Role of Neutrophils in Enhancing Vascular Reactivity to Angiotensin II in Preeclampsia

Nikita Mishra
Virginia Commonwealth University

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ROLE OF NEUTROPHILS IN ENHANCING VASCULAR REACTIVITY TO ANGIOTENSIN II IN PREECLAMPSIA

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

by

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<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>A.A</td>
<td>antibiotics antimycotics</td>
</tr>
<tr>
<td>Ang II</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>APMA</td>
<td>amino-phenyl mercuric acetate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DMT</td>
<td>Danish myo technologies</td>
</tr>
<tr>
<td>D-PBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FST</td>
<td>Fine Science Tools</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloride</td>
</tr>
<tr>
<td>H₃PO₄</td>
<td>phosphoric acid</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>Hyp</td>
<td>hypoxanthine</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>ICAM-1</td>
<td>intercellular cell adhesion molecule-1</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL-8</td>
<td>interleukin-8</td>
</tr>
<tr>
<td>IUGR</td>
<td>intrauterine growth restriction</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
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<tr>
<td>M-199</td>
<td>medium-199</td>
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<tr>
<td>MCV</td>
<td>Medical College of Virginia</td>
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<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
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<td>MLC</td>
<td>myosin light chain</td>
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<td>MMP-1</td>
<td>matrix metalloproteinase-1</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MYPT1</td>
<td>myosin phosphatase target regulatory protein 1</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NP-40</td>
<td>nonyl phenoxylpolyethoxylethanol-40</td>
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<tr>
<td>ONOO⁻</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>PASM</td>
<td>placental arterial smooth muscle</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
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<tr>
<td>PGF₂α</td>
<td>prostaglandin-F₂α</td>
</tr>
<tr>
<td>pMLC</td>
<td>phosphorylated myosin light chain</td>
</tr>
<tr>
<td>pMYPT1</td>
<td>phosphorylated MYPT1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N’, N’ tetramethylethylenediamine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TTC</td>
<td>tris-triton-calcium</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule -1</td>
</tr>
<tr>
<td>VCU</td>
<td>Virginia Commonwealth University</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WBC</td>
<td>white blood cell</td>
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<tr>
<td>XO</td>
<td>xanthine oxidase</td>
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List of Units of Measurements

L  liter
ml  milliliter
µl  microliter
m  meter
mm  millimeter
µm  micrometer
g  gram
mg  milligram
µg  microgram
ng  nanogram
pg  picogram
M  molar (moles/liter)
mM  millimolar (millimoles/liter)
µM  micromolar (micromoles/liter)
°C  degrees Celsius
IU  international unit
rpm  revolutions per minute
Abstract

ROLE OF NEUTROPHILS IN ENHANCING VASCULAR REACTIVITY TO ANGIOTENSIN II IN PREECLAMPSIA

By Nikita Mishra, MBBS

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

Virginia Commonwealth University, 2010

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

Women with preeclampsia have enhanced vascular reactivity to Angiotensin II (Ang II) and extensive vascular infiltration of neutrophils. The primary mechanism to enhance vessel reactivity is RhoA kinase that phosphorylates MYPT1 to inhibit myosin light chain (MLC) phosphatase. Therefore, MLCs remain phosphorylated and increase sensitivity to calcium. Neutrophils release reactive oxygen species (ROS), which can activate this pathway, so we hypothesized that neutrophils would enhance vessel reactivity to Ang II. Omental vessels from normal pregnant women were used to study vascular reactivity. Ang II dose response (0.001-10µM) was significantly enhanced with perfusion of neutrophils (<2000/mm³, activated with IL-8) or ROS. Addition of
superoxide dismutase (SOD)/Catalase to quench ROS or 3µM Y-27632, a specific RhoA kinase inhibitor, blocked enhancement. Vascular smooth muscle expression of pMYPT1 and pMLC in cell culture was significantly increased by neutrophils or ROS. The increase was prevented by Y-27632. RhoA kinase activity assay showed a 3-fold increase in RhoA kinase activity in omental vessels treated with ROS. Similarly, ROS also enhanced vessel reactivity to another vasoconstrictor, norepinephrine, via RhoA kinase. In preeclamptic women, increased neutrophil infiltration is associated with increased vascular expression and production of matrix metalloproteinase-1 (MMP-1). MMP-1 activates protease activated receptor-1 (PAR-1), which could cause endothelial endothelin-1 release, so we considered a novel hypothesis that MMP-1 might cause vasoconstriction and enhance vessel reactivity to Ang II via PAR-1. Omental vessels perfused with activated MMP-1 (0.025-25ng/ml) showed dose-dependent vasoconstriction. Perfusion of activated MMP-1 (2.5ng/ml) significantly enhanced dose response to angiotensin II. MMP-1 mediated vasoconstriction and enhanced vessel reactivity to Ang II was abolished by co-perfusion of 10µM SCH-79797, a specific PAR-1 blocker, and by 5µM BQ-123, a specific endothelin-1 type A receptor blocker. These data are the first to show that activated neutrophils enhance vascular reactivity to Ang II via ROS and the RhoA kinase pathway. They are also the first to show that MMP-1 induces vasoconstriction and enhances vessel reactivity to Ang II. Thus, vascular neutrophil infiltration leading to ROS and MMP-1 generation could be an important mechanism for hypertension in preeclampsia.
CHAPTER 1

GENERAL INTRODUCTION

A. Preeclampsia

Preeclampsia is a hypertensive disorder of pregnancy, which results in considerable maternal and fetal morbidity and mortality. Sixteen percent of all pregnancy related deaths in the US from 1991-1999 were the result of pregnancy related hypertension\(^1\). The incidence of preeclampsia is 7.6% in nulliparous pregnancies and 3.3% of all nulliparous women experience severe disease\(^2\). Preeclampsia is a disorder more common at both extremes of age and also in the first pregnancy. Diastolic blood pressure above 95 mmHg significantly increases fetal death rate. African American women are 3 times more likely to die from preeclampsia as compared to Caucasian women\(^3\). In developing nations the perinatal mortality is more than six times higher in infants of preeclamptic women as compared to infants of normal pregnant women\(^4\) (WHO defines perinatal mortality as death of the fetus between 22 weeks of gestation and seventh day of life and includes fetal deaths during childbirth). Minimum defining criteria for preeclampsia are BP $\geq 140/90$ mm Hg after 20 weeks’ gestation in a previously known normotensive woman along with proteinuria of 300 mg/day or more\(^5\). Complications include HELLP (hemolysis, elevated liver enzyme levels, and a low platelet count) syndrome, eclampsia characterized by seizures with or without cerebral
hemorrhage and risk of maternal death. Currently, the only definitive treatment is delivery of the fetus and removal of the placenta. Since preeclampsia is usually seen early in pregnancy (< 34 weeks of gestation), delivery of the fetus results in a high incidence of pre-term infants. Pre-term infants are often low birth weight babies with high rates of mortality and morbidity.

**Pathophysiology of Preeclampsia:** Preeclampsia is a topic that has been vastly studied and yet a lot remains to be determined. A number of theories have been proposed to explain pathogenesis of preeclampsia and I will attempt to discuss the relevant literature to date.

1. **Endothelial Dysfunction:** The role of endothelium in the pathogenesis of preeclampsia is complex and involves a number of factors that ultimately lead to inappropriate endothelial cell activation, also known as endothelial cell dysfunction\(^6\). Endothelial activation results in increased production of vasoconstrictors and pro-coagulants like Von Willebrand’s factor (vWF)\(^7\), endothelin-1 (ET-1)\(^8\), thromboxane, prostaglandin-F2\(\alpha\) (PGF2\(\alpha\))\(^9\) and platelet derived growth factor (PDGF) along with a decrease in production of vasodilators\(^10\) and anti-coagulants like prostacyclins\(^11, 12\), nitric oxide (NO)\(^13\) and endothelium derived hyperpolarizing factor. This imbalance results in a vasoconstricted state that causes hypertension and reduced capacity of the vascular system in preeclampsia. The mitogenic activity of PDGF results in vessel hyperplasia and thickening\(^14\), akin to atherosclerosis. Moreover, placental factors in preeclampsia disrupt
endothelial junction molecules and increase endothelial permeability\textsuperscript{15}, which can explain the edema and proteinuria in preeclampsia\textsuperscript{16}. Endothelial activation also releases chymase, interleukin-8 (IL-8) and P-selectin and up-regulates expression of vascular cell adhesion molecule-1 (VCAM-1)\textsuperscript{17}. IL-8 is a potent chemo-attractant for neutrophils, while VCAM-1 and P-selectin\textsuperscript{18} promote attachment and infiltration of immune cells across the endothelium, thus promoting neutrophil infiltration and vascular inflammation in preeclampsia. Endothelial release of chymase increases local levels of angiotensin II (Ang II) by catalyzing conversion of Angiotensin I to Ang II\textsuperscript{19}.

2. **Oxidative stress:** Oxidative stress is a state characterized by an excess of oxidants as compared to anti-oxidants. The major oxidants are free radicals and reactive oxygen species (ROS). Free radical is defined as a molecule containing one or more unpaired electrons and capable of independent existence. ROS is a collective term for oxygen derived free radicals as well as non-radical derivatives of oxygen such as hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})\textsuperscript{20}. Antioxidants are compounds that can react with ROS and free radicals to convert them into non-oxidizing molecules. Major cellular antioxidant enzymes are catalase, glutathione peroxidase and superoxide dismutase (SOD). SOD dismutates the superoxide anion (O\textsubscript{2}\textsuperscript{−}) to H\textsubscript{2}O\textsubscript{2} and oxygen\textsuperscript{21}. Activity of SOD is significantly decreased in preeclampsia\textsuperscript{22, 23}. Catalase and glutathione peroxidase catalyse the conversion of H\textsubscript{2}O\textsubscript{2} to water and oxygen\textsuperscript{21}. Other known antioxidants are Vitamins A, E and C, as well as uric acid, transferrin, ceruloplasmin and quinines. In preeclampsia, there is a reduction in the total antioxidant capacity of the serum\textsuperscript{24} along with an increase in superoxide production.
In preeclampsia, levels of nitric oxide (NO) metabolites and endothelial nitric oxide synthase (eNOS) are elevated\textsuperscript{25}. Although this would seem to be protective as NO is a vasodilator, overproduction of $O_2^-$ by neutrophils\textsuperscript{26} results in endothelial cell injury because endothelial NO reacts with $O_2^-$ to form peroxynitrite (ONOO$^-$)\textsuperscript{27}. Peroxynitrite levels are elevated in preeclampsia\textsuperscript{28} and can induce eNOS uncoupling which causes the NO synthesizing enzyme to produce superoxide\textsuperscript{29}. Peroxynitrite thus interferes with NO dependent relaxation and can also decompose into the hydroxyl ion (OH$^-$)\textsuperscript{20}. Hydroxyl ion is a potent oxidizing agent that can directly impair cellular function and mediate the vascular response in preeclampsia\textsuperscript{20}. Levels of 8-epi-PGF$_2$\alpha, a marker of lipid peroxidation, are increased in preeclamptic women\textsuperscript{30}. Also abnormal mitochondria in preeclamptic placentas\textsuperscript{31} are indicative of oxidative stress at the cellular level\textsuperscript{32, 33}. Oxidative stress and lipid peroxidation of the endothelial cell membrane alter its fluidity and result in an increase in endothelial cell junction permeability\textsuperscript{34}.

3. Thromboxane/Prostacyclin Imbalance: In 1985, Walsh first demonstrated the presence of thromboxane/prostacyclin imbalance\textsuperscript{35}. Within the placenta, lipid peroxides are formed from the interaction of polyunsaturated fatty acids with free radicals in normal pregnancy\textsuperscript{36}. In placentas from preeclamptic pregnancies there is significantly more lipid peroxide formation\textsuperscript{37, 38}. Lipid peroxides are known to stimulate cyclooxygenase production of prostaglandins and thromboxanes\textsuperscript{39}. Lipid peroxides also inhibit the prostacyclin synthase enzyme\textsuperscript{40, 41}. Further evidence comes from studies showing decreased plasma concentrations and urinary excretion of prostacyclin (PGI$_2$) metabolites
by preeclamptic women\textsuperscript{11}. Thus, enhanced lipid peroxide formation in preeclamptic placental tissue results in thromboxane/prostacyclin imbalance favoring thromboxane\textsuperscript{42}. Thromboxane A\textsubscript{2} is a potent vasoconstrictor\textsuperscript{43} while PGI\textsubscript{2} is a potent vasodilator and inhibitor of platelet aggregation\textsuperscript{43, 44}. Thus, the thromboxane/prostacyclin imbalance induces placental as well as systemic vasoconstriction and can explain the placental hypoperfusion and intra-uterine growth restriction seen in preeclampsia.

4. **Vasomotor tone and reduced blood supply:** Lipid peroxides have been shown to cause vasoconstriction in the systemic vasculature\textsuperscript{45} and could explain maternal hypertension in preeclampsia. Lipid peroxides also increase calcium influx within the cells\textsuperscript{45}. The thromboxane/prostacyclin imbalance and increased sensitivity to usual pressors\textsuperscript{46} in preeclampsia could also partially explain the maternal hypertension and rise in basal tone. In preeclampsia, plasma volume is reduced, however levels of atrial natriuretic peptide (ANP) are elevated\textsuperscript{47} and plasma renin activity is low\textsuperscript{48}. This suggests that the vasculature is overfull even though there is low circulating plasma volume\textsuperscript{49} and that there is an even greater reduction in the capacity of the vasculature than the reduction of plasma volume. This is explained by the combined result of vasoconstriction and the formation of microthrombi in the vasculature secondary to the pro-coagulant state in preeclampsia. The end result is hypertension and reduced blood supply to all organs.

5. **Abnormal placentation:** Evidence for a role of abnormal placentation in the pathogenesis of preeclampsia is evident from a case report describing a woman with an
abdominal pregnancy and retained placenta. She remained preeclamptic for many months until the placenta was absorbed\textsuperscript{50}. Thus the presence of a placenta is a prerequisite to the development of preeclampsia but obviously not sufficient cause of preeclampsia in itself. It has been suggested that the underlying cause of preeclampsia is reduced placental perfusion. During normal pregnancy, the spiral arterioles of the non-pregnant state undergo considerable vascular remodeling to supply the inter-villous space of the developing placenta. These small muscular arteries increase considerably in diameter and lose their muscle and inner elastic lamina\textsuperscript{51}. During the first ten weeks of gestation high metabolic requirements of the fetus result in a hypoxic stimulus within the placenta which leads to the release of vascular endothelial growth factor (VEGF) from decidual macrophages. VEGF is an angiogenic factor that acts on the VEGF receptors on cytotrophoblasts and transforms them into endothelial-like cells. The lumen and vessel walls of the spiral arteries are invaded by extracellular trophoblast. This trophoblast invasion extends into the inner third of the myometrium and is completed by 20 weeks of gestation\textsuperscript{52}. These changes do not proceed in a normal manner in preeclamptic pregnancies. In preeclampsia, these changes may be absent or restricted to only the superficial decidual segment of the spiral arteries\textsuperscript{51}. This leads to decreased placental perfusion in preeclampsia.

B. Neutrophils in Preeclampsia:

Neutrophils are the most abundant white blood cells (WBC) in the circulation (65\% of all WBC). They are the first line of defense against bacterial and fungal
infections to be recruited to sites of infection and they contain an impressive armory of cytotoxic mechanisms capable of killing a wide range of microbial organisms. Inappropriate activation of these cytotoxic mechanisms can lead to tissue damage. Therefore, the process of neutrophil emigration and activation is controlled at several stages\textsuperscript{53}. In preeclampsia all these controls are overcome to result in neutrophil infiltration and activation in the absence of any underlying infection.

Neutrophil numbers are increased up to 2.5 fold in normal pregnancy\textsuperscript{65}. Luries et al reported further increase neutrophil numbers in preeclampsia as compared to normal pregnancy\textsuperscript{54}. Greer et al were the first to present evidence for neutrophil activation in preeclampsia by the demonstration of elevated levels of neutrophil elastase, a marker of neutrophil activation, in plasma from preeclamptic women as compared to that from normal pregnant women\textsuperscript{55}. Increased neutrophil elastase levels in preeclamptic women are independent of the absolute neutrophil count\textsuperscript{56} which is the number of neutrophils per mm\textsuperscript{3} of whole blood. The substrates for neutrophil elastase are elastin, collagen, fibrinogen and proteoglycans\textsuperscript{56}. Thus, along with other proteases it can cause vascular basement membrane damage and facilitate tissue infiltration of neutrophils. Vascular basement membrane damage could also contribute to the edema and proteinuria of preeclampsia.

Neutrophil activation in preeclampsia is confined to the maternal circulation\textsuperscript{57}. Additional evidence of neutrophil activation in preeclampsia came with demonstration of increased neutrophil expression of CD11b; a surface marker of neutrophil activation\textsuperscript{58}. Also, neutrophils from preeclamptic women generated significantly increased levels of
ROS and reduced expression of L-selectin (CD62L)\textsuperscript{59, 60}. Recently, it was shown that neutrophils isolated from preeclamptic women generated significantly more superoxide than the neutrophils from normal pregnant women but did not differ significantly from neutrophils of non-pregnant women\textsuperscript{61}. Also, the exposure to plasma from normal pregnant women inhibited superoxide production by non-pregnant neutrophils but did not affect superoxide production by preeclamptic neutrophils\textsuperscript{61}, suggesting altered response of preeclamptic neutrophils to the protective anti-oxidant factors in plasma from normal pregnant women.

Neutrophil gelatinase associated lipocalin (NGAL), a matrix glycoprotein of human neutrophil granules\textsuperscript{62}, is strongly induced during neutrophil activation and inflammation\textsuperscript{63}. Elevated levels of NGAL have been reported in women predestined to develop preeclampsia. NGAL levels showed a strong positive correlation with development of hypertension and proteinuria\textsuperscript{64}; providing additional evidence to support the role of neutrophils in the pathogenesis of preeclampsia. Luppi et al reported 4.5-fold increase in the DNA binding activity of NF-κB in preeclamptic women as compared to normal pregnant women not in labor\textsuperscript{60}. Even when compared to normal women in labor, there was a 3.5 and 2 fold increase in the nuclear translocation of NF-κB p50 and p65 subunits, respectively. Nuclear translocation of NF-κB subunits initiates transcription of genes involved in regulation of inflammation\textsuperscript{65}

\textbf{Source of activation of neutrophils:}
Neutrophil activation by placental lipid peroxides was first suggested by Walsh in 1994\textsuperscript{36}. The primary source of lipid peroxides in the placenta is the trophoblast layer of cells that bathe the maternal blood\textsuperscript{38}. Thus the neutrophils circulating within the intervillous space are exposed to high levels of lipid peroxides \textsuperscript{36} (Fig 1). Lipid peroxidation of leukocytes results in sustained activation\textsuperscript{66}. Lipid peroxide production is significantly higher in placentas of preeclamptic women\textsuperscript{38}. Placenta is also a rich source of linoleic acid\textsuperscript{67}, which is known to stimulate placental release of thromboxane. Thromboxane in turn stimulates TNF\textsubscript{α} and superoxide production by neutrophils\textsuperscript{68}. Placental secretion of TNF\textsubscript{α} can also directly activate neutrophils circulating within the intervillous space. Placental mRNA expression and synthesis of TNF\textsubscript{α} is significantly higher in preeclamptic placentas\textsuperscript{69}. Increased placental production and circulation of synctiotrophoblast microvillus membrane micro-particles (STMB), known to activate neutrophils via increased expression of CD11b and superoxide production, has been reported in preeclampsia\textsuperscript{70}.

Brinkmann et al reported that neutrophil activation by IL-8, LPS or phorbol myristate acetate resulted in the release of granule proteins and chromatin which formed a web of extracellular fibril matrix called neutrophil extracellular traps (NET)\textsuperscript{71}. NET is composed of a chromatin DNA backbone with histones, granular peptides and enzymes like elastase and myeloperoxidase\textsuperscript{71, 72}. Recently it was reported that placentally derived STMB and soluble factors like IL-8 efficiently activate neutrophils to generate NET’s
rich in DNA, elastase and histones, which trap STMB particles. Preeclamptic placentae showed significantly increased numbers of NET’s within the intervillous space. NET’s are also released within the maternal circulation and may explain the increased concentrations of maternal cell free DNA in preeclamptic women reported by Zhong. They also cause physical obstruction to blood flow within the placenta and contribute to placental hypo-perfusion and IUGR.

ii) **Cytokines and cell adhesion molecules**

Neutrophils are also activated by TNFα. Increased levels of circulating VCAM-1 and intercellular cell adhesion molecule-1 (ICAM-1) lead to increased neutrophil adherence to the endothelium. TNFα enhances neutrophil superoxide production and levels of TNFα and its soluble receptor are elevated in plasma and amniotic fluid of preeclamptic women. Elevated levels of IL-6 in preeclampsia are also associated with increased circulating ICAM-1 and increased endothelial expression of VCAM and E-selectin. Increased VCAM, E-selectin and ICAM-1 facilitate neutrophil adherence to the endothelium. IL-1 and IL-8 are potent activators of neutrophils while IL-6 and ET-1 prime neutrophils for superoxide production.

iii) **Small dense LDL and VLDL**
In preeclampsia concentrations of small dense LDL and VLDL-1 are increased. Small dense LDL is readily oxidized. Oxidized LDL induces VCAM-1 expression on endothelial cells\textsuperscript{89} and CD11b/CD18 expression on neutrophils promoting neutrophil adhesion to the endothelium\textsuperscript{90}.

iv) ET-1

Levels of ET-1 are increased in preeclampsia. ET-1 is involved in neutrophil superoxide production\textsuperscript{91}, intracellular calcium release\textsuperscript{92} and neutrophil aggregation\textsuperscript{93} and adhesion\textsuperscript{94}.

Activated neutrophils in turn release TNF\(\alpha\), IL-6 and superoxide, which can then generate feed forward cycles of neutrophil activation.

**Evidence of neutrophil infiltration of systemic vasculature in preeclampsia:**

Immunohistochemistry has shown the presence of significantly increased numbers of neutrophils flattened onto the endothelium, infiltrating within the intima and within muscular layers of resistance size arteries of the systemic circulation in preeclamptic women as compared to that in the vasculature of normal pregnant women\textsuperscript{95, 96}. Using the CD66b marker for granulocytes (96\% of granulocytes are neutrophils), it has been shown that there is significantly greater neutrophil infiltration within the systemic vasculature in preeclamptic women as compared to normal pregnant women\textsuperscript{96, 97}. Furthermore,
activation of the neutrophils infiltrating the vasculature was demonstrated with markers for activated NF-κB, COX-2 expression\textsuperscript{95}, IL-8 and ICAM-1\textsuperscript{96}. Of these, IL-8 is a potent chemo-attractant for neutrophils. Increased expression of IL-8 in the VSMC’s establishes a concentration gradient of IL-8 across the endothelium, promoting neutrophil emigration across the endothelium\textsuperscript{96}. Increased endothelial ICAM-1 expression promotes neutrophil adhesion and activation\textsuperscript{85}. Neutrophil activation and release of ROS along with cytokines like TNFα and IL-1 can induce NF-κB activity and trigger a cascade of inflammatory reactions\textsuperscript{65}. NF-κB activation triggers up-regulation of genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines and enzymes like COX-2\textsuperscript{98} causing inflammation.

In the presence of inflammation, endothelial cells express antigens that recruit, sequester and transfer circulating cells to the site of inflammation. This is a crucial and necessary role of endothelial cells to effect the neutrophil emigration. In preeclampsia, there is increased expression and circulating concentrations of VCAM-1, ICAM-1\textsuperscript{99} and P-selectin\textsuperscript{100}. Endothelial activation by placental derived factors results in up-regulation of adhesion molecules, such as P-selectin and E-selectin\textsuperscript{101}. Both are involved in the early phases (capture and rolling) of neutrophil/endothelial and platelet/endothelial interaction and adhesion. The increased ICAM-1 expression on endothelial cells along with increased CD11b and IL-8 receptor expression on neutrophils promotes neutrophil emigration in preeclampsia.

In preeclampsia inappropriate endothelial activation or endothelial dysfunction results in translocation of vWF and fibronectin from the abluminal surface to the luminal
surface of endothelial cells. vWF and fibronectin are both pro-coagulant molecules with adhesive properties and when on the luminal surface they interact with platelets and leukocytes in the circulation to promote neutrophil emigration\textsuperscript{102}.

Neutrophil degranulation on endothelial cells results in degradation of fibronectin and spread of fibronectin fragments on endothelial cells. Degraded fibronectin stimulates the neutrophils and can induce further fibronectin breakdown setting up a feed forward inflammatory loop. Loss of the fibrillar architecture of fibronectin on endothelial cells is a marker of endothelial injury\textsuperscript{103}. Circulating levels of fibronectin are elevated in preeclampsia\textsuperscript{104} suggesting increased neutrophil degranulation and fibronectin degradation in preeclampsia. Neutrophil degranulation at the endothelial surface leads to endothelial damage and dysfunction.

**Neutrophils and oxidative stress:**

Neutrophil activation and degranulation is capable of generating considerable oxidative stress as activated neutrophils make large quantities of superoxide via the NADPH oxidase enzyme system\textsuperscript{105}. Also, 1-4\% of oxygen used in the mitochondrial electron transport pathway is converted to superoxide\textsuperscript{106}. Wright et al first provided direct evidence of nitric oxide generation by human neutrophils. In neutrophils, nitric oxide is produced by oxidation of one of the terminal nitrogens of arginine via calcium-dependent inducible nitric oxide synthase (iNOS-Type II)\textsuperscript{107, 108}. The interactions and effects of neutrophil generation of reactive oxygen species and reactive nitrogen intermediates relevant to preeclampsia are illustrated in Fig 2. Hydrogen peroxide (H\(_2\)O\(_2\)) can easily
pass across cell membranes\textsuperscript{109}. However, within the neutrophils in the presence of myeloperoxidase, which is abundant in primary azurophilic granules of neutrophils\textsuperscript{110,111}, H\textsubscript{2}O\textsubscript{2} primarily reacts with halides like Cl\textsuperscript{−} to form hypochlorous acid (HOCl). HOCl is known to inactivate sulfhydryl dependent transporter systems within cell membranes\textsuperscript{112}. Peroxynitrite is a potent and reactive oxidant\textsuperscript{113} that can damage endothelial cells directly\textsuperscript{114,115} and impair vascular permeability\textsuperscript{116}. In the presence of H\textsubscript{2}O\textsubscript{2}, the half life of ONOO\textsuperscript{−} is prolonged\textsuperscript{117}. Transition metals catalyze formation of hydroxyl ion (OH\textsuperscript{−}) and nitronium ion (NO\textsubscript{2}\textsuperscript{+}) from ONOO\textsuperscript{−}. Hydroxyl ion is a highly reactive oxidant that can directly impair cellular function\textsuperscript{20}.

NET’s generate oxidative stress in the placenta by interfering with blood flow through intervillous space increasing the degree of hypoxia\textsuperscript{73}. Hypoxia promotes placental release of inflammatory cytokines like IL-6 and IL-8. These cytokines are known to stimulate NET production by neutrophils\textsuperscript{118} resulting in a vicious cycle. Thus, neutrophils play a role in the pathogenesis of preeclampsia by promoting placental hypoxia, systemic endothelial dysfunction and generation of local oxidative stress in the vasculature.

C. Enhanced Vascular Reactivity to Ang II in Preeclampsia:

Women with preeclampsia have hypertension by definition. Though normal human pregnancy is characterized by increased levels of circulating angiotensin II\textsuperscript{119}, normal pregnant women are relatively resistant to the effects of infused angiotensin II\textsuperscript{120}. 
In contrast, the vasculature of preeclamptic women is highly sensitive to the vasoconstrictive effect of angiotensin II and also other vasopressors\(^\text{46, 121, 122}\). In a prospective clinical study of primigravid women (women who are pregnant for the first time), Norman Gant and colleagues demonstrated enhanced vascular responsiveness to Angiotensin II (Ang II) in women who go on to develop preeclampsia\(^\text{123}\). Primigravid women who went on to develop pregnancy-induced hypertension required a lower dose of Ang II infusion in order to elicit a pressor response of \(> 20\) mmHg diastolic pressure as compared to those who remained normotensive throughout pregnancy. Results of the study are depicted graphically in Figure 3. This classic paper established that increased sensitivity to Ang II induced pressor response preceded the development of preeclampsia\(^\text{123}\). Most studies following this work reported that neither renin, renin substrate nor Ang II were elevated in preeclamptic women\(^\text{48, 124}\) supporting a role of enhanced vascular reactivity to Ang II in preeclampsia. In this section I will briefly review the regulation and mechanism of action of Ang II and go on to explain how neutrophil release of ROS could enhance the vasoconstrictive action of Ang II via activation of the RhoA kinase pathway.

i. Angiotensin II and VSMC Contraction:

a. Angiotensin II (Ang II) Generation
i. **ACE pathway:** Renal renin catalyses the conversion of angiotensinogen to angiotensin I (Ang I). Angiotensin converting enzyme (ACE) within the lung vasculature catalyses the release of histidyl-leucine from the COOH-terminus of Ang I to yield the octapeptide vasopressor, Ang II.\(^{125}\)

ii. Another mechanism of Ang II generation resides within the heart and systemic vasculature, the chymase/non-ACE pathway: Chymase or chymotrypsin like serine protease (CLP) was first found in mast cells.\(^ {126}\) Subsequently, it was shown to be present within the heart and isolated human arteries.\(^ {127}\) Eighty percent of the Ang II in the human heart comes from chymase dependent conversion of Ang I to Ang II.\(^ {19}\)

b. **Mechanism of Action:**

The vascular effects of Ang II are predominantly mediated by the AT1 receptor (Fig. 4). The downstream cell-signaling molecules involved are phospholipase C, rise in intracellular calcium levels, protein kinase C (PKC)\(^ {128}\), tyrosine kinases\(^ {129}\), mitogenesis associated protein kinase (MAPK) and the janus kinase (JAK) pathways. Tyrosine kinase and MAPK modulate contraction in smooth muscle through a variety of mechanisms.\(^ {129}\)

i) **VSMC contraction:** Myosin light chain kinase (MLCK) is a Ca-calmodulin dependent enzyme that phosphorylates the regulatory myosin light chain (MLC) permitting its activation by actin.\(^ {130}\) Cross-bridge cycling of phosphorylated MLC and actin results in VSMC contraction. Agonists like Ang II and ET-1 activate Ca\(^ {++}\) influx
through ligand-gated and store operated \( \text{Ca}^{++} \) channels in VSMC\textsuperscript{131}. However, the work of Fujihara suggests that agonist induced tonic contraction is largely a result of \( \text{Ca}^{++} \) sensitizing effect of activated RhoA/Rho kinase pathway\textsuperscript{132} with a possible small contribution of \( \text{Ca}^{++} \) influx that maintains \([\text{Ca}^{++}]_i\) above basal levels. Ang II induced VSMC contraction and hypertrophy is mediated via AT1-R [G-Protein coupled receptor (GPCR)] activation of Rho/RhoA kinase and protein kinase C (PKC) pathway\textsuperscript{133}.

ii) **Calcium sensitization pathways:** The RhoA/Rho kinase pathway is a major mechanism of \( \text{Ca}^{++} \) sensitization that acts by inhibition of smooth muscle myosin phosphatase (Fig 4)\textsuperscript{134}. In VSMCs, several agonists including Ang II stimulate Rho kinase activity by increasing GTP-bound Rho A\textsuperscript{150}. Rho activation also up-regulates AT1-R mRNA expression, thus enhancing Ang II effects via receptor up-regulation\textsuperscript{135}. Activated RhoA kinase phosphorylates the regulatory subunit of MLC phosphatase, MYPT1. This inhibits MLC phosphatase and slows de-phosphorylation of MLC\textsuperscript{134, 136, 137}. Increased levels of pMLC lead to \( \text{Ca}^{++} \) sensitization. Ang II induced Rho/RhoA kinase activation requires a tyrosine kinase, PYK-2, and its upstream activator, PKC-\( \delta \). PYK-2 may signal to Rho via phosphorylation and activation of Rho GEF\textsuperscript{138}. Y-27632, a selective RhoA kinase inhibitor\textsuperscript{139}, can induce \( \text{Ca}^{++} \)- desensitization by inhibiting Ang II induced MYPT1 phosphorylation at threonine 696\textsuperscript{133}.

Evidence for a role of PKC in \( \text{Ca}^{++} \) sensitization of the contractile apparatus of VSMC’s comes from sustained VSMC contraction by direct activation of PKC by phorbol esters without significant changes in \([\text{Ca}^{++}]_i\)\textsuperscript{140, 141}. PKC activation results in
activation of CPI-17, a phosphatase-1 inhibitor, which inhibits MLC phosphatase leading to Ca\textsuperscript{++} sensitization\textsuperscript{142, 143}. ET-1 increases PKC activity in VSMC\textsuperscript{144} and thereby increases vascular reactivity to Ang II.

Calcium desensitization is mediated by NO and cyclic guanosine monophosphate (cGMP)\textsuperscript{145, 146}. cGMP mediated protein kinase (PKG) antagonizes RhoA/Rho kinase pathway by reversal of G-protein coupled inhibition of MLC phosphatase by PKG activation\textsuperscript{147, 148}. Cyclic AMP and cGMP activate A-kinase & G-kinase\textsuperscript{149} respectively, which phosphorylate site A of MLCK, decreasing its affinity for Ca-calmodulin 10-fold\textsuperscript{150}. In this way NO and cGMP reduce MLCK activity leading to Ca\textsuperscript{++} desensitization.

iii) Sympathetic Nervous System: In vivo, vascular smooth muscle is under the influence of the sympathetic system. Ang II inhibits K\textsuperscript{+} currents in neurons leading to increased Ca\textsuperscript{++} current and increased neuronal firing leading to norepinephrine (NE) release and activation of the sympathetic system\textsuperscript{151}. Although sympathetic activity is decreased during normal pregnancy it is reversibly increased in stress and in women with preeclampsia\textsuperscript{152}. Stress related sympathetic stimulation and NE release might have a contributory role in the development of preeclampsia\textsuperscript{153-155}. Treatment of cultured sympathetic cells with preeclamptic plasma results in significant increase in NE release\textsuperscript{156}. NE is the most important neurotransmitter of the sympathetic system. In subcutaneous resistance arteries of preeclamptic women, NE induced Ca\textsuperscript{++} sensitization\textsuperscript{157, 158}, which was previously shown to be associated with an increase in pMLC as a result of decreased MLC phosphatase activity mediated via PKC\textsuperscript{159}. However,
as opposed to Ang II induced hypertension, NE induced hypertension did not increase superoxide production or oxidase expression\(^{160}\).

ii. **Sources of Ang II in Preeclampsia:**

During pregnancy, apart from the renal renin angiotensin aldosterone system (RAAS), there is also the placental RAAS\(^{161}\). Placental hypoxia in preeclampsia can induce increased ACE mRNA expression and activity in the venous endothelial cells of the stem villous which is the main site of conversion of Ang I to Ang II in the placenta\(^{162}\). The levels of ACE and Ang II are increased in placental tissues and umbilical venous plasma of preeclamptic women\(^{163}\). Shah introduced the concept that due to reduced perfusion of the utero-placental unit in preeclampsia, the placenta behaves like the clipped kidney of the 2 kidney 1 clip Goldblatt model\(^{164}\). In concurrence with the implication of placental RAAS activation (akin to the clipped kidney) in preeclampsia, renin gene expression and Ang II levels were 3-fold higher in the decidua and chorionic villi of preeclamptic women\(^{165}\). Preeclampsia is characterized by up-regulation of AT1R in the decidua\(^{166}\), however, the aminopeptidase-A (AP-A) enzyme cleaves N-terminal aspartyl acid residue of Ang II to form its inactive metabolite Ang III\(^{167}\) so the enzyme acts as a feto-maternal barrier to transport of Ang II across the placenta\(^{168}\). AP- A is induced by Ang II via AT1 and AT-2 receptors. The AP-A system is ideally localized within the basal zone of the syncytiotrophoblast to effectively remove Ang II from the fetal circulation (up to 90%)\(^{169}\).
Apart from placental Ang II, endothelial cells can be a source of local release of Ang II on VSMCs. Earlier studies have shown renin substrate, renin activity, Ang I and Ang II generation within endothelial cells\textsuperscript{170, 171}. Increased CLP and chymase activity is seen in women with preeclampsia\textsuperscript{172, 173}. Thus, CLP/chymase dependent generation of Ang II and/or ET-1 may regulate contractile function of the VSMC in preeclampsia. Also, increased basal release of angiotensin II and endothelial CLP/chymase activation was reported in response to stimulation by preeclampsia placental conditioned medium (PE-CM)\textsuperscript{172}. Basal release of Ang II increased in a concentration dependent manner with application of apical chymotrypsin was inhibited by chymotrypsin inhibitor and unaffected by ACE-I. This suggests additional angiotensin II generation by the endothelial cells via the chymase/non-ACE pathway right onto the vascular smooth muscle cells of the vessel walls in preeclamptic women. In preeclampsia high levels of Ang II locally can induce phosphodiesterase 1A1 (PDE1A1) which degrades cGMP to 5'-GMP\textsuperscript{174}. Inactivation of cGMP by AngII may also play a role in the development of Ca\textsuperscript{++}-sensitization in VSMCs. PE-CM also induced translocation of the AT-1 receptor from the cytosol to the nuclei which suggests AT-1 receptor up-regulation by the PE-CM\textsuperscript{172} potentially leading to increased sensitivity to Ang II.

iii. Role of Angiotensin II in preeclampsia:

Angiotensin II contributes to ROS production and inflammatory responses via activation of NADPH oxidase and NF-κB in trophoblasts and VSMCs\textsuperscript{175, 176}. This effect is important physiologically as even a low dose of Ang II (10\textsuperscript{-10} M) has been shown to
increase NADPH oxidase derived ROS in cultured VSMCs\textsuperscript{177, 178}. Baseline ROS production by vascular NADPH oxidase is low. On stimulation, ROS production by vascular NADPH oxidase is only a third of what neutrophils produce, but it is sustained for a longer duration\textsuperscript{179}. Long term (hours to days) ROS generation is mediated by p22phox gene up-regulation\textsuperscript{180} while short term ROS production probably involves assembly of component subunits into a functional oxidase and/or subunit biochemical modifications\textsuperscript{181}. Increased amount of superoxide produced in VSMCs combines with NO to form ONOO\textsuperscript{-182}. Peroxynitrite contributes to vasoconstriction and vascular injury\textsuperscript{183}. Increase in NO degradation rather than reduced production impairs endothelium dependent vasodilation\textsuperscript{184}. Angiotensin II stimulated ROS production within the VSMC induces degradation of I-κB leading to NF-κB activation\textsuperscript{185, 186}. Ang II and AT1-AA also stimulate endothelial cell expression of tissue factor (TF)\textsuperscript{187}. Enhanced TF activity in preeclampsia is associated with enhanced cell adhesion and migration of immune cells\textsuperscript{188} promoting neutrophil adhesion, emigration and synthesis of TNF-α and IL-6\textsuperscript{189}. Angiotensin II also promotes expression of soluble fms-like tyrosine kinase (sFlt-1) in decidual tissue, sFlt-1 is an antagonist of VEGF and placental growth factor\textsuperscript{190} believed to play a role in the pathogenesis of preeclampsia by interference with placentation and vascular remodeling.

**D. Role of neutrophils in enhanced vascular reactivity to Ang II in preeclampsia**

There are a number of ways neutrophils could cause vasoconstriction or enhance vascular reactivity to Ang II. Direct vasoconstrictive effects of thromboxane A\textsubscript{2} released
by activated neutrophils infiltrating the systemic vasculature of preeclamptic women\textsuperscript{191} could contribute to hypertension in preeclampsia in addition to ROS generation by neutrophils which impairs stability of the endothelium derived vascular relaxing factor, NO\textsuperscript{192}. Neutrophil production of hydroxyl radicals, superoxide anions and iron dependent reactive species can impair endothelium dependent relaxation\textsuperscript{193}. High concentrations of ROS inhibit the PI3K/Akt pathway decreasing endothelial NO production and thus increasing vascular tone\textsuperscript{194,195}. ROS are known to activate the Rho kinase pathway in a rat model of Ang II induced hypertension\textsuperscript{196}. Neutrophil release of ROS acting via the Rho kinase pathway could increase $[\text{Ca}^{++}]_{i}$ sensitivity to Ang II. ROS can also stimulate release of ET-1\textsuperscript{197}. ET-1 is a vasoconstrictor that also enhances $[\text{Ca}^{++}]_{i}$ sensitivity in VSMC’s\textsuperscript{198}. Relaxin is a hormone which when incubated with human neutrophils causes a reduction in CD11b expression, superoxide generation, $[\text{Ca}^{++}]_{i}$, release of granules and chemotactic migration\textsuperscript{199}. Relaxin is a potent vasodilator and its levels decline with advancing gestation. In preeclampsia, low levels of relaxin are associated with vasoconstriction\textsuperscript{200} and possibly neutrophil activation secondary to loss of the regulatory control of relaxin.

Neutrophil release of pro-inflammatory cytokines like IL-6 and TNF-\textalpha can activate the sympathetic nervous system\textsuperscript{201}. Three-fold higher sympathetic nerve activity is seen in preeclamptic women as compared to normotensive pregnant women\textsuperscript{202}. IL-6 treated rats have higher plasma renin activity, enhanced vasoconstriction, decreased pressure natriuresis and they develop hypertension\textsuperscript{203}. TNF-\textalpha infusion causes hypertension associated with transcriptional up-regulation of the ET-1 gene in the kidney,
placenta and vasculature\textsuperscript{204}. TNF-\(\alpha\) released by neutrophils can contribute to ROS production and oxidative stress generation by specifically stimulating superoxide production at the CoQ site of the electron transport chain in the mitochondria\textsuperscript{205}. Small concentrations of TNF-\(\alpha\) and IL-6 cause a reduction in acetylcholine induced vasodilation and increased production of endothelium derived contracting factors like ET-1\textsuperscript{206, 207}. Also, estradiol, levels of which are high in pregnancy, enhances leukocyte binding to endothelial cells via an increase in TNF-\(\alpha\) induced adhesion molecules promoting further neutrophil infiltration within the systemic vasculature of preeclamptic women. Neutrophil release of TNF\(\alpha\) might increase basal vascular tone by decreasing NO production and by interfering with insulin-mediated vasodilatation in preeclampsia\textsuperscript{208}.

**E. Role of Matrix Metalloproteinase-1**

Along with neutrophil infiltration and vascular inflammation, MMP-1 expression is significantly increased in the vasculature of preeclamptic women (Estrada-Gutierrez, Walsh, unpublished). Immunohistochemistry showed significantly greater staining for MMP-1 in the endothelium, vascular smooth muscle and infiltrating leucocytes. Consistent with a 7-fold increase in MMP-1 gene expression, pro-MMP-1 as well as activated MMP-1, were more abundant in omental vessels from preeclamptic women as compared to normal pregnant women. Moreover, plasma level of MMP-1 is also significantly higher in preeclamptic women. Thus we wanted to examine a role for MMP-1 in preeclampsia.
MMP-1 is known for its collagenolytic properties and has been widely studied with respect to its role as a collagenase. However, in recent years MMP-1 has been shown to activate the protease activated receptor-1 (PAR-1)\textsuperscript{209, 210}, also known as thrombin receptor. PAR-1 is a G-protein coupled receptor (GPCR) that is involved in a number of cellular regulatory and signaling pathways. MMP-1 cleaves the N-terminal end of PAR-1 two amino acids distal to the cleavage site of thrombin. This forms a new N-terminus that binds to the tethered ligand binding site leading to PAR-1 activation\textsuperscript{211-213}. MMP-1 induced N-terminal proteolytic cleavage resulting in irreversible PAR-1 activation is unique to PARs. Therefore PAR signaling is tightly regulated by rapid desensitization at the plasma membrane by β-arrestins\textsuperscript{214}, rapid phosphorylation and receptor trafficking\textsuperscript{215}. In endothelial cells PAR-1 undergoes rapid agonist-induced internalization, is delivered to lysosomes for degradation, and receptors from subcellular location are translocated to the surface upon PAR-1 agonist stimulation\textsuperscript{216}.

Our lab recently reported significantly increased PAR-1 expression in human omental vessels from preeclamptic women with little to no PAR-1 expression in normal pregnant women (Estrada-Gutierrez, Walsh, unpublished). Also endothelial cells express PAR-1 and MMP-1 can cause PAR-1 mediated endothelial activation\textsuperscript{217}. Thus in normal pregnant women, MMP-1 can activate endothelial PAR-1 leading to endothelial cell activation and release of endothelial cell granules containing vWF, IL-8 and ET-1\textsuperscript{18}. Endothelin-1 is the only endothelin produced by endothelium and it acts on VSMC via the ET\textsubscript{A} receptor to cause vasoconstriction and smooth muscle proliferation. Endothelin-1
also contributes to increased vessel reactivity to vasoconstrictors like Ang II and NE. Thus, MMP-1 can contribute significantly to the pathogenesis of preeclampsia by PAR-1 mediated endothelial activation and release of ET-1.

In preeclampsia neutrophils could be a source of MMP-1\textsuperscript{218}, as immunohistochemistry staining for MMP-1 and neutrophils co-localize. However, our lab has shown diffuse staining for MMP-1 within the vascular smooth muscle and that neutrophils and neutrophil products like ROS and TNF-\alpha induce MMP-1 expression by VSMC in vitro (Estrada-Gutierrez, Walsh, unpublished). Vascular smooth muscle cells express PAR-1\textsuperscript{219}. Protease activated receptor-1 has also been implicated in increased leucocyte endothelial adherance and rolling, migration of leukocytes from the vasculature into inflamed tissues\textsuperscript{220} and causing edema via a breach in endothelial integrity\textsuperscript{221}. Via PAR-1 activation, our lab has shown that MMP-1 induces VSMC production of IL-8 and collagen fragments, which are chemotactic to neutrophils and promote neutrophil infiltration and vascular inflammation (Estrada-Gutierrez, Walsh, unpublished). Thus, neutrophils and MMP-1 may be co-conspirators in the pathogenesis of preeclampsia.

F. Purpose of Investigation

Women who develop preeclampsia have enhanced vascular reactivity to Ang II and decreased sensitivity to insulin. Neutrophil infiltration and vascular inflammation was recently described within the systemic vasculature of preeclamptic women. Neutrophils release toxic compounds like reactive oxygen species (ROS) and tumor necrosis factor-alpha (TNF-\alpha) that could adversely affect the vasculature.
Neutrophil release of ROS might enhance vessel reactivity to Ang II. The primary mechanism to enhance vessel reactivity is the RhoA kinase (ROK) pathway. ROK phosphorylates MYPT1, the regulatory protein of MLC phosphatase. This inhibits MLC phosphatase and thus inhibits de-phosphorylation of myosin light chains (MLC). The resultant increase in phosphorylated MLC (pMLC) enhances vascular smooth muscle cell sensitivity to intracellular calcium. ROS activate ROK, so neutrophils could enhance vessel reactivity by release of ROS leading to activation of the ROK pathway.

Neutrophil release of ROS and TNFα might also adversely affect vascular function by promoting insulin resistance as a result of down-regulating phosphatidylinositol 3-kinase (PI3K) and thus inhibiting GLUT-4 receptor translocation in vascular smooth muscle cells. TNFα could down-regulate the activity and/or expression of PI3K because it is the direct downstream target of insulin receptor substrate-1 and it is also upstream of phosphorylated Akt, both of which are inhibited by TNFα. ROS might down-regulate PI3K by activating RhoA, which inhibits PI3K/Akt signaling in endothelial and vascular smooth muscle cells.

The following are the specific aims and hypotheses for my thesis dissertation, which are designed to elucidate the role of neutrophils and neutrophil products in the development of enhanced vessel reactivity to Ang II, vasoconstriction and insulin resistance in preeclampsia.

G. Overall Hypothesis
Specific Aim 1: To determine if neutrophils or neutrophil products, ROS and TNFα, enhance vessel reactivity to vasoconstrictors like Ang II and NE.

Hypothesis 1: Activated neutrophils enhance vessel reactivity to Ang II.

Neutrophils isolated from healthy non-pregnant women will be activated with IL-8 and perfused at physiologic concentration through the lumen of human omental resistance arteries mounted on a myograph system. The vasoconstrictive response to Ang II will be compared to the response in the absence of neutrophils. To determine the mechanisms underlying enhanced vessel reactivity superoxide dismutase/catalase will be used to quench ROS and Y-27632 to inhibit ROK.

Hypothesis 2: Neutrophil products, ROS and TNFα, will enhance vessel reactivity to Ang II.

To test this hypothesis, human resistance arteries will be dissected from omental fat obtained from normal pregnant women and mounted on a myograph system to assess vasoconstrictive response to Ang II in the presence and absence of neutrophil products, ROS and TNFα. Pharmacologic inhibitors, superoxide dismutase/catalase and Y-27632 will be used to determine the mechanism underlying neutrophil mediated enhanced vessel reactivity to Ang II.
Hypothesis 3: Neutrophils or neutrophil products will activate RhoA kinase in human vascular smooth muscle cells as evidenced by increased phosphorylation of MYPT1 and MLC.

To test this hypothesis, primary cultures of human vascular smooth muscle cells will be co-cultured with neutrophils (activated using arachidonic acid) or treated with neutrophil products, ROS and TNFα, for 24 hrs, after which cells will be harvested. The cell lysates will be analysed using Western blotting to quantify the relative amounts of phosphorylated and non-phosphorylated MLCs. Western Blotting using a specific antibody to pMYPT1-Thr696 will be done for quantification of MYPT1 phosphorylation.

To test this hypothesis ex-vivo, human omental vessels will be dissected and treated with ROS or Dulbecco’s phosphate buffered saline (D-PBS) alone. Within 5 minutes, the vessels will be flash frozen in liquid nitrogen and processed for determination of RhoA kinase activity using radioisotope labeled adenosine tri-phosphate (ATP) assay.

Hypothesis 4: ROS will enhance vessel reactivity to NE.

To test this hypothesis, human resistance arteries will be dissected from omental fat obtained from normal pregnant women and mounted on a myograph system to assess vasoconstrictive response to NE in the presence and absence of ROS. Pharmacologic inhibition of RhoA kinase with Y-27632 will be used to determine if activation of RhoA kinase underlies ROS mediated enhanced vessel reactivity to NE.
**Specific Aim 2:** To determine if MMP-1 causes vasoconstriction or enhances vessel reactivity to Ang II via activation of PAR-1 and ET-1 release from endothelium.

**Hypothesis 1:** MMP-1 causes dose-dependent vasoconstriction in human omental vessels.

To test this hypothesis, human resistance arteries will be dissected from omental fat obtained from normal pregnant women and mounted on a myograph system to assess vasoconstrictive response to varying doses of MMP-1. To determine the mechanisms underlying MMP-1 induced vasoconstriction, SCH-79797, a PAR-1 inhibitor, and BQ-123, an ET<sub>A</sub> receptor blocker, will be used.

**Hypothesis 2:** MMP-1 enhances vessel reactivity to Ang II.

To test this hypothesis, human omental fat arteries from normal pregnant women will be used to assess vasoconstrictive response to varying doses of Ang II in the presence and absence of a low dose of MMP-1. To determine the mechanism underlying enhanced vessel reactivity, SCH-79797 and BQ-123 will be used.

**Specific Aim 3:** To determine if neutrophils or neutrophil products could cause insulin resistance by affecting PI3K.

**Hypothesis 1:** Neutrophils and/or neutrophil products will decrease protein expression of the regulatory (p85α) and catalytic (p110α) subunits of PI3K.

To test this hypothesis, human vascular smooth muscle cells will be co-cultured with neutrophils (activated using arachidonic acid) or treated with neutrophil products,
ROS and TNFα, for 48 hours, after which cells will be harvested and frozen at -80°C until analysis. The protein cell lysate will be analyzed by Western Blotting using specific antibodies for the regulatory (p85α) and catalytic (p110α) subunits of PI3K. All comparisons will be made to the control with culture medium alone.

In summary, my dissertation will examine if infiltration of neutrophils into the systemic vasculature of preeclamptic women can explain the enhanced reactivity to Ang II and insulin resistance characteristic of preeclampsia. It will also explore the mechanisms by which neutrophils and neutrophil products enhance reactivity to Ang II and determine if these phenomena are generalized or specific to Ang II. It will also explore the role of MMP-1 in vasoconstriction and enhancement of vessel reactivity to Ang II.

H. Significance of this research

If neutrophils or neutrophil products, ROS or TNF-α, are found to enhance vessel reactivity to Ang II, then neutrophil infiltration and inflammation can explain the increased vascular reactivity to Ang II in preeclampsia and provide the first explanation for development of hypertension in preeclampsia. Moreover, if MMP-1 causes dose dependent vasoconstriction or enhances vascular reactivity to Ang II, it may provide alternate or additional evidence for explaining development of hypertension in preeclampsia. If increased vessel reactivity to Ang II by neutrophils and ROS is mediated
via the RhoA kinase pathway, the role of RhoA kinase inhibitors could be explored as
treatment options for preeclampsia. If MMP-1 causes vasoconstriction and enhances
vessel reactivity to Ang II via PAR-1 and ET-1, then PAR-1 inhibitors and ET_{A} receptor
blockers could be studied for treatment of preeclampsia. If neutrophils are implicated in
the pathogenesis of preeclampsia, neutrophil-neutralizing antibodies are another
possibility that may be useful in preventing development or arresting progression of
preeclampsia by prevention of neutrophil adhesion and infiltration within the maternal
systemic vasculature.
Figure 1. Theory of neutrophil activation in the intervillous space in placenta.

Neutrophils are activated as they circulate through the inter-villous space by lipid peroxides secreted by the placental trophoblast cells. Neutrophils return to the maternal compartment to cause vascular damage by generation of reactive oxygen species and oxidative stress. (Used with permission of artist. From Walsh, S. W. The role of oxidative stress and anti-oxidants in preeclampsia. Contemporary OB/GYN, 1997; 42: 113-124)
Figure 2. Pathways of neutrophil induced oxidative stress production. Interactions between reactive oxygen intermediates and reactive nitrogen intermediates resulting in nitric oxide (NO) scavenging by superoxide (O$_2^-$) production leading to formation of peroxynitrite (ONOO$^-$).
Figure 3. Data redrawn from Gant et al (The Journal of Clinical Investigation, Nov 1973, Vol52, pg 2682-2689). This study clearly established development of sensitivity to the pressor response of Angiotensin II during pregnancy as a predecessor to the development of preeclampsia.
Figure 4. RhoA kinase pathway. Release of reactive oxygen species (ROS) by activated neutrophils may activate RhoA kinase (ROK) to result in phosphorylation (p) of the regulatory myosin light chain (MLC) phosphatase target subunit 1 (MYPT1), which inhibits the MLC phosphatase enzyme (MLCP). Inhibition of MLCP leads to decreased MLC dephosphorylation, an accumulation of phosphorylated myosin light chains and thus enhanced contraction for a given change in intracellular calcium level by the same agonist concentration. (CaM-calcium calmodulin complex, MLCK- myosin light chain kinase).
Figure 5. Matrix metalloproteinase-1 (MMP-1) activation of PAR-1. MMP-1 induced cleavage of the N-terminal end of the PAR-1 receptor generates a new N-terminal end that allows inter-molecular interaction of the tethered ligand to the tethered ligand binding site on the exo-domain of PAR-1, which results in activation of PAR-1. Activation of endothelial PAR-1 can lead to endothelial activation with release of endothelial cell granule contents like von Willebrand’s factor (vWF) and endothelin-1 (ET-1).
CHAPTER 2

MATERIALS AND METHODS

A. Myograph System:

i. Collection of Fat

Omental fat biopsies (approximately 3 cm x 2 cm x 0.5 cm) were collected from normal pregnant women and women with preeclampsia undergoing cesarean section at Medical College of Virginia (MCV) Hospitals, Virginia Commonwealth University (VCU) Medical Center. The fat was placed in a labeled bottle with isotonic normal saline. The bottle was kept in a refrigerator and transferred to the laboratory on ice as soon as possible. After the vessel arrived, it was immersed in cold (0-4°C) Dulbecco’s Phosphate Buffered Saline (D-PBS) containing glucose, sodium pyruvate, calcium chloride and magnesium chloride (Gibco, Invitrogen) on a silicone dissection dish pre-cooled to 4°C.

ii. Vessel Dissection
The fat biopsy was pinned down on the silicone dissection dish in cold (0-4°C) D-PBS to visualize the vasculature under a dissection microscope (Olympus, B061, zoom 1:7.5). A vessel of approximately 200-500 µ diameter was selected for dissection. The fat around the vessel up to a length of 2 cm was dissected and discarded, while being careful to leave about 1 mm of fat around the vessel to avoid damaging the vessel. Then, the vessel was fixed on the dissection dish using two pins at either end. The artery was identified from the vein (often the artery and vein ran together) (Fig.6). Fine dissection was done using a 0.075 mm tip scissors (Fine Science Tools (FST) No.15001-08). Tissue was held with Dumont #55 forceps (FST no.11255-20) and gently pulled away from the artery. The scissors were used to cut the fat as close to the artery as possible, keeping the cutting edge of the scissors parallel to the length of the artery and the curved tip of the scissors pointing away from the artery. The artery was cleaned of adherent fat on all sides making note of branch points and being careful not to puncture the vessel at any point during dissection. A 1 cm length of vessel, free of branch points or potential punctures was selected for mounting.

iii. Mounting of the vessel

To mount the vessel on the myograph chamber (Fig.7) of the pressure myograph (Danish Myo Technologies (DMT), Denmark, Netherlands, Fig.8), four double looped ties were prepared using the suture tying forceps (FST 18025-10). Individual fibers isolated from embroidery thread (Walmart Superstores) were used to prepare the ties. Ten
ml D-PBS was added to the myograph chamber. The glass cannulae at both ends were tested for patency before mounting the vessel. The high vacuum grease which seals the transducer pin hole was inspected before beginning the experiment and replaced every 2 weeks as per DMT recommendations. The myograph chamber was placed under the dissecting microscope and two ties were looped over the P2 side glass cannula. Once the selected vessel was mounted onto the tip of the P2 cannula, the ties were pulled over the vessel and tightened by gentle pulling as excessive force can break the tip of the glass cannula. Then, D-PBS was perfused through the vessel to gently flush out blood from the vessel lumen. The process was repeated to secure the other end of the vessel on the P1 glass cannula (Fig.9). The myograph chamber was then placed on the stage of an inverted microscope (Nikon Eclipse, TS100). Pressure and flow were applied using either the DMT flow regulator or 10 ml syringes mounted at heights above the level of the vessel so as to generate 45 and 42 mmHg pressures at the inlet and outlet, respectively. Syringes reduced the amount of perfusate required to perfuse the vessel lumen to 10-15 ml as compared to 250 ml required for the Schott bottle of the DMT flow regulator system. When syringes were used flow was initiated from the P1 end with an inlet pressure of 45 mmHg with the syringe placed at a height of 61.2 cm above the vessel (13.6 mm H2O = 1 mmHg pressure) till D-PBS was seen to drip from the P2 end. Then, an outlet pressure of 42 mmHg with the syringe placed at a height of 57.12 cm above the vessel level was applied at the P2 end.

iv. Endothelium denudation
For endothelium denudation, one end of the vessel was mounted onto the P1 side of a glass cannula. The vessel was then gently pulled over the tip of the glass cannula over its entire length and rolled gently. It was pulled back off the glass cannula leaving about 2 mm vessel length mounted onto the tip of the glass cannula. Then the two ties were pulled over the vessel to secure the vessel onto the glass cannula, after which the vessel lumen was gently flushed using D-PBS in a 1 ml syringe from the P1 end to flush out the endothelial cells. The other end of the vessel was then mounted and secured onto the P2 side glass cannula.

v. Data Recording

The microscope focus was adjusted to obtain a clear view of the vessel. After connecting the Myo-Interface to the myograph chamber, the Myoview program on the computer was started. The microscope focus was adjusted to obtain a clear view of the vessel edges and lumen on the computer screen. Target inlet and outlet pressures were set to 45 and 42 mmHg, respectively, on the computer screen. A box was drawn around the vessel by clicking and dragging using the mouse for calibration by loading the 4X file (i.e. objective lens magnification used). A new box was drawn around the clean edges of the vessel for analysis by the software. The graph configuration was set to a time range of 3600 seconds, data rate at 10 seconds, inflow pressure 0-60 mmHg, outflow pressure 0-60 mmHg, vessel diameter 200-600 µ, lumen diameter 50-500 µ, cross-sectional area 0-
25000, and temperature to 0-40°C. After graph configuration, history was reset and data logging was started.

vi. Pressure and Force calibration

Pressure and force calibration was performed periodically using a Big Ben manometer and the weights as described in the DMT Manual (Model 110P & 111P, version 3.2, pg 63-69).

B. PASM isolation and cell culture

Placental arterial smooth muscle (PASM) cells were isolated as described by Leik et al.\textsuperscript{222}. Briefly, placentas were obtained from normal pregnant women after informed consent (IRB# HM12361). The chorionic plate arteries were identified and dissected away from the placental tissue. The vessel was cut longitudinally to open the arterial lumen. The arterial sheet was cut into small pieces 3-5 mm, which were placed endothelial surface down onto 10 cm culture plates. Five ml of Medium-199 (M-199, Gibco, Invitrogen)) with 10% fetal bovine serum (FBS), 1% antibiotics and antifungotics (100X Anti-Anti, Gibco Cat#15240-062, penicillin 100 U/ml, streptomycin 100 µg/ml, amphotericin B 25 µg/ml) was added to the culture plates taking care not to disturb the adherent explants. Cells started to grow from the explants at the end of 1 week and were
confluent by about 4 weeks. At this point, they were exposed to M-199 without FBS for 24 hours to eliminate contaminating endothelial cells and fibroblasts, which do not survive without serum. After 24 hours, PASM cells were replated with M-199 with 10% FBS and then passaged or frozen. Cells were used between passage 2 and 6 (Fig.10).

C. Neutrophils, oxidative stress and TNF-α treatments:

Control: Control treatments were fresh M-199 medium with 10% FBS and 1% antibiotics/antimycotics.

Neutrophils: Neutrophils were isolated from whole blood obtained from normal non-pregnant or normal pregnant women using Histopaque density gradient. For neutrophil isolation, 3 ml of Histopaque 1119 (Sigma, Cat#11191) was added to 15 ml conical tube. It was carefully layered with 3 ml of Histopaque 1077 (Sigma, Cat#10771) and then 6 ml of whole blood. This was centrifuged at 700 x g for 30 minutes at room temperature (18-26°C) to form 5 distinct layers comprised of plasma, mononuclear cells with platelets, histopaque 1077, granulocytes, histopaque 1119, and red blood cells (RBC) from top to bottom [Fig.11]. Fluid to within 0.5 cm of the granulocyte layer was aspirated and discarded. Cells from the granulocyte layer were aspirated and transferred to a new tube labeled granulocytes. Ten ml phosphate buffered saline (PBS) was added and the cells were centrifuged for 10 minutes at 200 x g at 4°C. The PBS was removed with gentle aspiration and the cell pellet re-suspended in 10 ml of PBS and centrifuged for 10 minutes at 200 x g at 4°C. If the cell pellet had RBC’s, 3 ml of ice-cold ddH₂O was added
to the cell pellet followed by vortexing for 30 seconds to lyse the RBC’s. After 30 seconds, 1 ml of 0.6 M potassium chloride (KCl) was added to restore tonicity followed by centrifugation at 200 x g at 4°C for 4 minutes. The supernatant was discarded and the cell pellet re-suspended in 1 ml of medium.

To count the neutrophils, 10 µl of cell suspension was added to 90 µl of trypan blue dye and pipetted several times to mix well (dilution factor of 10). Ten µl of this 100 µl cell suspension with dye was loaded onto a hemocytometer for counting. Neutrophils in all four quadrants of the hemocytometer were counted and added together \[4X= X_1+X_2+X_3+X_4\] (Fig.12). This number \[4X\] was divided by four to get the average number of cells per square \[X= X_1+X_2+X_3+X_4/4\]. The concentration of neutrophils was calculated as average number of cells per square x dilution factor x 10^4 neutrophils/ml.

Neutrophil activation was done using either 10^{-8} M IL-8 or 100 µM arachidonic acid. For activation using IL-8, 10 µg of recombinant human IL-8 from R&D Systems (Cat# 208-IL) is reconstituted in 1 ml of sterile phosphate buffered saline containing 1% bovine serum albumin and aliquoted into aliquots containing 1 µg/100 µl. For a final concentration of 10^{-8} M IL-8, 80 µl of 1 µg/100µl aliquot is used per 10 ml of D-PBS containing neutrophils. For activation using arachidonic acid, 10 µl of a 50 mg in 100 µl ethanol solution was added to 1.08 ml of PBS to form Solution A. Then, 16.6 µl of solution A was added to the 1 ml cell suspension and incubated in a rotator for 30 minutes at 37°C.
ROS: A reactive oxygen species (ROS) generating solution was made using 0.05 mM hypoxanthine (Hyp, Sigma, H-9377) and 0.003 U/ml xanthine oxidase (XO, Calbiochem, Cat#682151) in M-199 with 10% FBS. A 1 mM hypoxanthine stock solution was prepared by dissolving 0.0027 gm of Hyp (MW 136.1) in 20 ml of M-199 with 1% antibiotics-antimycotics and 10% FBS. Sonication for 30 minutes in a water bath was used for dissolving the Hyp. For a final concentration of 0.05 mM, 250 µl of 1mM Hyp solution was added to 4.75 ml of M-199 with 1% antibiotics/anti-mycotics and 10% FBS. XO (Calbiochem) was purchased as a 20 U/ml stock solution made from buttermilk. For culture, 0.75 µl of 20 U/ml XO was added to a T-25 flask containing 5 ml of M-199 with 1% antibiotics/anti-mycotics and 10% FBS for a final concentration of 0.003 U/ml.

TNF-α: To make a 1 ng/ml solution of TNF-α, 0.5 µl of 10 µg/ml recombinant human TNF-α (R&D Systems, Cat#210-TA) was added to a T-25 flask containing 5 ml of media [M-199 with 1% antibiotics/anti-mycotics and 10% FBS].

D. Protein extraction and quantification for Western blot

To detect phosphorylated proteins phospho-MYPT1 and phospho-MLC along with total MYPT1 and MLC by Western blot, culture medium was carefully removed from adherent PASM cells in T-25 flasks. One ml of ice cold PBS was added to each T-25 flask and aspirated to remove FBS. M-PER (237 µl, Pierce 78501) with 3 µl of Halt protease inhibitor cocktail (Pierce 78415) and 60 µl of phosphatase inhibitor cocktail set
III (Calbiochem) was added to each T-25 flask. The flasks were placed on a shaker for 5 minutes. Cells were then scraped and the lysate transferred to a microcentrifuge tube to be centrifuged at 14000 x g for 10 minutes at 4°C. The supernatant was collected and frozen at -80°C. Protein concentration in the collected supernatant was quantified using the Pierce BCA Protein Assay Kit.

E. Western blot methodology:

For all Western blots, the Invitrogen Xcell Surelock system was used for electrophoresis and the Odyssey (Licor) system was used for scanning fluorescence tagged secondary antibodies.

i. Sample Preparation: For MYPT1 and MLC, equal concentrations of protein for every sample were calculated and volumized to 45 µl. Three µl of 20X reducing agent and 12 µl of 5X Laemli’s were added. Samples were placed in boiling water at 100°C for 15 minutes followed by cooling on ice and centrifugation at 4°C for 1 minute. The samples were then applied to the wells of the gel using gel loading pipette tips. Biorad All Blue protein standards were run in the first lane of every Western blot.

ii. Antibodies, electrophoresis and transfer for phospho-MYPT1 & total MYPT1:

a) Primary antibodies:
i) Rabbit polyclonal phospho-MYPT1 antibody (Cell signaling technologies, Cat#4563) in a 1:300 dilution.

ii) Rabbit polyclonal MYPT1 antibody (Cell signaling technologies, Cat#2634) in a 1:1000 dilution.

iii) Mouse monoclonal anti-β actin Ig (Sigma, A2228) in a 1:10,000 dilution.

b) Secondary antibodies:

i) IRDye 800 anti-mouse antibody (Licor) in a 1:30,000 dilution

ii) AlexaFluor 680 goat anti-rabbit antibody (Invitrogen) in a 1:2500 dilution

Tris-Glycine SDS gels (6%) were cast using the Biorad casting system and 1.5 mm combs. For a 15 ml gel, 7.9 ml ddH2O, 3.0 ml 30% acrylamide mix, 3.8 ml 1.5 M Tris at pH 8.8, 0.15 ml of 10% SDS, 0.15 ml of 10% ammonium persulfate (Fischer, BP179-25) and 12 µl of TEMED (N, N, N’, N’- Teramethylethylenediamine from Sigma, T9281). With sample loading of at least 50 µg protein/well, electrophoresis was run at 125V for 1 hour 50 minutes. After electrophoresis, the gel was washed with ddH2O followed by equilibration in methanol free transfer buffer for 15-20 minutes. PVDF membrane (0.45µm pore size, Millipore) was wetted in 100% methanol for 30 seconds followed by rinsing in ddH2O and equilibration in methanol free transfer buffer for 15 minutes. Ice cold methanol free transfer buffer was used to soak the blotting pads taking care to remove all air bubbles. The sandwich of blotting pads, gel and PVDF membrane was assembled and
transfer was run for 2.5 hours at 70V. Coomasie blue staining of the gel was used to confirm optimal transfer of protein.

iii. Antibodies, electrophoresis and transfer for phospho-MLC and total MLC:

a) Primary antibodies:
   i) Rabbit polyclonal phospho myosin light chain 2 (Ser19) antibody (Cell signaling technologies, Cat#3671) in a 1:3000 dilution.
   ii) Rabbit polyclonal myosin light chain 2 antibody (Cell signaling technologies, Cat#3672) in a 1:1000 dilution.
   iii) Mouse monoclonal anti-β actin Ig (Sigma, A2228) in a 1:10,000 dilution.

b) Secondary antibodies:
   i) IRDye 800 anti-mouse antibody (Licor) in a 1:30,000 dilution.
   ii) AlexaFluor 680 goat anti-rabbit antibody (Invitrogen) in a 1:10,000 dilution.

Tris-Glycine SDS gels (12%) were cast using the Biorad casting system and 1.5 mm combs. For a 15 ml gel, 4.9 ml ddH₂O, 6.0 ml 30% acrylamide mix, 3.8 ml 1.5 M Tris at pH 8.8, 0.15 ml of 10% SDS, 0.15 ml of 10% ammonium persulfate (Fischer, BP179-25) and 6 µl of TEMED (Sigma, T9281). With sample loading of at least 50 µg protein/well, electrophoresis was run at 125V for 1 hour 50 minutes. After electrophoresis, the gel was washed with ddH₂O followed by equilibration in methanol free transfer buffer for 15-20 minutes. PVDF membrane (0.2µ pore size, Millipore) was wetted in 100% methanol till
the opaque membrane turned a translucent gray color, followed by rinsing in ddH$_2$O and equilibration in 40% methanol transfer buffer for 15 minutes. Ice cold 40% methanol transfer buffer was used to soak the blotting pads taking care to remove all air bubbles. The sandwich for transfer was assembled and transfer run for 1.5 hours at 25V. Coomassie blue staining of the gel was used to confirm optimal transfer of protein. The membrane was immersed in 0.25% glutaraldehyde at room temperature for 45 minutes to fix the myosin light chains and washed with PBS before proceeding with blocking.

**iv. Primary and secondary antibody incubation**

For all Western blots, the membrane was marked with pencil, cut or fold to identify the face of the membrane in approximation with the gel. The membrane was washed in ddH$_2$O for 5 minutes and blocked in 1:1 diluted Odyssey Blocking buffer (Licor) and PBS for 1 hour at room temperature. The membrane was then washed once with PBS for 5 minutes. After that, the membrane was incubated overnight at 4°C in the primary antibody solution comprised of 1:1 PBS and Odyssey Blocking Buffer with 0.1% Tween-20. The next day the membrane was washed 4 times for 5min each in PBS with 0.1% Tween-20. Henceforth, foil wrap was used to protect the membrane from light. The membrane was incubated for 1 hr at room temperature with secondary antibody solution in 1:1 PBS and Odyssey blocking buffer with 0.1% Tween-20 and 0.01% w/v of SDS. The membrane was washed 4 times for 5 minutes each in PBS with 0.1% Tween-20.
Then the membrane was washed 4 times for 5 minutes each in PBS alone and twice for 5 minutes each in ddH₂O. The membrane was scanned using the Odyssey system. The glass and silicon mat were cleaned before placing the membrane on the Odyssey scanner.

v. Membrane stripping using low pH:

A stripping buffer of 25 mM glycine-HCl at pH 2.0 with 1% SDS was prepared by addition of 1.88 gm glycine and 10 gm SDS to 600 ml ddH₂O and titrated to a pH of 2.0 using 1 N HCl. It was then volumized to 1 L with ddH₂O. For membrane stripping, the membrane was placed in the stripping buffer for 30 minutes with agitation and then washed in PBS for 10 minutes twice. After stripping, blocking of the membrane for the next antigen detection step was performed.

vi. Densitometric analysis

Quantification of bands obtained on Western blots was done by densitometric analysis of the bands in the ‘Details View’ of the Odyssey software using the median method for calculation of background. The average intensity obtained for each band was normalized to that of β-actin for the same lane. The band intensity of each treatment was then calculated as a percent value of the normalized value of the control lane for that experiment. The percent value of the control was used for statistical analysis.
Figure 6. Identification of the artery. The artery can be identified from the vein by looking for branch points. Branches off the artery are sharp and shaped like a V while those from veins are U-shaped. The vein is collapsible on pressure and shows slow or no return of blood flow when pressed against. Pressing the artery against the silicone dish to judge thickness of the vessel wall and return of blood flow damages the vessel, so a length of vessel distal or proximal but not including the area pressed should be used for the experiment. The artery is usually the narrower of the two vessels.
Figure 7. Myograph Chamber. Pressure myograph system with in-built pressure and force transducers and a perfusion system with inlet (P1) and outlet (P2) (From DMT manual, model 110P & 111P, version 3.2).
Figure 8. DMT Pressure Myograph. The equipment comprised of the myograph chamber placed on the stage of the inverted microscope and connected with the Myointerface and the flow and pressure regulator placed on top of the Myointerface with Schott bottles for perfusion of the vessel. The Myointerface connects the myograph chamber to the computer enabling data recording and control of the myograph chamber through the computer program settings.
Figure 9. Myoview image of the mounted vessel, showing the tip of the glass cannula within the vessel lumen.
Table 1. Configuration settings of the software used for myograph experiments

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Figure 10. Placental arterial smooth muscle (PASM) cell culture. PASM cells growing from chorionic plate arterial explants at A) 1 week, B) 2 weeks, C) 3 weeks and D) 4 weeks.
Figure 11. Neutrophil Isolation by Histopaque Gradient Separation. The top layers up to 0.5 cm above the granulocyte (neutrophil) layer were aspirated and discarded. The neutrophil layer was then aspirated and transferred to a new tube to be washed with PBS.
Figure 12. Cell counting grid of hemocytometer: Count all neutrophils within the red boxes and add them together (4X). Divide this number by 4 to get X. The volume of each square with the coverslip in place is 0.1 mm$^3$. Therefore, cells per ml = average count per square x dilution factor x 10$^4$. 
Chapter 3

NEUTROPHILS ENHANCE VASCULAR REACTIVITY TO ANGIOTENSIN II
VIA REACTIVE OXYGEN SPECIES ACTIVATION OF THE RHOA KINASE
PATHWAY

A. Introduction

Preeclampsia is a hypertensive disorder of pregnancy that complicates 5-7% of all pregnancies resulting in significant maternal and fetal mortality and morbidity\(^5\). Recently, we showed significant infiltration of neutrophils into systemic vasculature of women with preeclampsia, which was associated with marked vascular inflammation\(^95,96\). We also found a similar vascular infiltration of neutrophils in obese women\(^97\) which may be of interest because obesity is a risk factor for preeclampsia, as well as chronic hypertension\(^223\).

In 1973, Gant et al described enhanced vascular reactivity to angiotensin II (Ang II) in women predestined to develop preeclampsia\(^123\). However, mechanisms underlying this increased vascular reactivity remained elusive. The major mechanism of increased vascular reactivity is the RhoA kinase (ROK) pathway and reactive oxygen species
(ROS) activate this pathway\textsuperscript{134}. When ROK is activated, it phosphorylates MYPT1 which inhibits myosin light chain phosphatase so myosin light chains (MLC) remain phosphorylated which enhances vascular reactivity.\textsuperscript{134} Therefore, for a given concentration of Ang II, vessel responsiveness would be enhanced. Neutrophils release inflammatory compounds that could cause vasoconstriction or enhance vessel reactivity to vasoconstrictors. For example, neutrophil release of ROS may enhance vessel reactivity by RhoA kinase pathway activation.

In this study we used human omental vessels and cultured human vascular smooth muscle cells to test the hypothesis that neutrophils or neutrophil products (ROS, TNF-\(\alpha\)) cause vasoconstriction by enhancing vessel reactivity to Ang II via the RhoA kinase pathway.

\textbf{B. Materials and Methods}

\textit{Study Subjects}

Omental fat biopsies (approximately 2 cm x 4 cm x 2 cm) were collected from normal pregnant women undergoing term C-section at MCV Hospitals, Virginia Commonwealth University Medical Center. Omental fat is vascularized tissue representative of the systemic vasculature. Neutrophils were isolated from whole blood of pregnant or non-pregnant female subjects using dual Histopaque (Sigma, St. Louis, MO) density gradient centrifugation as previously described\textsuperscript{191}. Demographic data on the
subjects were (Mean±SD): age (27.5±6 years), systolic blood pressure (127±16 mmHg),
diastolic blood pressure (74±11 mmHg), gestational age (39±1 weeks), birth weight
(3387±506 grams). Twenty-one subjects were primiparous and 19 were multiparous.
Mean lumen diameter of omental vessels studied was 287 ± 99 µ. The Office of Research
Subjects Protection of Virginia Commonwealth University approved this study and all
subjects gave informed consent. IRB No. HM12361

Myograph Experiments

The omental fat biopsy was placed in Dulbecco’s phosphate buffered saline (D-PBS, Gibco Invitrogen, Carlsbad, CA) on a silicone dissection dish pre-cooled to 4°C. A 2 cm length of a vessel 200-500 µ diameter was dissected and mounted on glass micro-cannulae of a myograph system (Model 110P, Danish Myo Technologies (DMT), Denmark, Netherlands). Endothelium was denuded by passing a fine metal wire through the vessel lumen. The vessel was immersed in 10 ml of D-PBS in the myograph chamber and secured at both ends using two 11-O silk suture ties at each end. The myograph chamber temperature was maintained at 37°C and the vessel pressures maintained at constant inlet (45 mmHg) and outlet (42 mmHg) pressures to achieve flow through the vessel. Ang II dose response was run in 10-fold increments in concentrations 0.001-10 µM. The vessel was challenged to 60µM KCl at beginning, end and in between treatments to ascertain vessel viability and to recharge intracellular calcium stores. Isolated neutrophils were re-suspended in 1 ml of D-PBS and counted using a hemocytometer. Neutrophils were activated using human recombinant interleukin-8 (IL-
8, 10^{-8} M final concentration, R&D Systems, Minneapolis, MN). Neutrophils were perfused through the vessel in a physiologic concentration of ~2000/mm^3 and after noting response to neutrophils alone the Ang II dose response was repeated. The experiment was repeated in the presence of either superoxide dismutase (SOD, 5000 IU/ml, Sigma, St. Louis, MO) and catalase (150 U/ml, Sigma, St. Louis, MO) or Y-27632 (3 µM, Calbiochem, Gibbstown, NJ), a specific RhoA kinase inhibitor, in the D-PBS bath along with perfusion of activated neutrophils in lumen. ROS generating solution composed of 0.05 mM hypoxanthine (Hyp, Sigma, St. Louis, MO) and 0.003 U/ml xanthine oxidase (XO, Calbiochem, Gibbstown, NJ) was added to the vessel chamber and after a period of stabilization, Ang II dose response was repeated. Ang II dose response was repeated again in the presence of either SOD/catalase or Y-27632 along with ROS in the bath. Ang II dose response was also tested in the presence of TNF-α (1 ng/ml). To determine if ROS mediated enhanced reactivity was specific to Ang II or a non-specific phenomenon, norepinephrine (NE) dose response was run in 2-fold increments (0.15625 µM to 5 µM) and repeated in the presence of ROS generating solution. Response to Y-27632 and ROS was tested at a single dose of 1.25 µM NE.

**Western Blot**

Primary human vascular smooth muscle cells (VSMC) were isolated from placental chorionic plate arteries and cultured as described in Chapter 2. VSMC were grown to confluence in T-25 flasks in M-199 with 10% FBS. Cells were treated for 5 minutes with Ang II alone or with Ang II plus activated neutrophils (50,000
neutrophils/ml) or Ang II plus ROS with and without Y-27632 (3 µM). ROS generating solution was composed of 0.05 mM Hyp and 0.003 U/ml XO. Western blot was performed for Ser19-pMLC and total MLC (Cell Signaling technologies, 1:2000, Beverly, MA) along with Thr696-pMYPT1 (Santa Cruz, 1:1000) and total MYPT1 (Cell Signaling technologies, 1:500). Blots were scanned and quantified using the Licor Odyssey system. β-Actin was used to normalize data.

**Immunokinase Assay to determine RhoA Kinase Activity**

Equal amounts of omental vessels were dissected and weighed into two 0.5 ml centrifuge tubes and treated with D-PBS alone (control) or D-PBS with a ROS generating solution composed of 0.05 mM Hyp and 0.003 U/ml XO for 5 minutes. At the end of 5 minutes the vessels were flash frozen in liquid nitrogen and stored at -80°C until the RhoA Kinase activity assay was run as described previously. Briefly, 20 µl of Rho kinase immunoprecipitates were added to the reaction mixture containing 1 mM adenosine triphosphate (ATP) and 10 µCi of [γ-32P] ATP (3000 Ci/mol) along with 5 µg of myelin basic protein, followed by incubation for 15 min at 37°C. Phosphorylation of myelin basic protein was absorbed onto phosphocellulose disks, and free radioactivity was removed by washing 3 times with 75 mM phosphoric acid (H₃PO₄). The amount of radioactivity on the disks was measured using liquid scintillation. The results are expressed as counts per minute per milligram protein.

**Data Analysis**
The myograph experimental data were analyzed by one-way ANOVA and Bonferroni post-hoc test for each dose of Ang II due to significant interaction detected by the two-way ANOVA. The myograph experimental data for each dose of NE were analyzed using repeated measures two-way ANOVA with Bonferroni post-test. The western blot data were quantified using the average intensity measurements normalized to β-actin and then calculated as a percentage of the control value. The RhoA kinase immunoassay data were analyzed using the Mann Whitney test. A statistical software program was used (Prism 4, GraphPad software, San Diego, CA). All data are presented as mean ± SE.

C. Results

Effect of Neutrophils

As shown in Figure 13, dose response to Ang II alone ranged from a 4 µ decrease in vessel diameter with $10^{-9}$ M Ang II up to a 14 µ decrease with $10^{-6}$ M Ang II. When neutrophils were perfused through the vessel lumen and Ang II dose response was repeated, vascular reactivity to Ang II was significantly enhanced at all doses (Fig.14). The vessel diameter decreased by an average of 46 µ with $10^{-5}$ M Ang II in the presence of activated neutrophils in the vessel lumen. Addition of SOD/Catalase to the myograph chamber to quench ROS significantly blocked enhanced vascular reactivity to Ang II (Fig.15). The specific RhoA kinase inhibitor, Y-27632, also abolished enhanced vascular
reactivity to Ang II in the presence of neutrophils (Fig.16). Perfusion with either IL-8 alone or un-activated neutrophils did not affect the Ang II dose response.

**Effect of ROS and TNF-α**

As shown in Figure 17, addition of a ROS generating solution to the myograph chamber significantly enhanced vascular reactivity to Ang II similar to that seen with perfusion of activated neutrophils. Endothelium intact vessels also showed a significant increase in vessel reactivity to Ang II in the presence of ROS. However, the response was attenuated because of endothelial compensatory mechanisms as would be expected (Fig.17 B). Also similar to neutrophils, addition of SOD/Catalase (Fig.18) or Y-27632 (Fig.19) significantly inhibited ROS induced enhancement of vascular reactivity to Ang II. ROS generating solution by itself caused contraction of only -2.67±1.61 μ. Contraction induced by ROS alone was excluded for measurement of enhanced vascular reactivity to Ang II. In contrast to ROS, TNF-α, another neutrophil inflammatory product did not alter vascular reactivity to Ang II (Fig.20, N=4, p>0.1).

**Effect of Neutrophils and ROS on RhoA kinase activity and expression of pMYPT1 and pMLC**

Human omental vessels treated with a ROS generating solution for 5 minutes showed a significant increase in RhoA kinase activity as compared to control (Fig.21). To confirm increased RhoA kinase activity, the effect of ROS and neutrophils on phosphorylation of MYPT1 and MLC was evaluated by Western blot in VSMC.
Treatment with ROS plus Ang II or neutrophils plus Ang II increased phosphorylation of MYPT1 which is the protein directly phosphorylated by RhoA kinase. Y-27632 inhibited phosphorylation of MYPT1 by ROS and neutrophils (Fig.22). As shown in Fig.23, treatment of VSMC with ROS or neutrophils in the presence of Ang II significantly increased phosphorylation of MLC, which is consistent with inhibition of myosin light chain phosphatase by pMYPT1. Phosphorylation of MLC was reduced by Y-27632. Treatment of VSMC with Ang II alone did not significantly increase phosphorylation of MYPT1 or MLC over control.

_Norepinephrine Dose Response_

As shown in Fig. 24A, ROS significantly enhanced the vasoconstrictive response to all doses of NE tested (N=6, p<0.001) except the 0.3125 µM dose. At 1.25 µM dose the increase in vasoconstrictive response to NE by ROS was significantly inhibited by addition of Y-27632 (Fig. 24B, N=4, p<0.05).

_D. Discussion_

In this study we demonstrate that vascular reactivity of human omental vessels to Ang II is markedly increased in the presence of ROS in both endothelium intact and endothelium denuded vessels. Enhancement of vascular reactivity was less in the presence of endothelium as would be expected due to endothelial compensatory factors such as nitric oxide and prostacyclin. Removal of the endothelium enabled the study of direct effects of
ROS on the vascular smooth muscle contractile apparatus without activation of endothelial pathways, and so was used for neutrophil experiments. Increased vessel reactivity to Ang II was due to ROS because co-treatment with SOD/catalase to quench ROS completely inhibited enhanced vessel reactivity to both ROS and neutrophils. The enhancement to ROS was via the RhoA kinase pathway because the enhanced response was abolished by using a specific RhoA kinase inhibitor. This was confirmed with ex-vivo omental vessels treated with ROS showing increased Rho kinase activity as well as in cell culture experiments using human vascular smooth muscle cells which demonstrated increased phosphorylation of MLC and MYPT1 in the presence of ROS or neutrophils. The enhanced vessel responsiveness to Ang II was specific to ROS as another neutrophil product, TNFa, was ineffective. The ability of ROS to enhance vascular reactivity via the RhoA kinase pathway was not limited to Ang II as ROS also enhanced vascular reactivity to NE, another vasoconstrictor. NE release is induced by stress and sympathetic activation, factors reported to be associated with preeclampsia\textsuperscript{153-155}. Our findings of enhanced vessel reactivity to Ang II and NE in the presence of ROS or neutrophils may explain hypertension in pregnancy and why women who are destined to develop preeclampsia have an increased sensitivity to Ang II before clinical symptoms of hypertension are manifest\textsuperscript{123}.

Our findings may extend to hypertension in non-pregnant as well as pregnant subjects because RhoA kinase has been shown to play a critical role in chronic hypertension\textsuperscript{139, 225}. Obesity is a known risk factor for chronic hypertension, as well as preeclampsia\textsuperscript{223}, and we recently reported extensive systemic vascular infiltration of
neutrophils in obese women\textsuperscript{97}. The vascular phenotype of the obese women was similar to that of preeclamptic women. Thus, vascular infiltration of neutrophils may help explain the relationship between obesity and hypertensive disorders, such as chronic hypertension and preeclampsia.

Our recent finding of extensive vascular infiltration of neutrophils in women with preeclampsia\textsuperscript{95, 96} challenged conventional thinking because neutrophils are usually thought of as part of the innate immune system and the first line of defense against infection at the site of a wound\textsuperscript{53, 226}. A role for neutrophils in non-infectious disease has not been widely studied, but accumulating evidence indicates a role for neutrophils in "sterile" inflammatory diseases\textsuperscript{227}.

The reason neutrophils may be playing an important role in pregnancy is that in normal pregnancy, the number of neutrophils increases 2.5-fold by 30 weeks of gestation\textsuperscript{228} and this number increases further in preeclampsia\textsuperscript{54}. The human placenta produces oxidized lipids and secretes them into the maternal circulation\textsuperscript{36, 38, 69, 159, 229}. Oxidized lipids are potent activators of neutrophils\textsuperscript{66, 68, 191}. In women with preeclampsia, placental production of oxidized lipids is significantly higher than in women with normal pregnancy\textsuperscript{38}. One possibility for neutrophil activation occurs as they circulate through the intervillous space and are directly exposed to oxidized lipids released by the placenta\textsuperscript{36, 230, 231}. Activated neutrophils in the maternal systemic circulation adhere to the endothelium and could cause vascular inflammation and dysfunction by release of toxic compounds. Neutrophil release of matrix metalloproteinase-8 and myeloperoxidase could compromise vascular integrity, and that coupled with increased hydrostatic pressure as a
result of enhanced vascular reactivity to Ang II, could promote extravasation of proteins. Thus, neutrophil infiltration may contribute not only to hypertension, but also to edema and proteinuria in preeclampsia.

This is the first study to show that neutrophils enhance vascular reactivity by activating RhoA kinase pathway and the first to show that ROS activate RhoA kinase in human vessels. Our findings are highly significant with respect to hypertension, as resistance is inversely proportional to the fourth power of the radius, so even small decreases in vessel diameter cause large increases in resistance. For example, in our study the highest dose of Ang II caused a 28% increase in resistance. In the presence of ROS or neutrophils, Ang II caused a 101% or 115% increase in resistance, respectively, which was approximately a 4 fold increase in resistance over that with Ang II alone.

**Perspectives**

These data are the first to show that both ROS and neutrophils enhance vessel contractility to Ang II and NE and provide evidence that the mechanism of ROS and neutrophil enhanced Ang II reactivity is by activating RhoA kinase. These data support the idea that neutrophil infiltration into systemic vasculature of preeclamptic women may be a cause of hypertension. There is no definitive treatment for preeclampsia, but these new data could provide novel avenues for treatment based on regulation of neutrophil infiltration. Potential treatments that are becoming available for clinical studies are neutralizing antibodies against adhesion molecules and selective RhoA kinase inhibitors.
Angiotensin II facilitates norepinephrine release from sympathetic nerve endings and prevents norepinephrine re-uptake and thus enhances sympathetic adrenergic activity.
Figure 13. Vessel reactivity to Ang II alone. Ang II (0.001 µM) caused a mean vasoconstriction of 7 µ while maximum mean vasoconstriction of 20 µ was obtained with 1 µM Ang II (N=20).
Figure 14. Vessel reactivity to Ang II in the presence of neutrophils. Perfusion of activated neutrophils (<2000/mm³) through the vessel lumen significantly enhanced vessel reactivity to Ang II. Ang II (0.001 µM) caused a mean vasoconstriction of 12 µ while maximum mean vasoconstriction of 50 µ was obtained with 10 µM Ang II in the presence of neutrophils (N=10, ** p<0.01, *** p<0.001).
Figure 15. Role of ROS in enhancing vessel reactivity to Ang II by neutrophils. Addition of SOD/catalase to quench ROS abolished enhancement of vessel reactivity to Ang II by neutrophils. (N= 4, ** p<0.01, *** p<0.001).
Figure 16. Role of RhoA kinase pathway in enhancing vessel reactivity to Ang II by neutrophils. Addition of Y-27632, a specific RhoA kinase inhibitor, abolished enhancement of vessel reactivity to Ang II by neutrophils. (N= 4, * p<0.05, ** p<0.01).
Figure 17. Vessel reactivity to Ang II in the presence of ROS. Addition of a ROS generating solution in the bath significantly enhanced vessel reactivity to Ang II, similar to that seen with neutrophils. A) In endothelium denuded vessels, Ang II (0.001 µM) caused a mean vasoconstriction of 12 µ while maximum mean vasoconstriction of 48 µ was obtained with 10 µM Ang II in the presence of ROS (N=17, ** p<0.01, *** p<0.001). B) In endothelium intact vessels, Ang II (0.001 µM) caused a mean vasoconstriction of 8 µ while maximum mean vasoconstriction of 25 µ was obtained with 10 µM Ang II in the presence of ROS (N=6, * p<0.05, ** p<0.01).
Figure 18. Specificity of ROS in enhancing vessel reactivity to Ang II. Addition of SOD/catalase to quench ROS abolished enhancement of vessel reactivity to Ang II. (N=4, * p<0.05, ** p<0.01).
Figure 19. Role of RhoA kinase pathway in enhancing vessel reactivity to Ang II by ROS. Addition of Y-27632, a specific RhoA kinase inhibitor, abolished enhancement of vessel reactivity to Ang II by ROS. (N= 4, * p<0.05, ** p<0.01).
Figure 20. Vessel reactivity to Ang II in the presence of TNF-α. Addition of 1 ng/ml of TNF-α in the bath did not significantly affect vessel reactivity to Ang II (0.001 µM to 10 µM, N=17).
Figure 21. RhoA kinase activity in human omental vessels. RhoA kinase activity in omental vessels treated with ROS showed a 3-fold increase as compared to control (N=5, ** p<0.01).
Figure 22. Thr-696 pMYPT1 (phosphorylated regulatory target sub-unit of myosin light chain phosphatase) expression in cultured human vascular smooth muscle cells. A) Representative Western blot of pMYPT1 and total MYPT1 normalized to β-actin in cultured human VSMC. B) Mean band density of pMYPT1 expression normalized to β-actin and plotted as percentage of control. VSMC were treated with control media, Ang II alone, Ang II plus ROS generating solution or activated neutrophils with and without Y-27632 (Y) for 5 min. Treatment with ROS or activated neutrophils significantly enhanced phosphorylation of MYPT1 which was inhibited by Y-27632 (N=3, * p<0.05).
Figure 23. Expression of Ser-19 pMLC (phosphorylated myosin light chain) in cultured human vascular smooth muscle cells. A) Representative Western blot of pMLC and total MLC normalized to β-actin in cultured human VSMC. B) Mean band density of pMLC expression normalized to β-actin and plotted as percentage of control. VSMC were treated with control media, Ang II alone, Ang II plus ROS generating solution or activated neutrophils with and without Y-27632 (Y) for 5 min. Treatment with ROS or activated neutrophils significantly enhanced phosphorylation of MLC which was inhibited by Y-27632 (N=5, * p<0.05).
Figure 24. Vessel reactivity to norepinephrine (NE). A) Dose response of vessel reactivity to NE was significantly enhanced in the presence of ROS compared to NE alone (N=6, ***p<0.001). B) Addition of Y-27632 (Y) abolished enhanced vessel reactivity to NE in response to ROS at 1.25 \( \mu \text{M} \) dose of NE (N=4, *p<0.05).
A. Introduction

Preeclampsia is a hypertensive disorder of pregnancy that complicates 5-7% of all pregnancies resulting in significant maternal and fetal mortality and morbidity\textsuperscript{5}. Recently, we showed significant increase in plasma MMP-1 level and MMP-1 expression in the vasculature of women with preeclampsia (Estrada-Gutierrez, Walsh, unpublished). Consistent with a 7-fold increase in MMP-1 gene expression, pro-MMP-1 as well as activated MMP-1 were more abundant in omental vessels from preeclamptic women as compared to normal pregnant women. Immunohistochemistry showed significantly greater staining for MMP-1 in the endothelium, vascular smooth muscle and infiltrating leukocytes in subcutaneous fat blood vessels of preeclamptic women.

Increased vascular expression of MMP-1 may be responsible for the edema and protein leakage in preeclampsia due to its ability to degrade type 1 collagen. However, MMP-1 has recently been shown to activate protease activated receptor-1 (PAR-1) also
known as thrombin receptor$^{209, 210, 212, 213}$. Activation of endothelial PAR-1 results in release of endothelin-1 (ET-1)$^{217}$, which is a potent vasoconstrictor and can enhance vascular reactivity to angiotensin II (Ang II)$^{198}$.

In this study, we tested a novel hypothesis that MMP-1 has vasoconstrictive properties via activation of PAR-1 and release of ET-1. To test this hypothesis we used human omental vessels and a pressure myograph system to record real time changes in vessel diameter in response to MMP-1. We also tested if MMP-1 enhances vessel reactivity to Ang II.

**B. Materials and Methods**

**Study Subjects**

Omental fat biopsies (approximately 2 cm x 4 cm x 2 cm) were collected from 21 normal pregnant women undergoing term C-section at MCV Hospitals, Virginia Commonwealth University Medical Center. Omental fat is a highly vascularized tissue containing resistance vessels representative of the systemic vasculature. Demographic data on the subjects were (Mean±SD): age (28.5±6 years), systolic blood pressure (126±15 mmHg), diastolic blood pressure (77±10 mmHg), gestational age (39±1 weeks), birth weight (3379±444 grams). Sixteen subjects were primiparous and 5 were multiparous. Mean lumen diameter of omental vessels studied was 281 ± 70 μ. The
Office of Research Subjects Protection of Virginia Commonwealth University approved this study and all subjects gave informed consent. IRB No. HM12361.

**MMP-1 Activation**

All MMPs are produced in a latent form (pro-MMP or zymogen) and require activation. Commercially obtained human pro-MMP-1 (Calbiochem 444208; Stock 80 µg/ml) was activated using amino-phenyl mercuric acetate (APMA, Calbiochem 164610). A 10 mM APMA solution was prepared by dissolving 35.18 mg APMA in 0.1 M sodium hydroxide (NaOH). This 10 mM APMA is stable for 1 week at 4°C. To initiate activation, 5 µl of pro-MMP-1 (400 ng) was diluted with 85 µl of Tris-Triton-Calcium (TTC) buffer and 10 µl of 10 mM APMA for a final volume of 100 µl. This was incubated for 2 hours at 37°C. TTC buffer was 50 mM Tris-hydrochloride (Tris-HCl) at pH 7.5 with 1 mM calcium chloride (CaCl₂) and 0.05% Triton X-100. For APMA elimination, a Microcon Centrifugal Filter Device, Ultracel YM-10 (10,000 MW), was blocked with 100 µl of 1 mg/ml bovine serum albumin (BSA) for 30 min at 37°C. Then, after addition of 100 µl of D-PBS, it was centrifuged at 11,000 x g for 20 minutes at 4°C. The filter device was then inverted onto a new tube, spun briefly and left to dry. Then, 100 µl of MMP-1 mixture was transferred to the blocked filter device, and after adding 200 µl of D-PBS, it was centrifuged at 11,000 x g for 20 minutes at 4°C to wash out the APMA. The filtrate was discarded and this step repeated to ensure maximal removal of APMA. The filter device was then inverted on a new tube and spun briefly. The
recovered activated MMP-1 was diluted to a total volume of 100 µl to give a final concentration of 400 ng/100 µl.

Myograph Experiments

The omental fat biopsy was placed in Dulbecco’s phosphate buffered saline (D-PBS, Gibco Invitrogen, Carlsbad, CA) on a silicone dissection dish pre-cooled to 4°C. A 1 cm length of a vessel 200-500 µ diameter was dissected and mounted on glass micro-cannulae of a myograph system (Model 110P, Danish Myo Technologies (DMT), Denmark, Netherlands). For some experiments, endothelium was denuded by passing a fine metal wire through the vessel lumen. The vessel was immersed in 10 ml of D-PBS and secured at both ends using two 11-O silk suture ties at each end. The myograph chamber temperature was maintained at 37°C and the vessel pressures maintained at constant inlet (45 mmHg) and outlet (42 mmHg) pressures to achieve flow through the vessel. The MMP-1 dose response was run in 10-fold increments in concentrations 0.025-25 ng/ml in endothelium intact as well as endothelium denuded omental vessels. The MMP-1 dose response was repeated with perfusion of 10 µM PAR-1 inhibitor SCH-79797 (Tocris, Ellisville, Missouri) or 5 µM ET-1 Type A receptor blocker, BQ-123 (Sigma-Aldrich, St.Louis, Missouri). The Ang II dose response was run alone in 10-fold increments (0.001-10 µM) and in the presence of 2.5 ng/ml of MMP-1. The Ang II dose response in the presence of MMP-1 was repeated with perfusion of 10 µM SCH-79797 or 5 µM BQ-123.
Data Analysis

The myograph experiment data were analyzed by one-way ANOVA and Bonferroni post-test (MMP-1 dose response data) or two-way ANOVA with Bonferroni posttests (Ang II dose response plus MMP-1 data) using a statistical software program (Prism 4, GraphPad software, San Diego, CA). Data are presented as mean ± SE.

C. Results

MMP-1 dose response

As shown in Figure 25, when MMP-1 was perfused through the vessel lumen in endothelium intact human omental vessels it caused dose dependent vasoconstriction ranging from an average decrease of 5 µ with 0.025 ng/ml MMP-1 to an average decrease of 40 µ with 25 ng/ml MMP-1. When MMP-1 was perfused through the vessel lumen in endothelium denuded human omental vessels, it did not cause dose dependent vasoconstriction.

Role of PAR-1 and ET-1 in MMP-1 induced vasoconstriction

As shown in Figure 26, co-perfusion of a PAR-1 inhibitor (10 µM SCH-79797) with MMP-1 in endothelium intact omental vessels abolished MMP-1 induced vasoconstriction. As shown in Figure 27, co-perfusion of 5 µM BQ-123, an ETA receptor blocker, with MMP-1 in endothelium intact omental vessels also abolished MMP-1 induced vasoconstriction.
MMP-1 mediated enhancement of vascular reactivity to Ang II

As shown in Figure 28, dose response to Ang II alone in endothelium intact vessels ranged from a 4 µ mean decrease in vessel diameter with 0.001 µM Ang II and up to a 16 µ mean decrease with 10 µM Ang II. When activated MMP-1 (2.5 ng/ml) was perfused through the vessel lumen and the Ang II dose response was repeated, vascular reactivity to Ang II at all doses (0.001 µM to 10 µM) was significantly enhanced. Vessel diameter decreased by an average of 10 µ with 0.001 µM Ang II and an average of 43 µ with 10 µM Ang II in the presence of MMP-1. Vasoconstriction to MMP-1 alone was excluded to calculate the change in vessel diameter induced by the Ang II dose.

Role of PAR-1 and ET-1 in MMP-1 induced enhanced vascular reactivity to Angiotensin II

Co-perfusion of a 10 µM SCH-79797, a specific PAR-1 inhibitor, with Ang II plus MMP-1 abolished MMP-1 enhanced vascular reactivity to Ang II (Fig. 29). Similarly, co-perfusion of 5µM BQ-123, an ET_A receptor blocker, abolished enhanced vascular reactivity to Ang II induced by MMP-1 (Fig.30).

D. Discussion

In this study we demonstrated for the first time that MMP-1 perfusion caused dose-dependent vasoconstriction in endothelium intact human omental vessels and also enhanced vascular reactivity to Ang II. Both of these effects were abolished by co-
perfusion with a PAR-1 inhibitor or an ET\textsubscript{A} receptor blocker. These data indicate that MMP-1 induced vasoconstriction and enhanced vascular reactivity to Ang II is mediated by PAR-1 activation leading to release of ET-1 acting on the ET\textsubscript{A} receptor.

MMP-1 is best known as a collagenase. However, in recent years it has been reported to activate the thrombin receptor, PAR-1, by cleaving the N-terminal peptide sequence just two amino acids distal to the thrombin cleavage site\textsuperscript{209, 210, 212, 213}. PAR-1 is expressed on the surface of endothelial cells and its activation by either MMP-1 or thrombin results in endothelial cell activation as evidenced by release of chymase, IL-8, P-selectin and ET-1 from the Weibel Palade bodies within the endothelial cells\textsuperscript{18, 217, 232}. Thus, MMP-1 can mediate its vasoconstrictive effects by PAR-1 mediated release of ET-1, a potent vasoconstrictor. PAR-1 activation also up-regulates expression of activated MMP-2\textsuperscript{233}, which is known to cleave big endothelin to ET-1\textsuperscript{234}. ET-1 is a vasoconstrictor and has been shown to enhance vascular reactivity to Ang II\textsuperscript{198} via activation of ET\textsubscript{A} receptors on vascular smooth muscle cells\textsuperscript{235}.

In normal pregnancy, basal levels of MMP-1 and ET-1 may have a role in maintaining vascular tone\textsuperscript{198}. However, in preeclampsia, levels of MMP-1 and ET-1\textsuperscript{236, 8} are significantly greater than in normal pregnancy. Neutrophils can produce MMP-1\textsuperscript{218}, thus, activated neutrophils in the maternal circulation are a potential source of elevated plasma levels of MMP-1 in preeclampsia. Another source of elevated plasma levels is release of MMP-1 by vascular smooth muscle cells resulting from release of TNF-\alpha by infiltrating neutrophils\textsuperscript{237}. Our recent findings of greater gene and protein expression of
MMP-1 in omental and subcutaneous fat vessels in preeclamptic women support a role for MMP-1 in the pathogenesis of preeclampsia.

In this study, we demonstrated that the presence of activated MMP-1 in the lumen of resistance vessels caused dose dependent vasoconstriction and enhanced the vasoconstrictive response to Ang II via PAR-1 activation and ET-1. In preeclamptic women elevated levels of MMP-1 might explain enhanced vascular reactivity to Ang II. MMP-1 induced narrowing of vessels, even at normal Ang II levels, would lead to increased intravascular hydrostatic pressure and reduced capacity of the vascular system resulting in high blood pressure and a tendency towards extravasation of intravascular fluid leading to edema, features common to preeclampsia.

E. Perspectives

These data are the first to show that activated MMP-1 causes vasoconstriction and enhances vascular reactivity to Ang II. These new data could provide novel avenues for treatment of preeclampsia by targeting PAR-1 or ET_{A} receptor. Potential treatments that are available and being used clinically for other indications are the class of ET_{A/B} antagonists, like Bosentan, and SCH 530348, an orally active PAR-1 inhibitor\[^{238}\].
Figure 25. MMP-1 induced vasoconstriction: Role of endothelium. MMP-1 perfusion into the vessel lumen caused dose dependent vasoconstriction (0.25-25 ng/ml) in endothelium intact human omental vessels (N=12). MMP-1 perfusion did not induce vasoconstriction in the absence of endothelium (N=4). (*p<0.05, ** p<0.01, *** p<0.001)
Figure 26. Role of protease activated receptor-1 (PAR-1) in MMP-1 induced vasoconstriction. In endothelium intact human omental vessels, perfusion of 10 µM SCH-79797, a specific PAR-1 blocker inhibited MMP-1 induced vasoconstriction (N=5, * p<0.05, **p<0.01, *** p<0.001).
Figure 27. Role of ET-1 in MMP-1 induced vasoconstriction. In endothelium intact human omental vessels, perfusion of 5 µM BQ-123, a specific endothelin type-A (ETₐ) receptor blocker, inhibited MMP-1 induced vasoconstriction (N=5, *p<0.05, *** p<0.001).
Figure 28. Vascular reactivity to Ang II in the presence of MMP-1 in endothelium intact human omental vessels. Perfusion of MMP-1 (2.5 ng/ml) through the vessel lumen significantly enhanced vascular reactivity to Ang II in endothelium intact human omental vessels at all doses of Ang II tested. (N=12, *p<0.05, ** p<0.01, *** p<0.001).
Figure 29. Effect of PAR-1 inhibitor on vascular reactivity to Ang II in the presence of MMP-1 in endothelium intact human omental vessels. Perfusion of 10 μM SCH-79797, a specific PAR-1 blocker, abolished MMP-1 mediated enhancement of vascular reactivity to Ang II. (N=4, *p<0.05, ** p<0.01).
Figure 30. Effect of ET\textsubscript{A} receptor blocker on vascular reactivity to Ang II in endothelium intact human omental vessels. Perfusion of 5 µMBQ-123, a specific ET\textsubscript{A} receptor blocker abolished MMP-1 mediated enhancement of vascular reactivity to Ang II. (N=4, *p<0.05, ** p<0.01).
A. Introduction

Women with preeclampsia have increased incidence of insulin resistance and show changes similar to that of metabolic syndrome. For example, Martinez et al reported 73% higher fasting plasma insulin concentrations and four-fold higher post-load plasma insulin levels in preeclamptic women. Kaaja et al showed the presence of hypertriglyceridemia, low HDL cholesterol and hyperuricemia in the blood of preeclamptic women.

Insulin Resistance

i. **Definition:** Insulin resistance (IR) is the condition in which normal amounts of insulin are inadequate to produce a normal insulin response from fat, muscle and liver cells (en.wikipedia.org/wiki/Insulin_resistance). Compensatory increase in insulin production by the pancreatic β-islet cells leads to hyperinsulinemia. Hyperinsulinemia
protects against the rise in blood glucose levels until the compensatory ability of the pancreas is overwhelmed and overt diabetes mellitus (DM Type II) is manifest. Thus, insulin resistance is a prelude to diabetes and maybe present in the absence of glucose intolerance.

\[\text{Insulin resistance} \rightarrow \text{Glucose Intolerance} \rightarrow \text{DM Type II}\]

ii. **Insulin Signaling Pathway:**

Insulin rapidly stimulates glucose uptake by the muscle cells and adipocytes by accelerating GLUT-4 translocation. GLUT-4 transporters mediate facilitated glucose uptake into the cell. The rate-limiting step at which insulin stimulates glucose uptake is translocation of GLUT-4 transporters to the plasma membrane\(^{241}\). GLUT-4 translocation involves synthesis of specialized vesicles containing GLUT-4 transporters by the endosomal system, transport of these vesicles from the perinuclear region towards the plasma membrane, followed by fusion with the plasma membrane\(^{242}\).

Phosphatidylinositol 3-kinase (PI3K) dependent Pathway (Fig.31): The insulin receptor is a hetero-tetrameric membrane protein composed of two identical \(\alpha\) and \(\beta\) chains. Insulin docking at its binding site between the two \(\alpha\) chains results in autophosphorylation of the tyrosine residues within the \(\beta\) subunits. This recruits insulin receptor substrates (IRS) which bind to the activated insulin receptor via phosphotyrosine binding. This results in tyrosine phosphorylation of IRS by the activated insulin receptor\(^{243}\). Tyrosine phosphorylation of insulin receptor substrates recruits the regulatory
subunit p85 of PI3K and brings the catalytic subunit p110α close to the membrane. PI3K p110α generates phosphatidyl inositol 3,4,5-triphosphate (PIP3) from phosphatidyl inositol (4,5)-bisphosphate. The PIP3 then activates 3’ phosphoinositide-dependent kinase-1 (PDK-1). Activation of PDK-1 leads to phosphorylation and activation of protein kinase Akt (protein kinase B) and also protein kinase C λ/ζ. Wortmannin and L-294002, PI3K inhibitors, as well as biological blockade of PI3K signaling pathway using dominant negative mutants inhibit insulin stimulated GLUT-4 translocation and glucose uptake. Phosphorylation of Akt substrate 160 (AS160) by protein kinase Akt activates AS160. Activation of AS160 activates the GTPase activating domain (GAP) for Rabs, which are small G-proteins important for vesicle formation, movement and fusion. The GTPase activating domain for a Rab on AS160 is essential for GLUT-4 translocation.

Khan et al hypothesized the existence of an alternative PI3K independent pathway for insulin mediated GLUT-4 translocation acting via changes in the cytoskeleton and lipid raft organization. The contribution of this pathway, if any, would be minimal and the PI3K dependent pathway remains to date the most important pathway for insulin mediated GLUT-4 translocation.

iii. Evidence of Insulin Resistance in Preeclampsia:

Women with preeclampsia show hyperglycemic responses to both oral and intravenous glucose load tests. Women with pregnancy-induced hypertension have been shown to have higher fasting levels of insulin and increased insulin
response to oral glucose load as compared to the normotensive women\textsuperscript{239, 253}. The higher insulin levels with similar glucose tolerance curves demonstrate development of insulin resistance. Martinez et al showed that though the glucose tolerance curves did not differ significantly, fasting plasma insulin concentrations were significantly higher and post-load plasma insulin values fourfold higher in the preeclamptic group than normal pregnant group\textsuperscript{239}. Sowers et al showed that women who subsequently developed preeclampsia had higher fasting plasma insulin levels at 20 weeks than control who maintained normotensive values\textsuperscript{252}.

Insulin resistance along with obesity characterize metabolic syndrome. One-third of all preeclamptic pregnancies have metabolic syndrome\textsuperscript{254}. Higher serum insulin levels have been shown to persist for as long as 17 years after preeclamptic pregnancy\textsuperscript{255}. Development of preeclampsia may contribute to an increased risk of cardiovascular diseases in later life\textsuperscript{256, 257}. Obesity is a risk factor for preeclampsia and is also characterized by inflammation of the vasculature, insulin resistance, hypertension, hypertriglyceridemia, hyperlipidemia, low HDL\textsubscript{2} and hyperuricemia\textsuperscript{240} forming a clinical picture of metabolic syndrome in preeclampsia.

iv.  **Preeclampsia and Insulin Resistance: The Common Link - Inflammation?**

Inflammation of the systemic vasculature is a common feature of obesity and preeclampsia. Walsh and colleagues have shown the presence of increased numbers of activated neutrophils in the vasculature of normal weight preeclamptic women\textsuperscript{95, 96, 258} and non-pregnant obese women\textsuperscript{97}. The degree of inflammation correlates with the extent
of obesity. This state of chronic inflammation is associated with oxidative stress and the local release of cytokines like TNFα, which interferes with insulin signal transduction and possibly GLUT-4 translocation.

Oxidative stress within neutrophils generates NO₂⁻ (nitronium ion). NO₂⁻ nitrates protein tyrosine residues to 3-nitrotyrosine in a reaction catalyzed by superoxide dismutase (SOD). Tyrosine nitration may have the potential to interfere with tyrosine phosphorylation involved in insulin signaling thus contributing to insulin resistance in preeclampsia.

v. Role of TNF-α in development of insulin resistance:

Studies in knock out mice of TNF-α gene, as well as its two receptors, provided direct evidence of TNF-α as an inhibitor of the insulin-signaling pathway. In ob/ob mice and dietary induced obese mice, ablation of TNFα improved insulin sensitivity and prevented the obesity related insulin resistance in muscle and adipose tissue. Plomgaard et al demonstrated that TNFα infusion in healthy humans induced insulin resistance without an effect on endogenous glucose production. TNFα specifically induces insulin resistance by increasing serine phosphorylation of insulin receptor substrate-1 (IRS-1). This converts IRS-1 to an inhibitor of tyrosine kinase activity. As discussed earlier, IRS-1 plays an important role in effecting insulin mediated GLUT-4 translocation and glucose uptake in skeletal muscle. TNFα inhibits AS160 phosphorylation. Phosphorylation of AS160 promotes GLUT-4 exocytosis to the plasma
membrane facilitating glucose uptake. Thus, TNFα inhibits GLUT-4 translocation, and as a result, promotes insulin resistance.

Though there is no direct evidence showing down-regulation of PI3K in response to TNFα, it is likely that TNFα down-regulates the activity and or expression of PI3K as PI3K is the direct downstream target of tyrosine phosphorylated IRS-1 and upstream of pAkt, both of which have been shown to be inhibited by TNFα. In preeclamptic women the levels of TNFα and its soluble receptors are significantly higher than those in normal pregnant women. TNFα is a major secretory product of purified cytotrophoblast cells exposed to hypoxic conditions in vitro and in villous explants. Walsh and Wang reported increased TNFα levels and mRNA expression in preeclamptic placentas.

In 2005, Vaughan and Walsh showed that neutrophils from normal pregnant women when activated by oxidative stress and linoleic acid released TNF-α. In preeclampsia, there are elevated levels of linoleic acid, oxidative stress and neutrophil infiltration within the systemic vasculature. Thus, local release of TNFα by activated neutrophils infiltrating the systemic vasculature could mediate the insulin resistance seen in preeclampsia. We hypothesized that activated neutrophils and neutrophil products, like TNFα and ROS, will impair PI3K expression in vascular smooth muscle cells thus contributing to insulin resistance in preeclampsia.

B. Materials and Methods
Placental arterial smooth muscle (PASM) cells were grown in culture and treated with control (medium M-199), neutrophils activated using arachidonic acid (50µM), TNF-α (1 ng/ml) or ROS generating solution in medium M-199, described in detail in Chapter 2. To quantify PI3K p85α and p110α by Western blot, PASM cells were grown and treated with cell treatments in T-25 flasks overnight. The media was aspirated followed by scraping the cells in 1 ml of ice cold sterile PBS. The cells scraped in PBS were centrifuged at 2000 x g at 4°C for 5 minutes. After discarding the supernatant, 200 µl of M-PER buffer was added and mixed well. The cell suspension with M-PER buffer was placed on a shaker for 10 min and then centrifuged at 14,000 x g for 20 minutes. The supernatant was collected and frozen to -80°C. The pellet of cell debris was discarded. Protein concentration in the collected supernatant was quantified using the Pierce BCA Protein Assay Kit.

To prepare 2 ml of M-PER buffer, 80 µl of protease inhibitor (PI) stock solution and 40 µl of 100 mM sodium orthovanadate (SOV) was added to 1.880 ml of M-PER (Pierce). PI stock solution was prepared by dissolving 2 Complete PI tablets (Roche) in 4 ml of ddH₂O and stored as 150 µl aliquots at -20°C for up to 12 wks. Solution of SOV was prepared as 10 ml of 100 mM SOV in ddH₂O in a 50 ml polypropylene tube. The pH was adjusted to 10.0 using 1 N hydrochloric acid (HCl) and warmed in a beaker of boiling water till the solution turned clear (approx 20 minutes). After the solution had cooled to room temperature, it was stored in 100 µl aliquots at -20°C. For Western blotting, the Invitrogen Xcell Surelock system was used for electrophoresis and the Odyssey (Licor) system was used for scanning fluorescence tagged secondary antibodies.
Sample Preparation: Equal concentrations of protein per sample were calculated and volumized to 22.5 µl with ddH₂O. To this 1.5 µl of 20X reducing agent and 6 µl of 5X Laemli’s Buffer were added. Samples were placed in boiling water at 100°C for 15 minutes followed by cooling on ice and centrifugation at 4°C for 1 minute. The samples were then applied to the wells of the gel using gel loading pipette tips. Biorad All Blue protein standards were run in the first lane of every Western blot.

Antibodies, Electrophoresis and Transfer for PI3K p85α, p110α: Primary rabbit monoclonal anti-PI3K p110α antibody (Cell signaling technologies, 1:500) and anti-PI3K p85α antibody (Millipore, 1:1000) were used for overnight incubation at 4°C. Primary mouse monoclonal anti-β actin Ig (Sigma, 1:10,000) was added to control for variable protein loading. Secondary antibodies, IRDye 800 anti-mouse antibody (Licor, 1:30,000) and AlexaFluor 680 goat anti-rabbit antibody (Invitrogen, 1:10,000) were incubated for 45 minutes at room temperature. When stored at 4°C, both primary and secondary antibody solutions could be re-used several times.

For electrophoresis, precast 8% Novex Tris-Glycine mini-gels from Invitrogen were used with sample loading of 25 µg protein/well and electrophoresis was run at 125V for 1.5 hours. After electrophoresis, the gel was washed with ddH₂O followed by equilibration in methanol free transfer buffer for 15-20 minutes. A 0.45 µ pore size PVDF membrane (Millipore) was wetted in 100% methanol for 30 seconds followed by rinsing in ddH₂O and equilibration in methanol free transfer buffer for 15 minutes. Ice cold methanol free transfer buffer was used to soak the blotting pads taking care to remove all air bubbles. The sandwich for transfer composed of 3 blotting pads, filter paper, gel,
PVDF membrane, filter paper and 3 blotting pads was assembled and transfer was run for 3 hours at 35V. Coomassie blue staining of the gel was used to confirm optimal transfer of protein.

Quantification of PI3K was done using densitometric analysis by Odyssey software. The values were normalized to β-actin and calculated as a percentage of control to be compared by one-way ANOVA and the Newman Keuls post-test.

C. Results

i. PI3K p85α Western Blot

As shown in Fig. 32, expression of the regulatory sub-unit of phosphatidyl inositol 3-kinase, p85α, in primary human vascular smooth muscle cell culture was significantly decreased when co-cultured with activated neutrophils overnight (N=4, p<0.05). Similarly neutrophil products like ROS (N = 6, * p<0.05) and TNFα (N = 7, ** p<0.01) also significantly decreased expression of PI3K p85α.

ii. PI3K p110α Western Blot

As shown in Fig. 33, expression of the catalytic sub-unit of phosphatidyl inositol 3-kinase, p110α, in primary human vascular smooth muscle cell culture was significantly decreased when co-cultured with activated neutrophils overnight (N=4, p<0.05). Similarly neutrophil products like ROS (N = 5, * p<0.05) and TNFα (N = 6, ** p<0.01) also significantly decreased expression of PI3K p110α.
D. Discussion

Our data show that activated neutrophils and the neutrophil products, ROS and TNF-α, significantly decrease expression of the PI3K regulatory and catalytic subunits, p85α and p110α, respectively, in primary human vascular smooth muscle cell culture. The catalytic subunit of PI3K, p110α, is important for GLUT-4 mediated glucose uptake (Fig. 31). It is interesting to note that TNF-α inhibits insulin signaling and glucose metabolism in humans\(^ {271, 272}\) but not in rodents\(^ {273}\). This brings to light inter-species differences in the sensitivity to cytokines, which may be one of the reasons preeclampsia is a disorder unique to human pregnancy.

TNF-α is known to affect intracellular insulin signaling in fat, skeletal muscle and other insulin responsive tissues by inhibiting kinase activity in the proximal part of the insulin-signaling pathway\(^ {260, 274, 275}\). TNF-α also affects a similar signaling pathway in vascular endothelium that results in production of nitric oxide (NO)\(^ {276}\), which mediates insulin-stimulated vasodilation\(^ {277-279}\). We show that neutrophils and ROS also affect insulin-signaling pathway by decreasing the regulatory (p85α) and catalytic (p110α) subunits of PI3K. Thus, in addition to TNF-α, neutrophils and ROS also contribute to insulin resistance and vascular dysfunction in preeclampsia.

Neutrophils could be involved in maintaining a vicious cycle perpetuating insulin resistance by down-regulation of PI3K. TNFα release by activated neutrophils in the maternal circulation or within the vasculature would inhibit the PI3K/Akt pathway.
Inhibition of the PI3K/Akt pathway within the vascular smooth muscle cells would inhibit GLUT-4 translocation and thus induce insulin resistance\textsuperscript{259, 263, 264, 272}.

Although hypertension of preeclampsia falls rapidly with delivery of the placenta, Fuh et al reported that it took 2 months after delivery for the insulin insensitivity to decrease\textsuperscript{280}. Jacober et al reported no difference in insulin sensitivity 3-6 months post-partum\textsuperscript{281}. With delivery of the placenta, a major source of lipid peroxides is lost, reducing oxidative stress. Without the placenta, there is no ongoing neutrophil activation and TNF\(\alpha\) release. Eventually, new neutrophils are released from the bone marrow and the state of vascular inflammation can be reversed. Thus, neutrophil infiltration and release of TNF\(\alpha\) could explain the reversible nature of the insulin resistance seen in preeclamptic women.
Figure 31. Phosphatidyl inositol 3-kinase (PI3K) dependent insulin mediated GLUT-4 translocation pathway. Insulin mediated activation of insulin receptor causes tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) which activates the regulatory (p85α) and catalytic subunits (p110α) of PI3K. Activated PI3K phosphorylates its substrate, membrane phosphatidyl inositol 3,4,5 triphosphate, to result in phosphorylation of Akt and Akt substrate 160 (AS160). Activation of AS160 activates the GTPase activating domain (GAP) for Rabs, which are small G-proteins important for vesicle formation, movement and fusion. Activation of AS160 activates the GTPase activating domain (GAP) for Rabs, which are small G-proteins important for vesicle formation, movement and fusion. The GTPase activating domain for Rab on AS160 is essential for GLUT-4 translocation. When in the plasma membrane, GLUT-4 receptors facilitate glucose uptake by the cells.
Figure 32. Expression of the regulatory sub-unit of phosphatidylinositol 3-kinase, p85α, in vascular smooth muscle cell culture. Expression of p85α is significantly decreased with treatments of activated neutrophils (N=4, * p<0.05) or neutrophil products, ROS (N=6) and TNFα (N = 7,** p<0.01).
Figure 33. Expression of the catalytic sub-unit of phosphatidylinositol 3-kinase, p110α, in vascular smooth muscle cell culture. Expression of p110α is significantly decreased with treatments of activated neutrophils (N=4, * p<0.05) or neutrophil products, ROS (N=5, * p<0.05) and TNFα (N = 6, ** p<0.01).
CHAPTER 6

DISCUSSION

This investigation is the first to demonstrate a cause for the development of hypertension in preeclampsia. It goes on to explain the pathways by which neutrophils and neutrophil products can give rise to the pathogenesis of preeclampsia. We began by noting the significantly increased neutrophil infiltration and inflammation within the systemic vasculature of preeclamptic women\(^96\) and proposed that this neutrophil infiltration leads to increased vessel reactivity to Ang II, which is seen in women predestined to develop preeclampsia\(^123\).

We began our investigation by testing vessel reactivity to Ang II in omental vessels from normal pregnant women with perfusion of activated neutrophils through the vessel lumen. The presence of neutrophils significantly increased vessel reactivity to Ang II. Increased vessel reactivity to Ang II leads to hypertension and reduced circulatory volume in preeclampsia.

Activated neutrophils release a number of inflammatory mediators, such as ROS and TNF-\(\alpha\). To elucidate the mechanism by which neutrophils increase vessel reactivity to Ang II, we tested vessel reactivity to Ang II with perfusion of neutrophils in the presence of SOD/catalase to quench the ROS generated. This abolished the increased vessel reactivity observed with neutrophils. To confirm the role of ROS, and to exclude
the role of other neutrophil products like TNF-α, we repeated the Ang II dose response in
the presence of a ROS generating solution and also with TNF-α. While ROS enhanced
vessel reactivity to Ang II similar to that seen with neutrophil perfusion, TNF-α did not
affect vessel reactivity to Ang II.

A major mechanism of increasing vessel reactivity to Ang II in vascular smooth
muscle cells is the RhoA kinase pathway. ROS can activate RhoA kinase\textsuperscript{134}. Therefore, to
determine whether ROS were activating RhoA kinase to result in enhanced vessel
reactivity, we used Y-27632, a specific RhoA kinase inhibitor. Addition of Y-27632
significantly reduced the increased vessel reactivity to Ang II in the presence of
neutrophils or ROS. To confirm the role of the RhoA kinase pathway, we measured
RhoA kinase activity using a previously validated assay in omental vessels treated with
ROS\textsuperscript{224}. Omental vessels exposed to ROS showed a 3-fold increase in RhoA kinase
activity as compared to control vessel segments.

We also quantified expression levels of Thr-696 pMYPT1 and Ser-19 pMLC in
vascular smooth muscle cell culture treated with Ang II plus ROS or neutrophils, with
and without Y-27632. Both ROS and neutrophils significantly increased expression
levels of pMYPT1 and pMLC in the absence of Y-27632, but not in the presence of Y-
27632. Thus, we conclude that neutrophil release of ROS activates RhoA kinase to
increase vessel reactivity to Ang II.

To determine if ROS activation of RhoA kinase could affect vessel reactivity to
other vasoconstrictors, we tested NE in the presence of ROS, with or without Y-27632.
We found that ROS activation of RhoA kinase also enhanced vessel reactivity to NE, so ROS enhancement of vessel reactivity appears to be a generalized mechanism.

In light of the recent findings of increased expression of MMP-1 within the systemic vasculature of preeclamptic women with increased MMP-1 gene expression in omental vessels and increased plasma levels of MMP-1 in preeclamptic women (Estrada-Gutierrez, unpublished), we proposed a role of MMP-1 in the pathogenesis of preeclampsia. We found that MMP-1 caused direct dose-dependent vasoconstriction and enhanced vessel reactivity to Ang II in endothelium intact vessels. Both effects were mediated via PAR-1 endothelial activation and release of ET-1.

Women with preeclampsia also have insulin resistance\textsuperscript{240}. To determine whether neutrophils or neutrophil products could play a role in the development of insulin resistance, we quantified protein expression levels of the regulatory (p85\(\alpha\)) and catalytic (p110\(\alpha\)) subunits of PI3K in vascular smooth muscle cell culture with treatments of activated neutrophils, ROS, TNF-\(\alpha\) and control. Neutrophils, ROS and TNF-\(\alpha\) significantly decreased expression of the catalytic subunit (p110\(\alpha\)) of PI3K. Neutrophils and TNF-\(\alpha\) also significantly decreased expression of the regulatory subunit (p85\(\alpha\)) of PI3K. PI3K plays a pivotal role in insulin-mediated GLUT-4 translocation to the plasma membrane\textsuperscript{244, 247}, so a neutrophil mediated decrease in PI3K would interfere with cellular glucose uptake. Decreased glucose uptake by cells is a hallmark of insulin resistance.

Our results demonstrate that neutrophils and neutrophil products act as the common denominator in the pathogenesis of preeclampsia contributing to increased vessel reactivity and vasoconstriction leading to hypertension along with insulin
resistance forming a clinical picture of preeclampsia (Fig.35). Our recent finding of extensive vascular infiltration of neutrophils in women with preeclampsia\textsuperscript{95,96} challenged conventional thinking because neutrophils are usually thought of as part of the innate immune system and the first line of defense against infection at the site of a wound\textsuperscript{53,226}. A role for neutrophils in a non-infectious disease, like preeclampsia, has not been widely studied. However, accumulating evidence indicates a role for neutrophils in “sterile” inflammatory diseases\textsuperscript{227}. The reason neutrophils may be playing an important role in pregnancy is that in normal pregnancy, the number of neutrophils increases 2.5-fold by 30 weeks of gestation\textsuperscript{228} and this number increases further in preeclampsia\textsuperscript{54}. The human placenta produces oxidized lipids and secretes them into the maternal circulation\textsuperscript{36,38,69,159,229}. Oxidized lipids are potent activators of neutrophils\textsuperscript{66,68,191}. In women with preeclampsia, placental production of oxidized lipids is significantly higher than in women with normal pregnancy\textsuperscript{38}. One possibility for neutrophil activation occurs as they circulate through the intervillous space and are directly exposed to oxidized lipids released by the placenta\textsuperscript{36,230,231}. Activated neutrophils in the maternal systemic circulation adhere to the endothelium and could cause vascular inflammation and dysfunction by release of toxic compounds. Neutrophil release of matrix metalloproteinase-8 and myeloperoxidase could compromise vascular integrity.

Neutrophils also express and produce MMP-1\textsuperscript{218}. Moreover, our lab has shown that neutrophil and neutrophil products stimulate VSMC to produce MMP-1 and IL-8 (Estrada-Gutierrez, Walsh, unpublished). IL-8 being a chemo attractant to neutrophils
will attract more neutrophils into the vasculature forming a self-perpetuating cycle of ongoing neutrophil infiltration leading to increasing production of MMP-1 contributing to increasing vasoconstriction and worsening of hypertension. Constant production of MMP-1 by VSMC under the influence of neutrophils could be another mechanism to explain the increased vessel reactivity to Ang II. The vascular effects of MMP-1 were mediated via PAR-1. PAR-1 expression is significantly increased in preeclamptic omental vessels as compared to normal pregnant women; and in VSMC neutrophils, ROS and TNF-α significantly increase PAR-1 expression (Estrada-Gutierrez, Walsh, unpublished). Thus, the effects of MMP-1 would likely be magnified many fold in preeclamptic women with both endothelial and VSMC expressing PAR-1. One of the limitations of the present study is that vessels from preeclamptic women were not evaluated. It would be interesting to repeat these experiments in vessels from preeclamptic women and evaluate the role of PAR-1 mediated direct intracellular calcium entry into VSMC as future studies. Also, MMP-1 is a collagenase, like MMP-8 (neutrophil collagenase), and could compromise vascular integrity which when coupled with increased hydrostatic pressure as a result of enhanced vascular reactivity to Ang II, would lead to extravasation of protein. Thus, neutrophil infiltration and increased MMP-1 may be contributing not only to hypertension, but also to the edema and proteinuria seen in preeclampsia.

Apart from vasoconstriction and increased vascular reactivity leading to hypertension in preeclampsia, neutrophils and neutrophil products also inhibited VSMC expression of regulatory and catalytic subunits of PI3K which could explain insulin
resistance in preeclampsia. These studies were done in VSMC culture and future studies for PI3K activity in preeclamptic vessels compared to normal are warranted. Moreover, it would be interesting to do real time polymerase chain reaction for quantifying gene expression along with epigenetic studies to determine if neutrophils or neutrophil products lead to epigenetic alteration of the PI3K genes.

In conclusion, neutrophils may be playing a key central role in the pathogenesis of preeclampsia (Fig. 34). Neutrophil products like ROS and MMP-1 can increase vessel reactivity to cause hypertension, while ROS and TNF-α inhibit PI3K to cause insulin resistance. Moreover, MMP-1 can also cause direct vasoconstriction and compromise vascular integrity that could lead to edema and proteinuria.

The work presented in this thesis offers several options for development of preventive and therapeutic treatments for preeclampsia. For example, use of neutrophil neutralizing antibodies and antibodies against adhesion molecules could prevent neutrophil infiltration of the systemic vasculature and thus prevent development of clinical manifestations of preeclampsia, or even reverse signs of preeclampsia by preventing new neutrophil infiltration. RhoA kinase inhibitors could be studied to determine their ability to control blood pressure in preeclampsia by blocking enhancement of vessel reactivity to Ang II and NE. MMP-1 neutralizing antibodies could possibly impair the ability of MMP-1 to induce vasoconstriction, enhance vessel reactivity and compromise vascular integrity, thus potentially reducing blood pressure and reversing edema and proteinuria. Since the vasoconstrictive properties of MMP-1 are mediated via PAR-1 and ET-1 release, PAR-1 inhibitors and ET<sub>A</sub> receptor blockers
would be excellent drug development targets to pursue for blood pressure control in preeclampsia. Intracellular pharmacologic stimulators of PI3K could be developed and studied as potential drugs to regain insulin sensitivity. This could also find application in the treatment of obesity associated metabolic syndrome and Type II DM, in addition to the treatment of preeclampsia. Thus, the work presented in this thesis has multiple applications and serves to better understand the complex pathogenesis of preeclampsia, a leading cause of maternal and neonatal mortality and morbidity.
Figure 34. Neutrophils: The common denominator in the pathogenesis of preeclampsia. Vascular infiltration of neutrophils leads to production of reactive oxygen species that lead to increased vessel reactivity and insulin resistance. Neutrophil release of MMP-1 or stimulation of vascular MMP-1 leads to vasoconstriction, increased vessel reactivity and compromised vascular integrity leading to hypertension, edema and proteinuria. On the other hand, TNF-α, another neutrophil product, contributes to insulin resistance without affecting vessel reactivity. Thus, vascular infiltration of neutrophils could explain the development of hypertension, insulin resistance, edema and proteinuria in preeclampsia.
Literature Cited


140. SALAMANCA DA, KHALIL RA. Protein kinase C isoforms as specific targets for modulation of vascular smooth muscle function in hypertension. Biochem Pharmacol 2005;70:1537-47.


187. **Nishimura** H, **TsujI** H, **Masuda** H, et al. Angiotensin II increases plasminogen activator inhibitor-1 and tissue factor mRNA expression without changing that of tissue type plasminogen activator or tissue factor pathway inhibitor in cultured rat aortic endothelial cells. Thromb Haemost 1997;77:1189-95.


193. **Todoki** K, **Okabe** E, **Kiyose** T, **Sekishita** T, **Ito** H. Oxygen free radical-mediated selective endothelial dysfunction in isolated coronary artery. Am J Physiol 1992;262:H806-12.


207. ORSHAL JM, KHALIL RA. Reduced endothelial NO-cGMP-mediated vascular relaxation and hypertension in IL-6-infused pregnant rats. Hypertension 2004;43:434-44.


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