is a primary stimulus for neutrophil transendothelial migration during inflammation. IL-8 is a C-X-C or α-chemokine [142]. The C-X-C reflects separation of conserved cysteine residues by an amino acid. Neutrophils contain two C-X-C receptors (CXCR1 & CXCR2), which both couple to IL-8. Other C-X-C chemokines, however, only bind CXCR2 [143]. This may explain the potent and specific neutrophil chemoattraction to IL-8. Leukocytes, endothelial cells, and smooth muscle cells produce IL-8 under conditions of oxidative stress and upon stimulation with IL-1 or TNFα [142].

Investigators only recently began to look for neutrophil transendothelial migration into systemic tissues during preeclampsia. Initially, immunohistochemical staining of elastase and neutrophils demonstrated the presence of neutrophils in the decidua and spiral arteries of preeclamptic patients [125]. In systemic maternal tissue, Reister et al. illustrated neutrophil infiltration and described elevated IL-8 concentrations in the characteristic glomerulonephritis lesion of preeclampsia [146]. There was also histological evidence of neutrophil infiltration into liver tissue of women with hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome and eclampsia [147]. Finally, an animal model of endotoxin-induced preeclampsia had evidence of neutrophil transendothelial migration, showing infiltration of the liver and kidneys [131, 148, 149].

E. Summary

Preeclampsia is a multi-system disorder characterized by maternal vascular cell dysfunction. It is thought that oxidative stress, originating in the placental compartment, produces the various pathophysiological features of preeclampsia. Specifically, placental ischemia-reperfusion generates localized oxidative stress, which is then propagated within the placenta. As the placenta grows, maternal oxidative stress gradually increases as more placental-derived oxidant agents, including lipid peroxides, ROS, activated neutrophils, cytokines and STBM particles, enter the maternal circulation, linking the primary placental pathology to the maternal compartment. These oxidant agents interact with vascular smooth muscle cells, endothelial cells and neutrophils to overwhelm maternal antioxidant mechanisms and exacerbate oxidative stress. It is a vicious, self-propagating cycle of cell-cell and oxidant-cell activation that culminates in the clinical symptoms of preeclampsia. This complex interplay of placental-derived oxidant agents, maternal vascular cells, and pre-existing maternal circulatory health determines the clinical manifestation and progression of preeclampsia. Only when the primary stimulus, the placenta, is removed will the pathological process of preeclampsia cease.

F. Purpose of Investigation

This investigation examined one of the possibilities for vascular cell dysfunction in preeclampsia: Activation and transendothelial migration of neutrophils into systemic

vascular tissue. We used in vitro studies to explore mechanisms related to oxidative stress and hyperlipidemia that would create an environment conducive for transendothelial migration of neutrophils. We used subcutaneous fat biopsy samples to determine if transendothelial migration of neutrophils actually occurs in women with preeclampsia.

We first developed a methodology for isolating and culturing human vascular smooth muscle cells for in vitro studies (Chapter 2). Second, we used these cells to investigate whether oxidative stress in the presence of linoleic acid stimulates vascular smooth muscle cell production of IL-8 in culture (Chapter 3). We used linoleic acid because it is elevated in the diet and plasma of women with preeclampsia, and because it is the precursor for AA and its metabolites that mediate inflammation. Third, we evaluated the ability of oxidative stress in the presence of linoleic acid to activate neutrophils by measuring superoxide production in real-time (Chapter 4). Last, we used fat biopsy samples to examine whether there is vascular smooth muscle expression of IL-8, endothelial expression of ICAM-1, and neutrophil infiltration into systemic vascular tissue in women with preeclampsia (Chapter 5).

These studies could provide evidence that dysfunction of vascular smooth muscle, endothelium and neutrophils are all linked to the pathogenesis of preeclampsia by neutrophil transendothelial migration into systemic vascular tissue in response to vascular smooth muscle production of IL-8.

G. Hypotheses

1) Treatment of vascular smooth muscle cells with an oxidizing solution enriched with linoleic acid will lead to increased production of IL-8, a potent neutrophil chemokine.

2) Treatment of neutrophils with an oxidizing solution enriched with linoleic acid will stimulate neutrophils to produce superoxide.

3) In the systemic vascular tissue of women with preeclampsia, there will be: a) increased expression of IL-8 by vascular smooth muscle, b) increased expression of ICAM-1 by endothelial cells, and c) infiltration of neutrophils.

H. Significance of this Research

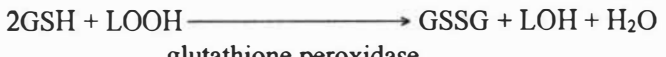
If our overall hypothesis is correct that neutrophils infiltrate into systemic vascular tissue in women with preeclampsia, this could explain vascular cell dysfunction and the clinical symptoms of preeclampsia. Such findings would suggest the use of antioxidants to reduce oxidative stress and dietary modifications, such as restriction of linoleic acid, to decrease neutrophil activation and vascular IL-8 production. These modifications could reduce vascular inflammation and inhibit neutrophil transendothelial migration to prevent preeclampsia. Furthermore, these studies would suggest a role for

neutralizing antibodies directed at IL-8 or cell adhesion molecules as novel treatments for preeclampsia.

Table 1. Reactive oxygen species and reactive nitrogen species.

Name	Symbol	Comments: Formation/Examples
Alkoxyl radical	$LO^{\bullet}$	Formed by interaction of superoxide and a lipid peroxide; capable of initiating lipid peroxidation $O_2^{\bullet -} + LOOH = LO^{\bullet} + OH^{\bullet} + O_2$
Hydrogen atom	$H^{\bullet}$	The simplest free radical
Hydrogen peroxide	H_2O_2	Formed by the dismutation of superoxide
Hydroxyl radical	$HO^{\bullet}$	Formed by metal-catalyzed decomposition of hydrogen peroxide; highly reactive; attacks all biological molecules
Lipid peroxide	LOOH	Formed by a peroxyl radical removing a hydrogen from an adjacent lipid $LOO^{\bullet} + LH = LOOH + L^{\bullet}$
Lipid radical	$L^{\bullet}$	Formed by the removal of a hydrogen (common agents: $ONOO^-$, $OH^{\bullet}$, $LOO^{\bullet}$), occurring preferentially at carbon-carbon double bonds of unsaturated fatty acids $LH + O_2^{\bullet -} = L^{\bullet}$
Nitric oxide	$NO^{\bullet}$	Formed in vivo by conversion of L-arginine into L-citrulline by nitric oxide synthase (NOS)
Peroxynitrite anion	$ONOO^-$	Formed by interaction of nitric oxide and superoxide; capable of initiating lipid peroxidation
Peroxyl radical	$LOO^{\bullet}$	Formed by the interaction of a lipid radical and molecular oxygen during lipid peroxide propagation or by the transition metal-catalyzed decomposition of lipid peroxide $L^{\bullet} + O_2 = LOO^{\bullet}$
Protein radical		Formed by removal of a hydrogen from a protein
Superoxide	$O_2^{\bullet -}$	Formed by enzymatic or non-enzymatic one electron reduction of oxygen $O_2 + e = O_2^{\bullet -}$
Transition metals	Fe, Cu	Powerful catalysts of free radical reactions because they can change their oxidation numbers

Table 2. Antioxidant enzymes and their actions.

Antioxidant Enzyme	Action
Superoxide Dismutase (SOD)	Reduces superoxide radical to hydrogen peroxide and molecular oxygen 
Catalase	Converts hydrogen peroxide to molecular oxygen and water 
Glutathione peroxidase	Uses glutathione (GSH) as a hydrogen donor to convert glutathione into glutathione disulfide (GSSG) to convert hydrogen peroxide to water and lipid peroxide to water and a fatty acid alcohol  

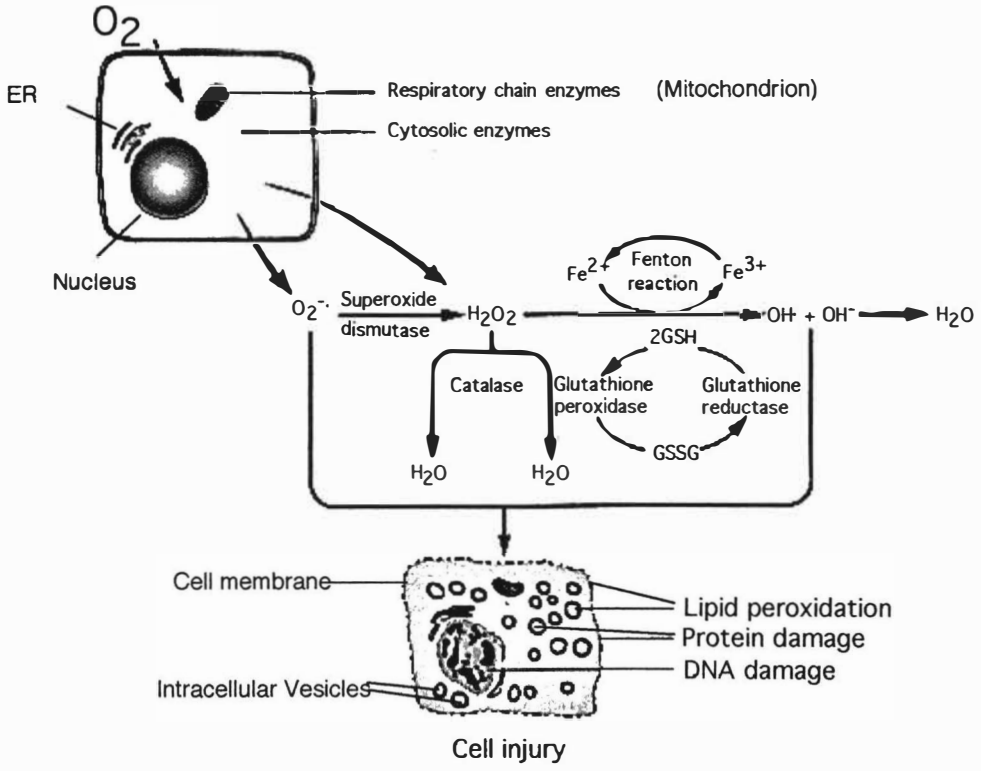


Figure 1. Cellular enzymatic antioxidant mechanisms.

The body has three primary enzymes to neutralize ROS, generated during oxidative metabolism, so to prevent cell injury. SOD dismutates $O_2^{\bullet -}$ to H_2O_2, which is then metabolized by catalase or glutathione peroxidase to H_2O. (This figure was adapted from Cellular Pathology I: Cell Injury and Cell Death. In: Cotran RS, Kumar V, Collins T, eds. Robbins Pathologic Basis of Disease. Philadelphia: WB Saunders Company, 1999, p. 13.)

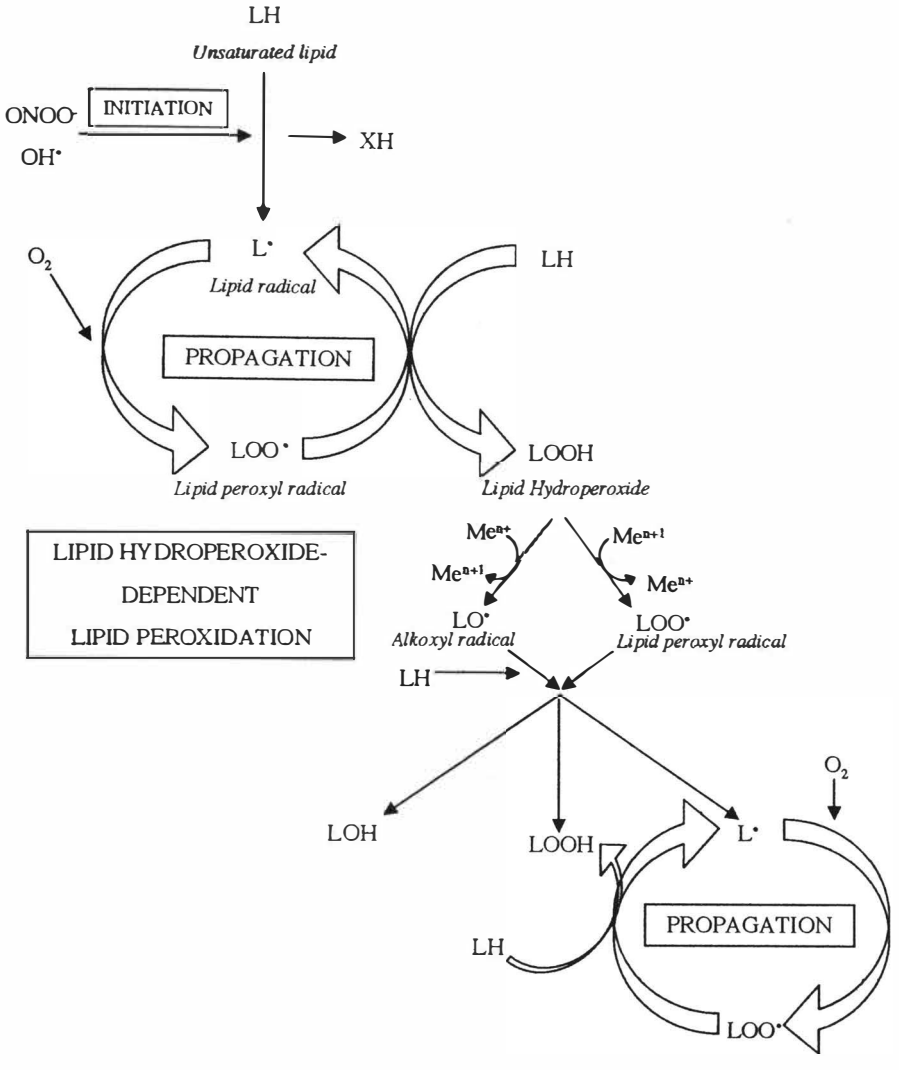


Figure 2. Free radical mechanism of lipid peroxidation.

Initiation of lipid peroxidation occurs when a free radical attacks a hydrogen atom of an unsaturated fatty acid to form a lipid radical. Propagation of lipid peroxidation occurs when that lipid radical reacts with O_2 to form a highly unstable lipid peroxyl radical, which attacks a nearby unsaturated fatty acid to form a lipid hydroperoxide and a new lipid radical, so that the cycle is repeated. Propagation of lipid peroxidation also occurs in the presence of transition metals. Definitions: XH = new unsaturated lipid; Me = metal, with varying valence electrons; LOH = lipid alcohol; all other definitions, see Table 1.

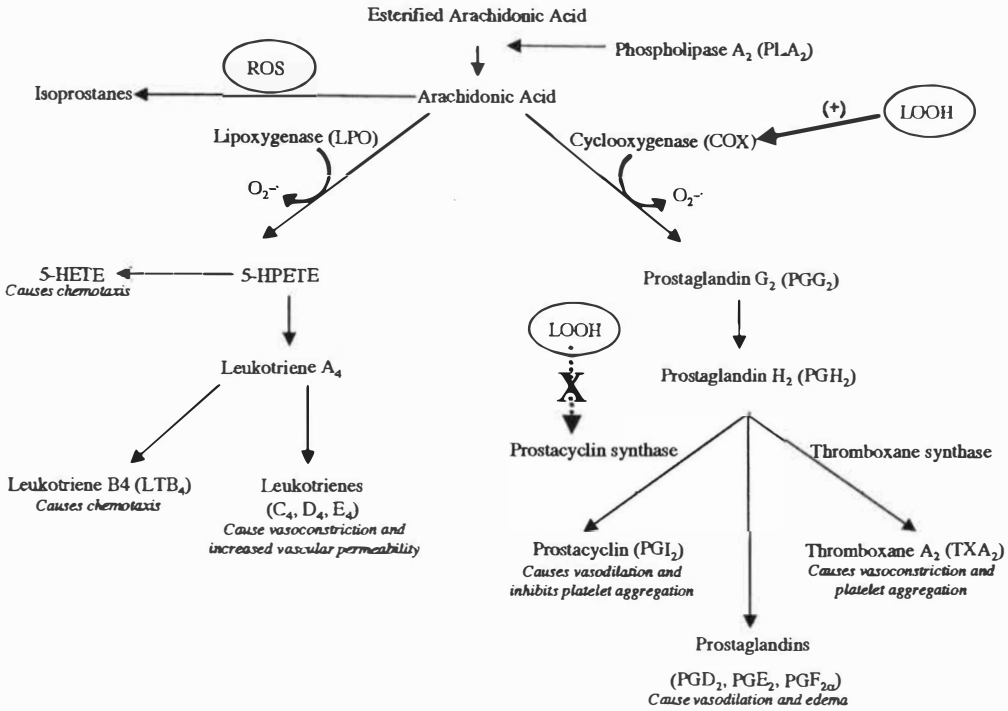


Figure 3. Stimulation and inhibition of arachidonic acid pathways by lipid peroxides and reactive oxygen species.

Lipid peroxides affect AA metabolism by stimulating COX and by inhibiting prostacyclin synthase leading to an increase of thromboxane metabolites and a decrease of prostacyclin metabolites, respectively. ROS react with AA to produce isoprostanes, which cause vasoconstriction.

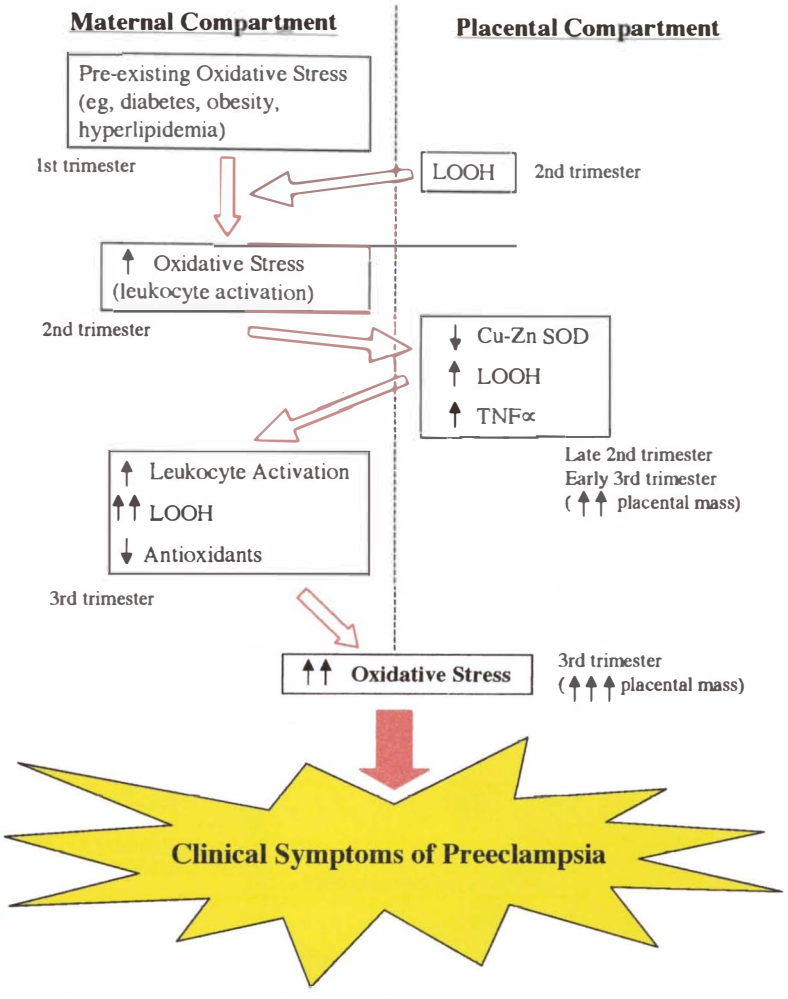


Figure 4. Hypothesized cascade in the development of clinical symptoms of preeclampsia.

Pregnancy is characterized by a gradual increase in oxidative stress due to secreted lipid peroxides by the placenta and activated leukocytes. In normal pregnancy, there is a parallel increase in antioxidants. In preeclampsia, there are increased levels of lipid peroxides, in addition to lower levels of antioxidants. These patients also tend to have pre-existing oxidative stress. Clinical symptoms of preeclampsia occur when oxidative stress overwhelms the maternal compartment. (Adapted from Walsh, S.W. Maternal-Placental Interactions of Oxidative Stress and Antioxidants in Preeclampsia. Seminars in Reproductive Endocrinology, 1998; 16:93-104.)

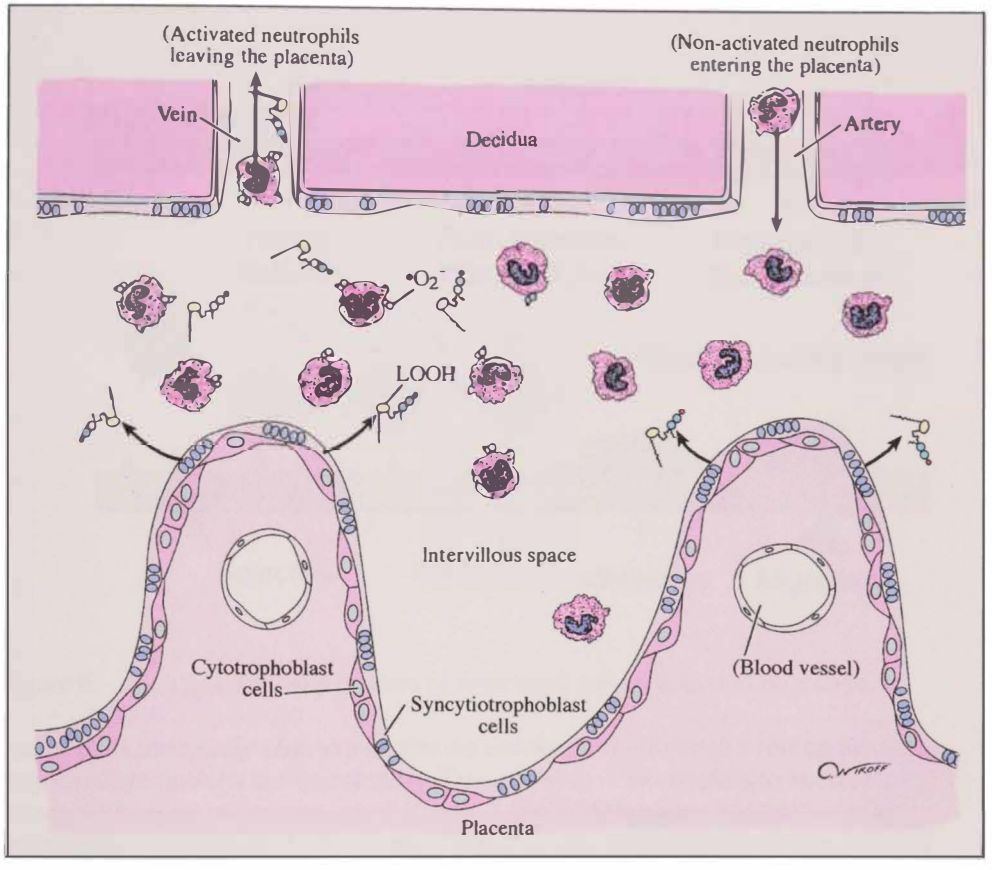


Figure 5. Theory of neutrophil activation in the intervillous space in preeclampsia.

Neutrophils are activated as they circulate through the intervillous space by lipid peroxides secreted by the placenta. Neutrophils return to the maternal compartment to produce vascular damage by producing superoxide and by adhering to endothelial cells. (Used with permission of artist. From Walsh, S.W. The role of oxidative stress and antioxidants in preeclampsia. Contemporary OB/GYN, 1997; 42:113-124.)

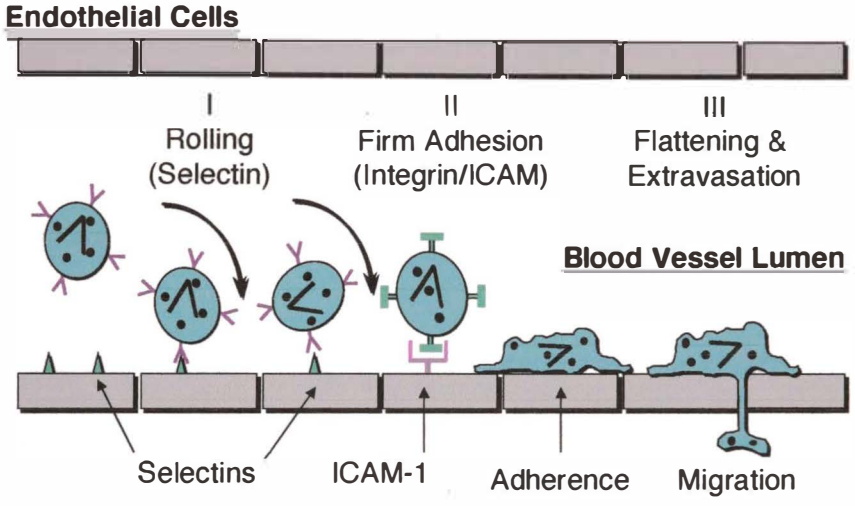


Figure 6. The multi-step process of neutrophil transendothelial migration.

Neutrophils transiently bind to selectins on endothelium, to create a rolling motion, as they circulate through the vasculature. Firm adhesion of neutrophils to endothelial cells occurs with increased expression of ICAM-1 and integrins upon stimulation with inflammatory mediators. A chemotactic signal causes neutrophils to flatten onto the endothelium and extravasate into systemic tissue.

Chapter 2

SIMPLE AND ECONOMICAL METHOD FOR ISOLATION AND CULTURE OF ARTERIAL SMOOTH MUSCLE CELLS FROM CHORIONIC PLATE ARTERIES

A. Introduction

Vascular smooth muscle cells regulate the diameter of blood vessels by modulating their contraction in response to neural, hormonal and chemical signals. Thus, vascular smooth muscle plays an important role in maintaining cardiovascular function. Hemodynamic regulation is especially critical during pregnancy when cardiac output increases while total peripheral resistance decreases. In preeclampsia, vascular smooth muscle function may be altered to cause hypertension.

To explore mechanisms for vascular smooth muscle dysfunction in preeclampsia, we considered studies with primary cultures of vascular smooth muscle. Primary cultures of adult vascular smooth muscle cells, however, can be difficult to obtain and commercially available cells are expensive. The age of the donor is often advanced and health problems of the donor may have altered the phenotype of the cells. There is always

a question as to whether donor cells are truly normal. A readily available source of normal vascular smooth muscle cells would be useful.

The purpose of this study was to develop a simple and economical method to obtain normal vascular smooth muscle cells for in vitro studies. This has been done for endothelial cells, which are commonly used for cardiovascular studies, by isolating endothelial cells from human umbilical veins. We applied a similar rationale and developed a simple and economical technique, based on the work of Graham et al. for establishing primary cultures of intestinal smooth muscle cells [150], to isolate and culture arterial smooth muscle cells from placental chorionic plate arteries.

B. Materials and Methods

i. Placental Arterial Smooth Muscle (PASM) Cell Isolation and Culture

Placentas were collected at the time of term delivery from normal pregnant women at MCV Hospitals, Virginia Commonwealth University Health System. Informed consent was obtained prior to delivery. This study was approved by the Virginia Commonwealth University Office of Research Subjects Protection.

In a sterile environment, the chorionic plate arteries were identified and excised from the chorionic plate. The vessels were rinsed three times in a solution of Hank's Balanced Salt Solution (HBSS) containing 2x strength antibiotic/antimycotic (100 U/mL of penicillin, 100 μg/mL streptomycin, 25 μg/mL amphotericin B, Life Technologies,

Long Island, NY). Next the vessels were placed in a sterile culture plate and cut longitudinally to expose the lumen. Vessels were dissected into small pieces of tissue (approximately 3-5 mm) and placed in a separate 100 mm culture plate with the lumen facing down. Approximately 20-25 explants were placed in each culture plate. Dulbecco's Modified Essential Media (DMEM, Life Technologies, Long Island, NY) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Long Island, NY) (5 mL) was carefully added to the culture plate so as not to disturb adhered explants. Culture plates were placed in a 37°C incubator (5% CO_2).

Media were removed and replaced with fresh media twice a week. At this time, culture growth and cell morphology were examined using an inverted light microscope. Cells were observed growing from the explant within one week. At approximately two weeks, the volume of media per 100 mm culture plate was increased to 10 mL.

Cells reached confluence after approximately 4 weeks. At confluence cells were placed in DMEM without serum for 24 hours to kill any contaminating cells, such as fibroblasts and endothelial cells, since these cells, in contrast to smooth muscle cells, do not survive without serum. After 24 hours, PASM cells were placed in DMEM with 10% FBS for trypsinization, passage or freezing according to standard protocols. PASM cells were used between passages two and seven.

ii. Verification of Vascular Smooth Muscle Cell Phenotype

Vascular smooth muscle cell phenotype for all PASM cultures (n = 5) was verified by immunoblotting for smooth muscle myofilament proteins: α-smooth muscle actin, β-tropomyosin, h-caldesmon, and vinculin. Both α-smooth muscle actin and h-caldesmon are specific only to smooth muscle. The antibody for vinculin also cross-reacted with metavinculin (Sigma, St. Louis, MO). PASM cell phenotype was compared to adult human aortic smooth muscle (HASM) cells, human intestinal smooth muscle (HISM) cells, and HUVECs. HASM cells were kindly supplied by Dr. Gary Bowlin, Department of Biomedical Engineering, Virginia Commonwealth University, Richmond, VA.

a. Cell Harvesting for Western Blot

Cells were seeded in 100 mm culture plates with 10 mL media (DMEM, 10% FBS) and allowed to grow to confluence. Cells were harvested six days after the last addition of fresh media. At confluence, the media were removed and exchanged with DMEM without serum for 24 hours. The following day cells were harvested with a cell scraper on ice in a sterile environment. The media were vacuumed off and the culture plates were rinsed twice with phosphate buffered saline (PBS), pH=7.4 (Life Technologies, Grand Island, NY). The plates were briefly scraped with a cell scraper to enhance cell lysing. Then 250 μL of fresh Laemmli's lysing buffer was added to the

culture plate. The plate was scraped again and the lysate was transferred to an Eppendorf tube with a 21-gauge needle. The cells were sheared a couple of times with the 21-gauge needle. The sample was then placed in a -70°C freezer until use.

b. Gel Electrophoresis and Western Blot

In the afternoon, a 9% polyacrylamide gel was prepared. Once it was determined that there were no leaks, the gels were covered with running buffer and plastic wrap overnight. In addition, running and blotting buffers were prepared and placed in the refrigerator until use. The next morning a 4% polyacrylamide stacking gel was prepared. While the stacking gel was polymerizing, the PASM lysate samples were thawed and protein concentration was determined by measuring light absorbance at 280 nm. The protein samples were balanced to ensure loading of equal protein concentrations to each lane. Sample volume was equalized with buffer containing blue dye to visualize sample migration in the gel (0.5 M Tris-HCl, 0.1% glycerol, 10% SDS, 0.05% 2-methanol, and a few grains of bromophenol blue powder) at a 1:4 ratio, respectively. A molecular weight marker (High Range Rainbow Molecular Weight Marker, Amersham Pharmacia, Piscataway, NJ) was prepared (8 – 10 μL of protein marker plus sample buffer equal to sample volumes). All samples and the protein marker were then boiled for five minutes. The samples were spun for 30 seconds, to spin down condensation due to boiling, and vortexed. The protein marker (20 μL) was added to the left most lane and then protein

samples (20 μL) were added from left to right. The gels were run at 165 volts for approximately two hours or until the blue dye front migrated to the end of the gel.

To confirm equal loading of the samples, one gel was then fixed (50 mL methanol, 10 mL acetic acid, 30 mL ddH_2O, 10 mL fixative agent) and stained with silver stain (Silver Stain Plus Kit, BioRad, Hercules, CA). The gel was watched closely while developing to prevent overdevelopment. Development was stopped by placing the gel in a 10% acetic acid solution.

The remaining gels were placed in electroblotting cassettes to transfer proteins from the gels to nitrocellulose membranes. Each gel was placed on top of a piece of filter paper, which lay on a fiber pad (Trans-Blot Fiber Pads, BioRad, Hercules, CA) that was on top of the cassette. Nitrocellulose paper was placed on top of the gel. A piece of filter paper and a fiber pad were placed on top of the nitrocellulose paper. The cassette was carefully closed to prevent entrapment of air bubbles and locked. Gel electrophoresis was performed in a cold room for 2.5 hours at 65 volts. At completion, nitrocellulose membranes were removed from cassettes and the visible protein marker was marked with a glow-in-the-dark crayon. Membranes were blocked with a 5% solution of dry milk and Tris buffered saline at 4°C overnight to prevent non-specific binding.

The next morning primary antibodies were diluted at 1:1000 in Tris buffered saline. Mouse monoclonal anti-human antibodies for smooth muscle myofilament proteins were directed at: α-smooth muscle actin (IgG2a), β-tropomyosin (IgG), h-caldesmon (IgG) and vinculin (IgG). To ensure equal loading of protein, gels were co-stained with a housekeeping protein, a mouse IgG anti-human monoclonal antibody

specific for β-actin (Sigma, St. Louis, MO). The gel stained for α-smooth muscle actin was not co-stained with β-actin because similar molecular weights would lead to overlapping expression and inability to compare α-smooth muscle actin expression among cell cultures. Membranes were incubated with primary antibody for one hour on a rotator at room temperature and followed by four 25-minute washings with Tris buffered saline and Tween-20 (0.1%). A goat monoclonal secondary antibody, conjugated to horseradish peroxidase, (Roche Molecular Biochemicals, Indianapolis, IN) was diluted 1:10,000 in Tris buffered saline and Tween-20 (0.1%) and incubated for one hour on a rotator at room temperature. The nitrocellulose membranes then were washed for two hours to remove excess secondary antibody. The wash solution (Tris buffered saline and Tween-20) was changed eight times during the wash period. The last wash solution was Tris buffered saline without Tween-20.

Western Lightning Chemiluminescence Reagent Plus Enhanced Luminol (PerkinElmer Life Sciences, Boston, MA) was used to develop the horseradish peroxidase. The membranes were incubated with 5 mL of the combined substrate for 60 seconds and then were quickly wrapped in plastic wrap, placed in a cassette, and exposed in a dark room using Kodak autoradiography film (Hyperfilm MP, Amersham Pharmacia, Piscataway, NJ).

C. Results

Cells were observed growing from chorionic plate vessel explants within one week (Figure 7). Primary cultures of PASM cells grew to confluence in approximately four weeks. At confluence, PASM cell cultures had a uniform cell morphology that was characterized by elongated cells in parallel rows, typical of smooth muscle cells (Figure 7). PASM cell cultures also showed evidence of overlapping layers forming ridges, which is characteristic of smooth muscle cell cultures. PASM cell morphology was retained after several passages.

All PASM cells expressed smooth muscle myofilament proteins: α-smooth muscle actin, β-tropomyosin, h-caldesmon, vinculin and metavinculin (Figures 8-9). PASM cultures showed similar protein expression to that of the other smooth muscle cells, HASM and HISM. All smooth muscle cell cultures strongly expressed α-smooth muscle actin. PASM cells were used between passages two and five and there was no difference in protein expression among various passages. HUVECs expressed vinculin and β-tropomyosin, but neither of the smooth muscle-specific myofilament proteins. Since PASM cells showed similar protein expression to HASM and HISM cells, but not to endothelial cells, these data indicated that cultured PASM cells were smooth muscle cells and not contaminated with endothelial cells. All lanes had similar expression of β-actin, confirming equal protein loading.

D. Discussion

In this study, we describe a simple and economical method to obtain human vascular smooth muscle cells for in vitro studies. Morphological and immunoblotting evidence confirmed that the established primary PASM cell cultures were vascular smooth muscle cells, and not contaminated with endothelial or fibroblast cells. PASM cell smooth muscle myofilament protein expression was similar to HASM cells. Since placentas are readily available to investigators and the health of the mother and sex of the baby are easily obtained, we suggest that PASM cells are a good alternative for expensive, commercially available vascular smooth muscle cells from adult donors. We used these cells in the next chapter to study the effects of oxidative stress and linoleic acid on vascular smooth muscle cell production of IL-8 and AA metabolites.

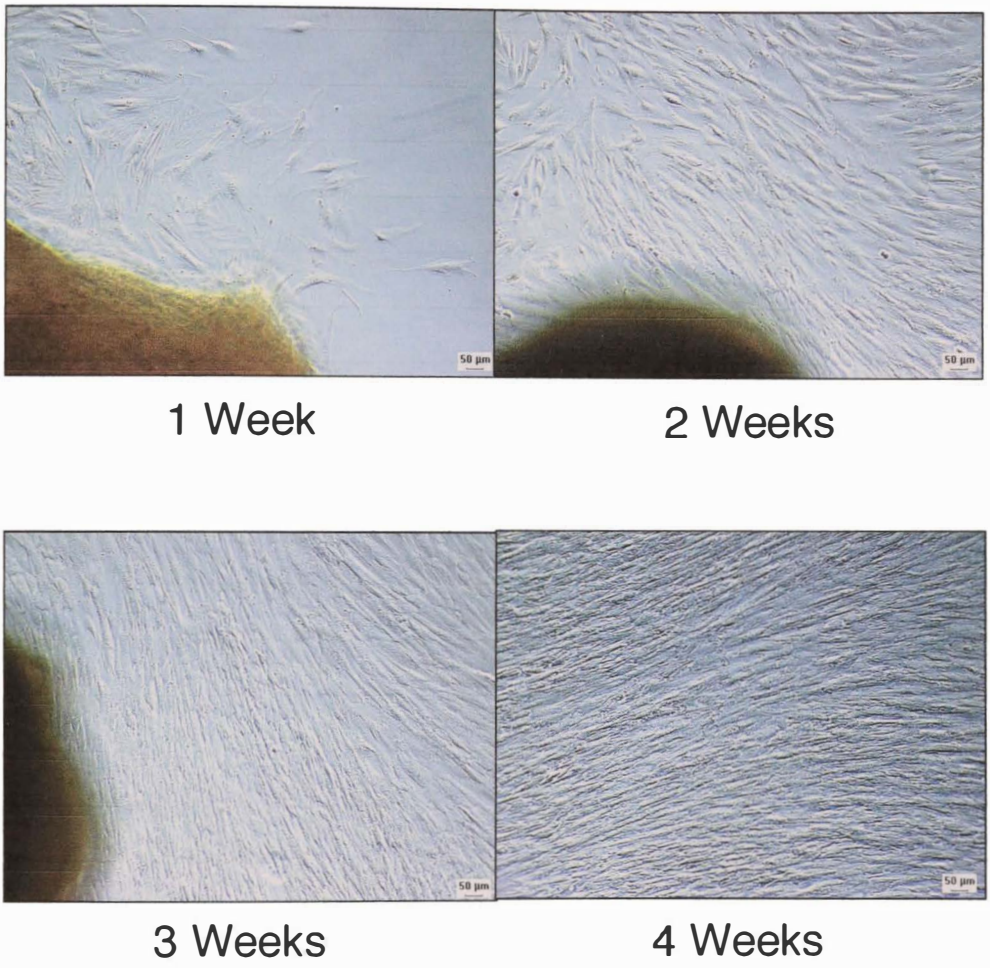


Figure 7. PASM explant growth to establish primary cultures of vascular smooth muscle cells.

PASM cells were observed growing from explants within one week. Cells grew to confluence over the next three weeks. Magnification is x100.

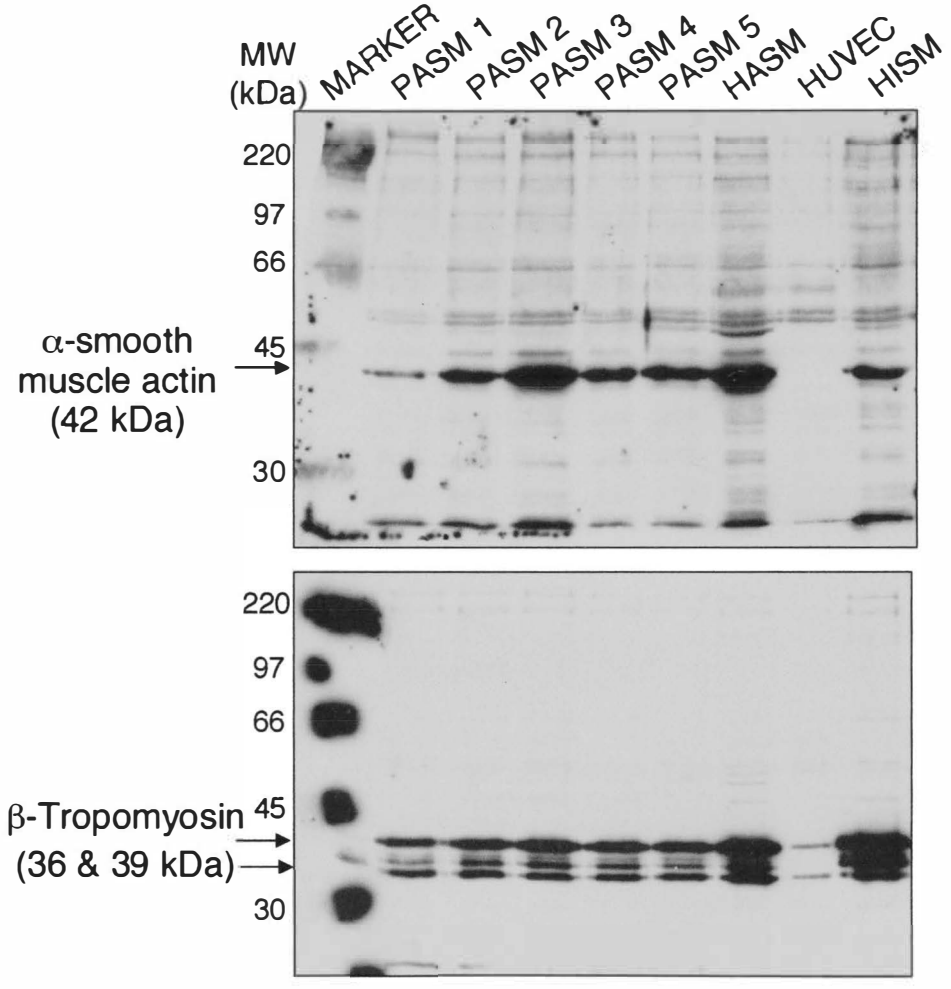


Figure 8. Western blot for smooth muscle myofilament proteins.

PASM, HASM and HISM cells, but not HUVECs, expressed α-smooth muscle actin and β-tropomyosin.

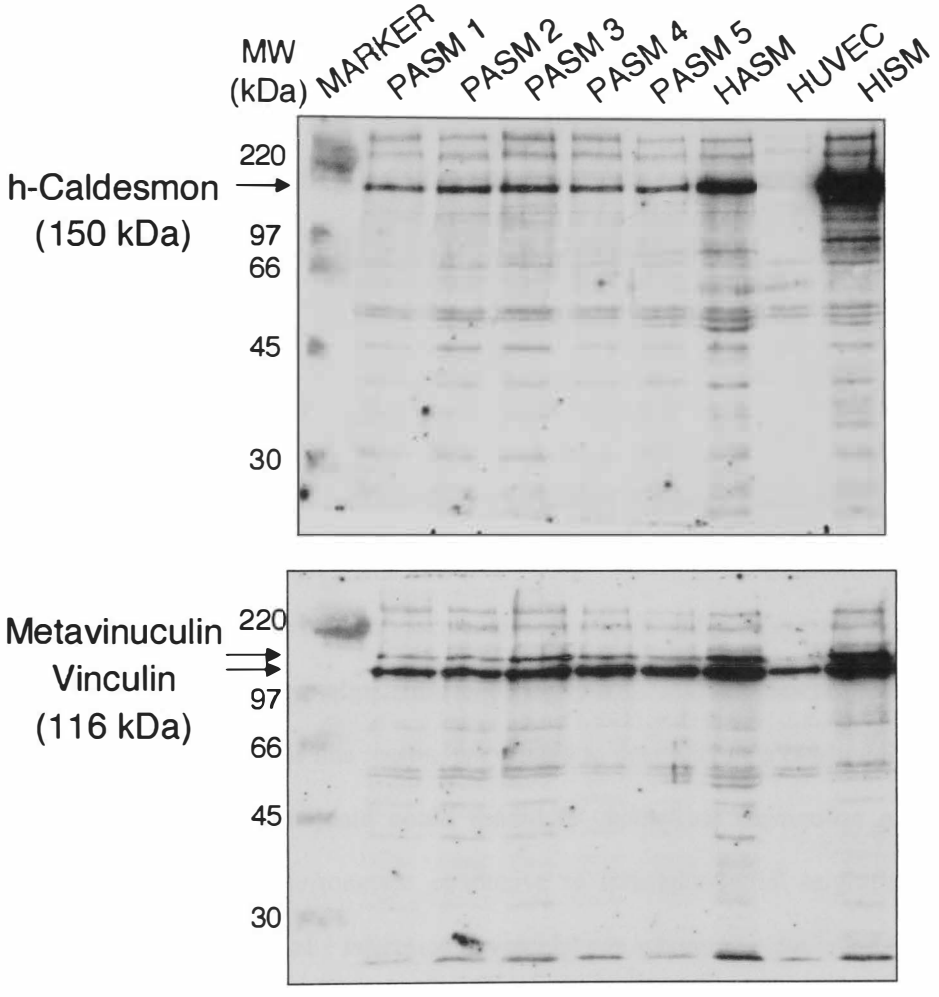


Figure 9. Western blot for smooth muscle myofilament proteins.

PASM, HASM and HISM cells, but not HUVECs, expressed h-caldesmon. All cells expressed vinculin.

Chapter 3

VASCULAR SMOOTH MUSCLE CELL PRODUCTION OF IL-8 IN RESPONSE TO OXIDATIVE STRESS AND LINOLEIC ACID

A. Introduction

In the previous chapter, we described a technique to isolate and culture human vascular smooth muscle cells. In this chapter, we used these cells to investigate the effect of oxidative stress and linoleic acid on vascular smooth muscle cell production of IL-8.

Oxidative stress and hyperlipidemia involving linoleic acid are present in women with preeclampsia [15, 98, 99]. For this reason, we wanted to determine if a combination of oxidative stress and linoleic acid could modulate chemokine expression of the vasculature to create an environment conducive to transendothelial migration of neutrophils. We were specifically interested in vascular smooth muscle regulation of IL-8, a potent neutrophil chemokine known to be upregulated by oxidative stress, because vascular smooth muscle expression of IL-8 would provide a chemotactic gradient from the circulation to the vascular smooth muscle for neutrophil infiltration. We chose linoleic acid for our in vitro studies because linoleic acid is elevated early in pregnancy in women destined to develop preeclampsia [98], linoleic acid is increased in the diets of

women that develop preeclampsia [105], and linoleic acid is the dietary precursor for AA and its metabolites which mediate inflammatory responses.

Previous research in our laboratory demonstrated that oxidative stress in the presence of linoleic acid increased IL-8 production, as well as AA metabolite production, by human intestinal smooth muscle cells [151, 152]. We questioned if oxidative stress plus linoleic acid also affected AA metabolite production by vascular smooth muscle cells, and possibly played a role in IL-8 expression. If the AA pathway was involved in vascular smooth muscle regulation of IL-8, then inhibition of AA metabolite production should result in a decrease in IL-8 expression.

We hypothesized that oxidative stress in the presence of linoleic acid would upregulate vascular smooth muscle expression of IL-8 and that IL-8 expression would be linked with AA metabolites.

B. Materials and Methods

PASM cells were isolated and characterized by smooth muscle myofilament proteins by techniques previously described (Chapter 2). Initially, an IL-8 time-course was conducted and AA metabolite production was analyzed to determine that PASM cells were functionally active.

PASM cells (passages two - seven) were suspended in DMEM, 10% FBS and were seeded into 24-well Costar tissue culture plates at 40,000 cells / well (Costar, Fisher Scientific, Malvern, PA). The following day, media were discarded and replaced with

fresh media to remove unattached cells. PASM cells grew for four days to confluence. On the fourth day, the media were discarded and replaced with DMEM without serum. Treatments were prepared the following day in M199 media, added for 18 hours and run in triplicate. Five to eight experiments were performed for each treatment group. PASM media were collected and stored at –20°C.

Cell viability was confirmed by Trypan blue exclusion staining and MTT viability and proliferation assay. Following collection of media, PASM cells were trypsinized, centrifuged, and resuspended in media. A small volume of the cell suspension was incubated with Trypan blue at a ratio of 4:1 and counted to determine percent viability. Trypan blue only permeates dead cells. Relative MTT concentrations were also used to evaluate cell viability between treatment groups [153]. MTT is a tetrazolium salt that is cleaved by mitochondrial dehydrogenase enzymes of living cells. In the presence of an acid-isopropanol solution, cleaved MTT will solubilize to yield a blue formazan product that can be measured spectrophotometrically. Following incubation with treatments and collection of media, PASM cells were incubated with 0.5 mg/mL MTT dissolved in PBS (1 mL) for four hours at 37°C. Cells then were incubated for 30 minutes with an acid-isopropanol solution composed of: isopropanol (45 mL), ultra pure water (5 mL), sodium dodecyl sulfate (0.25 g), and concentrated HCl (100 µL). Fluid suspension was collected and absorption was analyzed at 595 nm by a 96-well microplate reader (Tecan, Research Triangle Park, NC). This assay is a measurement of viable cells since MTT is only processed by mitochondria of living cells.

To mimic an environment of oxidative stress, we used an oxidizing solution (Ox) composed of: hypoxanthine (HX, 0.05 mM, Sigma, St. Louis, MO), xanthine oxidase (XO, 0.002 units / mL, Roche Molecular BioChemicals, Indianapolis, IN), and ferrous sulfate ($FeSO_4$, 5 μM, Sigma, St. Louis, MO). The oxidizing solution was enriched with linoleic acid (LA, 45 μM, Cayman Chemical, Ann Arbor, MI) to mimic the hyperlipidemic state of preeclampsia. We used nordihydroguaiaretic acid (NDGA, 5 μM, Sigma, St. Louis, MO) to inhibit the LPO pathway and indomethacin (Indo, 5 μM, Sigma, St. Louis, MO) to inhibit the COX pathway (Figure 10). PASM cells were treated for 18 h with the following solutions:

1) M199
2) LA (45 μM)
3) Ox
4) Ox enriched with LA (OxLA)
5) OxLA + NDGA (5 μM)
6) OxLA + Indo (5 μM)

Media were analyzed for IL-8, and the following AA metabolites representing the LPO pathway: leukotriene B_4 (LTB_4), and the COX pathway: prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2). Media were analyzed by commercially available enzyme-linked immunosorbant assays (ELISA) (R&D Systems, Minneapolis, MN). Data were analyzed by one-way analysis of variance (ANOVA) and Student-Newman-Keuls post

hoc test was used to determine differences between treatment groups. (*P<0.05, **P<0.01) Statistical analysis was performed using StatView software (StatView, Abacus Concepts, Inc., Berkeley, CA).

C. Results

PASM cells spontaneously produced IL-8 while cultured in M199 media. Concentrations of IL-8 increased progressively during 18 h of incubation (Figure 11). A culture time of 18 h was chosen for all subsequent experiments. Figures 12 and 13 show that PASM cells were viable following treatments.

PASM cells treated with OxLA produced significantly more IL-8 than M199 media, LA, or Ox (Figure 14). Both NDGA and Indo significantly inhibited IL-8 production induced by OxLA (Figure 15).

OxLA, but not LA, significantly increased production of LTB_4 by PASM cells as compared to M199 media or Ox (Figure 16). PASM cell LTB_4 production was significantly inhibited by NDGA, but not by Indo, demonstrating inhibition of the LPO pathway (Figure 16). Both LA and OxLA significantly increased production of COX metabolites as compared to M199 media or Ox (Figures 17 and 18). PASM cell production of PGE_2 was almost completely inhibited by Indo, but not by NDGA, demonstrating inhibition of the COX pathway (Figure 17). Surprisingly, PASM cell production of TXB_2 was inhibited by NDGA, as well as Indo, suggesting that NDGA

inhibits thromboxane synthase (Figure 18). This was an unexpected finding because NDGA is described as a selective LPO inhibitor.

D. Discussion

The presence of both LA and Ox were necessary to stimulate PASM cell production of IL-8. Neither LA alone nor Ox alone increased IL-8 production. To determine if AA metabolites were involved in the regulation of IL-8 production by vascular smooth muscle cells upon treatment with OxLA, we used NDGA to inhibit the LPO pathway and Indo to inhibit the COX pathway. Surprisingly, both NDGA and Indo inhibited IL-8 production. This suggested that AA metabolites were involved, but it did not differentiate between the LPO and COX pathways.

To distinguish which AA pathway mediated the IL-8 response, we measured representative AA metabolites of each pathway after treatment with LA, Ox, OxLA, and OxLA plus AA pathway inhibitors. OxLA, but not LA, significantly increased production of LTB_4 as compared to M199 media or Ox. In contrast, both OxLA and LA markedly stimulated the COX products, PGE_2 and TXB_2. Ox alone did not stimulate either LPO or COX metabolites. As expected, OxLA plus Indo inhibited PGE_2 and TXB_2, indicating inhibition of COX. Unexpectedly, OxLA plus NDGA not only inhibited LTB_4 indicating inhibition of LPO, but it also inhibited TXB_2 production, indicating NDGA also inhibits thromboxane synthase.

This unexpected finding made it more difficult to determine whether a LPO or COX metabolite was mediating IL-8 production. For an AA metabolite to mediate IL-8 production, it must be stimulated under conditions that stimulate IL-8, and it must be inhibited under conditions that inhibit IL-8. Since both TXB_2 and IL-8 were stimulated by OxLA and inhibited by OxLA plus NDGA and OxLA plus Indo, TXB_2 may be a mediator in IL-8 production. Experiments with specific thromboxane synthase inhibitors, thromboxane antagonists, and thromboxane mimetics will be necessary to prove the role of thromboxane in IL-8 production. Additional experiments with an inhibitor of LPO more specific than NDGA, will further clarify the role of LTB_4.

We have demonstrated that the combination of Ox and LA acts on vascular smooth muscle cells to stimulate production of IL-8 and AA metabolites. Our data suggest that IL-8 is regulated by AA metabolites, possibly by thromboxane, under conditions of oxidative stress. We speculate that oxidative stress and elevated levels of LA in preeclamptic women lead to increased IL-8 and AA metabolite production by vascular smooth muscle cells. The production of IL-8 by vascular smooth muscle would create an IL-8 concentration gradient from the circulation to the vascular smooth muscle, which would favor transendothelial migration of neutrophils. Since the AA metabolite, LTB_4, is also a neutrophil chemotactic agent, the production of LTB_4 under conditions of oxidative stress and hyperlipidemia involving LA would cause additional neutrophil chemotaxis from the circulation to vascular smooth muscle. Vascular smooth muscle also produces thromboxane in the presence of oxidative stress and LA. The production of

thromboxane by vascular smooth muscle cells, which contain thromboxane receptors, may be responsible for vasoconstriction leading to hypertension of preeclampsia.

In conclusion, oxidative stress and elevated plasma levels of LA during preeclampsia may act on vascular smooth muscle to produce vascular inflammation characterized by increased IL-8 and AA metabolite production, leading to neutrophil transendothelial migration and hypertension. In Chapter 5, we will show evidence that vascular smooth muscle express IL-8 in women with preeclampsia and that this is associated with neutrophil infiltration into systemic vasculature.

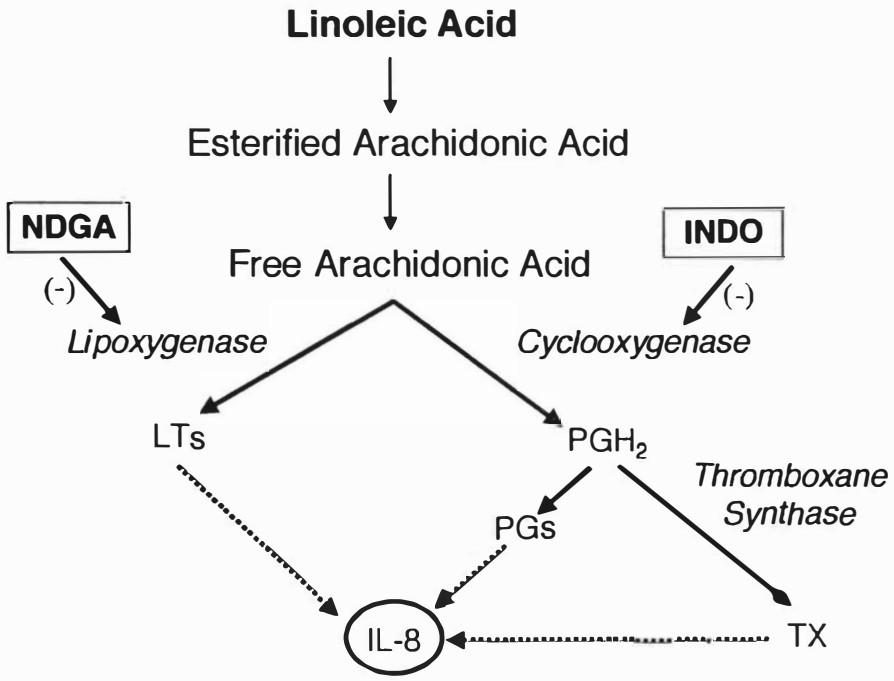


Figure 10. Inhibition of arachidonic acid cascade by NDGA and Indo.

LA incorporates into cell membranes to form esterified AA. PLA_2 releases AA from the cell membrane so that is can be acted upon by COX or LPO to produce prostaglandins (PGs) or leukotrienes (LTs), respectively. Thromboxane synthase converts PGH_2 into TX. We hypothesized that one of these AA metabolites mediates IL-8 production by vascular smooth muscle cells. Indo inhibited COX to prevent formation of PGs and TX. NDGA inhibited LPO to prevent formation of LTs. In the course of this study, we found that NDGA also inhibited thromboxane synthase.

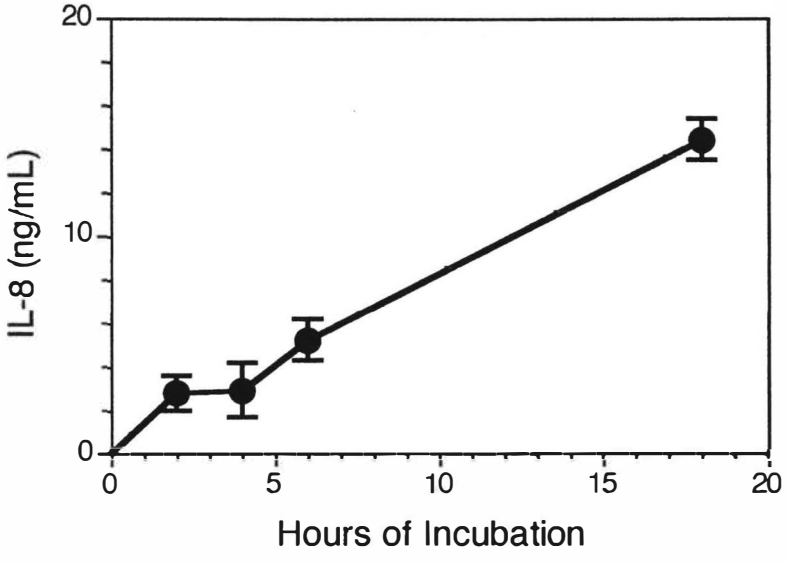


Figure 11. Time course for the spontaneous production of IL-8 by PASM cells cultured in M199 for 2, 4, 6, and 18 h.

Concentrations of IL-8 increased progressively during 18 h of incubation. A culture time of 18 h was chosen for all subsequent experiments.

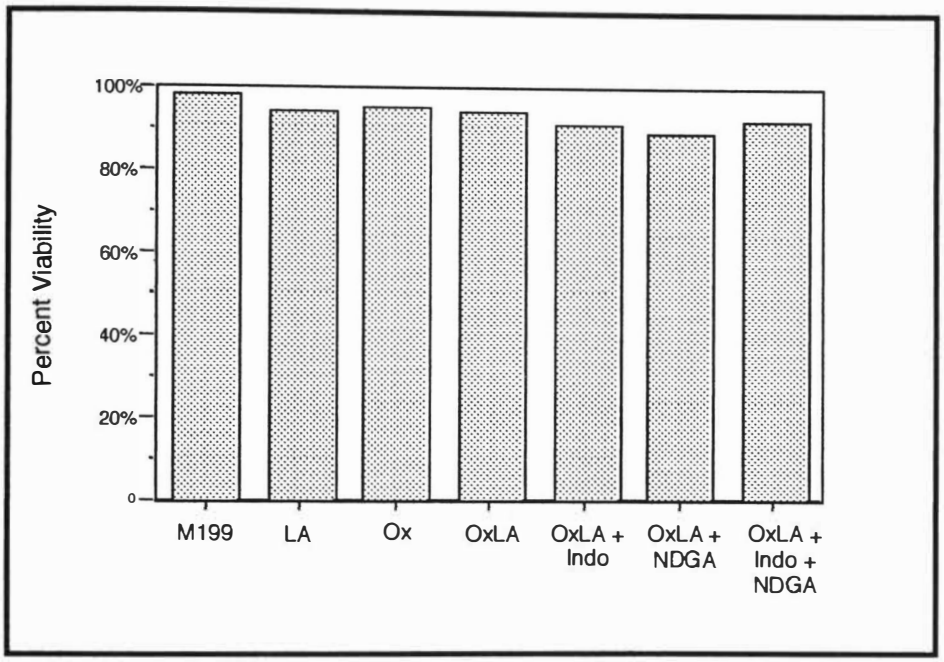


Figure 12. Percent viability of PASM cells following an 18 h treatment period using Trypan blue cell viability assay.

All treatment groups had a percent viability above 90%.

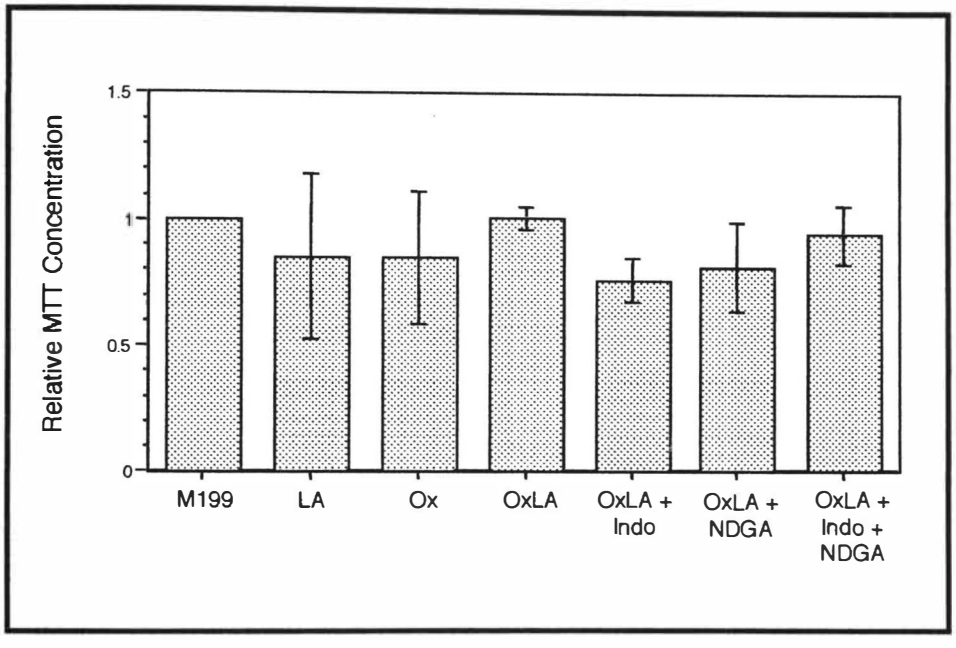


Figure 13. Relative MTT concentration of PASM cells following an 18 h treatment period.

There was no significant difference between treatment groups.

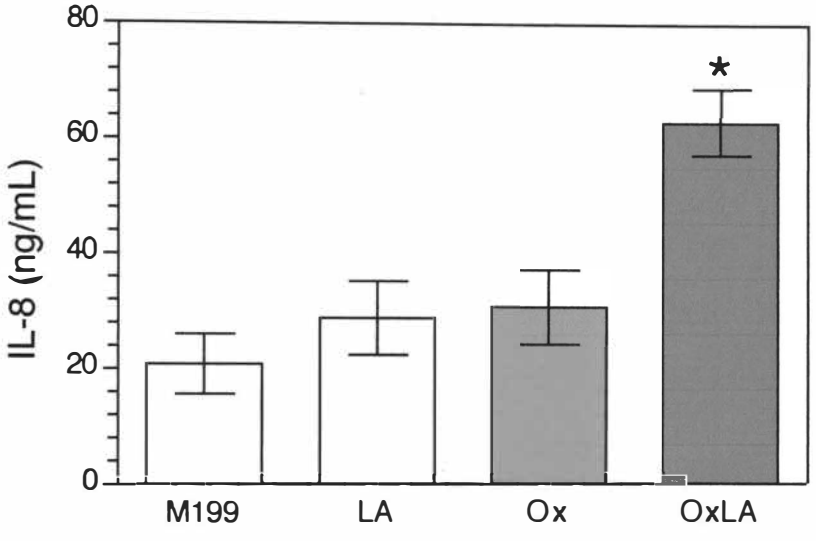


Figure 14. Production of IL-8 by PASM cells exposed to M199, LA, Ox, or OxLA for 18 h.

OxLA significantly increased IL-8 production as compared to M199 media, LA, or Ox. *$P < 0.05$ ($n = 5$)

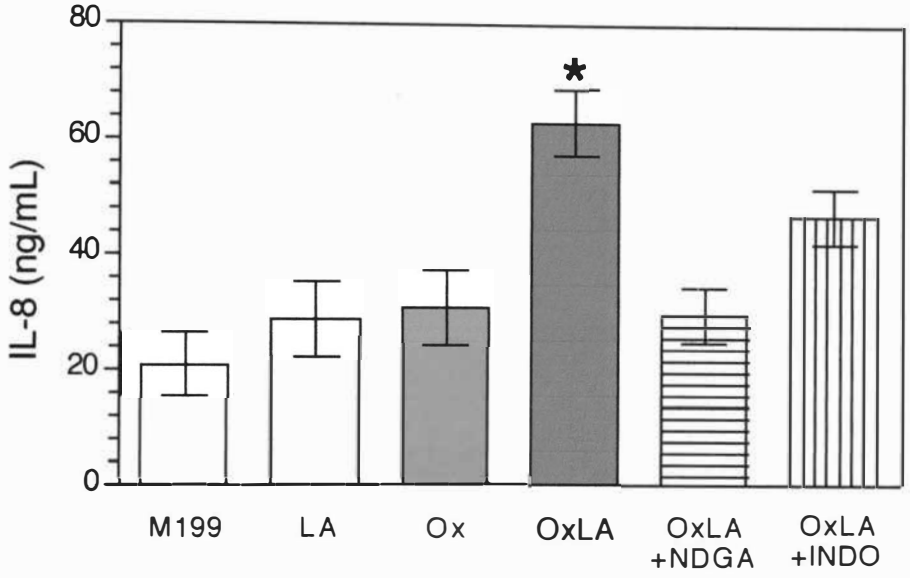


Figure 15. Production of IL-8 by PASM cells exposed to treatments with LA, Ox, OxLA, or OxLA with AA metabolite inhibitors for 18 h.

AA metabolite inhibitors (NDGA and Indo) significantly decreased IL-8 production as compared to OxLA. *$P < 0.05$ ($n = 5$)

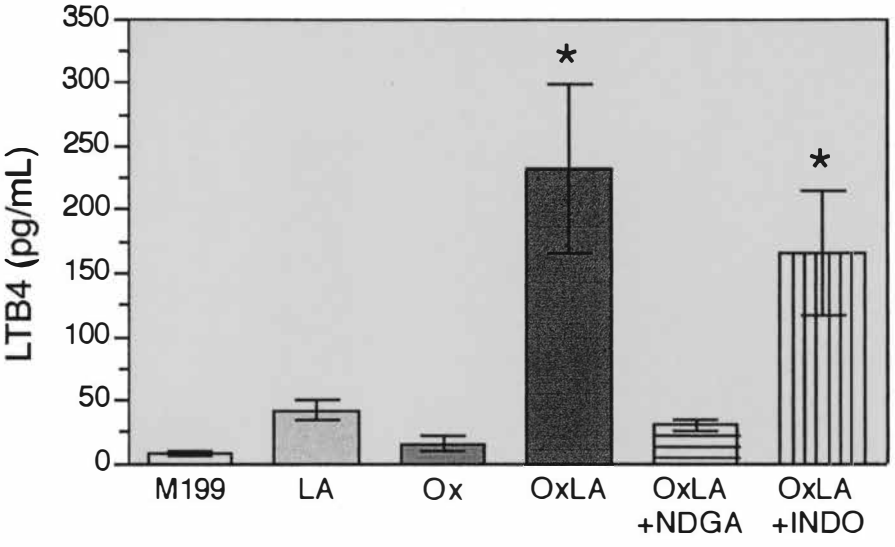


Figure 16. Production of LTB_4 by PASM cells exposed to M199, LA, Ox, OxLA, or OxLA with AA metabolite inhibitors for 18h.

LTB_4 production was significantly greater in the presence of OxLA as compared to M199 media control, LA, or Ox. NDGA significantly inhibited LTB_4 production, whereas Indo did not. *$P < 0.05$ ($n = 8$)

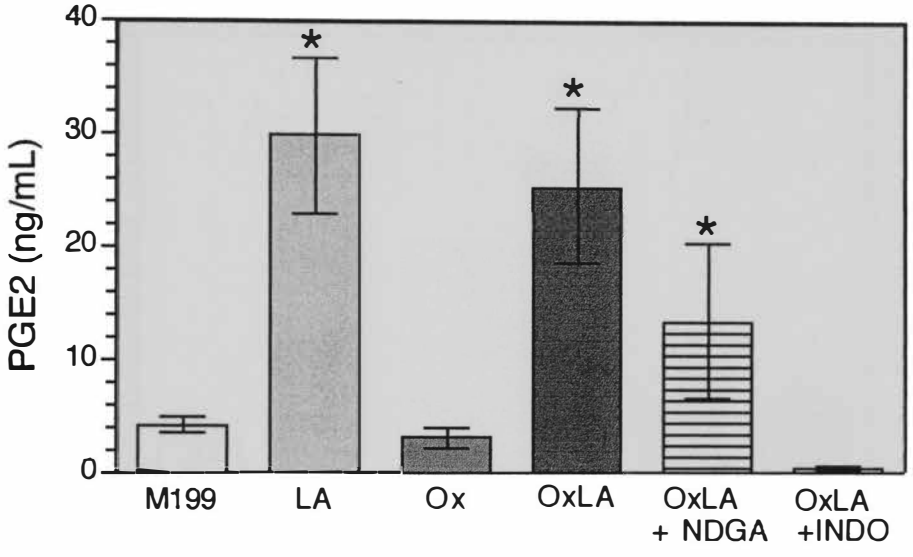


Figure 17. Production of PGE_2 by PASM cells exposed to M199, LA, Ox, OxLA, or OxLA with AA metabolite inhibitors for 18 h.

Both LA and OxLA significantly increased PGE_2 production as compared to M199 media control or Ox. Indo significantly decreased PGE_2 production, whereas NDGA did not. *$P < 0.05$ ($n = 8$)

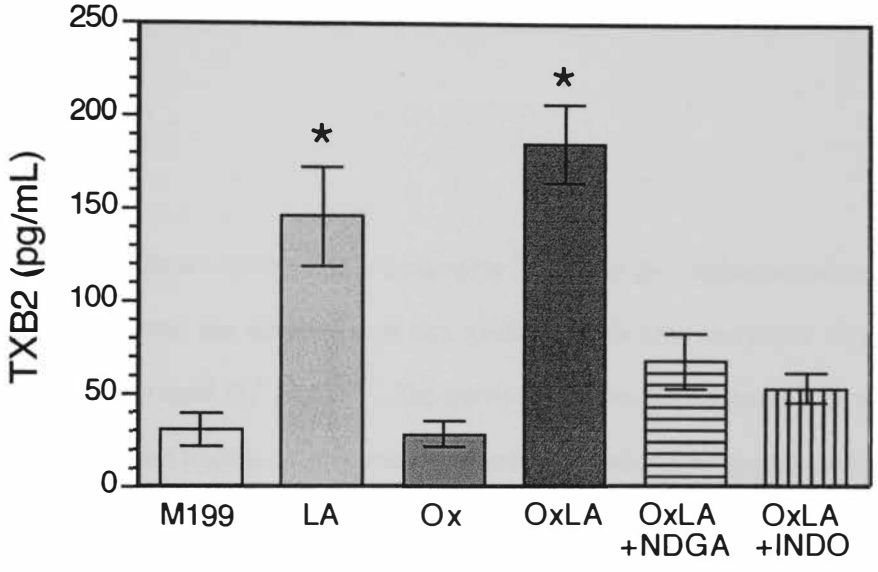


Figure18. Production of TXB_2 by PASM cells exposed to M199, LA, Ox, OxLA, or OxLA with AA metabolite inhibitors for 18 h.

Both LA and OxLA significantly increased TXB_2 production as compared to M199 media control or Ox. AA metabolites (NDGA and Indo) significantly decreased TXB_2 production as compared to LA or OxLA. *$P < 0.05$ ($n = 7$)

Chapter 4

THE NEUTROPHIL SUPEROXIDE BURST IN REAL TIME IN RESPONSE TO OXIDATIVE STRESS AND LINOLEIC ACID

A. Introduction

Neutrophils are activated in preeclampsia [122, 127], but the mechanism of activation is unclear. Research has demonstrated that oxidized lipids alter monocyte function to increase the neutrophil $O_2^{\bullet -}$ burst [139]. The previous chapter demonstrated that together oxidative stress and linoleic acid alter the expression of chemotactic agents, such as IL-8 and LTB_4, by vascular smooth muscle cells to create an environment conducive to transendothelial migration of neutrophils. Since oxidized lipids modulate monocyte function, we wanted to explore if an oxidizing solution enriched with linoleic acid would alter neutrophil $O_2^{\bullet -}$ production.

We used a novel chemiluminescent method to specifically measure neutrophil $O_2^{\bullet -}$ production in real time. Historically, ROS have been measured using a cytochrome C assay, which measures oxygen consumption over a period of time to indicate generation of ROS. Our methodology uses a synthesized chemiluminescent probe that specifically interacts with $O_2^{\bullet -}$ to produce photons of light that can be measured with a

photomultiplier. We used a synthesized analog of coelenterazine (2-(4-hydroxybenzyl)-6-(4-hydroxyphenyl)-8-benzyl-3, 7-dihydroimidazo[1,2-a] pyrazin-3-one), which is the biologically active component or light-producing molecule, of bioluminescent marine organisms. Specifically, we chose Detector "C" from the available synthesized analogs because it had the lowest background luminescence [154, 155].

We hypothesized that the neutrophil $O_2^{\bullet -}$ burst would be greater in response to oxidative stress plus linoleic acid than controls. If true, these data could explain neutrophil activation in preeclampsia and suggest a priming mechanism for the transendothelial migration of neutrophils.

B. Materials and Methods

i. Neutrophil Isolation

Healthy volunteers (n = 9) agreed to donate blood for the isolation of neutrophils. Signed consent was obtained. This protocol was approved by the Office of Research Subjects Protection, Virginia Commonwealth University. Whole blood (20 mL) was collected by vein puncture into two 16 x 100 mm sodium heparin vacutainer tubes (VWR Scientific Products, Pittsburgh, PA). The neutrophil isolation procedure was performed within one hour of blood collection.

Human peripheral blood neutrophils were isolated from whole blood as described by Boyum [156]. Histopaque 1119 (Sigma Chemical Company, St. Louis, Missouri) was

layered below Histopaque 1077 (Sigma Chemical Company, St. Louis, Missouri) in a 50 mL conical tube (Corning Incorporated, Corning, New York). Whole blood was slowly layered onto the upper gradient of Histopaque 1077 (Figure 19). The layered blood and Histopaque were centrifuged at 700 x g (Beckman J6-MC centrifuge, Beckman Instruments, Inc., Columbia, MD) for 30 minutes at room temperature (25°C). Two distinct layers of leukocytes were obtained; the upper layer contained monocytes, lymphocytes and platelets, and the lower layer contained granulocytes.

The upper layers of plasma, monocytes, lymphocytes, and Histopaque 1077 were aspirated and discarded to within 0.5 cm of the granulocyte layer. Granulocytes were aspirated and transferred to a new 50 mL conical vial. The granulocytes were washed to remove any remaining Histopaque by adding sterile PBS, pH 7.4 (Life Technologies, Grand Island, New York) and centrifuged for 10 minutes at 200 x g. PBS was aspirated and discarded up to the cell pellet and the washing procedure was repeated.

A pellet remained which contained neutrophils and residual erythrocytes. To lyse contaminating erythrocytes, 3 mL of ice-cold ddH_2O was added and the tube gently agitated. After exactly 30 seconds, 1 mL of ice-cold 0.6 M KCl was added to restore tonicity. The cell suspension was centrifuged at 200 x g for 4 minutes at 4°C to form a white pellet containing primarily neutrophils. Approximately 96% of the cells were neutrophils and only 4% were eosinophils and basophils.

Neutrophils were resuspended with 4 mL of Hanks' Balanced Salt Solution (HBSS) containing no Mg^{++} or Ca^{++} (Life Technologies, Grand Island, New York) and placed in a 15 x 100 mm non-adherent Teflon tube (Minisorp, Nunc, Rochester, NY).

The Teflon tube was placed on a slant rank attached to an orbital rotator (50800 Rotomix Rotator, Thermolyne, Dubuque, IA) in an incubator gassed with 5% CO_2 while a cell count was performed (Forma Scientific, Inc., Marietta, Ohio). Neutrophils were resuspended with DMEM/F12, 10% FBS and returned to the incubator until the experiment, approximately 4 hours. At the time of the experiment, cells were resuspended to a concentration of 2 x 10^6 cells/mL of PBS.

ii. Protocol for Oxidizing Linoleic Acid

Hypoxanthine (HX, 0.0123 g for a final concentration of 3.6 mM) (Sigma Chemical Company, St. Louis, Missouri) was added to a 50 mL conical vial containing 22.5 mL of M199 media (Life Technologies, Grand Island, New York) and sonicated for twenty-five minutes. Then 0.00187 g of xanthine oxidase (XO, activity of 0.067 units / mg solid) (Sigma Chemical Company, St. Louis, Missouri) was added for a final concentration of 0.005 units / mL. This solution was sterile filtered. A 0.02 M ferrous sulfate ($FeSO_4$, Sigma Chemical Company, St. Louis, Missouri) solution was made by sterile filtering 25 mL of ddH_2O and then adding 0.139 g of $FeSO_4$. Sixty-three µLs of the 0.02 M $FeSO_4$ solution was added to the HX / XO solution for a final concentration of 50 µM. This was our oxidizing solution (Ox).

A 0.11 M solution of linoleic acid (LA) was made fresh every 3 days by diluting 1 g of LA in 33 mL of 100% ethanol. A volume of 20.5 µL of the 0.11 M solution of LA was added to Ox to achieve a LA concentration of 90 µmol / L. This was followed by

addition of 2.5 mL FBS (heat-inactivated) for a total volume of 25 mL. XO generates $O_2^{\bullet -}$ during metabolism of HX to uric acid. LA in the Ox solution was oxidized by $O_2^{\bullet -}$ in the presence of Fe^{++} according to the Fenton reaction. We determined an optimum concentration for XO (0.05 units / mL) for $O_2^{\bullet -}$ production from previous experiments. This was our Ox solution enriched with LA (OxLA).

To compare the neutrophil $O_2^{\bullet -}$ burst upon stimulation with OxLA, we also stimulated neutrophils with known activators including, phorbol 12-myristate acetate (PMA, 8 μg/mL) and lipopolysaccharide (LPS, 100 ng/mL). Stock solutions of activators were prepared and an appropriate volume of stock solution was added to 1 mL of M199 to equal the final concentration noted above. This solution then was injected into the measuring tube.

To confirm cell viability, cell counts for Trypan blue uptake were performed following measurement of neutrophil $O_2^{\bullet -}$.

iii. Superoxide Measurement

The $O_2^{\bullet -}$ chemiluminescent measuring system was designed to directly measure $O_2^{\bullet -}$ production, as photons of light, from tissue maintained in a physiological environment (Figure 20). The measuring instrument was constructed to be virtually light-tight by Tom Gentry, Biomedical Engineering Department, Virginia Commonwealth University. Cells remained at 37°C and 95% air and 5% CO_2 using circulating warm water and appropriate gas tanks (15 psi). The photomultiplier tube was maintained at

–30°C by a LCT50 control unit with circulating cool water and was powered by a high voltage power unit set for 1000 volts. Photons of light were measured and the data were transmitted to a ctl counter timer computer program for analysis.

Neutrophils (500 μL, 2 x 10^6 cells / mL) were placed into a Teflon tube in a light-tight and thermostatically insulated chamber. Detector "C" (2 μL, 25 μM), the coelenterazine analog, was then added to the cell suspension. The sample was placed in the measuring chamber and covered with the measuring chamber cap containing treatment and gas injection tubes. Finally, the cap was secured with wing nuts to form a light-tight and thermostatically insulated chamber.

For control purposes, counts began with the photomultiplier shutter closed and the voltage source set for 0 volts. Initial counts were zeros. Upon enabling the voltage source to relay 1000 volts, the counts showed background noise. After several data counts, we opened the shutter to obtain baseline counts, which represented spontaneous $O_2^{\bullet -}$ production from unstimulated neutrophils. Injections consisted of 500 μl of oxidizing solution enriched with linoleic acid (90 μM). Since the injection was added to 500 μL of cell suspension, the final concentration of OxLA was 45 μM. OxLA injections were followed by an injection of oxidizing solution, which contained hypoxanthine, xanthine oxidase, and ferrous sulfate, for control purposes. Superoxide dismutase (Sigma, St. Louis, MO) was added following treatments to confirm that we were measuring $O_2^{\bullet -}$ anions and not artifact. The experiment concluded by repeating control steps in the opposite sequence: 1) the shutter was closed to measure background noise, and 2) the high voltage source was shut off. Data collection ended when counts were zeros.

C. Results

OxLA caused an immediate dose-dependent increase in photon counts, representative of $O_2^{\bullet -}$ production (Figure 21). Superoxide production upon stimulation with OxLA ranged from 210 - 764.4 picomoles. Injection of SOD caused an immediate decline in photon counts, verifying that we were measuring $O_2^{\bullet -}$. Figure 22 shows decreased photon counts when OxLA was injected into the measuring chamber in the absence of neutrophils. These photon counts represented $O_2^{\bullet -}$ produced by our Ox solution, and confirmed that the $O_2^{\bullet -}$ burst observed in the previous figure was from neutrophils, rather than Ox solution. In contrast to OxLA, LA or Ox alone did not induce a neutrophil superoxide burst (Figure 23).

Upon injection with OxLA, $O_2^{\bullet -}$ production peaked within 20 seconds and then declined to baseline with a half-life of 120 seconds (Figure 24). LPS caused a slower rise time in $O_2^{\bullet -}$ production as compared to OxLA (3 minutes vs. 20-30 seconds) (Figure 25). Treatment with PMA induced a slower rise time in $O_2^{\bullet -}$ production similar to LPS (data not shown).

D. Discussion

We measured the neutrophil $O_2^{\bullet -}$ burst upon stimulation with OxLA in real time using a novel chemilumiscent $O_2^{\bullet -}$ measuring system. OxLA rapidly stimulated neutrophil $O_2^{\bullet -}$ production. In contrast, neither Ox nor LA stimulated $O_2^{\bullet -}$ production by

neutrophils. Neutrophil $O_2^{\bullet -}$ production upon stimulation with OxLA differed from stimulation with LPS or PMA. LPS and PMA produced gradual increases in $O_2^{\bullet -}$ production, whereas stimulation with OxLA lead to a rapid and more intensive burst of $O_2^{\bullet -}$.

In conclusion, OxLA was a potent and immediate stimulator of neutrophil $O_2^{\bullet -}$ production. These data suggest that oxidative stress and elevated plasma levels of LA may be responsible for neutrophil activation in preeclamptic patients. This could contribute to the clinical symptoms of preeclampsia. Activated neutrophils would release $O_2^{\bullet -}$ and other ROS to propagate and exacerbate oxidative stress within the maternal vasculature. Transendothelial migration of activated neutrophils with production of ROS could oxidize lipid membranes and damage endothelial cells and vascular smooth muscle cells. In addition, generation of TNFα, MPO, and TX by neutrophils could cause inflammation of the vasculature and vasoconstriction. Systemic oxidative stress would favor neutrophil transendothelial migration by stimulating IL-8 and LTB_4 production by vascular smooth muscle cells (Chapter 3) and by increasing $O_2^{\bullet -}$ production by neutrophils.

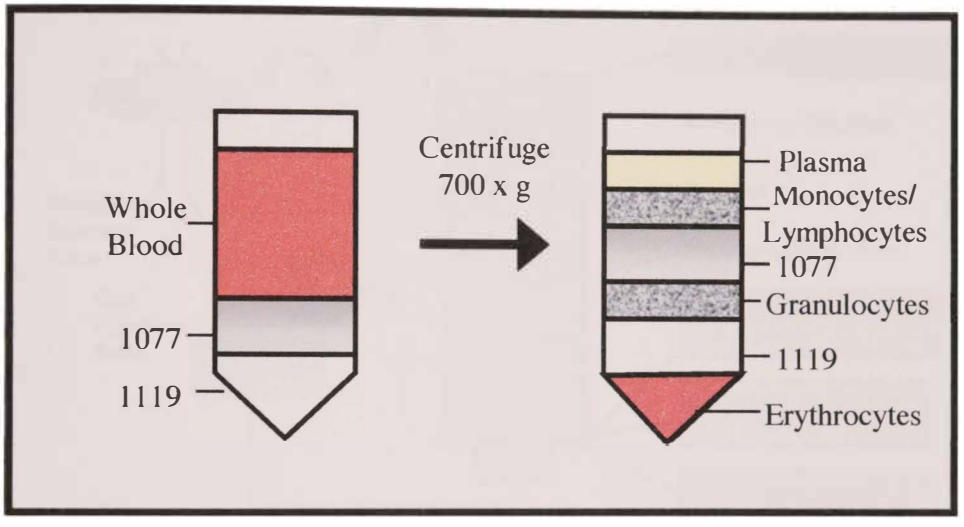


Figure 19. Neutrophil isolation from whole blood by histopaque density gradient separation.

Whole blood was layered upon two densities of histopaque. Centrifugation caused leukocytes to separate into layers by density, so that granulocytes (neutrophils) could be collected for experiments.

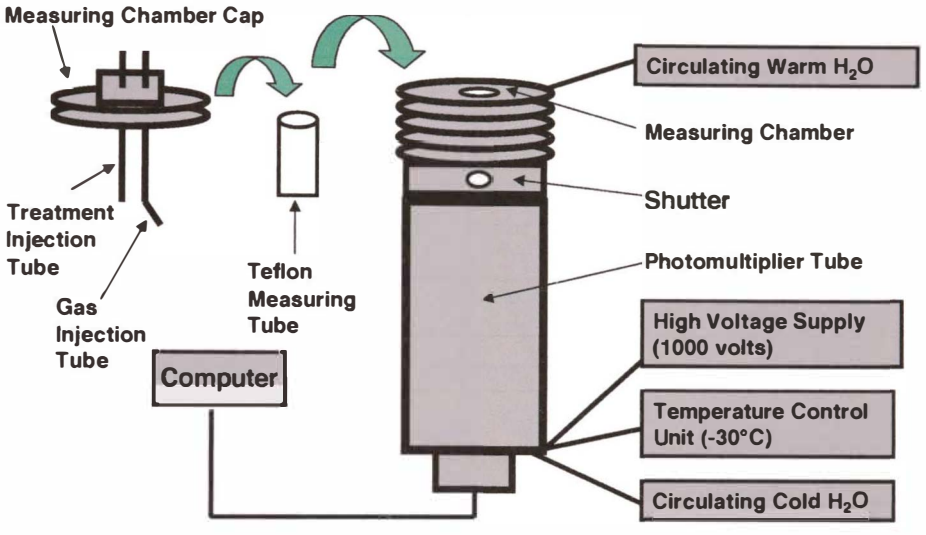


Figure 20. Superoxide chemiluminescent measuring system.

Neutrophils were placed into the Teflon measuring tube, which was placed into the measuring chamber. The measuring chamber was sealed with the measuring chamber cap. Treatments were injected through the treatment injection tube. The photomultiplier tube measured photons in real time. Photon counts were recorded by a computer and then transferred to Excel software for processing.

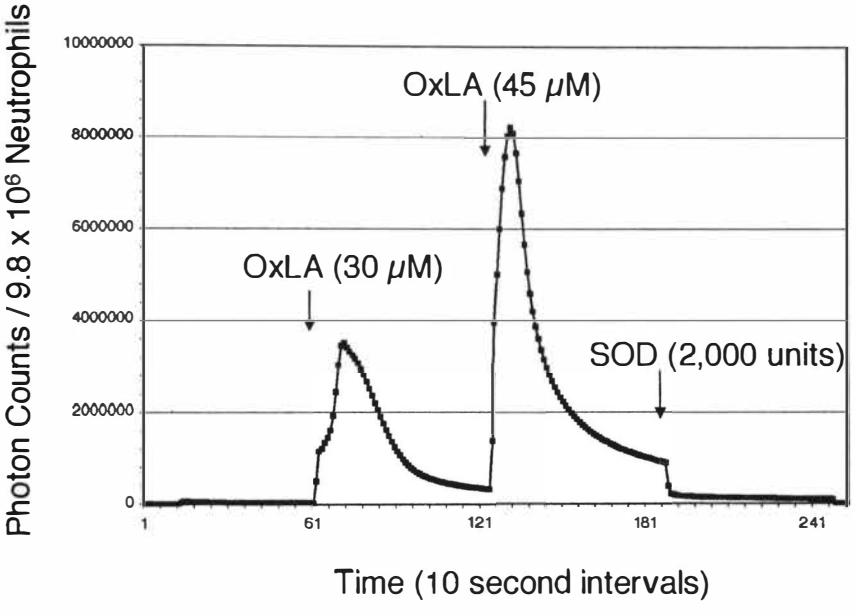


Figure 21. Neutrophil superoxide production upon stimulation with OxLA.

OxLA caused an immediate dose-dependent increase in neutrophil superoxide production. Superoxide dismutase (SOD) caused an immediate decline in photon counts verifying that we were measuring superoxide.

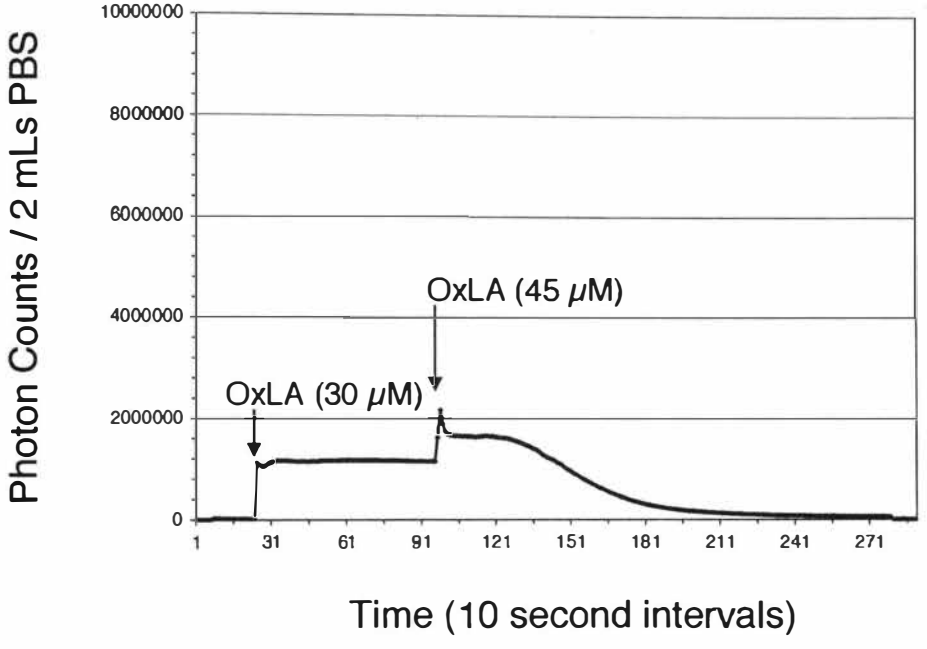


Figure 22. Injections of OxLA into the measuring chamber without neutrophils.

This graph illustrates that the superoxide burst observed in the previous figure was from neutrophils rather than oxidizing solution.

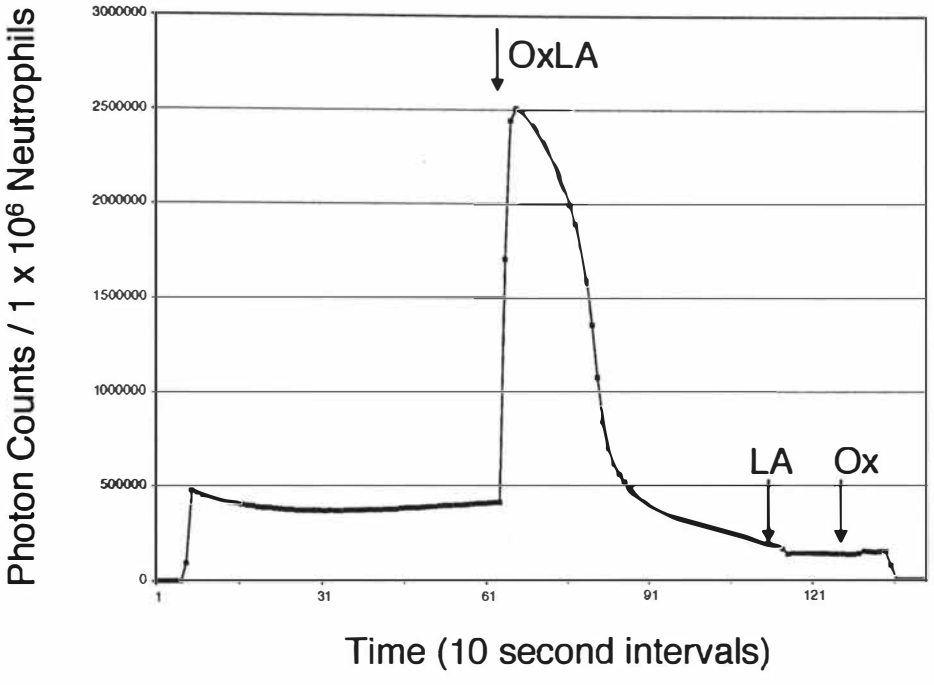


Figure 23. Comparison of neutrophil superoxide burst upon stimulation with OxLA, LA, and Ox.

In contrast to OxLA, LA or Ox alone did not induce a neutrophil superoxide burst.

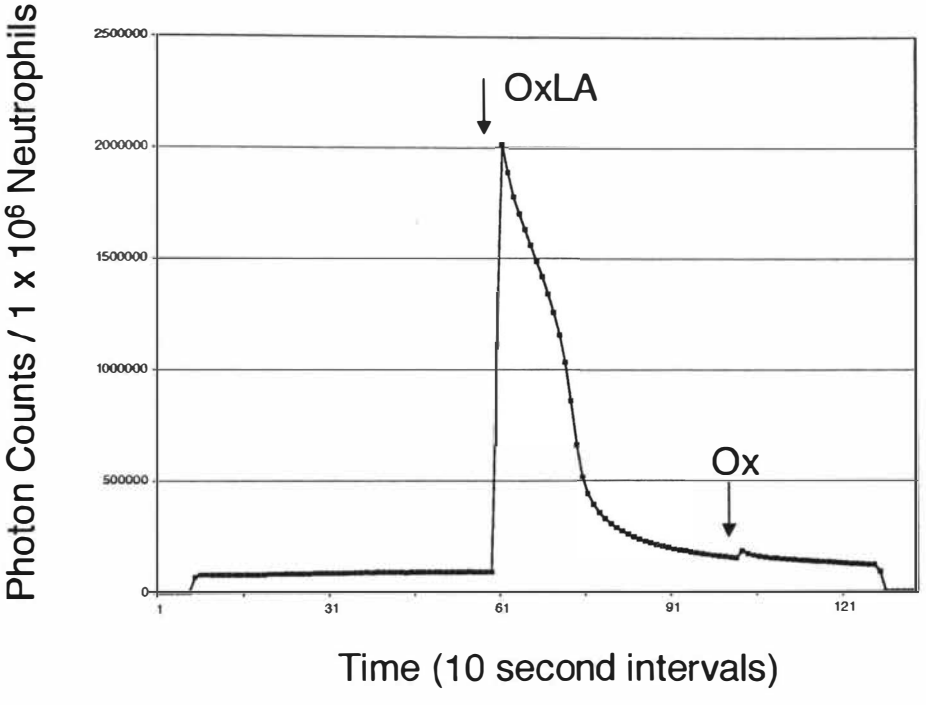


Figure 24. Time-course of neutrophil superoxide burst caused by OxLA.

OxLA caused an immediate increase in neutrophil superoxide production, which peaked within 20 seconds and then declined to baseline with a half-life of 120 seconds. As in the previous figure, Ox alone did not induce a superoxide burst by neutrophils.

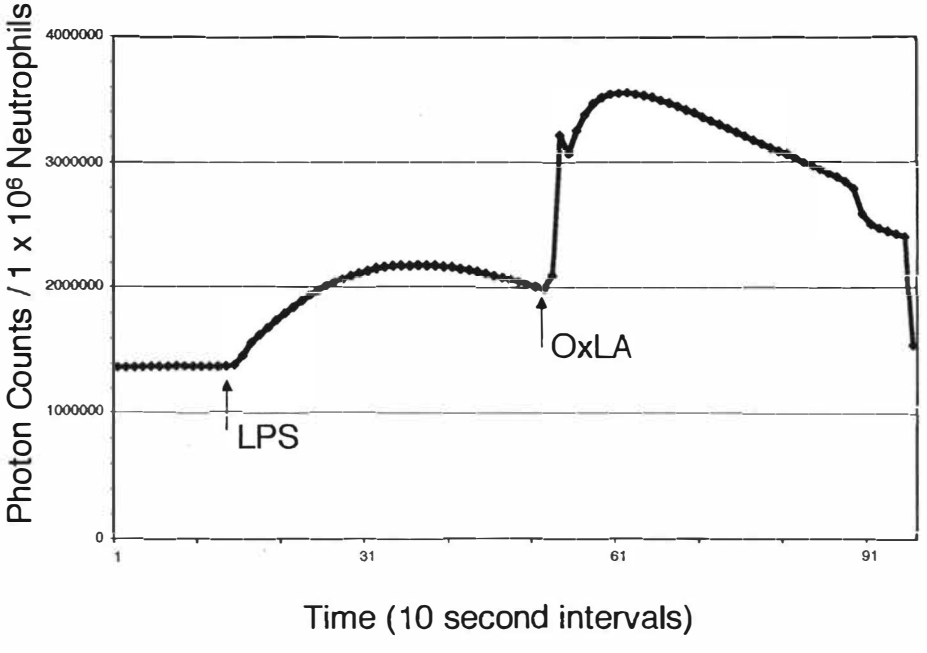


Figure 25. Time-course comparison of the neutrophil superoxide burst with OxLA as compared to LPS, a neutrophil activator.

LPS (100 ng/mL) caused a slower rise time in superoxide production than OxLA (3 minutes vs. 20-30 seconds). Similar to LPS, PMA (8 μg/mL) also produced a slower rise time in superoxide production (data not shown).

Chapter 5

INFILTRATION OF NEUTROPHILS INTO SYSTEMIC VASCULAR TISSUE IN WOMEN WITH PREECLAMPSIA IN ASSOCIATION WITH INCREASED EXPRESSION OF ENDOTHELIAL ICAM-1 AND VASCULAR SMOOTH MUSCLE CELL IL-8

A. Introduction

The previous chapters explored the relationship between oxidative stress, hyperlipidemia, and vascular cell dysfunction using in vitro models. These studies demonstrated increased vascular smooth muscle cell production of IL-8 and increased neutrophil $O_2^{\bullet -}$ production upon treatment with oxidative stress and linoleic acid. These data suggested a mechanism for neutrophil transendothelial migration in preeclampsia based on neutrophil activation and vascular smooth muscle production of IL-8.

Neutrophil infiltration into vascular tissue requires at least three things: 1) vascular expression of a chemotactic agent to attract neutrophils, 2) endothelial expression of adhesion molecules to bind neutrophils to the endothelium, and 3) neutrophil activation. Therefore, to study if neutrophils infiltrate vascular tissue in women with preeclampsia, we used immunohistochemistry with specific antibodies to

evaluate: 1) vascular smooth muscle expression of IL-8, 2) endothelial expression of ICAM-1, and 3) neutrophil activation and infiltration into systemic vascular tissue. We used subcutaneous fat biopsies for this study because subcutaneous fat is a highly vascularized tissue representative of the systemic vasculature.

We evaluated IL-8 because it is a potent chemotactic agent and activator of neutrophils. IL-8 activates neutrophils to enhance their chemotaxis, integrin expression, and respiratory burst activity during inflammation. IL-8 is upregulated in the presence of oxidative stress [144], and our previous in vitro experiments demonstrated that exposing vascular smooth muscle cells to oxidative stress and linoleic acid resulted in increased production of IL-8. Therefore, we reasoned that there would be increased expression of IL-8 by vascular smooth muscle in preeclampsia. This would provide a concentration gradient from the circulation to the vascular smooth muscle for neutrophil transendothelial migration.

We evaluated endothelial expression of ICAM-1 because it is an adhesion molecule that binds integrins (CD11b/CD18) on the neutrophil surface causing them to adhere and flatten onto the endothelium prior to infiltration. Stimuli similar to that for IL-8 increase ICAM-1 expression, such as oxidative stress. ICAM-1 is elevated in the plasma of preeclamptic women [83, 84] and ICAM-1 is upregulated upon treatment of HUVECs with plasma from preeclamptic women [112], suggesting a role for ICAM-1 in the pathogenesis of preeclampsia.

We evaluated neutrophils in preference of other leukocytes because: 1) they are the most abundant of the leukocytes, 2) their numbers increase in pregnancy [1], 3) their

numbers further increase in preeclampsia [121], and 4) they produce toxic substances (ROS, MPO, TX and TNFα), which could be responsible for vasoconstriction and vascular cell dysfunction in women with preeclampsia.

We hypothesized that in women with preeclampsia there would be increased expression of vascular smooth muscle IL-8 and endothelial ICAM-1 coincident with infiltration of neutrophils into maternal systemic vascular tissue. Figure 26 is a schematic representation of our hypothesis.

B. Materials and Methods

i. Study Subjects

Subcutaneous fat biopsies were collected from patients at MCV Hospitals, Virginia Commonwealth University Health System. Fat biopsies were collected at the time of cesarean section from normal pregnant patients (n = 6) and preeclamptic patients (n = 5) or at the time of abdominal or minimally invasive surgery from normal, non-pregnant patients (n = 4). Cesarean sections for normal pregnant women were performed because of previous c-section or secondary to latent herpes simplex virus or fetal malposition. Surgeries for normal non-pregnant women were performed for removal of uterine myomas (fibroids) or for tissue biopsies. The criteria for normal pregnancy were a maternal blood pressure of less than 140/90 mmHg, no proteinuria, non-smoker, and no other complications. The diagnosis of preeclampsia was based on ACOG

recommendations. Mild preeclampsia was defined as hypertension greater than 140/90 mmHg on at least two separate measurements six hours apart and proteinuria greater than 300 mg/24 hours or 1-2 plus dipstick. Severe preeclampsia was defined as hypertension greater than 160/110 mmHg and proteinuria greater than 5,000 mg/24 hours or 3-4 plus dipstick. One preeclamptic patient had gestational diabetes, which was diet controlled. Preeclamptic women were non-smokers. Women in active labor were excluded from the study. The criteria for normal, non-pregnant patients were a blood pressure of less than 140/90 mmHg, non-smoker, and no systemic inflammatory conditions, such as diabetes. Patients were matched for BMI. Informed consent was obtained prior to surgery. This study was approved by the Virginia Commonwealth University Office of Research Subjects Protection.

ii. Collection of Fat

Subcutaneous fat biopsies (approximately 1 cm x 1 cm x 1 cm) were collected at cesarean section or abdominal or minimally invasive surgery. In the operating room fat biopsies were snap-frozen in liquid nitrogen or placed immediately in 10% neutral buffered formalin. The tissue was further processed in the laboratory. Frozen tissue was wrapped in aluminum foil and stored at –70°C until tissue processing. Formalin-fixed samples were cut into smaller pieces, placed in tissue cassettes and returned to 10% neutral buffered formalin for five days. Fixation was confirmed by observation and touch.

Formalin-fixed samples were rinsed in ddH_20 and placed in 100 mM phosphate buffer, pH=7.5 until paraffin-embedding.

iii. Protocol for Frozen Tissue

Frozen tissue was used to perform immunohistochemical staining for IL-8. At the time of sectioning, a frozen biopsy was cut into smaller pieces and allowed to equilibrate to -30°C in a cryotome (Leica CM1100, Leica Microsystems Inc., Allendale, NJ). A small amount of frozen tissue matrix (Tissue-Tek O.C.T. Compound, VWR, Pittsburgh, PA) was placed on the cryotome chuck. As the matrix began to freeze, a piece of frozen tissue was placed on top and covered slowly with additional matrix. Once frozen, the mounted tissue was cut in 20 µm sections and placed on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Slides were warmed quickly to room temperature by pressing the slide against the palm of a gloved hand and fixed in cold acetone (-20°C) for three minutes. Slides were air-dried and placed in a -70°C freezer until staining.

On the day of staining, slides were warmed rapidly to room temperature by pressing the slide against the palm of a gloved hand and placed immediately in 100 mM phosphate buffer saline (0.9% NaCl), pH=7.5 for ten minutes. To quench endogenous tissue peroxidase, tissue sections were blocked with 0.3% H_2O_2 in methanol for ten minutes followed by a five minute wash in phosphate buffered saline.

The ABC technique was used for immunohistochemical staining. This methodology is based on the binding of a preformed avidin and biotin horseradish peroxidase macromolecular complex to a biotinylated antibody, which is bound to the primary antibody and antigen of interest. Visualization was produced by a buffered H_2O_2 and chromogen, 3,3'-diaminobenzidine tetrahydrochloride (DAB), solution.

Immunohistochemical staining was performed at room temperature using VECTASTAIN Elite ABC (Universal) and DAB Substrate Kits (Vector Laboratories, Burlingame, CA) with a ST 5050 automated staining machine (Vision Instruments, Australia). Every step was strictly controlled for each antibody. Each step of the staining cycle was followed by a rinse with phosphate buffered saline (PBS) + 0.05% Tween-20 (3 rinses; 5 minutes) (Gibco, Long Island, New York). For IL-8 staining, all solutions were prepared in 100mM PBS, pH=7.5, except for DAB substrate solution which was prepared in Grade 1 ultrapure H_2O. For ICAM-1 and CD66b staining, except for DAB substrate solution, all solutions were prepared in 100mM phosphate buffer, pH=7.5.

In preparation for staining, slides were covered briefly with PBS + 0.05% Tween-20, pH=7.4 (Gibco, Long Island, New York). Tissue was blocked with horse serum blocking solution for 20 minutes. Tissue was stained with a mouse IgG anti-human monoclonal antibody specific for IL-8 (1:50, BioSource International, Camarillo, CA). The negative control for IL-8 was a mouse IgG monoclonal isotype standard (pre-diluted, Zymed Laboratories, San Francisco, CA). To confirm intact endothelium and identify vascular smooth muscle, tissue was immunostained with a rabbit anti-human monoclonal antibody directed at Factor VIII (1:600, Zymed Laboratories, San Francisco, CA) and a

mouse anti-human monoclonal antibody directed at α-smooth muscle actin (1:6000, Sigma, St. Louis, MO). Their staining also was used to identify vessels and determine the location of IL-8, ICAM-1, and CD66b staining. Next the tissue sections were covered with biotinylated antibody (anti-mouse/anti-rabbit IgG made in horse) for 30 minutes. The enzyme conjugate (Vectastain ABC reagent) was incubated on the tissue for 30 minutes. The protein-antibody complex was localized with a DAB substrate chromogen mixture for five minutes.

Tissue was counterstained with filtered alcian blue stain, pH=2.5 (1% alcian blue dye + 3% acetic acid) for five minutes followed by rinsing in tap H_2O [157]. Slides then were dipped in methyl green (Vector Laboratories, Burlingame, CA), placed on a metal tray, and incubated for three minutes in a 60°C oven. Sections were rinsed in Grade 1 ultrapure water for one minute and dipped five times in 0.05% acetic acid in acetone. Sections were dehydrated (50%, 85%, 95%, 95%, 100%, 100%; 2 minutes each) and cleared two times for 3 minutes in Histoclear (National Diagnostics, Atlanta, Georgia). Slides were mounted with VectaMount (Vector Laboratories, Burlingame, CA) and covered with a coverslip.

iv. Protocol for Formalin-fixed Tissue

Formalin-fixed tissue was used for ICAM-1 and CD66b staining. Tissue was dehydrated in a graded alcohol series (70%, 80%, 95%, 95%, 100%, 100%, 100%), cleared in CitriSolv (Fisher Scientific, Malvern, PA), and paraffin-embedded overnight

using an automated tissue processor (Shandon Citadel 2000 Tissue Processor, Shandon Scientific Limited, Cheshire, England). The next day paraffin-embedded tissue was placed in embedding rings and covered with additional paraffin.

Tissue was cut in 8 μm sections using a microtome (820 Spencer Microtome, American Optical Company). Sections were floated on a preheated 42°C ddH_2O bath (Flotation Bath Model 135, Fisher Tissue Prep, Fisher Scientific, Malvern, PA), separated into tissue sections of three, and placed on Superfrost Plus glass slides (Fisher Scientific, Malvern, PA). Slides were dried on a 37°C slide warmer (C.S. & E Slide Warmer No. 26020, Clinical Scientific Equipment Co., Melrose Park, Illinois), and then placed in a 37°C oven overnight.

On the day of staining, tissue sections were cleared three times with Histoclear (National Diagnostics, Atlanta, Georgia) to remove paraffin (five minutes each) and then hydrated in a graded alcohol series (100%, 100%, 95%, 95%, 85%, 50%, 0%; two minutes each). Tissue sections were placed in 100 mM phosphate buffer, pH=7.5 for ten minutes. To quench endogenous tissue peroxidase, the tissue was blocked with 0.3% H_2O_2 in methanol for 30 minutes followed by washes in 100 mM phosphate buffer, pH=7.5 (5 minutes) and Grade 1 ultrapure water (6 minutes).

Antigen retrieval was performed with microwave citrate pretreatment. Five slides were placed upright in a polypropylene Coplin jar filled with citrate buffer (10 mM; pH = 6.0) and microwaved at 90°C for 10 minutes at 70% power. Slides remained in the covered container for 30 minutes, at which time they were transferred to 100 mM phosphate buffer, pH=7.5 for a minimum of five minutes prior to staining.

Tissue was incubated for 30 minutes with a primary antibody. Primary antibodies included: 1) a mouse IgG anti-human monoclonal antibody against ICAM-1 (CD54, 1:100, Zymed Laboratories, San Francisco, CA), 2) a mouse IgG monoclonal isotype standard used as a negative control for ICAM-1 (pre-diluted, Zymed Laboratories, San Francisco, CA), 3) a mouse IgM anti-human monoclonal antibody specific for CD66b (1:500, BD BioSciences, San Diego, CA), and 4) a mouse IgM monoclonal isotype standard used as a negative control for CD66b (1:500, BD BioSciences, San Diego, CA). CD66b is a granulocyte-specific glycosylphosphatidylinositol-linked membrane antigen that is upregulated and released upon granulocyte activation. CD66b is involved in granulocyte phagocytosis, cell adherence, and chemotaxis [158] and it promotes adhesion of granulocyte β_2 integrins (CD11b/CD18) to endothelial cells [159, 160]. Thus, CD66b staining not identified granulocytes, but diffuse CD66b staining also indicated secretion by activated granulocytes. Granulocytes from a normal non-pregnant individual were isolated by density centrifugation and used as a positive control for CD66b. Isolated monocytes and lymphocytes were used as negative controls. Granulocytes are comprised of neutrophils, eosinophils and basophils. Neutrophils comprise 96% of the granulocyte population. In addition, neutrophils respond to tissue inflammation, whereas eosinophils and basophils respond to parasitic diseases and various forms of allergy. Therefore, our data primarily represent neutrophils.

Tissue sections also were stained with a rabbit IgG anti-human antibody specific for Factor VIII (1:400) and a mouse IgG anti-human antibody directed at α-smooth muscle actin (1:3000). To increase cross-reactivity between primary and secondary

antibodies, since CD66b was an IgM, the concentration of the biotinylated antibody (anti-mouse/anti-rabbit IgG made in horse) was doubled. Slides were incubated for 30 minutes. The remainder of the staining methodology for formalin-fixed tissue was identical to that for frozen tissue.

v. Data Analysis

To quantify staining, an observer unaware of the patient's identity scanned the middle tissue section of each slide in a systematic manner. Lumen width was measured for each vessel and vessels were categorized as small (≤10 μm), medium (10 μm-39 μm) or large (≥40 μm). Vessels between 10 μm and 200 μm, which represent resistance-sized vessels, were analyzed for staining. For each patient, approximately 34 vessels were evaluated for IL-8 staining and 92 vessels for ICAM-1 staining. Vessels staining for IL-8 and ICAM-1 were graded using a visual score ranging from zero to four (from absent to intense) and also were evaluated by density measurements using image analysis software (Image Pro Plus, Media Cybernetics, Silver Spring, MD). The visual score was correlated to density measurements to verify objectivity of the visual score.

For CD66b staining, an average of 126 vessels were analyzed for each patient. Each vessel was graded using a visual score ranging from zero to four based on overall staining intensity and neutrophil infiltration. Since the CD66b protein is secreted from activated neutrophils, diffuse staining for CD66b also was evaluated for staining density using image analysis software. In addition to visual scores and density measurements,

CD66b staining of neutrophils was recorded as present within the lumen, along the endothelium, within the intima and vascular smooth muscle, and present on the outside of the vessel wall. The number of stained neutrophils was counted in each of these locations.

vi. Statistical Analysis

Kruskal-Wallis and Chi square were used to analyze visual score data for staining. Frequency distributions were completed for visual scores. For density measurements, one-way ANOVA was performed and Student-Newman-Keuls post-hoc test was used to determine differences between the patient groups. Regression analysis was performed to correlate visual scores with density measurements. Bar graph data are reported as mean ± SE.

For CD66b staining data, statistical analysis also was performed for the percent of vessels with staining in certain vessel locations: 1) along the endothelium, 2) within the intima, and 3) on the outside of the vessel, and for the number of infiltrated neutrophils per stained vessel. ANOVA was performed and Student-Newman-Keuls post-hoc test was used to analyze differences between patient groups. Kruskal-Wallis and Dunn's post hoc test were used when variances were not equal. Bar graph data are reported as mean ± SE.

C. Results

i. Subjects

The clinical data for normal non-pregnant, normal pregnant, and preeclamptic groups are summarized in Table 3. According to criteria, systolic and diastolic blood pressures and proteinuria were significantly greater for preeclamptic patients than for other groups. The preeclamptic group also was characterized by significantly lower parity, gestational age, and infant birth weight as compared to normal pregnant patients. Maternal age was not significantly different between normal pregnant and preeclamptic groups, and there was no difference in BMI among groups.

ii. Immunohistochemical Staining

Vessels of preeclamptic patients had intense IL-8 staining, whereas normal pregnant patients and normal non-pregnant patients had light or no IL-8 staining (Visual score: 2.8 ± 0.1 vs. 0.7 ± 0.1 vs. 0.7 ± 0.1, respectively, $P<0.001$, Figure 27). Optical density (OD) measurements confirmed that preeclamptic patients had significantly greater IL-8 staining as compared to normal pregnant or normal non-pregnant patients (126.0 ± 3.7 OD vs. 70.2 ± 2.4 OD vs. 67.5 ± 2.8 OD, respectively, $P<0.001$, Figure 28). Chi square analysis verified higher visual scores for preeclamptic patients ($P<0.0001$)

(Figure 29). The visual scores were highly correlated with the density measurements, r = 0.99 (Figure 30).

Preeclamptic patients had intense IL-8 staining in vascular smooth muscle cells, as well as in endothelial cells. In contrast, normal pregnant patients had light IL-8 staining in endothelial cells and vascular smooth muscle cells, and normal non-pregnant patients had little or no IL-8 staining of the vessel (Figure 31). Figure 32 shows intense staining for IL-8 in the vascular smooth muscle of preeclamptic patients, which is verified by co-localization of α-smooth muscle actin.

ICAM-1 was constitutively expressed in all groups. The visual scores for ICAM-1 staining were greater for preeclamptic patients than normal pregnant or normal non-pregnant patients (2.5 ± 0.1 vs. 1.9 ± 0.2 vs. 2.2 ± 0.1, respectively, $P<0.05$, Figure 33). Although the visual scores for ICAM-1 staining were statistically significantly greater for preeclamptic patients than normal pregnant or normal non-pregnant patients, the difference in staining was minimal and probably does not reflect physiological differences. The density measurements also showed that ICAM-1 expression was constitutive among all groups. Preeclamptic patients had more intense staining for ICAM-1 as compared to normal pregnant patients, but less staining than normal non-pregnant patients (116.7 ± 2.9 OD vs. 96.9 ± 7.8 OD vs. 136.2 ± 3.1 OD, respectively, $P<0.01$, Figure 34). Chi square analysis indicated higher visual scores for preeclamptic patients ($P<0.0001$) (Figure 35). There was a strong correlation between visual scores and density measurements, r = 0.95 (Figure 36).

In women with preeclampsia, ICAM-1 staining was present not only on the endothelium, but also on the vascular smooth muscle (Figures 37 and 38). Although there was ICAM-1 staining of endothelium and vascular smooth muscle in normal pregnant women, the staining was not as intense, nor as diffuse, as in preeclamptic women (Figure 37). Staining for ICAM-1 was localized to endothelium of normal non-pregnant women (Figure 37).

A visual score, representing intensity of CD66b staining, as well as quantity of neutrophil infiltration, was significantly greater for preeclamptic than normal pregnant patients or normal non-pregnant patients (1.9 ± 0.07 vs. 0.3 ± 0.03 vs. 0.3 ± 0.1, respectively, $P<0.01$, Figure 39). The visual score was verified by density measurements, which also indicated more intense CD66b staining for preeclamptic patients as compared with normal pregnant patients or normal non-pregnant patients (126 ± 2 OD vs. 68 ± 2 OD vs. 75 ± 5 OD, respectively, $P<0.01$, Figure 40). Chi square analysis indicated higher visual scores for preeclamptic patients ($P<0.0001$) (Figure 41). There was a strong correlation between visual scores and density measurements, $r = 0.90$ (Figure 42).

The percentage of vessels stained for CD66b was significantly greater for preeclamptic patients than normal pregnant patients or normal non-pregnant patients (76 ± 8% vs. 27 ± 7% vs. 23 ± 6%, respectively, $P<0.01$, Figure 43). Of these vessels, there was greater staining for CD66b in the preeclamptic patients in all vessel locations, except in the lumen. In preeclamptic patients as compared with normal pregnant patients and normal non-pregnant patients, there was greater adherence and flattening of neutrophils along the endothelium (55 ± 7% vs. 19 ± 6% vs. 15 ± 4%, respectively, $P<0.05$, Figure

44), infiltration into the intima (52 ±11% vs. 6 ± 2% vs. 2 ± 1%, respectively, P<0.01, Figure 45) and number of neutrophils on the outside of the vessel (26 ± 7% vs. 3 ± 1% vs. 2 ± 2%, respectively, P<0.05, Figure 46). The total number of neutrophils adhered to endothelium, infiltrated into intima, and present on the outside of the vessel per stained vessels was also greater for preeclamptic patients than normal pregnant patients or normal non-pregnant patients (7.3 ± 0.8 vs. 1.9 ± 0.4 vs. 1.3 ± 0.3 per 8 μm section of vessel, respectively, P<0.001, Figure 47). Table 4 summarizes these data.

CD66b specific staining of neutrophils was verified by staining of leukocytes isolated by Histopaque density centrifugation of whole blood. CD66b stained neutrophils, but not monocytes or lymphocytes (Figure 48). Representative sections of vessels are shown for CD66b staining of neutrophils in normal non-pregnant patients, pregnant patients, and preeclamptic patients (Figure 48). Figure 49 shows representative examples of neutrophil adherence and flattening on to endothelium, infiltration into the intima, and presence outside of the vessel wall of vessels from women with preeclampsia.

D. Discussion

Staining for IL-8, a potent neutrophil chemotactic agent, was significantly greater in preeclamptic women as compared to normal pregnant or normal non-pregnant women. IL-8 staining was observed on vascular smooth muscle, in addition to endothelium, in women with preeclampsia. This finding is significant because it demonstrates inflammation of vascular smooth muscle in preeclampsia. Increased expression of

vascular smooth muscle IL-8 in preeclampsia establishes a concentration gradient for IL-8 from the circulation to the vascular smooth muscle. Since neutrophils migrate along a concentration gradient to IL-8 [161], these data provide a mechanism for transendothelial migration of neutrophils to the vascular smooth muscle in preeclampsia.

Vascular ICAM-1 expression was significantly greater in preeclamptic patients than normal pregnant or normal non-pregnant patients. ICAM-1 was constitutively expressed on the endothelium in all groups, but in preeclamptic patients ICAM-1 was expressed intensely on vascular smooth muscle as well as on endothelial cells. This is further evidence, corroborating the IL-8 data, that there is inflammation of vascular smooth muscle in preeclampsia. Increased endothelial ICAM-1 expression provides evidence that endothelial cells are a likely source of elevated plasma levels of ICAM-1 in preeclampsia, as reported by other investigators [83, 84, 162]. They are also consistent with an in vitro study that demonstrated increased endothelial cell ICAM-1 expression upon incubation with preeclamptic plasma [112].

The percentage of vessels stained for CD66b was significantly greater in women with preeclampsia than in normal pregnant or normal non-pregnant women. There were significantly more neutrophils adhered and flattened along the endothelium, within the intima and present on the outside of the vessel wall in women with preeclampsia as compared to controls. The percentage of neutrophils present within different portions of the vessel correlated with an overall greater number of neutrophils per vessel. Diffuse vessel staining also was present in women with preeclampsia and, most likely, reflects secretion of CD66b from activated neutrophils.

These new data may explain previous findings in the field of preeclampsia. A study by Roggensack et al. showed increased expression of nitrotyrosine, suggestive of $ONOO^-$ formation, a strong prooxidant, in the vasculature of women with preeclampsia [110]. A simultaneous increase in the expression of endothelial NOS and a decrease in the expression of SOD also were observed. Our findings may explain the source of $O_2^{\bullet -}$ for the formation of $ONOO^-$ because the rapid interaction of neutrophil $O_2^{\bullet -}$ with endothelial NO• in the presence of deficient SOD would produce $ONOO^-$. Our data in conjunction with Roggensack's data strongly suggest that there is localized systemic oxidative stress throughout the maternal vasculature, which results in vascular lipid peroxidation leading to vascular inflammation and dysfunction.

Chappell et al. reported that vitamin E and vitamin C supplementation significantly decreased the incidence of preeclampsia in women at increased risk [48]. A decrease in the incidence of preeclampsia was associated with a decrease in urinary isoprostane, a marker of oxidative stress [49]. These data implicate oxidative stress in the pathogenesis of preeclampsia by using antioxidants to prevent development of the disorder. Since oxidative stress upregulates many components involved in neutrophil transendothelial migration, the prophylactic use of antioxidants during pregnancy may inhibit mechanisms involved in transendothelial migration of neutrophils to prevent development of preeclampsia.

Neutrophil adherence and infiltration were evident in resistance-sized vessels. Neutrophils are known to produce thromboxane, a potent vasoconstrictor, in the presence of oxidative stress [163]. Hypertension, therefore, may result as activated neutrophils deliver

thromboxane directly to the vascular smooth muscle. Vascular disruption and oxidative stress caused by the products of adhered and infiltrated neutrophils would also affect endothelial cell integrity to proteins [164], resulting in edema in the systemic circulation and proteinuria in the kidney.

In summary, the present study is the first to provide in vivo evidence of vascular smooth muscle inflammation and neutrophil infiltration into maternal systemic vascular tissue in preeclampsia. These new data suggest that there is total "vascular cell dysfunction", which includes vascular smooth muscle cells, in addition to endothelial cells, in preeclampsia. These data could explain the clinical symptoms of hypertension, proteinuria, and edema, and they suggest novel treatments for preeclampsia based on neutralizing antibodies to IL-8 or cell adhesion molecules.

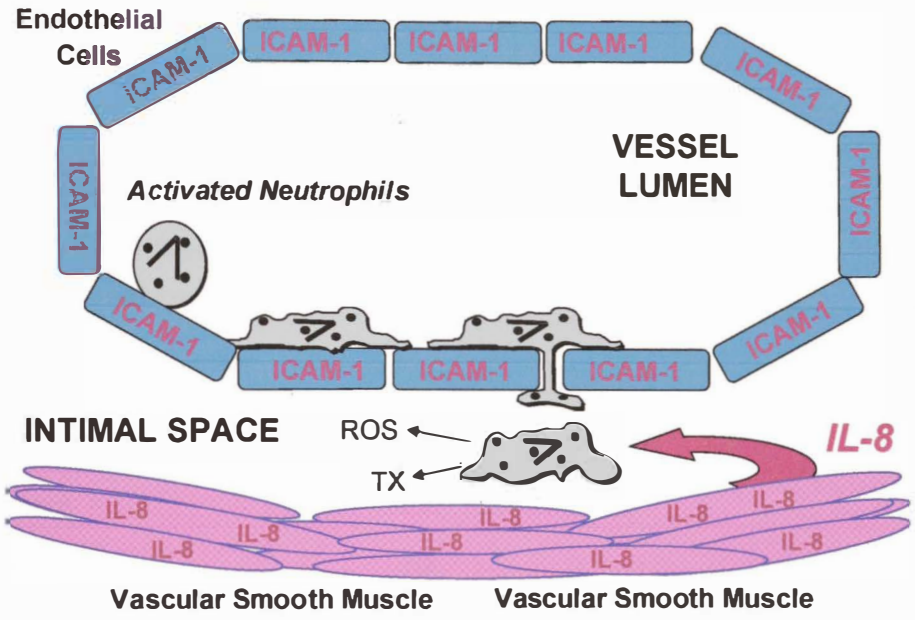


Figure 26. A visual representation of our hypothesis of increased expression of vascular smooth muscle IL-8 and endothelial ICAM-1 coincident with infiltration of neutrophils into maternal systemic vascular tissue in women with preeclampsia.

Activated neutrophils adhere and flatten onto endothelial cells by binding to ICAM-1. In response to IL-8 produced by vascular smooth muscle cells, neutrophils infiltrate into the intimal space. We speculate that neutrophil production of ROS, MPO or TX could result in vasoconstriction and vascular dysfunction.

Table 3. Clinical data for patient groups.

	Normal Non-pregnant (n = 4)	Normal Pregnant (n = 6)	Preeclamptic (n = 5)
Maternal Age	38.5 ± 5.4	27.5 ± 4.5	24.6 ± 5.5^{+}
Pre-pregnancy BMI	31.8 ± 4.4	26.6 ± 6.5	29.2 ± 7.9‡
Systolic Blood Pressure (mm Hg)	126.0 ± 9.2	119.5 ± 12.6	178.0 ± 12.8**
Diastolic Blood Pressure (mm Hg)	79.3 ± 9.2	76.7 ± 8.2	111.0 ± 12.3**
Proteinuria (mg / 24 h)	ND	ND	885 ± 374.8 (n = 2)
Dipstick	ND	ND	3.7 ± 0.6 (n = 3)
Parity	NA	2.8 ± 1.2	1.2 ± 1.1*
Gestational Age (wk)	NA	39.1 ± 0.8	33.7 ± 3.8**
Infant Birth Weight (g)	NA	3291 ± 533.8	2004 ± 1027*

Values are mean ± SD. ND indicates not determined. NA indicates not applicable.
*P<0.05, **P<0.01 compared with normal pregnant or normal non-pregnant.
$^{+}$ indicates no difference between preeclamptic and normal pregnant.
‡ indicates no difference among groups.

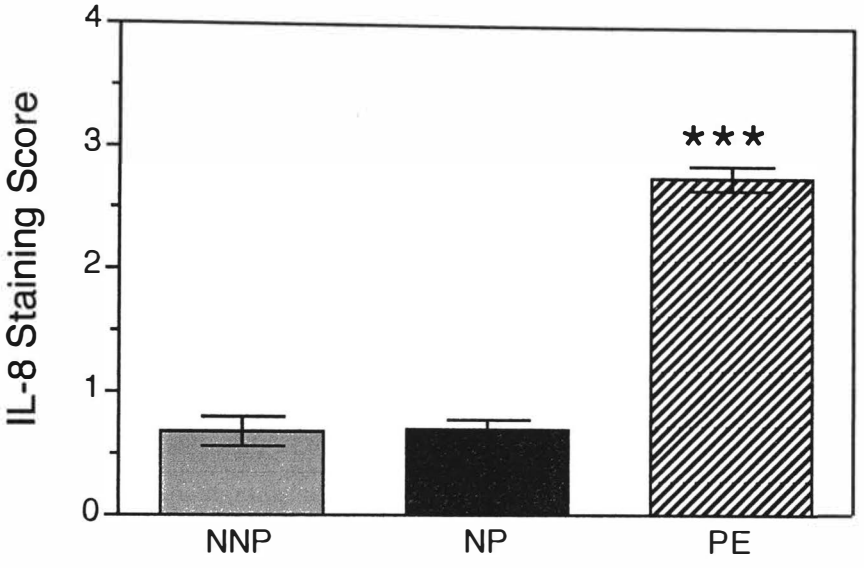


Figure 27. Summarized visual score results for IL-8 staining.

Preeclamptic patients had significantly greater IL-8 staining as compared to normal non-pregnant or normal pregnant patients. The visual score for preeclamptic patients reflected staining of both vascular smooth muscle cells and endothelial cells, whereas the visual scores for normal non-pregnant and normal pregnant reflected staining primarily of endothelial cells. ***$P < 0.001$ (NNP = 1, NP = 4, PE = 3)

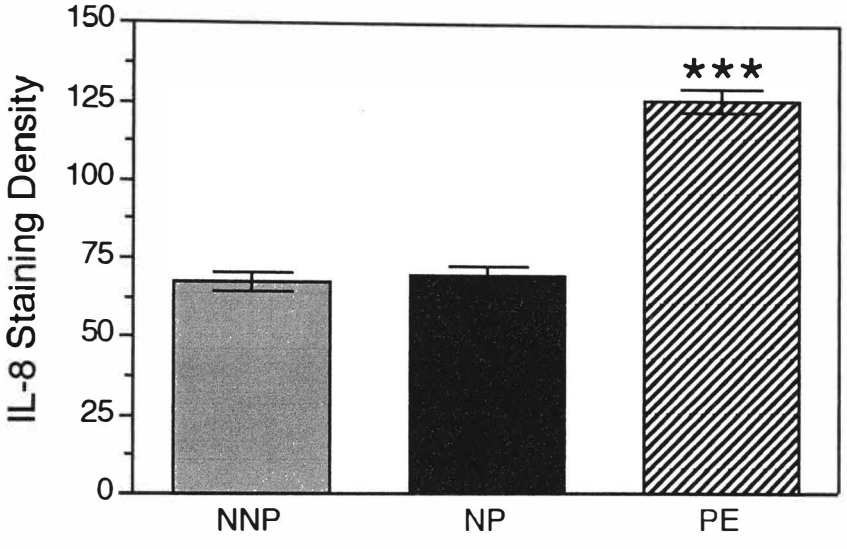


Figure 28. Summarized density measurements for IL-8 staining.

Density of IL-8 staining in preeclamptic patients was significantly greater than in normal non-pregnant or normal pregnant patients. *** $P < 0.001$ (NNP = 1, NP = 4, PE = 3)

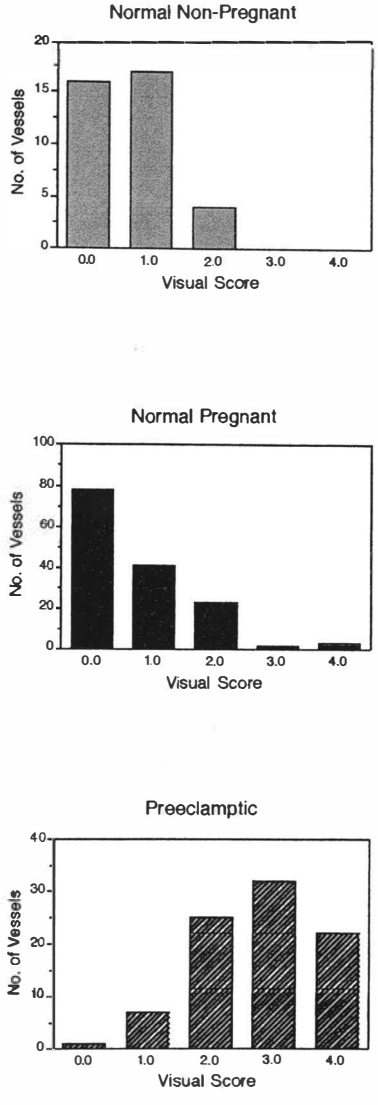


Figure 29. Frequency distributions of visual scores for IL-8 staining.

Preeclamptic patients had more vessels with higher visual scores for IL-8 staining than normal non-pregnant or normal pregnant patients. $\chi^2 < 0.0001$

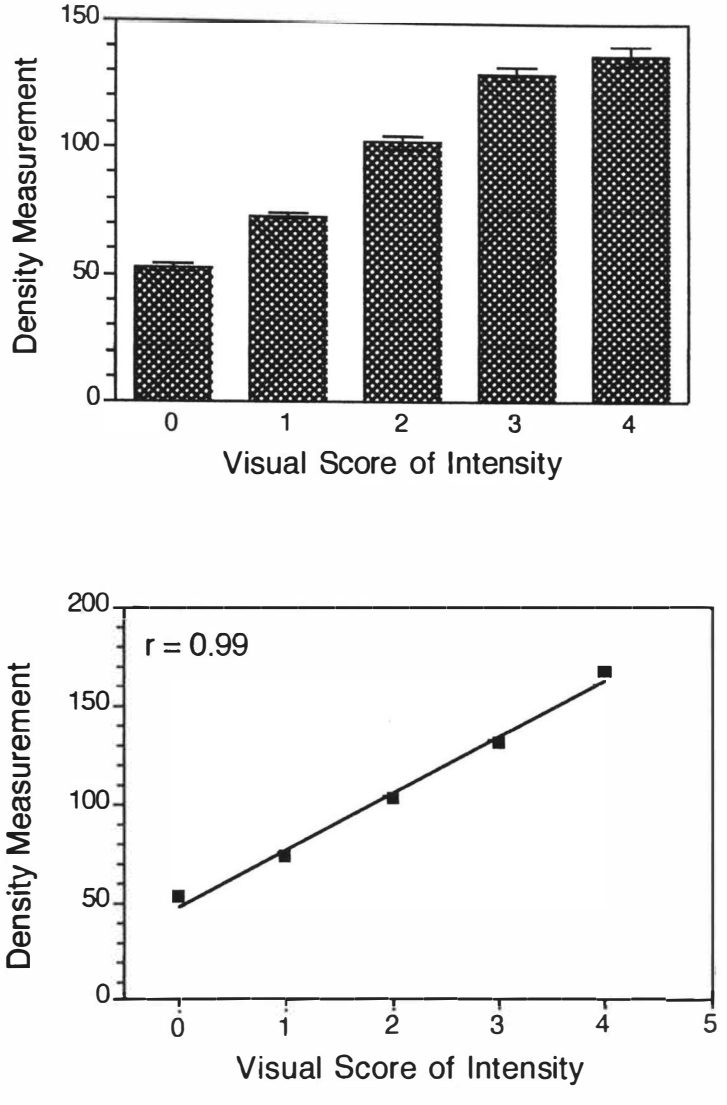


Figure 30. Correlation between visual scores and density measurements for IL-8 staining.

There was a positive correlation ($r = 0.99$) between visual scores and density measurements. These data show that visual scoring was objective and precise.

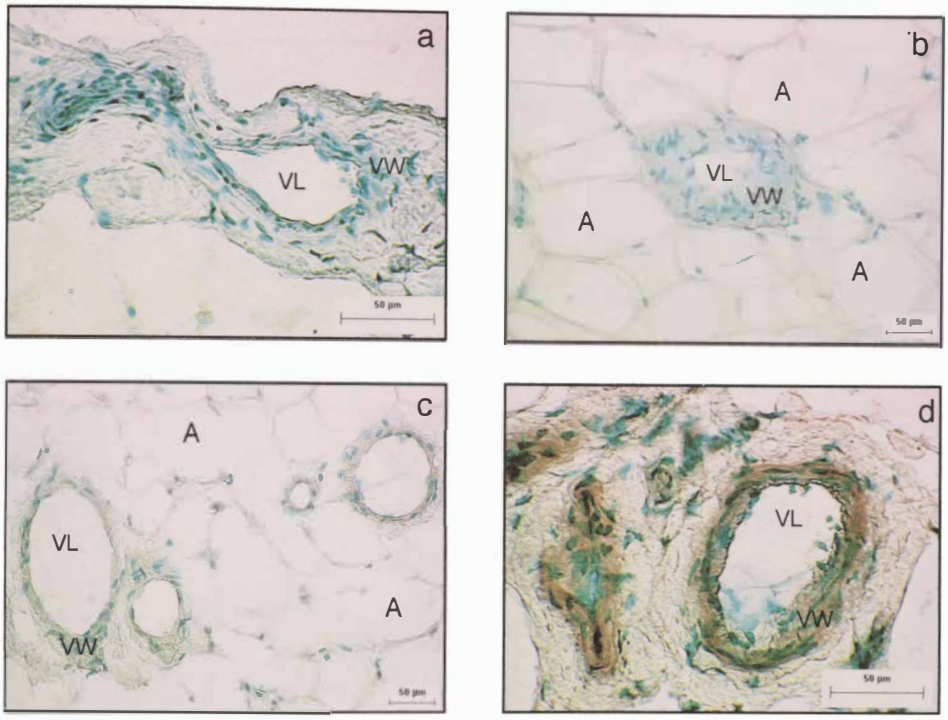


Figure 31. IL-8 immunohistochemical staining of vessels in subcutaneous fat.

a) IgG negative control, x600, b) Normal non-pregnant patient showing no vessel staining, x300, c) Normal pregnant patient showing light brown vessel staining, x300, d) Preeclamptic patient showing intense brown vessel staining, x600.

(A- adipocyte, VL- vessel lumen, VW- vessel wall)

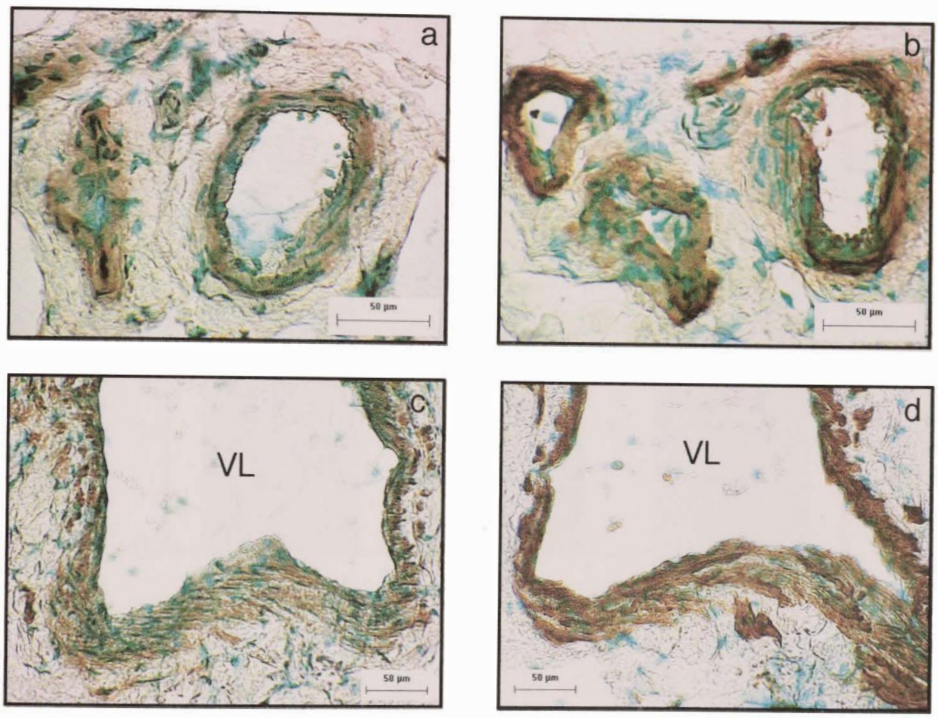


Figure 32. IL-8 immunohistochemical staining (panels a and c) contrasted with staining for α-smooth muscle actin (panels b and d) in preeclamptic patients.

IL-8 staining is clearly evident in the vascular smooth muscle of these preeclamptic patients. The longitudinal arrangement of the vascular smooth muscle is evident by IL-8 staining in panel c. a) IL-8, x600, b) α-smooth muscle actin, x600, c) IL-8, x400, d) α-smooth muscle actin, x400.

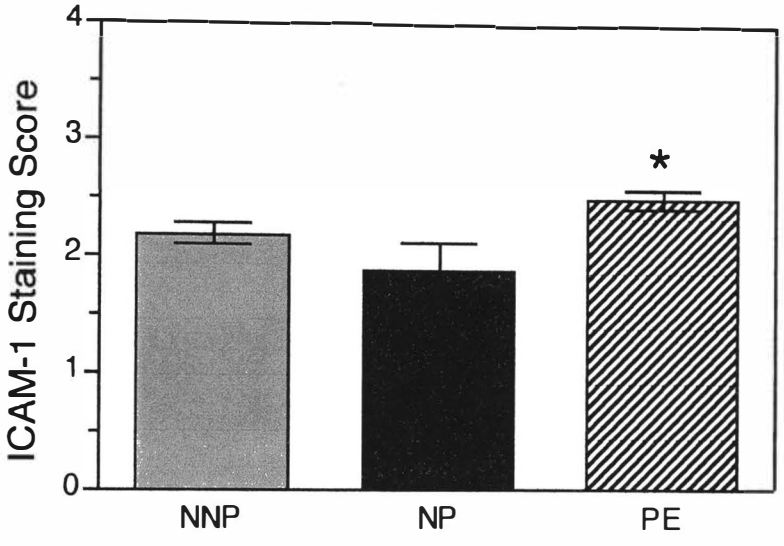


Figure 33. Summarized visual score results for ICAM-1.

ICAM-1 was constitutively expressed in all groups. Preeclamptic patients had significantly greater ICAM-1 staining as compared to normal non-pregnant or normal pregnant patients, primarily due to staining in vascular smooth muscle cells, as well as endothelial cells. There was no difference for ICAM-1 staining between normal non-pregnant and normal pregnant patients. *$P < 0.05$

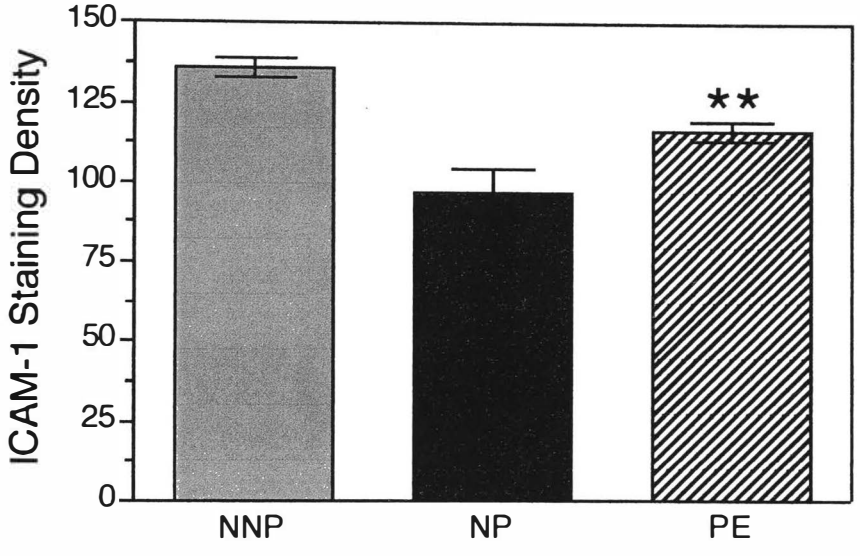


Figure 34. Summarized density measurements for ICAM-1.

Density of ICAM-1 staining in preeclamptic patients was significantly greater than in normal pregnant patients and significantly less than in normal non-pregnant patients. Staining in normal non-pregnant patients was limited to the endothelium, whereas in the normal pregnant patients and especially in the preeclamptic patients, staining was present in vascular smooth muscle, as well as endothelium. Density measurement, as opposed to visual score, did not reflect the spread of staining to vascular smooth muscle cells.
**$P < 0.01$

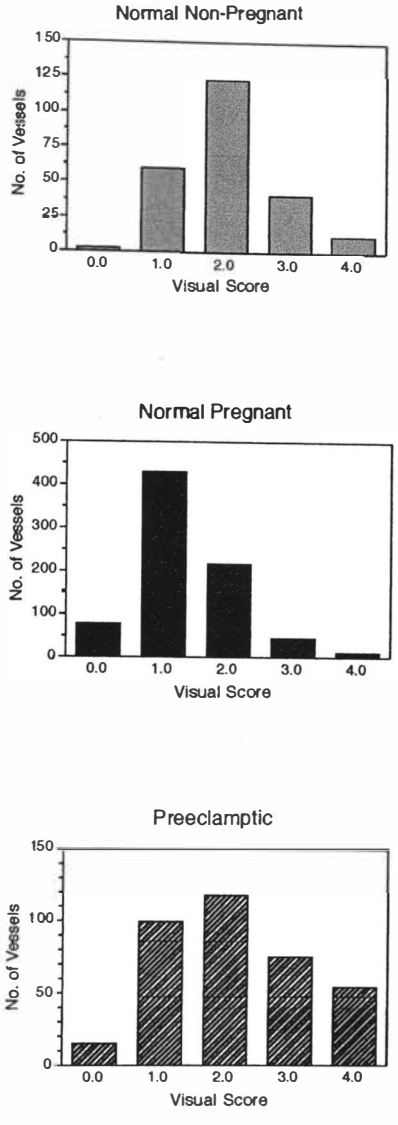


Figure 35. Frequency distributions of visual scores for ICAM-1 staining.

Preeclamptic patients had more vessels with higher visual scores for ICAM-1 staining than normal non-pregnant or normal pregnant patients. $\chi^2 < 0.0001$

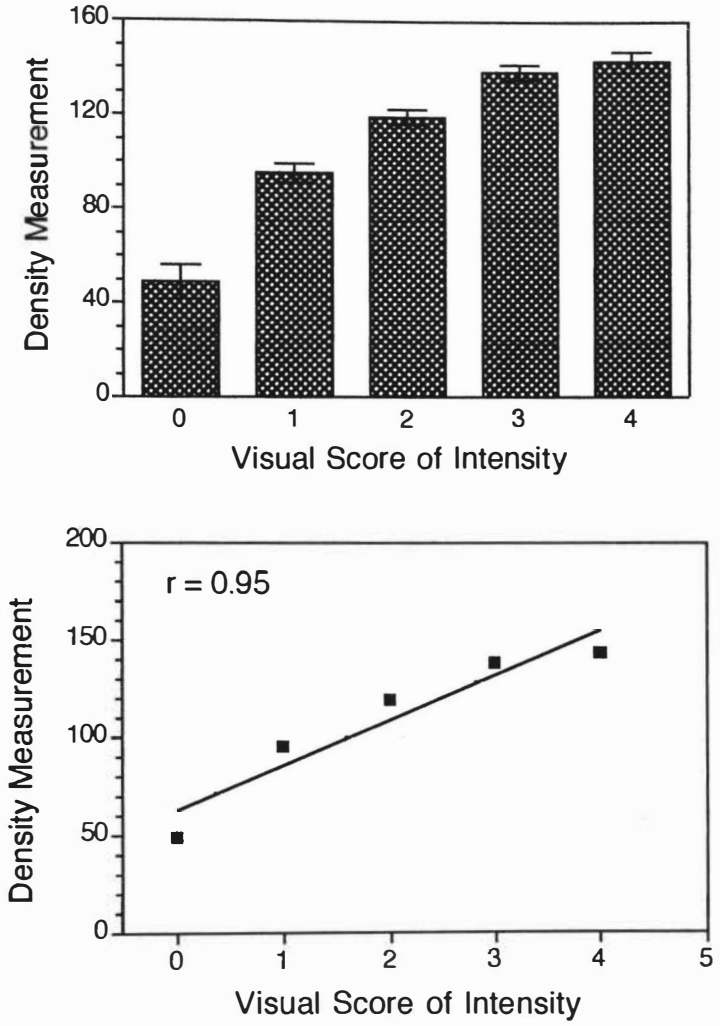


Figure 36. Correlation between visual scores and density measurements for ICAM-1 staining.

There was a positive correlation ($r = 0.95$) between visual scores and density measurements. These data show that visual scoring was objective and precise.

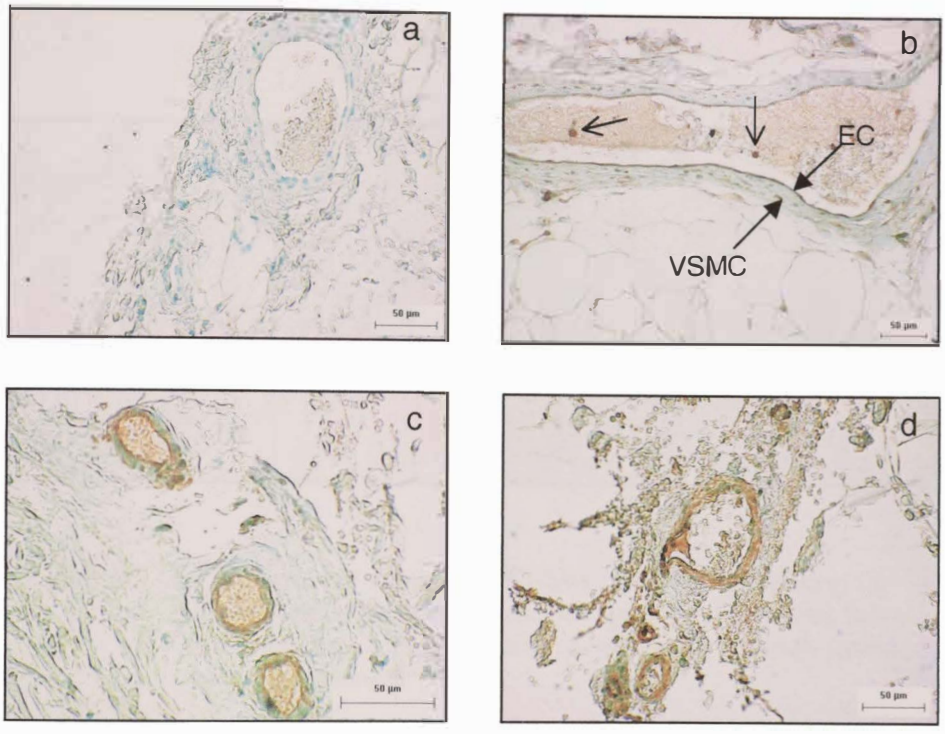


Figure 37. ICAM-1 staining of vessels in subcutaneous fat.

a) IgG Negative control, x400, b) Normal non-pregnant patient showing brown staining of the endothelium lining the vessel lumen, but not of the vascular smooth muscle. Leukocytes also express ICAM-1 and some are stained in the lumen (thin arrows), x300, c) Normal pregnant patient showing brown staining of the endothelium, as well as light staining of the vascular smooth muscle, x600, d) Preeclamptic patient showing intense brown staining of endothelium, as well as vascular smooth muscle, x400.

(EC- endothelial cells, VSMC- vascular smooth muscle cells)

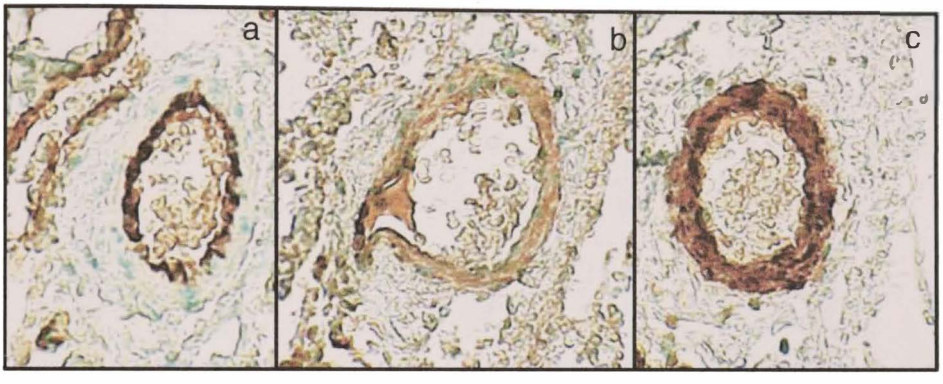


Figure 38. Comparison of staining for ICAM-1 (panel b) with staining for Factor VIII delineating endothelium (panel a) and staining for α-smooth muscle actin delineating vascular smooth muscle (panel c) in a preeclamptic patient.

Staining for ICAM-1 is clearly evident in the vascular smooth muscle, as well as in the endothelium, x400.

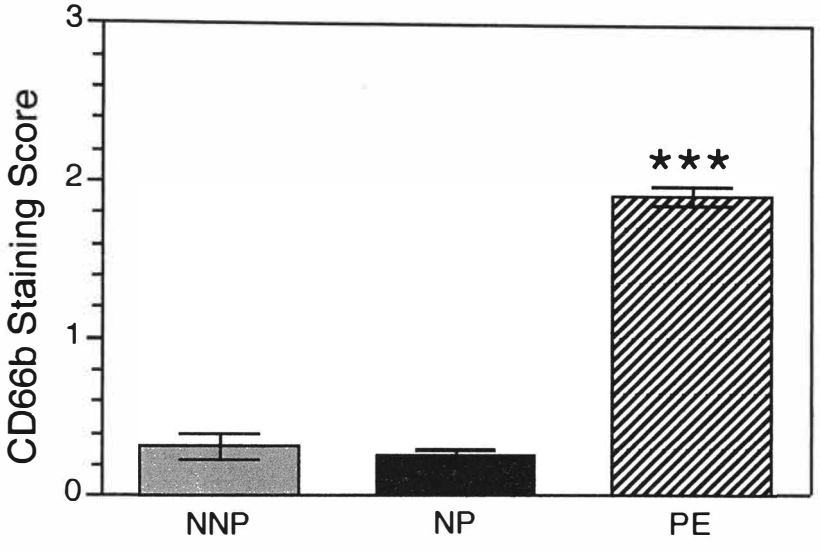


Figure 39. Summarized visual score results for CD66b staining.

Preeclamptic patients had significantly greater CD66b staining as compared to normal non-pregnant or normal pregnant patients. The visual score for CD66b was based on both staining intensity and quantity of neutrophil infiltration.
***$P < 0.001$

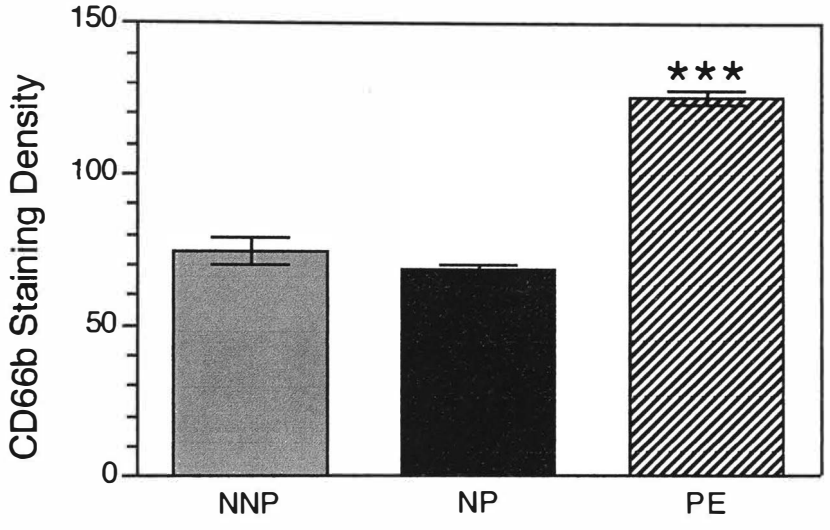


Figure 40. Summarized density measurements for CD66b staining.

Density of CD66b staining was significantly greater for preeclamptic patients as compared to normal non-pregnant or normal pregnant patients. ***$P < 0.001$

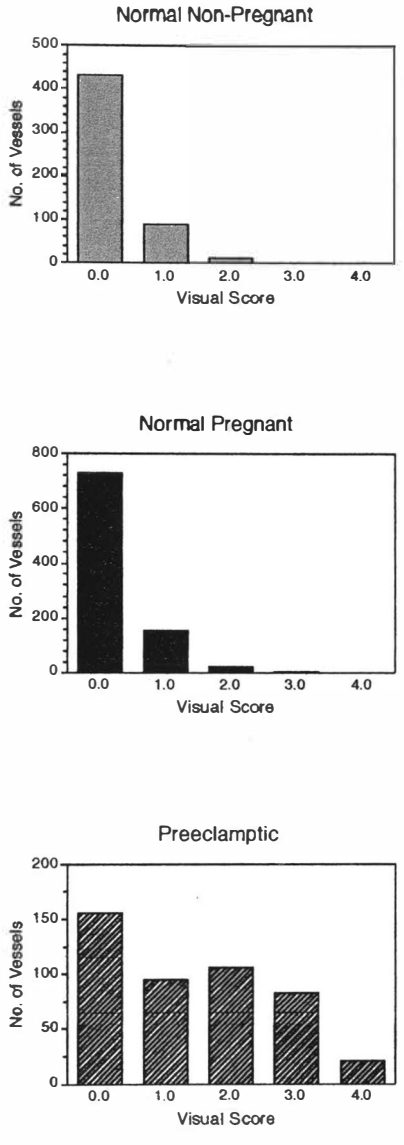


Figure 41. Frequency distributions of visual scores for CD66b staining.

Preeclamptic patients had more vessels with higher visual scores of CD66b staining than normal non-pregnant or normal pregnant patients. $\chi^2 < 0.0001$

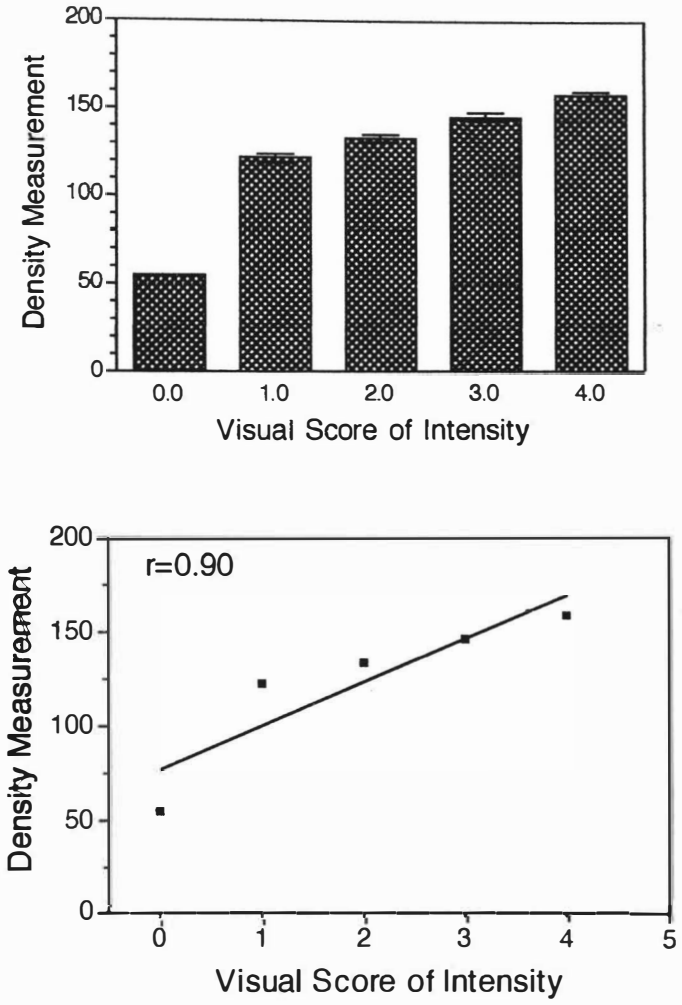


Figure 42. Correlation between visual scores and density measurements for CD66b staining.

There was a positive correlation (r = 0.90) between visual scores and density measurements. These data show that visual scoring was objective and precise.

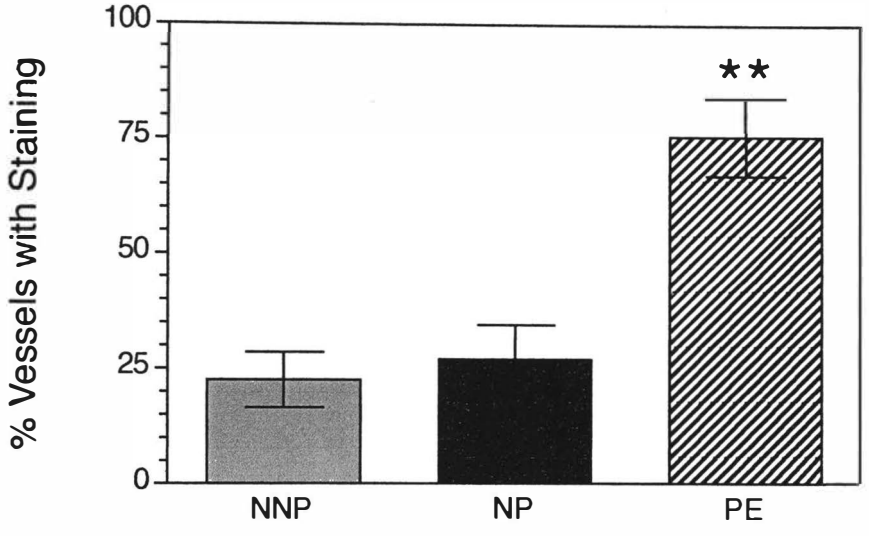


Figure 43. Percent of vessels stained with CD66b.

A mean of 76% of the vessels in preeclamptic patients stained for CD66b as compared to 27% for normal pregnant patients and 23% for normal non-pregnant patients. **P < 0.01

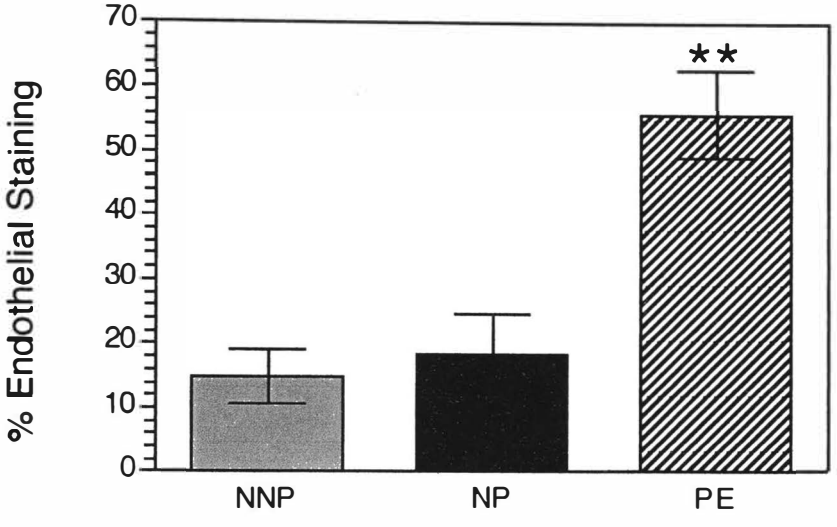


Figure 44. Percent of vessels with neutrophils adhered and flattened onto endothelial cells.

Over half of the vessels of preeclamptic patients had neutrophils adhered and flattened to the endothelium as compared to 19% for normal pregnant patients and 15% for normal non-pregnant patients. **P < 0.01

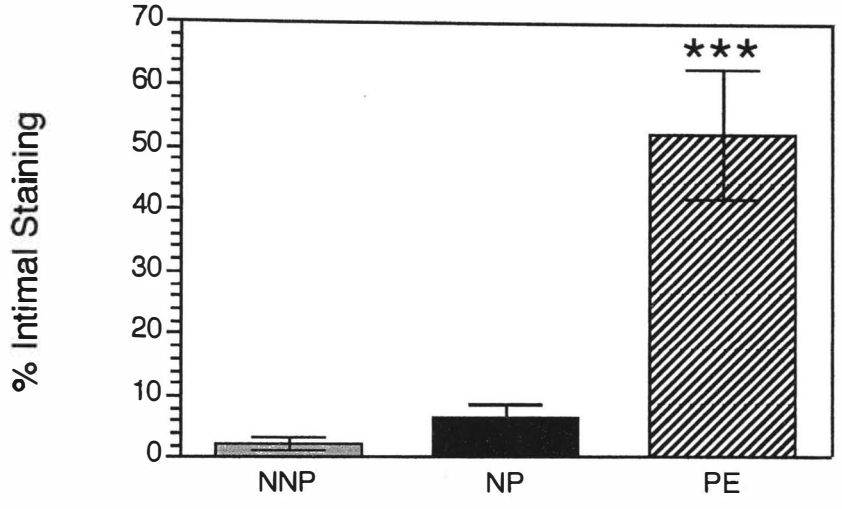


Figure 45. Percent of vessels with neutrophils infiltrated into the intimal space.

Over 50% of the vessels of preeclamptic patients showed neutrophil infiltration to the intima as compared to 6% for normal pregnant patients and 2% for normal non-pregnant patients. *** $P < 0.001$

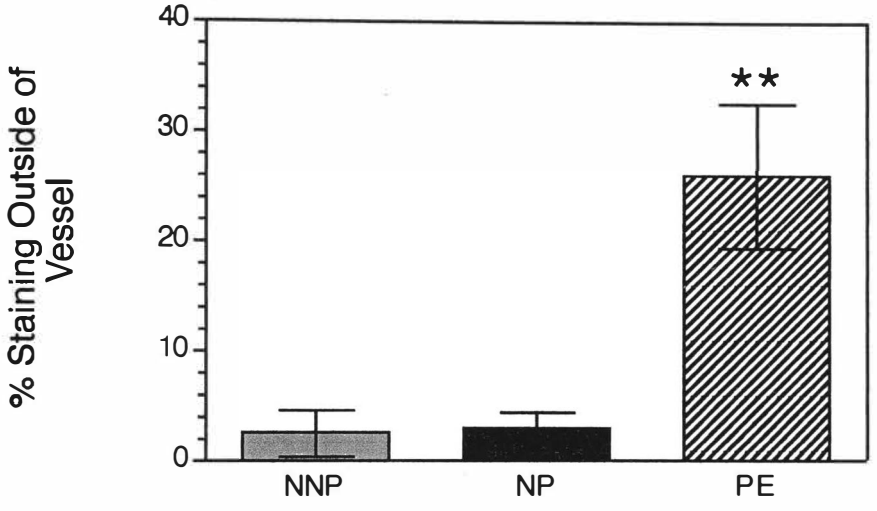


Figure 46. Percent of vessels with neutrophils present on the outside of the vessel.

Twenty-six percent of the vessels of preeclamptic patients had neutrophils present on the outside of the vessel as compared to 3% for normal pregnant patients and 2% for normal non-pregnant patients. **P < 0.01

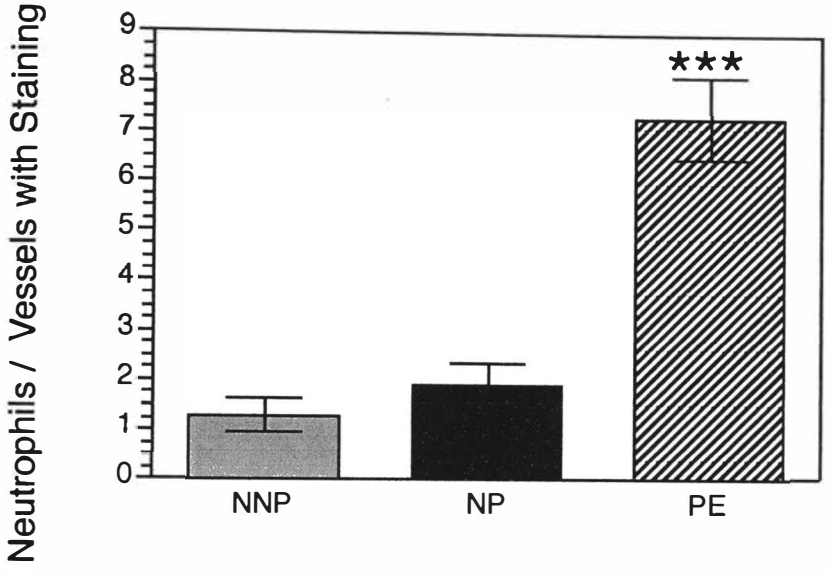


Figure 47. Average number of neutrophils adhered and flattened onto endothelium, infiltrated into intima space, and present on the outside of the vessel for a 8 μm section of tissue.

The average number of neutrophils per vessels with staining was 7.3 for preeclamptic patients as compared to 1.9 for normal pregnant patients and 1.3 for normal non-pregnant patients. Neutrophil staining for normal non-pregnant and normal pregnant patients primarily involved only endothelial cell adherence, whereas neutrophil staining for preeclamptic patients involved all aspects of the vessel. ***$P < 0.001$

Table 4. Summary of CD66b immunohistochemical staining for resistance-sized vessels (10 μm - 200 μm)

	Normal Non-Pregnant (n = 4)	Normal Pregnant (n = 6)	Preeclamptic (n = 5)
Total Vessels with Stained Cells	22.6 ± 6.1%	26.9 ± 7.5%	76.6 ± 8.4%**
Vessels with Stained Cells Adhered & Flattened on Endothelium	14.8 ± 4.2%	18.5 ± 6.2%	55.9 ± 6.9%**
Vessels with Stained Cells in Intima	2.3 ± 1.0%	6.5 ± 2.2%	52.3 ± 10.6%***
Vessels with Stained Cells on Outside of Vessel	2.5 ± 2.2%	3.0 ± 1.4%	26.0 ± 6.6%**
No. of Neutrophils / Vessel with Staining (8 μm section)	1.3 ± 0.3	1.9 ± 0.4	7.3 ± 0.8***

Values represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared with normal pregnant and normal non-pregnant.

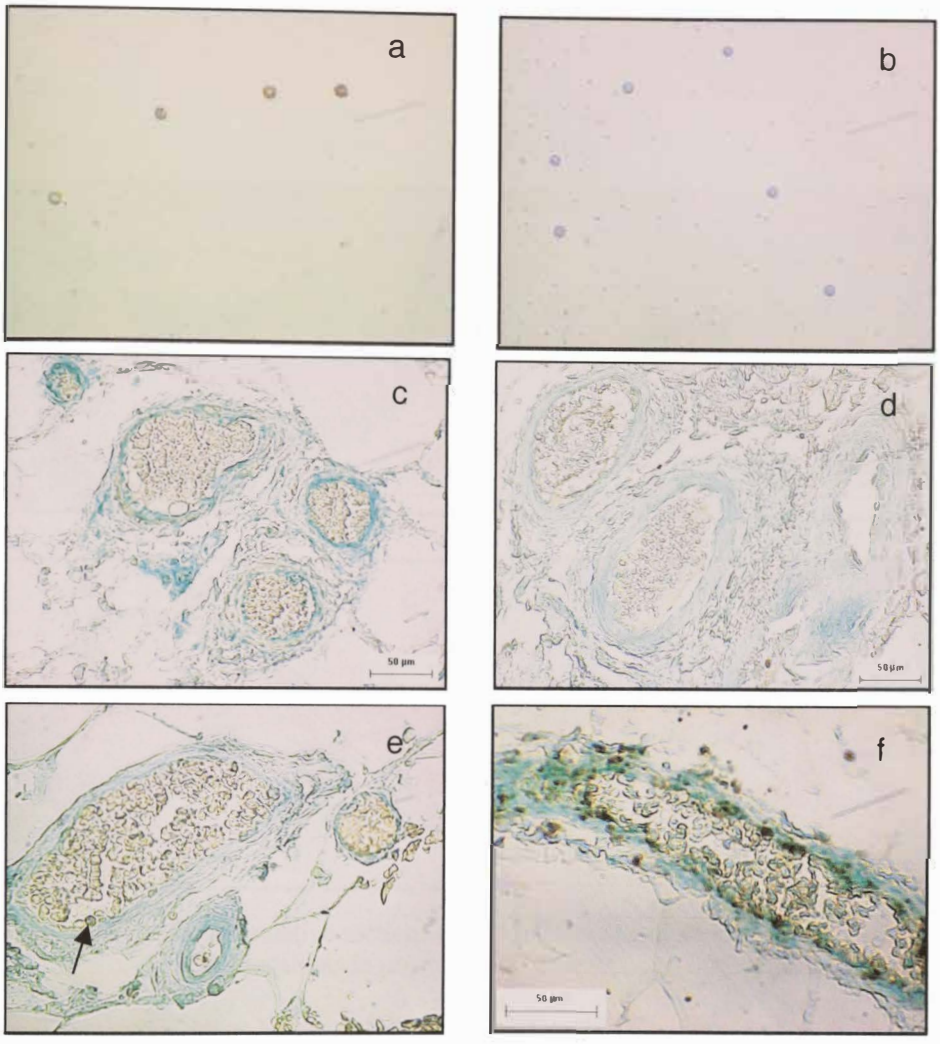


Figure 48. CD66b staining of leukocytes (panels a and b) and of vessels in subcutaneous fat (panels c-f).

a) Neutrophils isolated from blood showing brown staining for CD66b, x600, b) Monocytes and lymphocytes isolated from blood showing lack of staining for CD66b, x600, c) IgM negative control, x400, d) Normal non-pregnant patient showing no CD66b staining, x400, e) Normal pregnant patient showing a rounded cell lightly stained for CD66b on the endothelium (arrow), x600, f) Preeclamptic patient showing massive brown staining for CD66b along the endothelium and in the intima, x600.

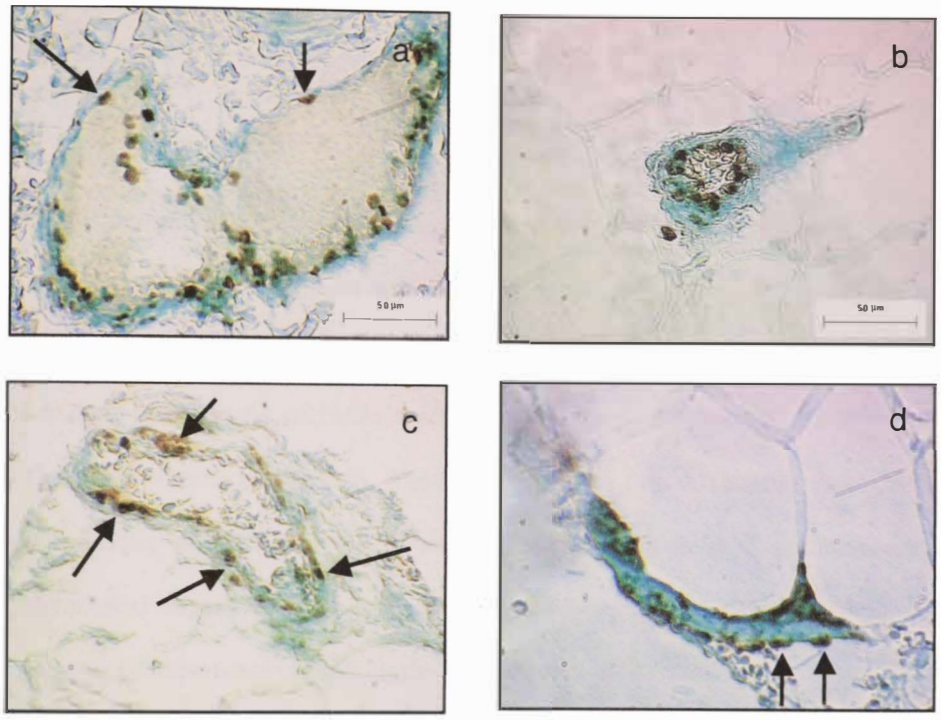


Figure 49. Representative sections of CD66b staining of neutrophils in various vessel locations in preeclamptic patients.

a) This section shows brown stained neutrophils within the lumen and along the endothelium. In the upper part of the vessel are two examples of neutrophils adhered and flattened onto the endothelium (arrows), x600, b) This vessel shows massive neutrophil involvement. Neutrophils are adhered to the endothelium and present within the intima. The entire circumference of the vessel is involved, x600, c) This vessel is an example of neutrophil staining in the intima (arrows), x900, d) This vessel also shows massive neutrophil involvement. Neutrophil staining is clearly evident on the outside of the vessel (arrows), x900.

Chapter 6

DISCUSSION

This investigation is the first to demonstrate neutrophil transendothelial migration into systemic vascular tissue in women with preeclampsia. It also suggests how transendothelial migration of neutrophils may be favored in an environment of oxidative stress and elevated levels of linoleic acid by activation of neutrophils and vascular smooth muscle expression of IL-8. The observations of this dissertation link vascular smooth muscle, endothelial, and neutrophil dysfunction to a single mechanism, transendothelial migration of neutrophils, that could explain the clinical symptoms of preeclampsia of hypertension, proteinuria, and pathological edema.

Oxidative stress and elevated plasma levels of linoleic acid are present in women with preeclampsia. Our in vitro data indicated that this combination favors a mechanism for neutrophil transendothelial migration. Specifically, our studies demonstrated that treatment of vascular smooth muscle cells with an oxidizing solution enriched with linoleic acid, but not either component alone, led to increased vascular smooth muscle cell production of IL-8. Similarly, this treatment solution rapidly activated neutrophils to produce superoxide. This favors neutrophil transendothelial migration because increased expression of IL-8 by vascular smooth muscle would create a concentration gradient for

IL-8 from the circulation to vascular smooth muscle to attract neutrophils, and rapid activation of neutrophils would prime them for transendothelial migration. The final study confirmed the predictions of the in vitro studies by demonstrating vascular smooth muscle cell expression of IL-8 coincident with neutrophil infiltration into systemic vascular tissue in women with preeclampsia. Inflammation of the vascular smooth muscle, as well as endothelium, was evident by expression of both IL-8 and ICAM-1 in women with preeclampsia.

Although there is little reason to believe that vasculature supplying subcutaneous fat differs from other systemic vasculature, it would be of interest to confirm neutrophil transendothelial migration in other systemic tissues, such as muscle or skin. This could be done in future experiments by taking small pieces of skin and abdominal muscle from the abdominal incision.

Another future investigation of our laboratory will be to determine the relationship between the degree of neutrophil infiltration and the severity of clinical symptoms of preeclampsia. We were unable to do this in the present study because the sample size was too small. However, analysis of samples from women with hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, considered a severe form of preeclampsia by many clinicians, provided some information regarding the relationship between neutrophil activity and clinical symptoms. Our patient group with HELLP had blood pressures characteristic of severe preeclampsia, but proteinuria levels characteristic of mild preeclampsia. Vessels of HELLP women had less neutrophil infiltration as compared to our preeclampsia group, which was composed primarily of

severe preeclamptics, suggesting that the degree of neutrophil infiltration was correlated with the severity of proteinuria (Figure 50). These observations suggest that as the number of vessels with neutrophil infiltration into the intima increase, so does the severity of proteinuria and clinical presentation. Future investigations in our laboratory intend to clarify the relationship between neutrophil involvement and disease severity. We plan on increasing our sample size to differentiate between mild and severe preeclampsia. We expect to observe a linear relationship between the level of neutrophil involvement and the progression of preeclampsia. We also plan to increase our sample size for HELLP patients to clarify if HELLP truly is a severe form of preeclampsia or is a distinct pathophysiological process.

Our sample population did not include any women with eclampsia, which is diagnosed when grand mal seizures accompany symptoms of preeclampsia. If neutrophil activation and transendothelial migration in the systemic circulation are responsible for clinical symptoms of preeclampsia, maybe a similar pathology in cerebral blood vessels is responsible for seizures of eclampsia. Belfort et al. proposed that increased cerebral perfusion pressure might cause eclamptic seizures in women with severe preeclampsia [165]. The work of this dissertation would support this hypothesis because neutrophil adherence and infiltration of cerebral blood vessels, accompanied with production of ROS, would lead to increased vascular permeability, cerebral edema and increased cerebral pressure. This idea could be tested in an animal model of preeclampsia, in which a brain biopsy could be obtained to determine if neutrophils infiltrate cerebral blood vessels.

Magnesium sulfate is the present treatment to prevent progression of preeclampsia to eclampsia. The mechanism of action of magnesium sulfate, however, is disputed and not understood [166]. Our data demonstrating neutrophil infiltration into systemic vascular tissue in women with preeclampsia may explain how magnesium sulfate prevents eclampsia. Neutrophil function and activation is modulated by magnesium. Magnesium deficiency increases neutrophil phagocytosis and ROS production in a rat model, whereas magnesium sufficiency inhibits neutrophil activation [167]. These results were confirmed using human neutrophils [168]. Another group showed a 70% decrease in plasma glutathione associated with neutrophil activation in magnesium deficient rats [169]. Decreased plasma glutathione levels indicate systemic oxidative stress. Magnesium sulfate for the prevention of eclampsia may act by inhibiting neutrophil activation, and subsequently, minimize oxidative stress and reduce neutrophil transendothelial migration of cerebral vessels. The findings of this dissertation also would explain why magnesium sulfate is significantly more effective at preventing seizures of eclampsia compared to nimodipine, a cerebral dilator [170].

During the course of our research we found evidence of neutrophil involvement and infiltration in normal pregnant and non-pregnant patients. Upon investigation of their medical records, we found that normal pregnant women with neutrophil infiltration were in labor when the biopsy was collected. Non-pregnant women with neutrophil involvement were found to be obese or obese and hypertensive. In laboring women as compared to non-laboring women, there were significantly more vessels with staining for neutrophils, and neutrophils flattened and adhered to the endothelium, infiltrated into the

intima, and adhered to the outside of the vessel (Figures 51 and 52). In obese women or obese women with hypertension, there was greater neutrophil vessel involvement than in non-obese women with normal blood pressures (Figure 53). These observations invite speculations regarding a role for neutrophils and neutrophil transendothelial migration in many normal, as well as abnormal physiological processes, and suggest future research directions.

We could speculate that parturition is an inflammatory process mediated by neutrophils. Neutrophils infiltrate the myometrium during labor, and the number of neutrophils present in the lower uterine segment increases with cervical dilatation [171, 172]. Increased expression of cell adhesion molecules and IL-8 with parturition indicates a mechanism for transendothelial migration of neutrophils into these tissues [172, 173]. Neutrophils infiltrated into the myometrium may be responsible for the production of prostaglandins and thromboxane that cause myometrial contractions. Neutrophils within the lower uterine segment may release matrix metalloproteinases to cause cervical ripening. Systemic vascular neutrophil activation and transendothelial migration during labor may demonstrate a significant role for neutrophils in the process of parturition. These data also may answer why preeclampsia is associated with preterm labor.

We could also speculate as to why neutrophil numbers increase and infiltrate systemic tissue during labor. Neutrophils may have a protective role in case of post-partum infection. This may explain why the infection rate is so low after vaginal delivery with episiotomy (0.35%) as compared to C-section (2%) [1, 174]. It is remarkable that women who have completed the laboring process are less likely to develop post-partum

infections, despite the unsterile conditions of vaginal delivery. This may be explained by the activation and systemic infiltration of neutrophils with labor that provide an immediate defense to any infection. A rise in neutrophil numbers and activation during labor is not harmful to most women, but some women may not be able to withstand the vascular insult. This may explain why some women with normal pregnancy develop preeclampsia in the post-partum period.

Our preliminary evidence for neutrophil activation and infiltration in obese and obese, hypertensive women are fascinating, especially considering the similarities in risk factors, pathology, and clinical symptoms between preeclampsia and cardiovascular disease. Cardiovascular disease, as preeclampsia, is characterized by endothelial cell dysfunction, oxidative stress, and generalized low-grade inflammation. Essential hypertension and preeclampsia share clinical symptoms of hypertension and proteinuria (microalbuminuria in the case of hypertension). Essential hypertension is unexplained hypertension caused by an increase in total peripheral resistance. A few recent studies have investigated a role for leukocytes, namely neutrophils, in the pathology of cardiovascular disease and essential hypertension. For example, increased white blood cell counts were shown to be strong predictors of coronary artery disease and stroke in post-menopausal women, and neutrophil count and neutrophil elastase were significantly elevated in subjects with essential hypertension [175, 176]. Neutrophils from patients with essential hypertension were more adhesive than neutrophils from normotensive individuals [177], as were neutrophils isolated from spontaneously hypertensive rats, which also exhibited neutrophil-mediated cytotoxicity to endothelial cells [178]. Another study

used scanning electron microscopy to demonstrate neutrophil adhesion to endothelial cells and invasion of endothelial cell junctions of cerebral vessels in hypertensive rats. The evidence suggests a role for neutrophils in the pathology of essential hypertension, but no one has demonstrated significant neutrophil involvement in systemic vasculature as shown by our preliminary data.

Our findings suggested that one of the risk factors for essential hypertension, obesity, is associated with increased vascular neutrophil involvement as compared to individuals of normal weight. In obese women, there were increased numbers of vessels with staining for neutrophils, and increased numbers of neutrophils adhered and flattened to endothelium, but not increased numbers infiltrated into the intima (Figures 52 and 53). The physical presence of increased numbers of neutrophils adhered and flattened onto endothelium would increase total peripheral resistance and could partly explain essential hypertension. The production of vasoconstrictors, such as TX and ROS, by activated neutrophils also could cause hypertension. It is evident that obesity shows early signs of a pathological process that will likely worsen over time and eventually may cause essential hypertension. These preliminary data for obese and hypertensive women suggest that preeclampsia may be a temporary, but an extreme and accelerated form of the pathological process responsible for essential hypertension.

The work presented in this dissertation, demonstrating a role for oxidative stress, elevated levels of linoleic acid and neutrophil transendothelial migration in the pathophysiology of preeclampsia, offers numerous options for preventive and therapeutic benefits. These findings can serve as rationale for dietary modifications or therapeutic

targets. Dietary modification would be a simple and inexpensive method to reduce the risk of preeclampsia. A diet low in omega-6 fatty acids and high in antioxidants prior to and throughout pregnancy would offset oxidative stress associated with pregnancy and, hopefully, prevent preeclampsia. This dissertation work showed that neutrophil activation and vascular smooth muscle production of IL-8 are enhanced by oxidative stress in the presence of linoleic acid, suggesting that if one could decrease oxidative stress, preeclampsia could be prevented or its severity lessened. Since linoleic acid is a polyunsaturated fatty acid and readily oxidizes, it causes lipid peroxidiation, which propagates oxidative stress. Linoleic acid is the primary constituent of most vegetable oils. Substitution of oils rich in oleic acid, such as olive oil, could substantially reduce one's intake of linoleic acid. Research in our laboratory demonstrated that oleic acid, a monounsaturated fatty acid, reduces lipid peroxidation [152]. Dietary modifications to decrease oxidative stress and the risk of preeclampsia are supported by the research of Chappell et al. [48, 49], who demonstrated that vitamin E and C supplementation in women at high-risk for preeclampsia led to decreased markers of oxidative stress and a lower incidence of preeclampsia.

Our observations also suggest that neutralizing antibodies to IL-8 or cell adhesion molecules might prevent or stop the progression of preeclampsia. Recently, a monoclonal antibody directed against α_4 integrins showed positive outcomes for the treatment of Crohn's Disease and multiple sclerosis in two large clinical trials [179, 180]. Neutralizing antibodies to IL-8, ICAM-1, or CD11/CD18 would block the process of neutrophil transendothelial migration. This treatment could prevent production of ROS, MPO, and

TX by neutrophils present within the intima, which we speculate results in vascular cell damage and vasoconstriction.

Another potential pharmacological intervention suggested by the results of this dissertation would be the use of AA metabolism inhibitors, such as aspirin, to reduce vascular inflammation and inhibit neutrophil transendothelial migration. Our in vitro data suggested that inhibition of thromboxane synthase would decrease production of IL-8 by vascular smooth muscle cells in the presence of oxidative stress and linoleic acid. A decrease in IL-8 production would reduce the chemotactic gradient for neutrophil transendothelial migration. The suggestion to use aspirin to prevent preeclampsia is not novel. Many small clinical studies showed that treatment with low-dose aspirin in women at high risk for preeclampsia reduced the incidence of preeclampsia [181-184]. However, two large clinical studies by the National Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Units did not demonstrate a beneficial effect with low-dose aspirin, although patient compliance was low [185, 186]. These unfavorable results lead to a recommendation against low-dose aspirin for the prevention of preeclampsia. The debate over treatment of preeclampsia with low-dose aspirin is not over. Recently, a meta-analysis of clinical trials using aspirin to prevent preeclampsia demonstrated that aspirin was beneficial in preventing preeclampsia [187].

These preventive modifications and therapeutic agents may also be beneficial in preventing essential hypertension. Dietary modifications of decreasing linoleic acid and increasing oleic acid with antioxidant supplementation would decrease available substrates for AA metabolism and inhibit oxidative stress, respectively. These

adjustments would inhibit many aspects of the mechanism of transendothelial migration of neutrophils.

In conclusion, this investigation demonstrated transendothelial migration of neutrophils into systemic vascular tissue in women with preeclampsia. In vitro studies complemented this observation, suggesting aspects of this mechanism, involving vascular smooth muscle cells, endothelial cells, and neutrophils, that are modified by oxidative stress and linoleic acid to favor neutrophil transendothelial migration. These new data provide evidence for total "vascular cell dysfunction" that could explain the clinical symptoms of preeclampsia. It is our hope that this work is beneficial to understanding the pathophysiology and the eventual treatment of preeclampsia. AMEN.

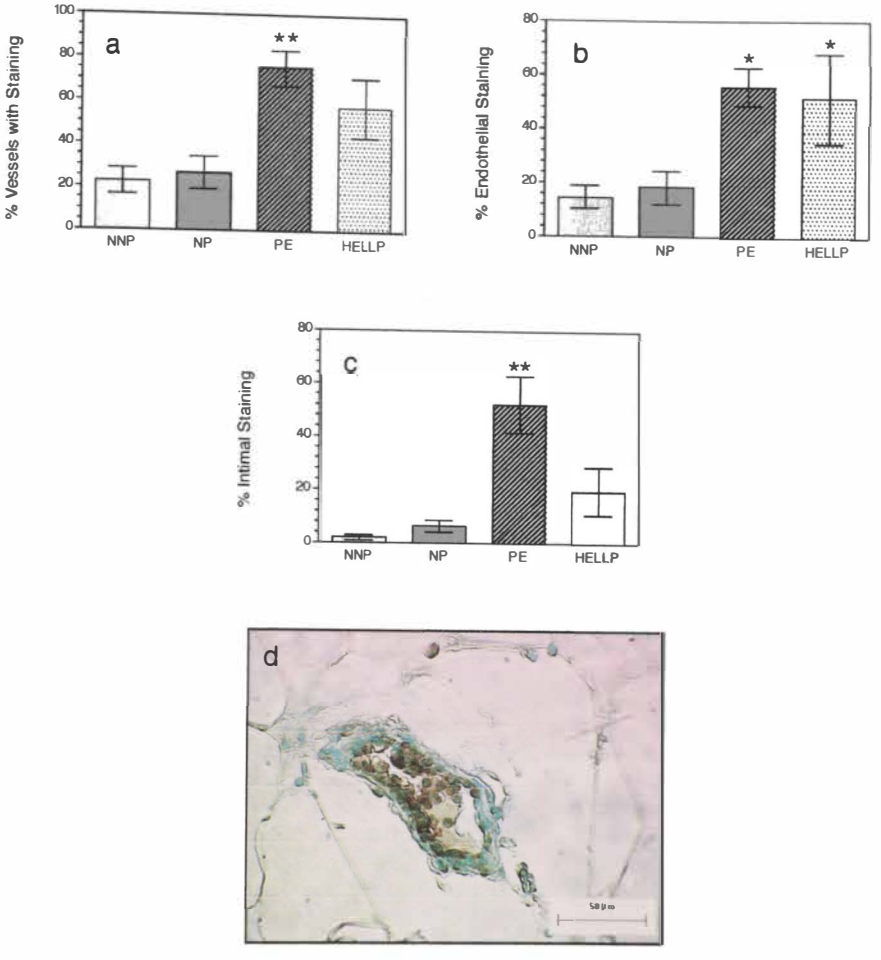


Figure 50. CD66b staining in vessels for HELLP patients.

HELLP patients (n = 3) were similar to preeclamptic patients with respect to percent of vessels with staining (panel a) and percent of vessels with neutrophils adhered and flattened onto endothelium (panel b). HELLP patients, who did not have as severe of proteinuria as the preeclamptic patients, did not have the same degree of neutrophils infiltrated into the intima (panel c). Panel d shows a vessel with a massive number of brown stained neutrophils adhering to the endothelium and occluding the lumen in a HELLP patient, x600. *P < 0.05, **P < 0.01

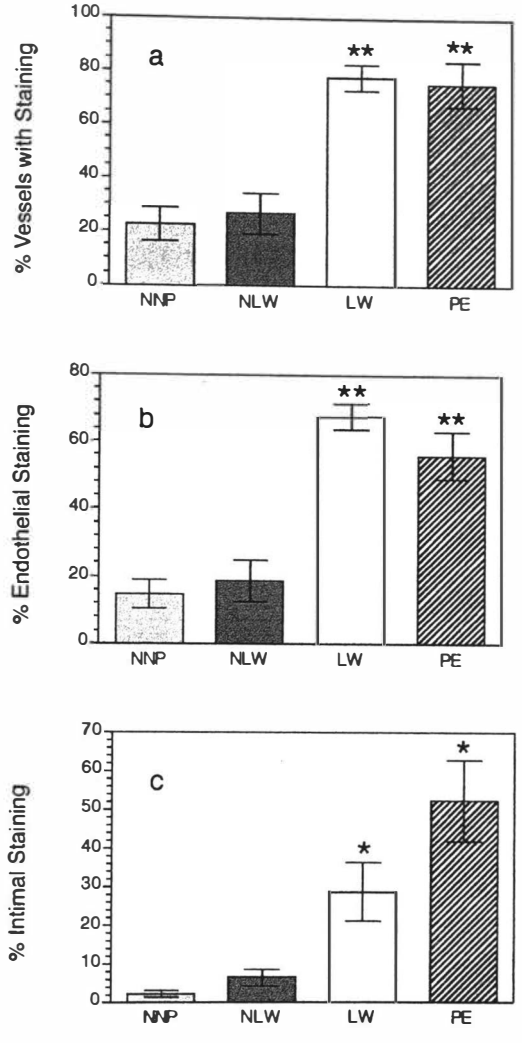


Figure 51. CD66b staining in vessels for laboring patients.

Laboring patients (n = 3) were similar to preeclamptic patients with respect to percent of vessels with staining (panel a) and percent of vessels with neutrophils adhered and flattened onto endothelium (panel b). Laboring patients, however, did not have the same degree of neutrophils infiltrated into the intima (panel c). *$P < 0.05$, **$P < 0.01$

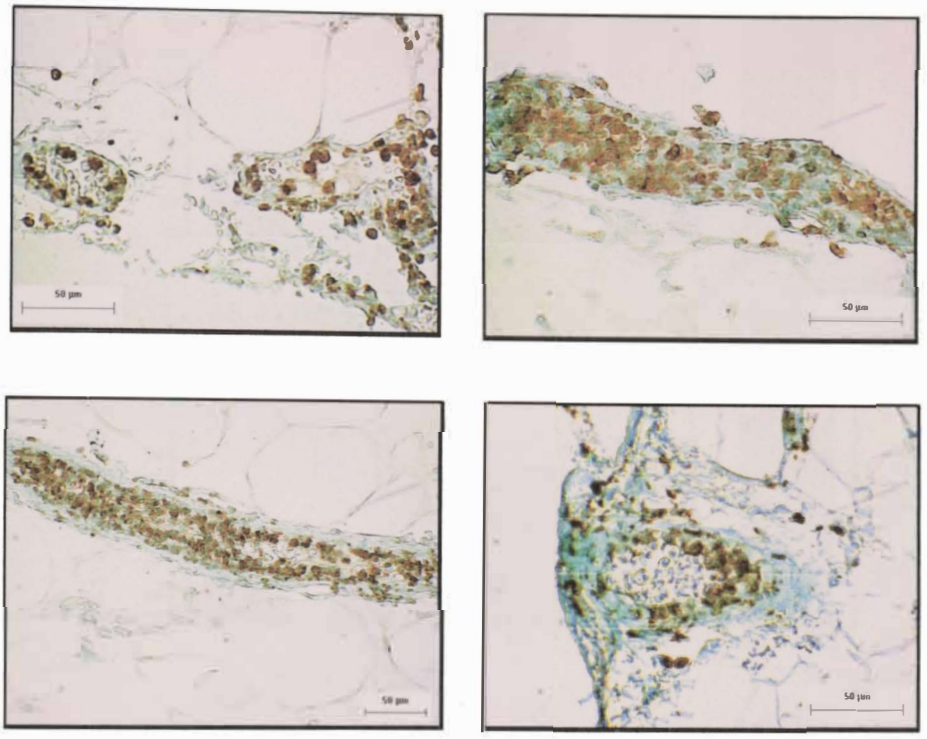


Figure 52. Representative sections of CD66b staining of neutrophils in vessels of laboring patients.

a) This section shows brown staining for neutrophils adhered to endothelium, x600, b) This vessels shows a vessel occluded with neutrophils, x600, c) This vessel shows massive neutrophil involvement. The entire endothelium is lined with neutrophils, x400, d) This vessel has dark brown staining of neutrophils accumulated along the endothelium, x600.

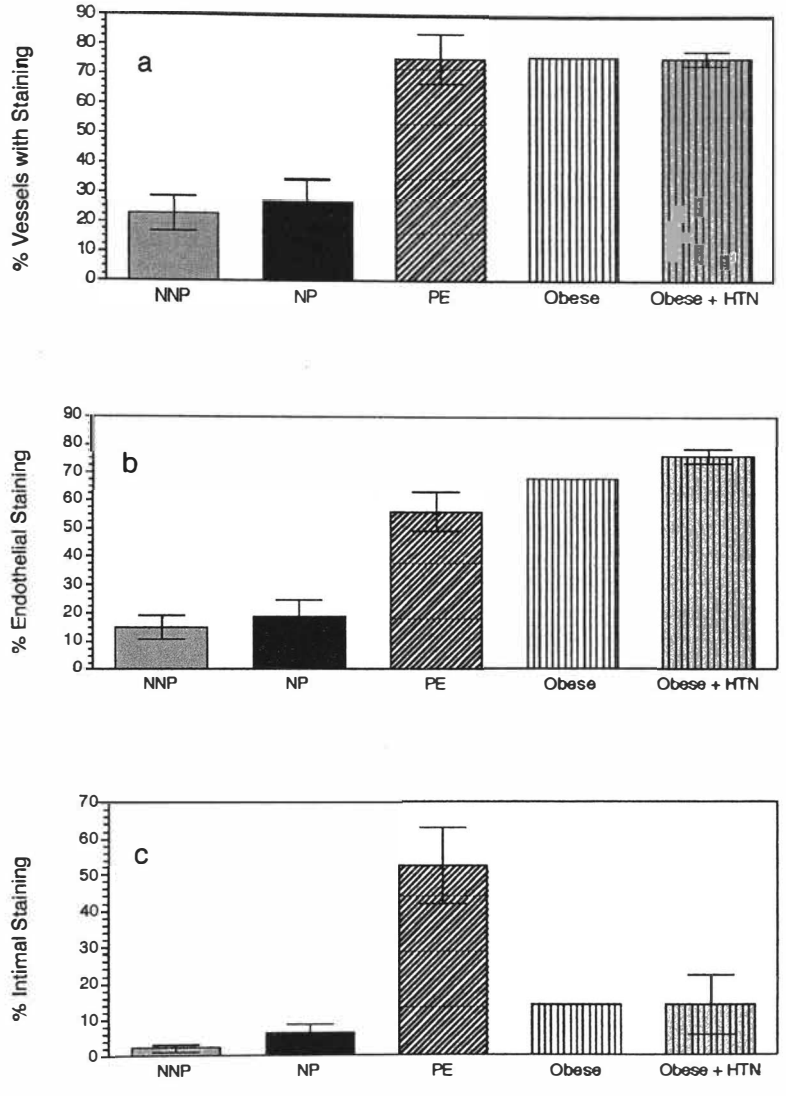


Figure 53. CD66b staining in vessels for obese and hypertensive patients.

Obese (n = 1) and obese, hypertensive (n = 2) patients were similar to preeclamptic patients with respect to percent of vessels with staining (panel a) and percent of vessels with neutrophils adhered and flattened onto endothelium (panel b). Obese patients, however, did not have the same degree of neutrophils infiltrated into the intima as compared to preeclamptic patients (panel c). These preliminary data may explain why obesity is a risk factor for preeclampsia, as well as essential hypertension.

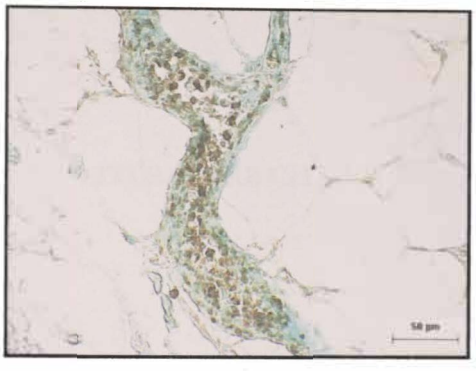


Figure 54. Representative section of CD66b staining of neutrophils in a vessel of an obese patient.

This longitudinal section shows massive brown staining of neutrophils throughout the length of the vessel, x400.

LITERATURE CITED

Literature Cited

1. CUNNINGHAM GF, MACDONALD PC, GANT NF, LEVENO KJ, GILSTRAP LC. Williams Obstetrics. Norwalk, Connecticut: Appleton and Lange, 1993.

2. ROBERTS JM. Preeclampsia: what we know and what we do not know. Semin Perinatol 2000;24:24-8.

3. LORENTZEN B, HENRIKSEN T. Plasma lipids and vascular dysfunction in preeclampsia. Semin Reprod Endocrinol 1998;16:33-9.

4. HALLIWELL B, GUTTERIDGE JM. Free Radicals in Biology and Medicine. London: Oxford University Press, 1999.

5. HALLIWELL B. Antioxidants and human disease: a general introduction. Nutr Rev 1997;55:S44-9; discussion S49-52.

6. GARRETT RH, GRISHAM CM. Biochemistry. Philadelphia: Saunders College Publishing, 1995.

7. GRIENDLING KK, SORESCU D, USHIO-FUKAI M. NAD(P)H oxidase: role in cardiovascular biology and disease. Circ Res 2000;86:494-501.

8. MYATT L, KOSSENJANS W, SAHAY R, EIS A, BROCKMAN D. Oxidative stress causes vascular dysfunction in the placenta. J Matern Fetal Med 2000;9:79-82.

9. HALLIWELL B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? [see comments]. Lancet 1994;344:721-4.

10. URSINI F, BINDOLI A. The role of selenium peroxidases in the protection against oxidative damage of membranes. Chem Phys Lipids 1987;44:255-76.

11. MEYDANI M. Vitamin E. The Lancet 1995;345:170-175.

12. WHITNEY EN, ROLFES SR. Understanding Nutrition. New York: West Publishing Company, 1996.

13. MACHLIN LJ, A. B. Free radical tissue damage: protective role of antioxidant nutrients. FASEB J. 1987;1:441-445.

14. WALSH SW. Lipid peroxidation in pregnancy. Hypertension in Pregnancy 1994;13:1-32.

15. HUBEL CA. Oxidative stress in the pathogenesis of preeclampsia. Proc Soc Exp Biol Med 1999;222:222-35.

16. SEKIBA K, YOSHIOKA T. Changes of lipid peroxidation and superoxide dismutase activity in the human placenta. Am J Obstet Gynecol 1979;135:368-71.

17. WANG Y, WALSH SW, KAY HH. Placental lipid peroxides and thromboxane are increased and prostacyclin is decreased in women with preeclampsia. Am J Obstet Gynecol 1992;167:946-9.

18. WALSH SW, WANG Y. Secretion of lipid peroxides by the human placenta. Am J Obstet Gynecol 1993;169:1462-1466.

19. WALSH SW, WANG Y. Trophoblast and placental villous core production of lipid peroxides, thromboxane, and prostacyclin in preeclampsia. J Clin Endocrinol Metab 1995;80:1888-93.

20. WALSH SW. Preeclampsia: An imbalance in placental prostacyclin and thromboxane production. American Journal of Obstetrics and Gynecology 1985;152:335-340.

21. WETZKA B, NUSING R, CHARNOCK-JONES DS, SCHAFER W, ZAHRADNIK HP, SMITH SK. Cyclooxygenase-1 and -2 in human placenta and placental bed after normal and pre-eclamptic pregnancies. Hum Reprod 1997;12:2313-20.

22. JOHNSON RD, SADOVSKY Y, GRAHAM C, ANTEBY EY, POLAKOSKI KL, HUANG X, NELSON DM. The expression and activity of prostaglandin H synthase-2 is enhanced in trophoblast from women with preeclampsia. J Clin Endocrinol Metab 1997;82:3059-62.

23. MATSUBARA S, SATO I. Enzyme histochemically detectable NAD(P)H oxidase in human placental trophoblasts: normal, preeclamptic, and fetal growth restriction-complicated pregnancy. Histochem Cell Biol 2001;116:1-7.

24. WALSH SW. Maternal-placental interactions of oxidative stress and antioxidants in preeclampsia. Seminars in Reproductive Endocrinology 1998;16:93-104.

25. WANG Y, WALSH SW. TNF alpha concentrations and mRNA expression are increased in preeclamptic placentas. J Reprod Immunol 1996;32:157-69.

26. WANG Y, WALSH SW. Placental mitochondria as a source of oxidative stress in pre-eclampsia. Placenta 1998;19:581-6.

27. WALSH SW, VAUGHAN JE, WANG Y, ROBERTS LJ, 2ND. Placental isoprostane is significantly increased in preeclampsia. Faseb J 2000;14:1289-96.

28. MORROW JD, ROBERTS LJ, 2ND. The isoprostanes. Current knowledge and directions for future research. Biochem Pharmacol 1996;51:1-9.

29. ZUSTERZEEL PL, RUTTEN H, ROELOFS HM, PETERS WH, STEEGERS EA. Protein carbonyls in decidua and placenta of pre-eclamptic women as markers for oxidative stress. Placenta 2001;22:213-9.

30. MYATT L, ROSENFIELD RB, EIS AL, BROCKMAN DE, GREER I, LYALL F. Nitrotyrosine residues in placenta. Evidence of peroxynitrite formation and action. Hypertension 1996;28:488-93.

31. WANG Y, WALSH SW. Antioxidant activities and mRNA expression of superoxide dismutase, catalase, and glutathione peroxidase in normal and preeclamptic placentas. J Soc Gynecol Investig 1996;3:179-84.

32. WALSH SW, WANG Y. Deficient glutathione peroxidase activity in preeclampsia is associated with increased placental production of thromboxane and lipid peroxides. Am J Obstet Gynecol 1993;169:1456-1461.

33. KNAPEN MF, PETERS WH, MULDER TP, MERKUS HM, JANSEN JB, STEEGERS EA. Glutathione and glutathione-related enzymes in decidua and placenta of controls and women with pre-eclampsia. Placenta 1999;20:541-6.

34. PORANEN AK, EKBLAD U, UOTILA P, AHOTUPA M. Lipid peroxidation and antioxidants in normal and pre-eclamptic pregnancies. Placenta 1996;17:401-5.

35. BOGGESS KA, OURY TD, KAY HH, CRAPO JD. Extracellular superoxide dismutase localization and activity within the human placenta. Placenta 1998;19:417-22.

36. SAHLIN L, OSTLUND E, WANG H, HOLMGREN A, FRIED G. Decreased expression of thioredoxin and glutaredoxin in placentae from pregnancies with pre-eclampsia and intrauterine growth restriction. Placenta 2000;21:603-9.

37. HOLLES SM, WANG Y, ROMNEY A, WALSH SW. Vitamin E Attenuates Peroxide-Induced Vasoconstriction in the Human Placenta. Hypertens Pregnancy 1997;16:389-401.

38. PORANEN AK, EKBLAD U, UOTILA P, AHOTUPA M. The effect of vitamin C and E on placental lipid peroxidation and antioxidative enzymes in perfused placenta. Acta Obstet Gynecol Scand 1998;77:372-6.

39. CUETO SM, ROMNEY AD, WANG Y, WALSH SW. beta-Carotene attenuates peroxide-induced vasoconstriction in the human placenta. J Soc Gynecol Investig 1997;4:64-71.

40. ISHIHARA M. Studies on lipoperoxide of normal pregnant women and of patients with toxemia of pregnancy. Clin Chim Acta 1978;84:1-9.

41. WICKENS D, WILKINS MH, LUNEC J, BALL G, DORMANDY TL. Free radical oxidation (peroxidation)products in plasma in normal and abnormal pregnancy. Ann Clin Biochem 1981;18:158-62.

42. UOTILA J, TUIMALA R, AARNIO T, PYYKKO K, AHOTUPA M. Lipid peroxidation products, selenium-dependent glutathione peroxidase and vitamin E in normal pregnancy. Eur J Obstet Gynecol Reprod Biol 1991;42:95-100.

43. WANG YP, WALSH SW, GUO JD, ZHANG JY. The imbalance between thromboxane and prostacyclin in preeclampsia is associated with an imbalance between lipid peroxides and vitamin E in maternal blood. Am J Obstet Gynecol 1991;165:1695-700.

44. HUBEL CA, MCLAUGHLIN MK, EVANS RW, HAUTH BA, SIMS CJ, ROBERTS JM. Fasting serum triglycerides, free fatty acids, and malondialdehyde are increased in preeclampsia, are positively correlated, and decrease within 48 hours post partum. Am J Obstet Gynecol 1996;174:975-82.

45. DIEDRICH F, RENNER A, RATH W, KUHN W, WIELAND E. Lipid hydroperoxides and free radical scavenging enzyme activities in preeclampsia and HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome: no evidence for circulating primary products of lipid peroxidation. Am J Obstet Gynecol 2001;185:166-72.

46. CHAVARRIA ME, LARA-GONZALEZ L, GONZALEZ-GLEASON A, GARCIA-PALETA Y, VITAL-REYES VS, REYES A. Prostacyclin/thromboxane early changes in pregnancies that are complicated by preeclampsia. Am J Obstet Gynecol 2003;188:986-92.

47. CHEN G, WILSON R, CUMMING G, WALKER JJ, MCKILLOP JH. Production of prostacyclin and thromboxane A2 in mononuclear cells from preeclamptic women. Am J Obstet Gynecol 1993;169:1106-11.

48. CHAPPELL LC, SEED PT, BRILEY AL, KELLY FJ, LEE R, HUNT BJ, PARMAR K, BEWLEY SJ, SHENNAN AH, STEER PJ, POSTON L. Effect of antioxidants on the occurrence of pre-eclampsia in women at increased risk: a randomised trial. Lancet 1999;354:810-6.

49. CHAPPELL LC, SEED PT, KELLY FJ, BRILEY A, HUNT BJ, CHARNOCK-JONES DS, MALLET A, POSTON L. Vitamin C and E supplementation in women at risk of preeclampsia is associated with changes in indices of oxidative stress and placental function. Am J Obstet Gynecol 2002;187:777-84.

50. CHEN G, WILSON R, BOYD P, MCKILLOP JH, LEITCH C, WALKER JJ, BURDON RH. Normal superoxide dismutase (SOD) gene in pregnancy-induced hypertension: is the decreased SOD activity a secondary phenomenon? Free Radic Res 1994;21:59-66.

51. LIOCHEV SI, FRIDOVICH I. How does superoxide dismutase protect against tumor necrosis factor: a hypothesis informed by effect of superoxide on "free" iron. Free Radic Biol Med 1997;23:668-71.

52. SUTHERLAND MW, GEBICKI JM. A reaction between the superoxide free radical and lipid hydroperoxide in sodium linoleate micelles. Arch Biochem Biophys 1982;214:1-11.

53. CRUIKSHANK DP, WIGTON TR, HAYS PM. Chapter 4: Maternal Physiology in Pregnancy. In: Gabbe SG, Niebyl JR, Simpson JL, eds. Obstetrics Normal and Problem Pregnancies. New York: Churchill Livingstone, 1986.

54. BEARCHELL MC, REDMAN CW, PYNE GJ, CADOUX-HUDSON T, CLARK JF. Vascular smooth muscle oxygen consumption is reversibly stimulated by sera from women with preeclampsia. Am J Obstet Gynecol 1998;179:1534-8.

55. THOMSON NF, THORNTON S, CLARK JF. The effects of placental extracts from normotensive and preeclamptic women on vasoconstriction and oxidative metabolism. Am J Obstet Gynecol 2000;183:206-10.

56. GREEN J, ASSADY S, NAKHOUL F, BICK T, JAKOBI P, ABASSI Z. Differential effects of sera from normotensive and hypertensive pregnant women on Ca(2+) metabolism in normal vasular smooth muscle cells. J Am Soc Nephrol 2000;11:1188-98.

57. JAFFE EA. Cell biology of endothelial cells. Hum Pathol 1987;18:234-9.

58. ROBERTS JM. Endothelial dysfunction in preeclampsia. Semin Reprod Endocrinol 1998;16:5-15.

59. ROBERTS JM, TAYLOR RN, MUSCI TJ, RODGERS GM, HUBEL CA, MCLAUGHLIN MK. Preeclampsia: an endothelial cell disorder. Am J Obstet Gynecol 1989;161:1200-4.

60. RODGERS GM, TAYLOR RN, ROBERTS JM. Preeclampsia is associated with a serum factor cytotoxic to human endothelial cells. Am J Obstet Gynecol 1988;159:908-14.

61. ROBERTS JM, EDEP ME, GOLDFIEN A, TAYLOR RN. Sera from preeclamptic women specifically activate human umbilical vein endothelial cells in vitro: morphological and biochemical evidence. Am J Reprod Immunol 1992;27:101-8.

62. ENDRESEN MJ, TOSTI E, LORENTZEN B, HENRIKSEN T. Sera of preeclamptic women are not cytotoxic to endothelial cells in culture. Am J Obstet Gynecol 1995;172:196-201.

63. FRIEDMAN SA. Preeclampsia: a review of the role of prostaglandins. Obstet Gynecol 1988;71:122-37.

64. PETRAK RA, BALK RA, BONE RC. Prostaglandins, cyclo-oxygenase inhibitors, and thromboxane synthetase inhibitors in the pathogenesis of multiple systems organ failure. Crit Care Clin 1989;5:303-14.

65. YAMAGUCHI M, MORI N. 6-Keto prostaglandin F1 alpha, thromboxane B2, and 13,14-dihydro-15-keto prostaglandin F concentrations of normotensive and preeclamptic patients during pregnancy, delivery, and the postpartum period. Am J Obstet Gynecol 1985;151:121-7.

66. FITZGERALD DJ, ENTMAN SS, MULLOY K, FITZGERALD GA. Decreased prostacyclin biosynthesis preceding the clinical manifestation of pregnancy-induced hypertension. Circulation 1987;75:956-63.

67. MILLS JL, DERSIMONIAN R, RAYMOND E, MORROW JD, ROBERTS LJ, 2ND, CLEMENS JD, HAUTH JC, CATALANO P, SIBAI B, CURET LB, LEVINE RJ. Prostacyclin and thromboxane changes predating clinical onset of preeclampsia: a multicenter prospective study. Jama 1999;282:356-62.

68. LORENTZEN B, ENDRESEN MJ, HOVIG T, HAUG E, HENRIKSEN T. Sera from preeclamptic women increase the content of triglycerides and reduce the release of prostacyclin in cultured endothelial cells. Thromb Res 1991;63:363-72.

69. ENDRESEN MJ, TOSTI E, HEIMLI H, LORENTZEN B, HENRIKSEN T. Effects of free fatty acids found increased in women who develop pre-eclampsia on the ability of endothelial cells to produce prostacyclin, cGMP and inhibit platelet aggregation. Scand J Clin Lab Invest 1994;54:549-57.

70. LIM KH, RICE GE, DE GROOT CJ, TAYLOR RN. Plasma type II phospholipase A2 levels are elevated in severe preeclampsia. Am J Obstet Gynecol 1995;172:998-1002.

71. DE GROOT CJ, DAVIDGE ST, FRIEDMAN SA, MCLAUGHLIN MK, ROBERTS JM, TAYLOR RN. Plasma from preeclamptic women increases human endothelial cell prostacyclin production without changes in cellular enzyme activity or mass. Am J Obstet Gynecol 1995;172:976-85.

72. GALLERY ED, ROWE J, CAMPBELL S, HAWKINS T. Effect of serum on secretion of prostacyclin and endothelin-1 by decidual endothelial cells from normal and preeclamptic pregnancies. Am J Obstet Gynecol 1995;173:918-23.

73. WANG JA, ZHEN EZ, GUO ZZ, LU YC. Effect of hyperlipidemic serum on lipid peroxidation, synthesis of prostacyclin and thromboxane by cultured endothelial cells: protective effect of antioxidants. Free Radic Biol Med 1989;7:243-9.

74. BAKER PN, DAVIDGE ST, BARANKIEWICZ J, ROBERTS JM. Plasma of preeclamptic women stimulates and then inhibits endothelial prostacyclin. Hypertension 1996;27:56-61.

75. FITZGERALD DJ, ROCKI W, MURRAY R, MAYO G, FITZGERALD GA. Thromboxane A2 synthesis in pregnancy-induced hypertension. Lancet 1990;335:751-4.

76. KLOCKENBUSCH W, SOMVILLE T, HAFNER D, STROBACH H, SCHROR K. Excretion of prostacyclin and thromboxane metabolites before, during, and after pregnancy-induced hypertension. Eur J Obstet Gynecol Reprod Biol 1994;57:47-50.

77. TAYLOR RN, DE GROOT CJ, CHO YK, LIM KH. Circulating factors as markers and mediators of endothelial cell dysfunction in preeclampsia. Semin Reprod Endocrinol 1998;16:17-31.

78. TAYLOR RN, VARMA M, TENG NN, ROBERTS JM. Women with preeclampsia have higher plasma endothelin levels than women with normal pregnancies. J Clin Endocrinol Metab 1990;71:1675-7.

79. CLARK BA, HALVORSON L, SACHS B, EPSTEIN FH. Plasma endothelin levels in preeclampsia: elevation and correlation with uric acid levels and renal impairment. Am J Obstet Gynecol 1992;166:962-8.

80. TAYLOR RN, CROMBLEHOLME WR, FRIEDMAN SA, JONES LA, CASAL DC, ROBERTS JM. High plasma cellular fibronectin levels correlate with biochemical and clinical features of preeclampsia but cannot be attributed to hypertension alone. Am J Obstet Gynecol 1991;165:895-901.

81. ISLAMI D, SHOUKIR Y, DUPONT P, CAMPANA A, BISCHOF P. Is cellular fibronectin a biological marker for pre-eclampsia? Eur J Obstet Gynecol Reprod Biol 2001;97:40-5.

82. BARDEN A, GRAHAM D, BEILIN LJ, RITCHIE J, BAKER R, WALTERS BN, MICHAEL CA. Neutrophil CD11B expression and neutrophil activation in pre-eclampsia. Clin Sci (Colch) 1997;92:37-44.

83. CLAUSEN T, DJUROVIC S, BROSSTAD FR, BERG K, HENRIKSEN T. Altered circulating levels of adhesion molecules at 18 weeks' gestation among women with eventual preeclampsia: indicators of disturbed placentation in absence of evidence of endothelial dysfunction? Am J Obstet Gynecol 2000;182:321-5.

84. AUSTGULEN R, LIEN E, VINCE G, REDMAN CW. Increased maternal plasma levels of soluble adhesion molecules (ICAM-1, VCAM-1, E-selectin) in preeclampsia. Eur J Obstet Gynecol Reprod Biol 1997;71:53-8.

85. DANIEL Y, KUPFERMINC MJ, BARAM A, GEVA E, FAIT G, LESSING JB. A selective increase in plasma soluble vascular cell adhesion molecule-1 levels in preeclampsia. Am J Reprod Immunol 1999;41:407-12.

86. DANIEL Y, KUPFERMINC MJ, BARAM A, JAFFA AJ, WOLMAN I, SHENHAV M, LESSING JB. Plasma soluble endothelial selectin is elevated in women with pre-eclampsia. Hum Reprod 1998;13:3537-41.

87. HALLER H, ZIEGLER E, HOMUTH V, DRAB M, EICHHORN J, NAGY Z, BUSJAHN A, VETTER K, LUFT FC. Endothelial Adhesion Molecules and Leukocyte Integrins in Preeclamptic Patients. Hypertension 1997;29 (Part 2):291-296.

88. KRAUSS T, KUHN W, LAKOMA C, AUGUSTIN HG. Circulating endothelial cell adhesion molecules as diagnostic markers for the early identification of pregnant women at risk for development of preeclampsia. Am J Obstet Gynecol 1997;177:443-9.

89. LYALL F, GREER IA, BOSWELL F, MACARA LM, WALKER JJ, KINGDOM JC. The cell adhesion molecule, VCAM-1, is selectively elevated in serum in pre-eclampsia: does this indicate the mechanism of leucocyte activation? Br J Obstet Gynaecol 1994;101:485-7.

90. CHAIWORAPONGSA T, ROMERO R, YOSHIMATSU J, ESPINOZA J, KIM YM, PARK K, KALACHE K, EDWIN S, BUJOLD E, GOMEZ R. Soluble adhesion molecule profile in normal pregnancy and pre-eclampsia. J Matern Fetal Neonatal Med 2002;12:19-27.

91. DE CATERINA R, SPIECKER M, SOLAINI G, BASTA G, BOSETTI F, LIBBY P, LIAO J. The inhibition of endothelial activation by unsaturated fatty acids. Lipids 1999;34 Suppl:S191-4.

92. DE CATERINA R, LIBBY P. Control of endothelial leukocyte adhesion molecules by fatty acids. Lipids 1996;31 Suppl:S57-63.

93. DE CATERINA R, LIAO JK, LIBBY P. Fatty acid modulation of endothelial activation. Am J Clin Nutr 2000;71:213S-23S.

94. BROWN AA, HU FB. Dietary modulation of endothelial function: implications for cardiovascular disease. Am J Clin Nutr 2001;73:673-86.

95. HUBEL CA, LYALL F, WEISSFELD L, GANDLEY RE, ROBERTS JM. Small low-density lipoproteins and vascular cell adhesion molecule-1 are increased in association with hyperlipidemia in preeclampsia. Metabolism 1998;47:1281-8.

96. POTTER JM, NESTEL PJ. The hyperlipidemia of pregnancy in normal and complicated pregnancies. Am J Obstet Gynecol 1979;133:165-70.

97. GRATACOS E, CASALS E, SANLLEHY C, CARARACH V, ALONSO PL, FORTUNY A. Variation in lipid levels during pregnancy in women with different types of hypertension. Acta Obstet Gynecol Scand 1996;75:896-901.

98. LORENTZEN B, DREVON CA, ENDRESEN MJ, HENRIKSEN T. Fatty acid pattern of esterified and free fatty acids in sera of women with normal and pre-eclamptic pregnancy. Br J Obstet Gynaecol 1995;102:530-7.

99. JENDRYCZKO A, DROZDZ M, WOJCIK A. Serum 18:2 (9, 11) linoleic acid in normal pregnancy and pregnancy complicated by pre-eclampsia. Zentralbl Gynakol 1991;113:443-6.

100. WANG YP, KAY HH, KILLAM AP. Decreased levels of polyunsaturated fatty acids in preeclampsia. Am J Obstet Gynecol 1991;164:812-8.

101. WILLIAMS MA, ZINGHEIM RW, KING IB, ZEBELMAN AM. Omega-3 fatty acids in maternal erythrocytes and risk of preeclampsia. Epidemiology 1995;6:232-7.

102. QIU C, WILLIAMS M, KING I, WALSH SW, FREDERICK I, KESTIN M, LEISENRING W, LUTHY D. A Prospective Study of Maternal Erythrocyte Omega-3 and Omega-6 Fatty Acids and Preeclampsia Risk. Am J Obstet Gynecol 2002;187:S77.

103. OGBURN PL, JR., WILLIAMS PP, JOHNSON SB, HOLMAN RT. Serum arachidonic acid levels in normal and preeclamptic pregnancies. Am J Obstet Gynecol 1984;148:5-9.

104. SHOUK TA, OMAR MN, FAYED ST. Essential fatty acids profile and lipid peroxides in severe pre-eclampsia. Ann Clin Biochem 1999;36:62-5.

105. CHUNG R, DAVIS H, MA Y, NAIVIKUL O, WILLIAMS C, WILSON K. Diet-related toxemia in pregnancy. I. Fat, fatty acids, and cholesterol. Am J Clin Nutr 1979;32:1902-11.

106. CLAUSEN T, SLOTT M, SOLVOLL K, DREVON CA, VOLLSET SE, HENRIKSEN T. High intake of energy, sucrose, and polyunsaturated fatty acids is associated with increased risk of preeclampsia. Am J Obstet Gynecol 2001;185:451-8.

107. WALSH SW. The role of oxidative stress and antioxidants in preeclampsia. Contemporary OB/GYN 1997;42:113-124.

108. GREER IA, LEASK R, HODSON BA, DAWES J, KILPATRICK DC, LISTON WA. Endothelin, elastase, and endothelial dysfunction in pre-eclampsia. Lancet 1991;337:558.

109. DAVIDGE ST. Oxidative stress and altered endothelial cell function in preeclampsia. Semin Reprod Endocrinol 1998;16:65-73.

110. ROGGENSACK AM, ZHANG Y, DAVIDGE ST. Evidence for peroxynitrite formation in the vasculature of women with preeclampsia. Hypertension 1999;33:83-9.

111. DAVIDGE ST, SIGNORELLA AP, HUBEL CA, LYKINS DL, ROBERTS JM. Distinct factors in plasma of preeclamptic women increase endothelial nitric oxide or prostacyclin. Hypertension 1996;28:758-64.

112. TAKACS P, KAUMA SW, SHOLLEY MM, WALSH SW, DINSMOOR MJ, GREEN K. Increased circulating lipid peroxides in severe preeclampsia activate NF-kappaB and upregulate ICAM-1 in vascular endothelial cells. Faseb J 2001;15:279-81.

113. WALKER JJ. Antioxidants and inflammatory cell response in preeclampsia. Semin Reprod Endocrinol 1998;16:47-55.

114. KNIGHT M, REDMAN CW, LINTON EA, SARGENT IL. Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. Br J Obstet Gynaecol 1998;105:632-40.

115. SMARASON AK, SARGENT IL, STARKEY PM, REDMAN CW. The effect of placental syncytiotrophoblast microvillous membranes from normal and pre-eclamptic women on the growth of endothelial cells in vitro. Br J Obstet Gynaecol 1993;100:943-9.

116. COCKELL AP, LEARMONT JG, SMARASON AK, REDMAN CW, SARGENT IL, POSTON L. Human placental syncytiotrophoblast microvillous membranes impair maternal vascular endothelial function. Br J Obstet Gynaecol 1997;104:235-40.

117. HELLEWELL PGW, T.J. The handbook of immunopharmacology. In: Page C, ed. Immunopharmacology of neutrophils. London: Harcourt Brace and Company, 1994.

118. CLARK P, BOSWELL F, GREER IA. The neutrophil and preeclampsia. Semin Reprod Endocrinol 1998;16:57-64.

119. ABRAMSON JS, WHEELER JG. The Neutrophil. New York: Oxford, 1993.

120. WEISS SJ. Tissue destruction by neutrophils [see comments]. N Engl J Med 1989;320:365-76.

121. LURIE S, FRENKEL E, TUVBIN Y. Comparison of the differential distribution of leukocytes in preeclampsia versus uncomplicated pregnancy. Gynecol Obstet Invest 1998;45:229-31.

122. GREER IA, HADDAD NG, DAWES J, JOHNSTONE FD, CALDER AA. Neutrophil activation in pregnancy-induced hypertension. British Journal of Obstetrics and Gynaecology 1989;96:978-982.

123. JANOFF A. Elastase in tissue injury. Annu Rev Med 1985;36:207-16.

124. GREER IA, DAWES J, JOHNSTON TA, CALDER AA. Neutrophil Activation Is Confined to the Maternal Circulation in Pregnancy-Induced Hypertension. Obstet Gynecol 1991;78:28-32.

125. BUTTERWORTH BH, GREER IA, LISTON WA, HADDAD NG, JOHNSTON TA. Immunocytochemical localization of neutrophil elastase in term placenta decidua and myometrium in pregnancy-induced hypertension. Br J Obstet Gynaecol 1991;98:929-33.

126. MELLEMBAKKEN JR, HOGASEN K, MOLLNES TE, HACK CE, ABYHOLM T, VIDEM V. Increased systemic activation of neutrophils but not complement in preeclampsia. Obstet Gynecol 2001;97:371-4.

127. TSUKIMORI K, MAEDA H, ISHIDA K, NAGATA H, KOYANAGI T, NAKANO H. The superoxide generation of neutrophils in normal and preeclamptic pregnancies. Obstet Gynecol 1993;81:536-540.

128. SACKS GP, STUDENA K, SARGENT K, REDMAN CW. Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis. Am J Obstet Gynecol 1998;179:80-6.

129. GERVASI MT, CHAIWORAPONGSA T, PACORA P, NACCASHA N, YOON BH, MAYMON E, ROMERO R. Phenotypic and metabolic characteristics of monocytes and granulocytes in preeclampsia. Am J Obstet Gynecol 2001;185:792-7.

130. SABATIER F, BRETELLE F, D'ERCOLE C, BOUBLI L, SAMPOL J, DIGNAT-GEORGE F. Neutrophil activation in preeclampsia and isolated intrauterine growth restriction. Am J Obstet Gynecol 2000;183:1558-63.

131. FAAS MM, SCHUILING GA, LINTON EA, SARGENT IL, REDMAN CW. Activation of peripheral leukocytes in rat pregnancy and experimental preeclampsia. Am J Obstet Gynecol 2000;182:351-7.

132. CROCKER IP, WELLINGS RP, FLETCHER J, BAKER PN. Neutrophil function in women with pre-eclampsia. Br J Obstet Gynaecol 1999;106:822-8.

133. CLARK CJ, CHETTIBI S, YOUNG JD, GREER IA, LYALL F. Locomotion of Human Neutrophils in Response to Plasma and Serum of Women with Preeclampsia. Hypertension in Pregnancy 1996;15:229-240.

134. CROCKER I, LAWSON N, DANIELS I, BAKER P, FLETCHER J. Significance of fatty acids in pregnancy-induced immunosuppression. Clin Diagn Lab Immunol 1999;6:587-93.

135. KUPFERMINC MJ, PEACEMAN AM, ADERKA D, WALLACH D, SOCOL ML. Soluble tumor necrosis factor receptors and interleukin-6 levels in patients with severe preeclampsia. Obstet Gynecol 1996;88:420-7.

136. SACKS GP, SCOTT D, TIVNANN H, MIRE-SLUIS T, SARGENT IL, REDMAN CW. Interleukin-12 and pre-eclampsia. J Reprod Immunol 1997;34:155-8.

137. VINCE GS, STARKEY PM, AUSTGULEN R, KWIATKOWSKI D, REDMAN CW. Interleukin-6, tumour necrosis factor and soluble tumour necrosis factor receptors in women with pre-eclampsia. Br J Obstet Gynaecol 1995;102:20-5.

138. VON DADELSZEN P, HURST G, REDMAN CW. Supernatants from co-cultured endothelial cells and syncytiotrophoblast microvillous membranes activate peripheral blood leukocytes in vitro. Hum Reprod 1999;14:919-24.

139. GOROG P. Activation of human blood monocytes by oxidized polyunsaturated fatty acids: a possible mechanism for the generation of lipid peroxides in the circulation. Exp. Path. 1991;72:227-237.

140. HAEGER M, UNANDER M, NORDER-HANSSON B, TYLMAN M, BENGTSSON A. Complement, neutrophil, and macrophage activation in women with severe preeclampsia and the syndrome of hemolysis, elevated liver enzymes, and low platelet count. Obstet Gynecol 1992;79:19-26.

141. SMARASON AK, SARGENT IL, REDMAN CW. Endothelial cell proliferation is suppressed by plasma but not serum from women with preeclampsia. Am J Obstet Gynecol 1996;174:787-93.

142. COLLINS T. Acute and Chronic Inflammation. In: Cotran RS, Kumar V, Collins T, eds. Robbins Pathologic Basis of Disease. Philadelphia: W.B. Saunders Company, 1999.

143. WAGNER JG, ROTH RA. Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. Pharmacol Rev 2000;52:349-74.

144. ROEBUCK KA. Oxidant stress regulation of IL-8 and ICAM-1 gene expression: differential activation and binding of the transcription factors AP-1 and NF-kappaB (Review). Int J Mol Med 1999;4:223-30.

145. LUSTER AD. Chemokines--chemotactic cytokines that mediate inflammation. N Engl J Med 1998;338:436-45.

146. REISTER F, FRANK HG, HEYL W, KOSANKE G, HUPPERTZ B, SCHRODER W, KAUFMANN P, RATH W. The distribution of macrophages in spiral arteries of the placental bed in pre-eclampsia differs from that in healthy patients. Placenta 1999;20:229-33.

147. HALIM A, KANAYAMA N, EL MARADNY E, MAEHARA K, TAKAHASHI A, NOSAKA K, FUKUO S, AMAMIYA A, KOBAYASHI T, TERAO T. Immunohistological study in cases of HELLP syndrome (hemolysis, elevated liver enzymes and low platelets) and acute fatty liver of pregnancy. Gynecol Obstet Invest 1996;41:106-12.

148. FAAS MM, SCHUILING GA, BALLER JF, VISSCHER CA, BAKKER WW. A new animal model for human preeclampsia: ultra-low-dose endotoxin infusion in pregnant rats. Am J Obstet Gynecol 1994;171:158-64.

149. FAAS MM, SCHUILING GA, BALLER JF, BAKKER WW. Glomerular inflammation in pregnant rats after infusion of low dose endotoxin. An immunohistological study in experimental pre-eclampsia. Am J Pathol 1995;147:1510-8.

150. GRAHAM MF, DIEGELMANN RF, ELSON CO, BITAR KN, EHRLICH HP. Isolation and culture of human intestinal smooth muscle cells. Proc Soc Exp Biol Med 1984;176:503-7.

151. WALSH SW, ALZOGHAIBI M, WILLEY A, GRAHAM MF. Linoleic acid specifically and markedly increases interleukin-8 secretion by human intestinal smooth muscle cells isolated from Crohn's bowel: evidence that modification of dietary fat may attenuate intestinal inflammation in Crohn's disease. Gasteroenterology (Suppl) 2002;122:A-146.

152. ALZOGHAIBI M, WALSH SW, WILLEY A, FOWLER III A, GRAHAM MF. Linoleic acid, but not oleic acid, upregulates the production of interleukin-8 by human intestinal smooth muscle cells isolated from patients with Crohn's disease. Clinical Nutrition 2003;In press.

153. MOSMANN T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.

154. TERANISHI K, SHIMOMURA O. Coelenterazine analogs as chemiluminescent probe for superoxide anion. Anal Biochem 1997;249:37-43.

155. SHIMOMURA O, WU C, MURAI A, NAKAMURA H. Evaluation of five imidazopyrazinone-type chemiluminescent superoxide probes and their application to the measurement of superoxide anion generated by Listeria monocytogenes. Anal Biochem 1998;258:230-5.

156. BOYUM A. Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest Suppl 1968;97:77-89.

157. BANCROFT JD, STEVENS A. Theory and practice of histological techniques. Edinburgh, New York: Churchill Livingstone, 1990.

158. SKUBITZ KM, KUROKI M, JANTSCHEFF P, SKUBITZ AP, GRUNERT F. CD66b. J Biol Regul Homeost Agents 1999;13:242-3.

159. SKUBITZ KM, CAMPBELL KD, SKUBITZ AP. CD66a, CD66b, CD66c, and CD66d each independently stimulate neutrophils. J Leukoc Biol 1996;60:106-17.

160. NAIR KS, ZINGDE SM. Adhesion of neutrophils to fibronectin: role of the cd66 antigens. Cell Immunol 2001;208:96-106.

161. SMART SJ, CASALE TB. Interleukin-8-induced transcellular neutrophil migration is facilitated by endothelial and pulmonary epithelial cells. Am J Respir Cell Mol Biol 1993;9:489-95.

162. BARDEN A. Circulating markers of oxidative stress are raised in normal pregnancy and pre-eclampsia. Br J Obstet Gynaecol 1999;106:1232.

163. WALSH SW, VAUGHAN JE, KAUMA SW. Oxidized linoleic acid stimulates neutrophil production of thromboxane by inducing PGHS-2. Journal of the Society for Gynecological Investigation 1999;6 (Supplement):194A (Abstract #582).

164. GRANGER JP, ALEXANDER BT, LLINAS MT, BENNETT WA, KHALIL RA. Pathophysiology of preeclampsia: linking placental ischemia/hypoxia with microvascular dysfunction. Microcirculation 2002;9:147-60.

165. BELFORT MA, VARNER MW, DIZON-TOWNSON DS, GRUNEWALD C, NISELL H. Cerebral perfusion pressure, and not cerebral blood flow, may be the critical determinant of intracranial injury in preeclampsia: a new hypothesis. Am J Obstet Gynecol 2002;187:626-34.

166. GREENE MF. Magnesium sulfate for preeclampsia. N Engl J Med 2003;348:275-6.

167. BUSSIERE FI, GUEUX E, ROCK E, GIRARDEAU JP, TRIDON A, MAZUR A, RAYSSIGUIER Y. Increased phagocytosis and production of reactive oxygen species by neutrophils during magnesium deficiency in rats and inhibition by high magnesium concentration. Br J Nutr 2002;87:107-13.

168. BUSSIERE FI, MAZUR A, FAUQUERT JL, LABBE A, RAYSSIGUIER Y, TRIDON A. High magnesium concentration in vitro decreases human leukocyte activation. Magnes Res 2002;15:43-8.

169. MAK IT, DICKENS BF, KOMAROV AM, WAGNER TL, PHILLIPS TM, WEGLICKI WB. Activation of the neutrophil and loss of plasma glutathione during Mg-deficiency--modulation by nitric oxide synthase inhibition. Mol Cell Biochem 1997;176:35-9.

170. BELFORT MA, ANTHONY J, SAADE GR, ALLEN JC, JR. A comparison of magnesium sulfate and nimodipine for the prevention of eclampsia. N Engl J Med 2003;348:304-11.

171. THOMSON AJ, TELFER JF, YOUNG A, CAMPBELL S, STEWART CJ, CAMERON IT, GREER IA, NORMAN JE. Leukocytes infiltrate the myometrium during human parturition: further evidence that labour is an inflammatory process. Hum Reprod 1999;14:229-36.

172. WINKLER M, FISCHER DC, RUCK P, MARX T, KAISERLING E, OBERPICHLER A, TSCHESCHE H, RATH W. Parturition at term: parallel increases in interleukin-8 and proteinase concentrations and neutrophil count in the lower uterine segment. Hum Reprod 1999;14:1096-100.

173. LEDINGHAM MA, THOMSON AJ, JORDAN F, YOUNG A, CRAWFORD M, NORMAN JE. Cell adhesion molecule expression in the cervix and myometrium during pregnancy and parturition. Obstet Gynecol 2001;97:235-42.

174. ISADA NB, GROSSMAN III JH. Perinatal Infections. In: Gabbe SG, Niebyl JR, Simpson JL, eds. Obstetrics Normal and Problem Pregnancies: Churchill Livingtone, 1991.

175. MARGOLIS KL, ASSAF A, BRAY PF, GREENLAND P, GRIMM RH, HOWARD B, MANSON JE, PRENTICE R, RODABOUGH R. Leukocyte Count as a Predictor of Cardiovascular Events in Post-Menopausal Women. Am Heart J 2002.

176. JACKSON MH, COLLIER A, NICOLL JJ, MUIR AL, DAWES J, CLARKE BF, BELL D. Neutrophil count and activation in vascular disease. Scott Med J 1992;37:41-3.

177. OLIVIERI O, LOMBARDI S, RUSSO C, GIRELLI D, GUARINI P, CARLETTO A, CORROCHER R. Neutrophil arachidonic acid level and adhesive capability are increased in essential hypertension. J Hypertens 1998;16:585-92.

178. OFOSU-APPIAH W, SFEIR G, SMITH D, RICHARD T. Neutrophil-mediated damage to vascular endothelium in the spontaneously hypertensive rat. Clin Immunol Immunopathol 1997;83:293-301.

179. GHOSH S, GOLDIN E, GORDON FH, MALCHOW HA, RASK-MADSEN J, RUTGEERTS P, VYHNALEK P, ZADOROVA Z, PALMER T, DONOGHUE S. Natalizumab for active Crohn's disease. N Engl J Med 2003;348:24-32.

180. MILLER DH, KHAN OA, SHEREMATA WA, BLUMHARDT LD, RICE GP, LIBONATI MA, WILLMER-HULME AJ, DALTON CM, MISZKIEL KA, O'CONNOR PW. A controlled trial of natalizumab for relapsing multiple sclerosis. N Engl J Med 2003;348:15-23.

181. WALLENBURG HC, DEKKER GA, MAKOVITZ JW, ROTMANS P. Low-dose aspirin prevents pregnancy-induced hypertension and pre-eclampsia in angiotensin-sensitive primigravidae. Lancet 1986;1:1-3.

182. SCHIFF E, PELEG E, GOLDENBERG M, ROSENTHAL T, RUPPIN E, TAMARKIN M, BARKAI G, BEN-BARUCH G, YAHAL I, BLANKSTEIN J, ET AL. The use of aspirin to prevent pregnancy-induced hypertension and lower the ratio of thromboxane A2 to prostacyclin in relatively high risk pregnancies. N Engl J Med 1989;321:351-6.

183. WALSH SW. Low-dose aspirin: treatment for the imbalance of increased thromboxane and decreased prostacyclin in preeclampsia. Am J Perinatol 1989;6:124-32.

184. WALSH SW. Physiology of low-dose aspirin therapy for the prevention of preeclampsia. Semin Perinatol 1990;14:152-70.

185. SIBAI BM, CARITIS SN, THOM E, KLEBANOFF M, MCNELLIS D, ROCCO L, PAUL RH, ROMERO R, WITTER F, ROSEN M, ET AL. Prevention of preeclampsia with low-dose aspirin in healthy, nulliparous pregnant women. The National Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Units. N Engl J Med 1993;329:1213-8.

186. CARITIS S, SIBAI B, HAUTH J, LINDHEIMER MD, KLEBANOFF M, THOM E, VANDORSTEN P, LANDON M, PAUL R, MIODOVNIK M, MEIS P, THURNAU G. Low-dose aspirin to prevent preeclampsia in women at high risk. National Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Units. N Engl J Med 1998;338:701-5.

187. COOMARASAMY A, HONEST H, PAPAIOANNOU S, GEE H, KHAN KS. Aspirin for prevention of preeclampsia in women with historical risk factors: a systematic review. Obstet Gynecol 2003;101:1319-32.

Vita