MECHANISMS OF CIRCULATING FACTORS IN ENDOTHELIAL DYSFUNCTION IN PREECLAMPSIA

by

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ABSTRACT

Preeclampsia, defined as new-onset hypertension after 20 weeks of gestation with concurrent proteinuria, is the leading cause of maternal mortality. Preeclampsia is a multi-system disorder with serious maternal and fetal morbidities, which affects 2 – 8% of pregnancies worldwide. Although the exact etiology is unknown, it is believed that defective trophoblast invasion in early pregnancy leads to placental hypoperfusion and local oxidative stress, which triggers the release of circulating factors and induces endothelial dysfunction in the maternal circulation. Previous literature has shown that circulating factors, and especially syncytiotrophoblast extracellular vesicles (STBEVs), from women with preeclampsia impair endothelial function in small resistance arteries. However, there is a still paucity of data on the mechanism(s) behind such findings. The Davidge laboratory has shown that circulating factors from preeclamptic plasma increase oxidative stress in isolated endothelial cells and impair vasodilation in small resistance arteries, via the activation of the lectin-like oxidized low density lipoprotein receptor-1 (LOX-1), which is upregulated in preeclampsia. In addition, LOX-1 is a scavenger receptor that binds oxidized low density lipoprotein as well as many other ligands, including cell debris, platelets, and bacteria. Therefore, we are interested in investigating: 1) the mechanism behind which circulating factors affect endothelium-dependent vasodilation, and 2) the effect of STBEVs on the maternal vasculature and whether it acts as a ligand for LOX-1. Using *ex vivo* myography experiments, we studied vascular responses in pregnant rat uterine arteries incubated with either preeclamptic plasma or STBEVs derived from human placentas in the absence or presence of various pharmacological agents to examine the involvement of reactive oxidative species, nitric oxide, and prostaglandin pathways. We also measured vascular superoxide levels in exposed arteries

using dihydroethidium staining. In addition, we examined the vascular expression of nitric oxide synthase isoforms to further understand the role of nitric oxide in preeclamptic plasma-induced endothelial dysfunction. Our studies support that circulating factors from women with preeclampsia lead to endothelial dysfunction by increasing oxidative stress and decreasing nitric oxide bioavailability. Contrary to our hypothesis, rather than reducing prostaglandin vasodilators, we have found that circulating factors contribute to an increase of prostaglandin H synthase dependent vasoconstrictors. We have also shown that, indeed, STBEVs impair endothelial vasodilation via activation of the LOX-1 receptor which is associated with a reduction in nitric oxide contribution. This thesis contributes to our understanding of the pathophysiology behind the role of circulating factors in vascular dysfunction in preeclampsia. We have also identified that LOX-1 contributes to endothelium-dependent impairment associated with STBEVs, which could potentially be reversed and/or prevented by the inhibition of the LOX-1, thereby making it a potential target of treatment.

PREFACE

This thesis is an original work by Cindy Kao. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name: “Pregnancy Complications”, No. AUP 00000242.

Some of the research conducted at this thesis forms part of an international research collaboration, with Professor Sandra T. Davidge being the lead investigator at the University of Alberta. The plasma samples utilized in chapter 2 was obtained from our collaborator Dr. Patricio Lopez-Jaramillo at the Universidad de Santander, Bucaramanga, Colombia, and Dr. Laura M. Reyes who is currently a Ph.D. student in Professor S. Davidge’s laboratory. The syncytiotrophoblast extracellular vesicles used in chapter 3 were provided by our collaborator Professor Ian L. Sargent and Dr. Dionne Tannetta at the University of Oxford, U.K. The LOX-1 receptor antibody was provided by Dr. Tatsuya Sawamura at the National Cerebral and Cardiovascular Centre Research Institute, Osaka, Japan.

Chapter 2 of this thesis is currently under revision for prospective publication in the journal Clinical Sciences, titled “Mechanism of Vascular Dysfunction Due to Circulating Factors in Women with Preeclampsia.” I was responsible for the design and performance of the experiments, data collection and analysis, and writing of the manuscript. J. Morton provided support for my techniques and data analysis. A. Quon performed the DHE staining and Western blots. S. Davidge supervised and assisted in the editing of the chapter.

Chapter 3 of this thesis is currently submitted for consideration of publication in the journal Hypertension, titled “Syncytiotrophoblast Extracellular Vesicles Impair Rat Uterine Vascular Function via the Lectin-Like Oxidized Low Density Lipoprotein Receptor-1.” I am a co-author with F. Spaans and we have contributed equally to the design and performance of the experiments, data collection and analysis, and writing of the manuscript. Specifically, F. Spaans contributed to the collection and analysis of data presented in Figure 4. S. Davidge supervised and assisted in the editing of the chapter.

DEDICATION

The proverb *"it takes a village to raise a child"* could not have been truer.

This thesis is dedicated to those who have helped me raise my children, Miya and Rylan, and to those who have always been by my side, helping me through the best and the worst times.

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This thesis would not have been possible without the support and guidance from Dr. Sandra Davidge. Her enthusiasm for science, balanced approach to mentorship, and continuous encouragement are only a few of the characteristics that make her one of the best role models I have ever had. The support and feedback from my committee members, Drs. Peter Mitchell, Jane Schulz, and Sue Chandra, have also allowed me to strive for the best I can.

The successful completion of this thesis is also attributable to the Davidge lab members. Dr. Jude Morton has shown me by example what it takes to be an excellent teacher: patience, perseverance, meticulous attention to details, and willingness to step away from her own busy days in order to help others. The cohesive and supportive environment provided by the lab members, Raven, Floor, Alison, Laura, Anita, Amin, Subhadeep, Mais and Christy, is what gets me through the tough days.

For any prospective students interested in joining the Davidge lab, here are some words of advice that I wish to share. First of all, the Davidge lab only offers lifetime memberships. Once you're in, you're in it *forever*. Be prepared to (want to) return for all the social events, (mandatory) birthday cake celebrations, and serious pumpkin carving contests. Secondly, it is a pre-requisite that you learn to speak several languages in order to understand the multi-cultural communication; these include, but are not limited to, the *Laura-ism*, the Australian, the Dutch, the British, and the American languages (and yes, they are all different languages,

believe me). Lastly, there is an endemic of a disease that you will likely contract once you join the lab, called *Judeitis.* The signs and symptoms of this disease consists of severe separation anxiety from Jude when she is away from her desk, sense of impending doom when she is not immediately available, and physical tremors and uncontrollable urge to scream *Juuuuuuuude* whenever your experiments are not working. I am a survivor of this disease, and you can be too.

Finally, I would like to acknowledge our funding agencies that have generously contributed to the making of this thesis. These include grants from the Canadian Institutes of Health Research (CIHR) and the Women and Children's Health Research Institute (WCHRI) through the contributions of the Stollery Children's Hospital Foundation and the Royal Alexandra Hospital Foundation. I am also supported by the Clinical Investigator Program funded by the Alberta Health Services. The collection of plasma samples are supported by the Departamento Administrativo de Ciencia, Tecnología e Innovación (COLCIENCIAS) and special thanks to Silvia L. Ruiz for her help in the sample collection in Colombia.

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ACE	Angiotensin converting enzyme
ACh	Acetylcholine
ALT	Alanine aminotransferase
Ang II	Angiotensin II
ANOVA	Analysis of variance
aPTT	Activated partial thromboplastin time
AST	Aspartate aminotransferase
AT1-AA	Angiotensin 1 receptor autoantibody
AT_1R	Angiotensin 1 receptor
AT_2R	Angiotensin 2 receptor
AUC	Area under curve
BMI	Body mass index
dBP	Diastolic blood pressure
cGMP	Cyclic guanosine monophophaste
COX	Cylooxygenase
DHE	Dihydroethidium
DNA	Deoxyribonucleic acid
EDF	End-diastolic flow
EDH	Endothelium-dependent hyperpolarization
EDHF	Endothelium-dependent hyperpolarization factor
EDTA	Ethylenediaminetetraacetic acid
E_{max}	Maximal response
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
ET_A-R	Endothelin receptor A
ET_B-R	Endothelin receptor B
FHR	Fetal heart rate
GFR	Glomerular filtration rate
GTP	Guanosine triphosphate

H_2O_2	Hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
HELLP	Hemolysis, elevated liver enzymes, and low platelets
HUVEC	Human umbilical vein endothelial cell
IgG	Immunoglobulins
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
INR	International normalized ratio
IRDYe	Near-infrared dye
IUGR	Intrauterine growth restriction
LDH	Lactate dehydrogenase
L-NAME	N-nitro-L-arginine methyl ester hydrochloride
LOX-1	Lectin-like oxidized low density lipoprotein receptor-1
MCh	Methacholine
Meclo	Meclofenamate
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NP	Normotensive pregnant
$O_2^{\bullet}$-	Superoxide
OCT	Optimal cutting medium
ONOO-	Peroxynitrite
oxLDL	Oxidized low density lipoprotein
PE	Preeclampsia
pEC_{50}	Effective concentration required to achieve 50% of maximal response
PG	Prostaglandin
PGHF1α	6-keto-prostaglandin F1α
PGH_2	Endoperoxide or prostaglandin H_2
PGHS	Prostaglandin H synthase

PGI_2	Prostacyclin
PIGF	Placental growth factor
PIH	Pregnancy-induced hypertension
PMSF	Phenylmethanesulfonylfluoride (serine protease)
ROS	Reactive oxygen species
RUPP	Reduced uterine perfusion pressure
RUQ	Right upper quadrant
sBP	Systolic blood pressure
sEng	Soluble endoglin
sFlt-1	Soluble fms-like tyrosine kinase-1
sLOX-1	Soluble lectin-like oxidized low density lipoprotein receptor-1
sGC	Soluble guanylyl cyclase
SOD	Superoxide dismutase
SOGC	Society of Obstetricians and Gynecologists of Canada
STBEV	Syncytiotrophoblast extracellular vesicle
TIA	Transient ischemic attack
TNF-α	Tumor necrosis factor alpha
TXA_2	Thromboxane
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
WBC	White blood cell
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1 Preeclampsia and its Clinical Impact

The understanding of the pathophysiology of preeclampsia has come a long way since the first mention in the medical history of pregnancy-related seizures dating from about 2200 BC. The term "preeclampsia", derived from Greek meaning "sudden flash or development," hints at how preeclampsia can be rapid onset, therefore often requiring urgent obstetrical care [1]. However, given the complicated multifactorial etiology of preeclampsia, the exact mechanism is still largely understudied.

1.1.1 Definition and Diagnosis of Preeclampsia

The definition of preeclampsia has evolved significantly through time. What has been historically known as toxemia or pregnancy-induced hypertension ("PIH") is now referred to as hypertensive disorders of pregnancy. This encompasses a wide spectrum of diagnoses involving problems with high blood pressure in pregnancy.

The Society of Obstetricians and Gynecologists of Canada (SOGC) provided a newly updated guideline in 2014 regarding the diagnosis and management of hypertensive disorders of pregnancy [2]. Many patients who have no pre-existing medical conditions can develop new-onset hypertension at ≥ 20 weeks of gestations, known as gestational hypertension. The new SOGC clinical guidelines suggest that hypertension in pregnancy can be diagnosed when systolic blood pressure (sBP) ≥ 140 mmHg and/or diastolic blood pressure (dBP) ≥ 90 mmHg, based on an average of at least 2 measurements, taken at least 15 minutes apart, using the same arm, in either an office or in-hospital setting (rather than home monitoring). Preeclampsia can develop

weeks after the onset of gestational hypertension or may arise *de novo* with new proteinuria or one or more adverse condition(s) or severe complication(s) (summarized in Table 1-1).

Table 1-1: Adverse conditions and severe complications of preeclampsia.

System	**Adverse conditions** ⇨ **Increase surveillance**	**Severe complications** ⇨ **Warrant delivery**
Central nervous system	Headaches Scotoma	Eclampsia Stroke, TIA Cortical blindness Retinal detachment
Cardiopulmonary	Chest pain, dyspnea Oxygen sat < 97%	Uncontrolled severe hypertension Oxygen sat < 90% Pulmonary edema Positive ionotropic support
Hematological	↑ WBC ↑ INR, aPTT ↓ Platelets	↓↓ Platelets < 50 x 10^9/L Transfusion of blood product
Renal	↑ Creatinine ↑ Uric acid	Acute kidney injury Dialysis
Hepatic	Nausea, vomiting RUQ or epigastric pain ↑ AST, ALT, LDH, bilirubin ↓ Albumin	Hepatic dysfunction (INR > 2) Hepatic hematoma or rupture
Feto-placental	Abnormal FHR IUGR Oligohydramnios Absent or reversed EDF	Abruption Reverse ductus venosus A wave Stillbirth

Adverse conditions and severe complications of preeclampsia affect multiple organ systems, including both the maternal and fetal compartments. Column 2 lists the adverse conditions including signs and symptoms necessitating increased surveillance. Column 3 lists severe complications requiring urgent obstetrical care and delivery. Modified from SOGC 2014 guideline [2].

Abbreviations: TIA, transient ischemic attacks; WBC, white blood cells; INR, international normalized ratio; aPTT, activated partial thromboplastin time; RUQ, right upper quadrant; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; FHR, fetal heart rate; IUGR, intrauterine growth restriction; EDF, end-diastolic flow.

Recognizing that many patients have pre-existing medical conditions, the SOGC also identifies a distinct category of patients whose hypertension begins prior to conception or at < 20 weeks of gestation [2]. This group of patients, with pre-existing chronic hypertension or chronic kidney disease, is at increased risk and requires careful surveillance and monitoring for the development of preeclampsia. In patients with pre-existing hypertension or kidney disease, superimposed preeclampsia is defined by the development of resistant hypertension requiring ≥ 3 antihypertensive medications, new or worsening proteinuria, or ≥ 1 adverse conditions or severe complications (Table 1-1).

Preeclampsia can be categorized depending on the timing of onset or severity. Traditionally, the onset of preeclampsia at < 34 weeks is referred to as "early onset" and those ≥ 34 weeks are known as "late onset". It is speculated that early onset preeclampsia is a different subtype of disease that might have a different pathogenesis from those with late onset disease [2]. Thus, this distinction is often made while interpreting results for clinical trials. In addition, it is also customary to classify patients with "severe" preeclampsia if the sBP is higher than 160 mmHg and/or the dBP is higher than 110 mmHg or if they present with severe complications such as eclampsia, hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, renal failure, or fetal compromise (Table 1-1). Recognizing the severity of preeclampsia is helpful in triaging the patients and expediting their management plan; however, it is also important to recognize that anyone with "mild" preeclampsia could rapidly progress into "severe" without warning and that "severe" preeclampsia is not always preceded by "mild" disease.

1.1.2 Clinical Manifestation of Preeclampsia

The clinical signs and symptoms of preeclampsia are complicated – hence, it is more appropriately referred to as a "syndrome" affecting multiple organ systems rather than a single disease entity. Not only does it affect the maternal brain, cardiovascular, pulmonary, renal, hepatic, and hematological systems, but it also impacts the growth and well-being of the fetus. The important signs and symptoms of preeclampsia are discussed in this section with the intention of providing an overview of the clinically relevant effects. The specific mechanisms of associated complications are not covered as they are beyond the scope of this thesis; a detailed literature on the pathogenesis of preeclampsia is reviewed in Chesley's Hypertensive Disorders in Pregnancy, 4th edition [3].

1.1.2.1 Cardiovascular and Pulmonary System

In a normal pregnancy, a decrease in both sBP and dBP is noted as early as 5 weeks of gestation and persists into the third trimester [3]. To accommodate a nearly 40% increase in blood volume to supply the growing uterus, placenta, and fetus, the maternal peripheral resistance decreases and the cardiac output increases by 35-50% during gestation [4]. In contrast for preeclampsia, in addition to an increased afterload due to increased peripheral resistance, there is also evidence for ventricular remodelling and diastolic dysfunction in 40% of women, predisposing them to heart failure or myocardial infarction [5]. Furthermore, an increase in endothelial-epithelial permeability results in a generalized edema and swelling in the periphery as well as pulmonary edema [3]. As a result of the fluid extravasating into the interstitial space, woman with preeclampsia are at risk of relative intravascular hypovolemia, even though they often present with a clinical picture of hypervolemia (swelling in the face and extremities). In the setting of postpartum hemorrhage, the loss of blood becomes imminently more dangerous than in

a normal pregnancy because the intravascular reservoir of blood volume is already depleted in preeclampsia. Therefore, preeclampsia imposes serious implications on the maternal cardiopulmonary system.

1.1.2.2 Renal System

During normal pregnancy, renal blood flow and glomerular filtration (GFR) rate are both increased while, in contrast, these are decreased in preeclampsia [4]. Clinically, this results in a rise in serum creatinine levels indicating a decrease in renal function. As a result of decreased GFR and depleted intravascular volume, urine output also decreases, which is an important clinical sign monitored in preeclamptic patients [4]. Unfortunately, due to the increase in vascular permeability, oliguria is often difficult to treat because intravenous crystalloid fluid only improves urine output temporarily while risks a worsening of pulmonary edema. In addition, glomerular damage in the kidney results in a "leak" of protein into the urine and thus proteinuria has become a hallmark in the diagnosis of preeclampsia [2]. Therefore, it is important to establish the absence or presence of proteinuria prior to conception or in early pregnancy in order to have a baseline for comparison if clinical suspicion for the development of preeclampsia arises. However, it is important to note that the severity of preeclampsia does not correlate with the severity of proteinuria and many who develop severe preeclampsia can do so without the development of proteinuria [3]. Hence, the presence of other adverse conditions or severe complications is now included in the diagnostic criteria by the SOGC clinical practice guideline in order to provide a more accurate method of diagnosing preeclampsia.

1.1.2.3 The Liver, the Blood, and HELLP syndrome

The involvement of the liver can vary in presentation but most often correlates with severe preeclampsia. Women with preeclampsia are at risk of hepatic hemorrhage and cellular

necrosis [3]. However, they can be asymptomatic or present with right upper quadrant to mid-epigastric pain. In most cases, elevations in serum hepatic aminotransferase (AST and ALT) levels are detected. In severe cases, hepatic hemorrhage from areas of cellular infarction can be identified in the form of intrahepatic or subcapsular hematoma. Although very rare, liver hemorrhage can be fatal and requires urgent operative management. Fortunately, in the majority of cases, liver dysfunction is usually non-lethal and improves within two to three days following delivery.

Another clinical sign correlated with the severity of preeclampsia is the presence of decreased platelet levels, or thrombocytopenia. Up to half of women with preeclampsia develop thrombocytopenia and their platelet level is inversely associated with the severity of preeclampsia [6]. The pathogenesis of thrombocytopenia is known to be multifactorial, however, increased platelet activation is believed to contribute to increased platelet clearance, as suggested by an elevated level of thromboxane A2 (TXA_2) metabolite in the urine of preeclamptic patients [7]. Furthermore, in severe preeclampsia, erythrocytes have been shown to undergo rapid hemolysis.

Together, this syndrome is clinically identified as hemolysis, elevated liver enzymes, and low platelets, or HELLP syndrome. The presence of HELLP syndrome is indicative of the severity of preeclampsia and, if left untreated, up to 40% of cases develop serious outcomes such as eclampsia (6%), placental abruption (10%), and pulmonary edema (10%) [8]. Therefore, HELLP syndrome is considered a severe complication in preeclampsia which requires immediate delivery of the fetus.

1.1.2.4 Central Nervous System

Several changes in the brain have been associated with severe preeclampsia and eclampsia. Headaches and visual changes commonly proceed eclamptic seizures (50-75% and 20-30% of cases, respectively) [9], and are also thought to correlate with hypertensive hemorrhage as reported by earlier autopsy studies. Cerebral edema, as a result of increased permeability of the blood brain barrier, can present as altered mental status and is very susceptible to sudden blood pressure elevations. Therefore, the control of severe hypertension is important in the management of preeclampsia to reduce the risk of stroke and eclampsia.

1.1.2.5 The Feto-Placental Unit

The pathogenesis of preeclampsia is thought to originate from defective trophoblast invasion of the maternal arteries which results in abnormal placentation and increased resistance in the uterine arteries [10]. Therefore, signs of placental insufficiency seen in preeclampsia can be detected by abnormal Dopplers on antenatal ultrasounds. Measurement of uterine artery blood flow velocity can be used to assess vascular resistance by comparing arterial systolic and diastolic waveforms. The mean resistance in uterine artery blood flow is found to be higher in women with preeclampsia compared with those in normotensive controls [11]. Specifically, an elevated resistance index in uterine artery blood flow in the second trimester of pregnancy is associated with the risk of developing severe preeclampsia [12]. However, the utility of using uterine Doppler flow as a diagnostic or prognostic tool for preeclampsia is still currently under clinical investigation.

Furthermore, as the placenta directly supplies the growing fetus, utero-placental insufficiency associated with preeclampsia could result in a decrease in placental perfusion, impaired fetal growth, and reduced fetal urine output which manifests in oligohydramnios [13].

The decreased uterine blood flow also impairs nutrient exchange to the fetus, which then preferentially shunts its limited resources away from the peripheral organs and toward the growth of vital organs such as the brain. As a result of reduced glucose transfer and hepatic storage, fetal liver size is reduced and this is reflected in a decrease in abdominal circumference with preservation in the growth of the brain and head circumference [14]. Thus, asymmetric intrauterine growth restriction (IUGR) can arise as a result of severe placental insufficiency. In cases where uterine blood flow to the placenta via the spiral artery is severely compromised, areas of the placenta can become necrotic and hemorrhage occurs in the utereo-placental interface, resulting in placental abruption and premature separation of the placenta from the uterine wall. Indeed, impaired trophoblast invasion with subsequent atherosis in the placenta has been linked to preeclampsia and placental abruption [15]. Increased inflammation has also been observed in placentas that separate prematurely in women who present with premature rupture of membrane or antepartum bleeding [16]. If obstetrical intervention is not pursued promptly, placental abruption can lead to life-threatening maternal hemorrhage and eventually intrauterine fetal demise.

1.1.3 Prevalence and Risk Factors of Preeclampsia

According to the World Health Organization (WHO), preeclampsia affects 2-8% of pregnancies worldwide. Globally, it accounts for 16.1% of maternal deaths in developed countries, 25.7% in Latin America and the Caribbeans, 9.1% in Africa, and 9.1% in Asia [17]. In low-resource countries, preeclampsia is the second most common etiology of maternal mortality, preceded by postpartum hemorrhage. However, in developed countries, preeclampsia is the most common cause of maternal mortality [17].

The wide range of risk factors for preeclampsia (summarized in Table 1-2) suggests that the pathogenesis of the disease may be multi-factorial. The majority of the risk factors associated with preeclampsia are also known risk factors for cardiovascular disease, such as age, pre-existing hypertension, diabetes, and obesity [18]. Furthermore, women with preeclampsia are at a 5- and 8-fold increased risk of premature mortality from stroke and cardiovascular event, respectively [19]. This suggests that a maternal predisposition to endothelial dysfunction, which also underlies their cardiovascular disease, may be an important determinant of the risk of developing preeclampsia.

Table 1-2: Risk factors and their relative risks for preeclampsia.

Risk Factor	Incidence or Relative Risk (95% CI)	Reference
Age ≥ 40	1.96 (1.36 – 2.87)	[18]
sBP ≥ 130 mmHg at 1st visit	2.37 (1.78 – 3.15)	[18]
dBP ≥ 80 mmHg at 1st visit	1.38 (1.01 – 2.37)	[18]
Pre-pregnancy renal disease	40-60% [1]	[20]
Pre-pregnancy diabetes	3.56 (2.54 – 4.99)	[18]
BMI > 35 kg/m^2	2.47 (1.66 – 3.67)	[18]
Weight gain during pregnancy	Increased [2]	[21]
Family history of preeclampsia	2.90 (1.70 – 4.93)	[18]
Personal history of preeclampsia	7.19 (5.85 – 8.83)	[18]
Inter-pregnancy interval > 59 months	1.83 (1.72 – 1.94)	[18]
Nulliparity	2.91 (1.28 – 6.61)	[18]
New paternity	Increased [2]	[21]
Use of barrier contraception	Increased [2]	[21]
Antiphospholipid antibodies	9.72 (4.34 – 21.75)	[18]
Multiple gestation	2.93 (2.05 – 4.21)	[18]
Molar pregnancy	Increased [2]	[21]
African American decent	11% [3]	[22]
Smoking[4]	0.51 (0.37 – 0.63) [4]	[23]

[1]In women with advanced chronic kidney disease, as many as 40-60% may be diagnosed with preeclampsia.

[2]The risk of preeclampsia is increased, but the relative risk is unknown.

[3]Among nulliparous women, the incidence of preeclampsia was 5% in white, 9% in Hispanic, and 11% in African-American descent.

[4]Smoking is the only known "protective factor" for preeclampsia, according to a large meta-analysis.

Abbreviations: BMI, body mass index; sBP, systolic blood pressure; dBP, diastolic blood pressure; BMI, body mass index.

The immune system is also thought to play a role in the pathogenesis of preeclampsia. Risk factors such as nulliparity, new partner, and use of barrier contraception strongly suggest that the introduction of a new antigen into the maternal circulation contributes to the development of preeclampsia [21]. An interesting observation has been made that men who father preeclamptic pregnancies with one woman are at 80% higher likelihood to father a subsequent preeclamptic pregnancy with another woman [24], suggesting a role for paternal factors in the pathogenesis. Other factors, such as family history and ethnicity, further suggest a genetic component to the etiology. In addition, risk factors such as multiple gestation and molar pregnancy suggest that excessive placental mass and relative placental hypoperfusion increase the risk of preeclampsia. Indeed, in women with twin pregnancies, the incidence of preeclampsia is increased to 13% versus 5% seen in singleton pregnancies [25].

1.1.3.1 Smoking and Preeclampsia

One of the most enigmatic factors in pregnancy-related complications is the fact that smoking appears to be protective in preeclampsia. According to a large meta-analysis published in 1999, smoking was a protective factor associated with a 51% reduction in the risk of developing preeclampsia [23]. This reduction in risk holds true across all categories of maternal BMI. According to *in vitro* studies, the speculated mechanism of cigarette smoking may be due to a reduction of anti-angiogenic factors (i.e. fms-like tyrosine kinase-1 or sFlt-1) and an increase in placental growth factor (PIGF) [26]. However, other studies have speculated that smoking

might have an idiosyncratic effect on angiogenesis and contribute to abnormal vascular development in the feto-maternal interface resulting in spontaneous termination of the embryo; therefore, the "protective" effect of smoking may be due to a survival bias [21]. Regardless, smoking is also strongly associated with several obstetrical complications, such as placenta previa, placental abruption, ectopic pregnancy, and premature prelabor rupture of membrane [23]. Thus, cessation of smoking in prenatal counselling remains to be an important issue.

1.1.3.2 Preeclampsia as a Cardiovascular Risk Factor

Not only does preeclampsia impact immediate maternal health, but it is also increasingly recognized as a major risk factor for cardiovascular diseases in later life. The prevalence of hypertension in women with a history of preeclampsia is > 50% at an average of 14 years after pregnancy [27]. These women are also at an increased risk of mortality from cardiovascular disease, which is even higher in women with a history of early-onset preeclampsia [28-30]. Thus, understanding the pathophysiology of preeclampsia will not only contribute to the improvement of immediate maternal and fetal health, but it will also help to modify their risk of developing cardiovascular disease in later life and improve the quality of life.

1.1.4 Treatment of Preeclampsia

Likely due to the heterogeneous nature of the disease, there is currently no cure *per se* for women with preeclampsia. Based on large clinical studies, there is insufficient evidence to recommend dietary and lifestyle changes, such as limited dietary salt intake, caloric restriction for obese women, exercise, work reduction, or stress reduction [2]. Counter-intuitively, the SOGC recommends *against* bed rest for women with preeclampsia due to the increased risk of thromboembolism and muscle wasting in those with prolonged bed rest [2]. However, in women with severe hypertension (sBP > 160 mmHg and/or dBP > 110 mmHg), the risk of eclampsia and

stroke is increased; therefore, medical treatment is recommended to achieve a therapeutic goal of sBP 130-155 mmHg and dBP 80-105 mmHg. The most commonly used anti-hypertensive agents include beta-blockers (labetolol), calcium channel blockers (nifedipine), and arterial vasodilator (hydralazine). Second line alternative treatments include the use of nitroglycerin infusion, alpha blocker (methyldopa), or an angiotensin converting enzyme inhibitor (captopril) if preeclampsia arises post partum. In addition, magnesium sulfate is also widely used in the prevention and treatment of eclampsia, although the mechanism by which magnesium acts is still unclear and beyond the scope of this thesis.

In terms of prevention strategies, among high risk women, calcium supplementation of at least 1000 mg/day in women with low dietary intake of calcium (< 600 mg/day) is recommended. It as been postulated that dietary calcium may reduce the severity of preeclampsia by increasing intracellular calcium with increases the activation of endothelial nitric oxide synthase contributing to relaxation of vascular smooth muscle cells. In addition, acetylsalicylic acid (ASA) is also recommended for high risk women and is associated with a 10-15% risk reduction in preeclampsia and prematurity when started prior to 16 weeks of gestation [31]. ASA works as an irreversible inhibitor of cyclooxygenase in platelets which likely contribute to the reduction in thromboxane and other prostaglandin vasoconstrictors. There is also evidence that folate supplementation may be beneficial by reducing homocysteine in women at high risk of preeclampsia [2] and this is currently being studied in a multi-centre, prospective, randomized clinical trial.

1.2 Preeclampsia and the Placenta

Due to the multi-factorial nature of the disease, the exact mechanism of preeclampsia is still largely unknown and, as mentioned, a myriad of factors has been shown to contribute to the

pathogenesis of the disease. The placenta, however, appears to be the heart of the problem: preeclampsia can occur in pregnancy without the fetus such as in hydatiform mole, without a uterus such as in abdominal ectopic pregnancy, and is resolved by delivery of the placenta [32]. The current dogma is in line with the theory that abnormal placentation in early pregnancy as the key inciting event and, in combination with maternal predisposition to endothelial dysfunction, leads to the development of preeclampsia. In order to understand abnormal placentation, I will first discuss normal placental development in a healthy pregnancy.

1.2.1 Normal Development of the Placenta in a Healthy Pregnancy

As described by Riddell [33], the placenta is essentially "the most important organ that you no longer need." The human placenta is a discoid-shaped organ that is critical for the development of the fetus in several aspects: 1) it provides oxygen and nutrients for growth, 2) it eliminates waste, 3) it maintains water and electrolyte balance in the amniotic fluid, and 4) it acts as a immunologic barrier, protecting the fetus from the maternal immune response as well as potentially toxic chemicals that circulate in the maternal blood [10, 33]. Differentiated early in embryo development, trophoblast cells are a unique cell type which is only present during pregnancy and contributes entirely to the development of the placenta. Being the primary organ at the feto-maternal interface, the placenta grows throughout gestation in order to supply the increasing demands of the fetus. Understanding how the placenta develops allows us to further explore how preeclampsia results from abnormal trophoblast development in early pregnancy.

1.2.1.1 Implantation and Trophoblast Differentiation

After fertilization, the zygote undergoes cleavage for three days while it travels within the fallopian tube. Once it reaches the uterine cavity approximately three days after fertilization, it becomes a 16-cell morula which then gradually develops into a blastocyst with accumulation of

fluid inside the cell. The blastocyst is differentiated into the *inner cell mass*, which later becomes the fetus, and *trophoblasts*, which develop into the placenta.

Six to seven days after fertilization, the embryo implants the uterine wall through three phases: (1) apposition, initial contact with the uterine wall, (2) adhesion, increased contact with uterine epithelium, and (3) invasion, penetration of trophoblast cells into the endothelium, inner third of the myometrium, and uterine vasculature [10]. By day eight after fertilization, the trophoblasts differentiate into a multinucleated syncytium called the *syncytiotrophoblast* and an inner mononuclear cell layer called *cytotrophoblast* (Figure 1-1). The syncytiotrophoblast is so named due to its unique structure of a multinucleated, joined cytoplasm without distinct cell orders which aids oxygen and nutrient transport from the placental to the fetus [10].

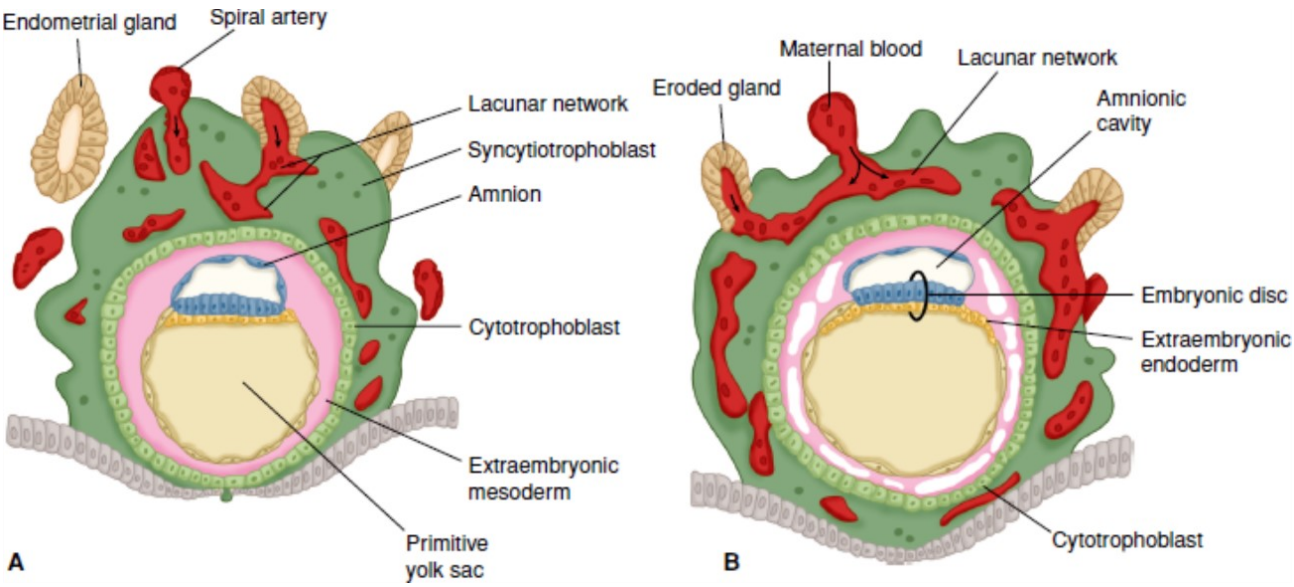

Figure 1-1: Implanted blastocyst and the development of syncytiotrophoblast.

(A) Around day nine after fertilization, the blastocyst wall facing the uterine lumen is a single layer of flattened cells. The inner cell mass is differentiated into a thick plate of primitive ectoderm and endoderm. As the embryo enlarges, maternal decidua basalis is invaded by syncytiotrophoblasts which develop into a large, multinucleated cell. **(B)** Around day 12, the syncytiotrophoblast

becomes permeated with channels of lacunar network and after successful invasion of the capillary walls, the lacunae become filled with maternal blood. Reproduced with permission from McGraw-Hill Education: Cunningham F, *et al.*, *Williams Obstetrics*; copyright obtained Dec 24, 2015 [10].

1.2.1.2 Early trophoblast invasion and Spiral Artery Remodelling

After implantation is complete, the trophoblast further develops into the villous and extravillous trophoblast [34]. The *villous* trophoblast gives rise to the chorionic villi, which transport oxygen, nutrients, and other compounds between the fetus and mother. The *extravillous* trophoblast migrates into the myometrium and penetrates the maternal vasculature [35]. The extravillous trophoblast then further develops into the interstitial and endovascular trophoblast. The *interstitial* trophoblasts invade the decidua and surrounds maternal spiral arteries while the *endovascular* trophoblasts penetrate the spiral artery lumen for vascular remodelling (Figure 1-2) [36].

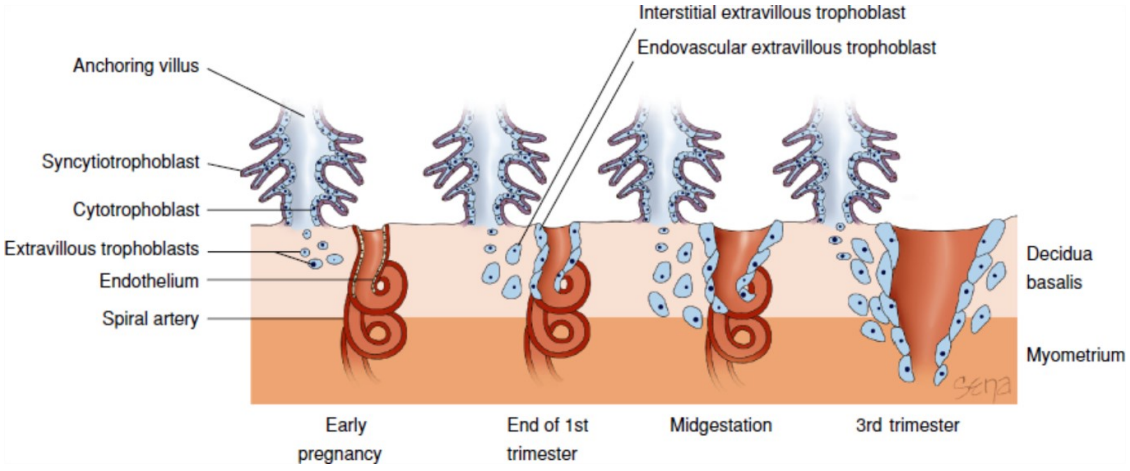

Figure 1-2: Early trophoblast invasion and Spiral Artery Remodelling.

The extravillous trophoblasts give rise to *interstitial trophoblasts*, which surround the maternal arteries, and *endovascular trophoblasts*, which invade and replace endothelium in maternal spiral arteries. As a result, a low resistance, wide capacitance vasculature develops throughout gestation. Reproduced with permission from McGraw-Hill Education: Cunningham F, *et al.*, *Williams Obstetrics*; copyright obtained Dec 24, 2015 [10].

The extravillous trophoblasts in early pregnancy are highly invasive, forming cell columns that extend from the endometrium into the inner third of the myometrium [10]. They secrete numerous proteolytic enzymes that digest extracellular matrix and activate proteinases in the endometrium to aid in the invasion of spiral arteries. The endovascular trophoblasts invade the spiral artery, destroy vascular endothelium, replace vascular smooth muscle with fibrinoid tissues, and regenerate endothelium within the spiral arteries [37]. As a result, the narrow, high resistance spiral arteries are remodelled into wide, low resistance, high capacitance arteries to facilitate the 20-fold increase in blood flow to the uterus, the placenta, and the fetus (Figure 1-2) [10].

1.2.2 Abnormal Placentation in Preeclampsia

In preeclampsia, the pathogenesis is thought to occur in two stages; the first stage of which consists of incomplete trophoblastic invasion. As a result of defective invasion, the cytotrophoblast cells fail to completely remodel the spiral arteries in the myometrium, resulting in a retained muscular tunica media layer, small caliber lumen, high resistance, and an altered responsiveness to vasoactive compounds (Figure 1-3) [11]. It has been shown that the mean external diameter of spiral arteries in placentas from preeclamptic women is only half of that seen in a placentas from healthy pregnancies [1]. In addition, the magnitude of defective trophoblastic invasion is thought to correlate with the severity of preeclampsia [38, 39]. As a result of the reduced lumen caliber in spiral arteries, there is a reduction in uterine blood flow and consequently placental hypoperfusion and hypoxia.

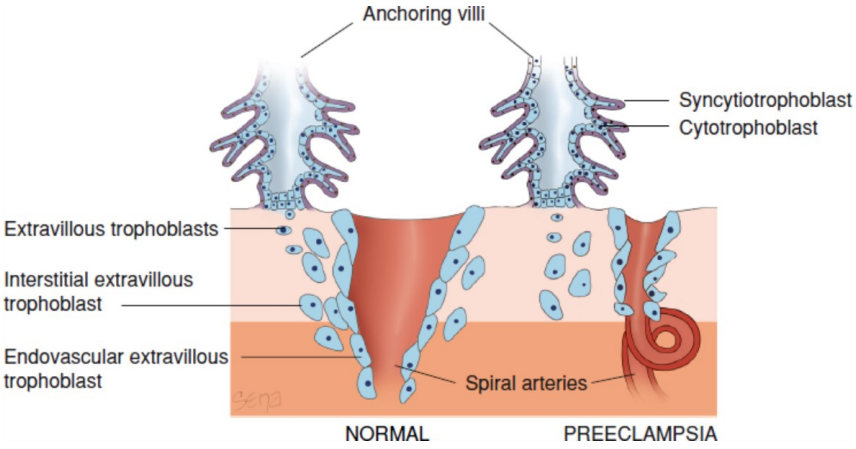

Figure 1-3: Incomplete trophoblast invasion in preeclampsia.

Defect trophoblast invasion of the maternal spiral arteries results in an incomplete replacement of the endothelium, retaining of muscular tunica media, and the development of high resistance, low capacitance arteries in preeclampsia. Reproduced with permission from McGraw-Hill Education: Cunningham F, *et al.*, *Williams Obstetrics*; copyright obtained Dec 24, 2015 [10].

In addition to a reduced arterial lumen and hypoperfusion, preeclampsia is also considered a state of "acute atherosis" of pregnancy. Previous studies examining arteries taken from preeclamptic placentas have shown that an increased accumulation of lipids is seen in the lumen, resulting in narrowing, atherosis, and infarcts of spiral arteries (Figure 1-4) [10]. The development of the lipid-laden vascular lesions that are seen in preeclampsia resemble the early stages of atherosclerosis, although they regress after delivery. In addition, many risk factors for metabolic syndrome are also risk factors for preeclampsia. For example, maternal weight is highly correlated with the risk of preeclampsia, increasing from 4.3 % in women with body mass index (BMI) < 20 kg/m^2 to 13.3% in those with BMI > 35 kg/m^2 [40]. Oxidized low density lipoprotein (oxLDL), which contributes to atherosclerotic plaque formation by binding scavenger receptors and activating foam cells, is also elevated in the vasculature in women with preeclampsia [41]. All in all, abnormal lipid metabolism is suggested to play a role in the

development of preeclampsia and the reduced spiral arteriolar lumen due to acute atherosis likely further worsens impaired placental blood flow and creates a relative local hypoxia at the placental interface.

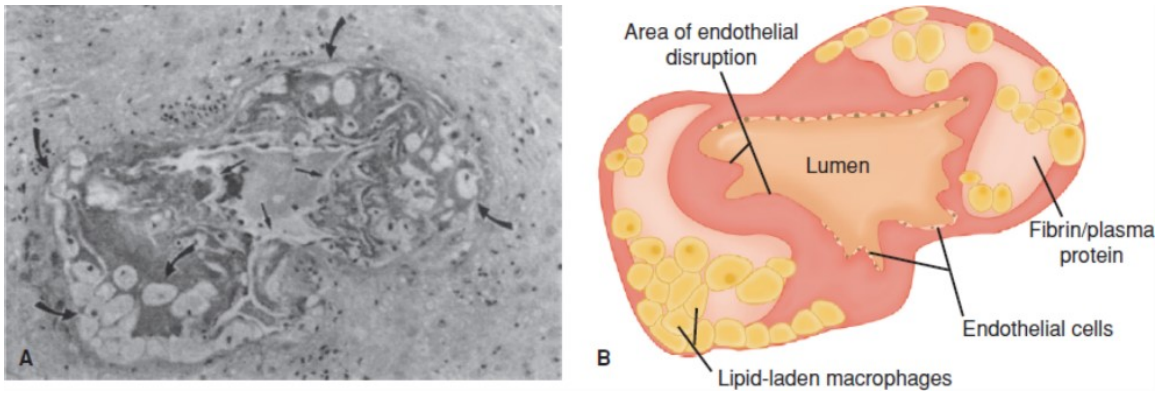

Figure 1-4: "Acute atherosis" of pregnancy in the placental bed from preeclampsia.

(A) Electron micrograph shows an accumulation of subendothelial lipids, plasma proteins, and foamy macrophages, resulting in a reduced arterial lumen and disrupted endothelium in a preeclamptic placenta. **(B)** A schematic diagram of A. Reproduced with permission from McGraw-Hill Education: Cunningham F, *et al.*, *Williams Obstetrics*; copyright obtained Dec 24, 2015 [11].

1.2.3 Consequences of Abnormal Placentation in Preeclampsia

The persistence of narrow-lumen, high-resistance spiral arteries leads to chronic placental hypoperfusion, which creates local hypoxia and oxidative stress and triggers the release of circulating factors into the maternal blood stream. In combination with a maternal predisposition due to genetic and/or environmental factors, these circulating factors induce endothelial dysfunction. As a result, diseased vessels exhibit increased contractility and result in increased vascular resistance which manifests as maternal hypertension, end organ damage, and abnormal

uterine blood flow. Ultimately, this mechanism feeds forward into a vicious cycle of worsening placental hypoperfusion and accentuates the development of preeclampsia (Figure 1-5).

Placental hypoperfusion, therefore, appears to be both a cause and a consequence of abnormal placentation. This is supported by studies that show that preeclampsia is more prevalent among women who live at high altitudes and who are chronically exposed to a relatively hypoxic environment compared to women living at sea level [42]. Women with vascular insufficiency, such as hypertension, diabetes, and systemic lupus erythematosus, are also at increased risk of preeclampsia, suggesting that maternal endothelial function is important in establishing normal placental implantation [2]. In addition, preeclampsia is

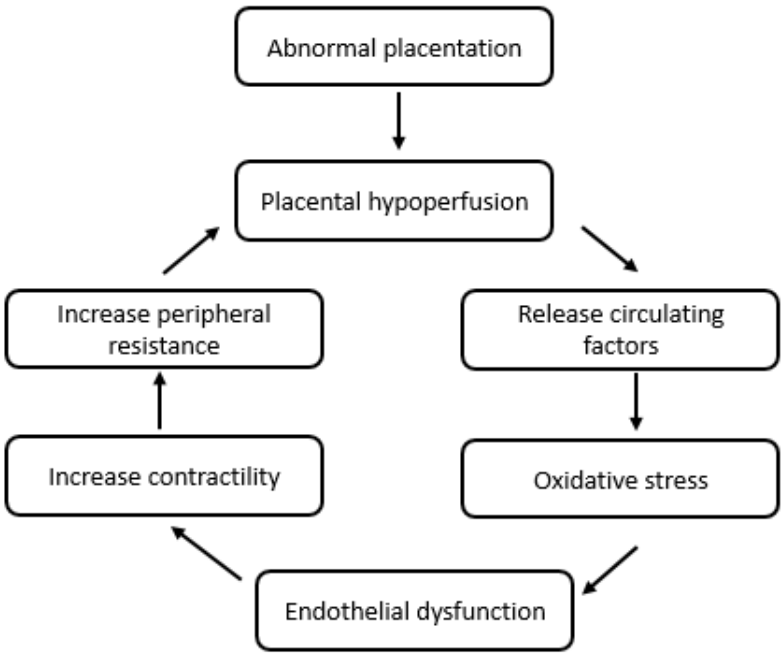

Figure 1-5: Positive feedback mechanism of the pathogenesis in preeclampsia.
Placental hypoperfusion as a result of incomplete trophoblast invasion triggers the release of circulating factors into maternal circulation. Not only does it cause local oxidative stress, it also induces systemic endothelial dysfunction. The diseased vessels, as a result, develop increased contractility and peripheral resistance leading to maternal hypertension and abnormal uterine blood flow. This further worsens placental hypoperfusion and feeds forward into a vicious cycle.

associated with conditions where an increased placental mass is not met by an increased placental blood flow resulting in relative ischemia, such as in twin gestation and hydatidiform molar pregnancy [43]. Similarly, in animal models, mechanically reduced uterine perfusion pressure (RUPP) in rats induces progressive placental ischemia as well as maternal signs of oxidative stress, inflammation, hypertension, and proteinuria – which are all hallmarks of preeclampsia [44]. Hence, placental hypoperfusion is strongly implicated in the pathogenesis of preeclampsia.

1.3 Circulating Factors

The second stage in the pathogenesis of preeclampsia is attributed to systemic inflammation and endothelial dysfunction as a result of the release of circulating factors triggered by diminished perfusion and a hypoxic environment near placentation. Interestingly, preeclampsia was initially termed "toxemia" at the end of the 19th century when the disease was first discovered when the ability to clinically measure blood pressure arose [1]. It was initially thought that a dysfunctional placenta could release "toxins" into the maternal circulation which "poison" the mother and trigger the signs and symptoms of preeclampsia (Taylor and Roberts, 1989). Interestingly enough, this theory remains the mainstream dogma for its pathogenesis today and the knowledge of identifying potential contributing factors has advanced significantly in the past few decades. The current dogma supports that the lack of remodelling of spiral arteries and high resistance flow in the placenta contribute to local hypoperfusion which triggers the release of circulating factors such as anti-angiogenic factors, cytokines, and other compounds into the maternal vasculature (Figure 1-6). These circulating factors then elicit maternal

inflammation and endothelial damage, leading to the syndrome of hypertension, vascular permeability, and end-organ damage.

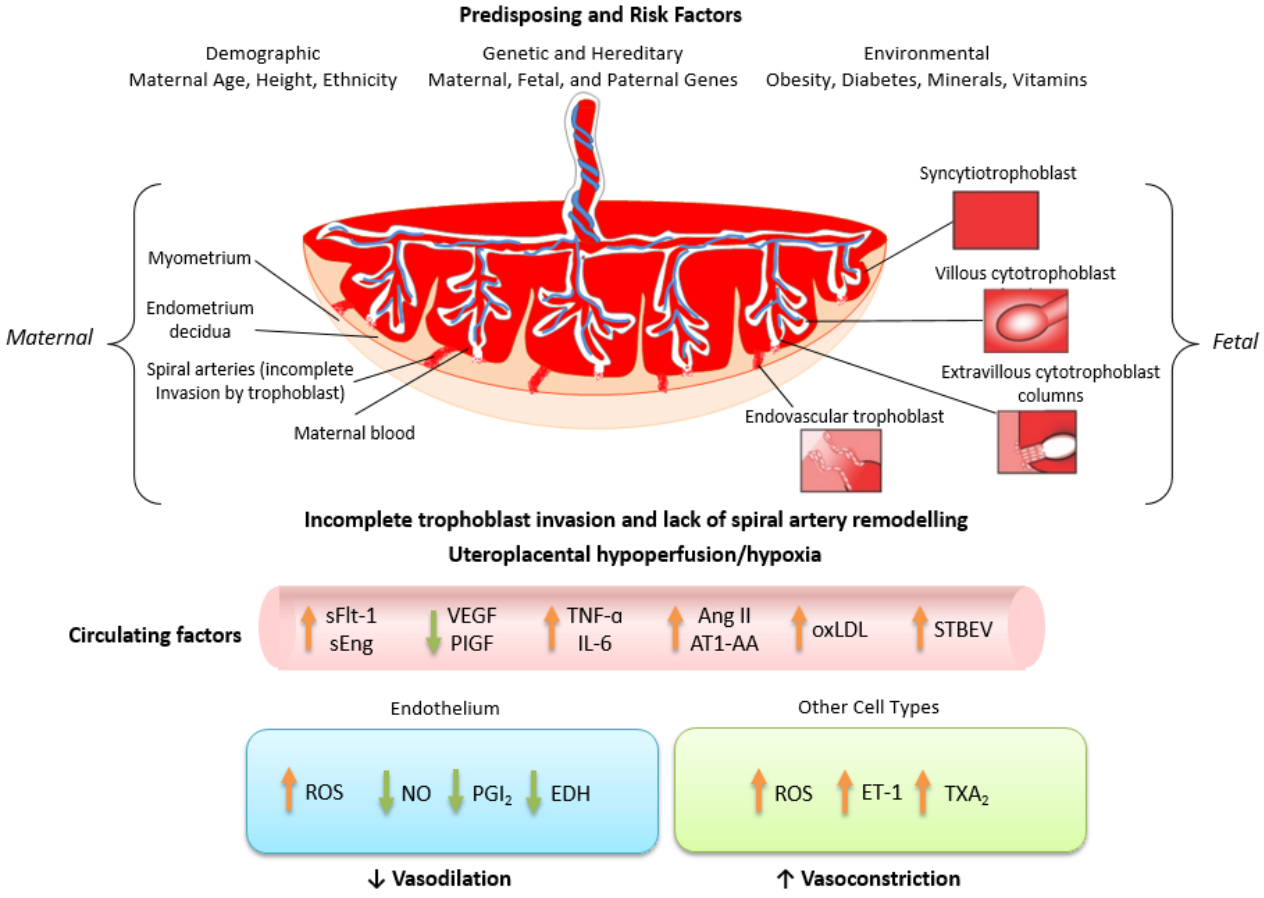

Figure 1-6: Overview of pathogenesis in preeclampsia.

Defective trophoblast invasion results in a lack of spiral artery remodelling and subsequent placental hypoperfusion and hypoxia. This triggers the release of myriad circulating factors into the maternal circulation, which affect the endothelium by increasing oxidative stress and altering the balance between vasodilators and vasoconstrictors. Modified from Shah, DA (2015) *Biochemical Pharmacology*;95:211 and Frost, JM (2010) PLoS Genet;6:e1001015.

Abbreviation: sFlt-1, soluble fms-like tyrosine kinase-1; sEng, soluble endoglin; VEGF, vascular endothelial growth factor; PIGF, placental growth factor; TNF-α, tumor necrosis factor alpha; IL-6, interleukin-6; Ang II, angiotensin II; AT1-AA, angiotensin receptor-1 autoantibodies; oxLDL, oxidized low density lipoprotein; STBEV, syncytiotrophoblast extracellular vesicles; ROS, reactive oxygen species; NO, nitric oxide; PGI_2, prostacyclin; EDH, endothelial derived hyperpolarization; ET-1, endothelin-1; TXA_2, thromboxane.

Although the exact causative factor of preeclampsia remains unknown, and it is unlikely that one single factor will be identified as the culprit responsible for the entire pathogenesis due to the heterogeneity of the disease, many studies have demonstrated that circulating factors released from the placenta are an important link to endothelial dysfunction in the maternal vascular system. Previous literature has shown that exposure of myometrial arteries from healthy pregnant women to plasma from preeclamptic women results in impaired endothelium-dependent vasodilation [45-47]. Similarly, arteries from pregnant rats exposed to plasma from RUPP animal model of preeclampsia exhibit impaired vasodilation [48], suggesting circulating factors released as a result of placental hypoperfusion are directly associated with endothelial dysfunction. Circulating factors in preeclampsia have also been shown to increase oxidative stress and activation of human umbilical vein endothelial cells (HUVECs) [41, 49]. However, the specific mechanism(s) by which circulating factors lead to endothelial dysfunction in preeclampsia remains unclear.

As previously eluded to, there has not been a single factor identified as the exact etiology of preeclampsia. In reality, the spectrum of the disease is heterogeneous and the development of preeclampsia is likely multi-factorial. Therefore, there are myriad circulating factors implicated in the pathogenesis of preeclampsia, including anti-angiogenic factors, cytokines, and other placental released factors, which will be briefly summarized in the following sections.

1.3.1 Anti-Angiogenic Factors

The human placenta undergoes extensive angiogenesis and vasculogenesis throughout its development. *Angiogenesis* is the process of neovascular branching from pre-existing blood

vessels and *vasculogenesis* is the process of blood vessel generation *de novo* from angioblast precursor cells, both of which are extremely important for normal placenta development [50].

In preeclampsia, there is an excess production of anti-angiogenic factors – soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) – both of which have been shown to contribute to the maternal syndrome of preeclampsia [27]. Placentas from preeclamptic pregnancies have increased expression of sFlt-1 and sEng and their circulating levels are also increased in the serum of preeclamptic women weeks before the onset of clinical disease [51]. The elevated circulating levels of sFlt-1 and sEng have also been shown to correlate with the severity of preeclampsia [51]. Interestingly, sFlt-1 overexpression in pregnant rodents induces hypertension, proteinuria, and glomerular changes in the kidneys, which are all hallmarks of preeclampsia [52]. Further, addition of sEng in this animal model induces a more severe phenotype, including cerebral edema, HELLP syndrome, and fetal growth restriction [53, 54]. Soluble Endoglin also interferes with endothelial nitric oxide synthase (eNOS) activation and impairs endothelial tube formation in HUVECs, which contributes to endothelial dysfunction [53].

Placental growth factors, such as PIGF and vascular endothelial growth factor (VEGF), are important for placental angiogenesis and endothelial health. In preeclampsia, an increase in circulating sFlt-1 binds PIGF and VEGF, which prevents them from interacting with membrane receptors on the endothelium [27]. Maynard *et al.* have demonstrated that increased circulating sFlt-1 in preeclamptic women is associated with decreased circulating levels of free PIGF and VEGF, which is associated with impaired angiogenesis *in vitro* that can be rescued by exogenous PIGF and VEGF [52]. PIGF has also been shown to increase uterine arterial vasodilation, promote endothelial cell growth and vasculogenesis in the placenta [55]. In addition, VEGF

increases intracellular calcium which stimulates eNOS activity in endothelial cells resulting in a decreased vascular tone and increased angiogenesis [55]. *In vitro* studies have also shown that treatment with VEGF inhibitors results in a loss of endothelial fenestrations, arterial patency, and blood flow in tumor cells [56]. Furthermore, VEGF mediates endothelium-dependent vasodilation via stimulation of nitric oxide (NO) formation and specific inhibition of VEGF receptor results in a rapid and sustained increase in blood pressure in healthy mice [57, 58]. Therefore, convincing evidence supports that the presence of elevated sFlt-1 and sEng decreases circulating levels of free PIGF and VEGF which contributes to a depression of the angiogenesis necessary for the growth of the placenta and endothelial function in pregnancy.

Clinically, an increase in sFlt-1 and a decrease of free PIGF and VEGF have been shown to precede the onset of the clinical signs and symptoms of preeclampsia [52, 59, 60]; therefore, the alteration in their serum levels may be a useful diagnostic or prognostic biomarker to predict the onset of preeclampsia. Indeed, Levine *et al.* have shown that the level of sFlt-1 is increased approximately five weeks prior to the onset of preeclampsia while the level of free PIGF is decreased at the beginning of the second trimester in women who later develop preeclampsia [59]. Furthermore, the magnitude of changes in the increasing levels of sFlt-1 and decreasing levels of free PIGF is also positively correlated with early onset preeclampsia and with preeclamptic women who have a small-for-gestational-age infant [59]. In addition, a high plasma sFlt-1/PIGF ratio has been shown to correlate with adverse maternal and perinatal outcomes in women at risk of preeclampsia presenting at < 34 weeks and correlates strongly with the duration of pregnancy [61]. In addition, in those presenting < 34 weeks of gestation, a low plasma PIGF/sFlt-1 ratio identifies patients who deliver within two weeks due to preeclampsia [62].

Therefore, alterations in (anti-)angiogenic factors may prove to be a diagnostic strategy; however, further clinical studies are required.

1.3.2 Inflammatory Responses and Circulating Cytokines

Normal pregnancy consists of a mild state of inflammation, which is evident beginning in the luteal phase of the menstrual cycle proceeding implantation and continues throughout pregnancy [63]. This mild increase in inflammation in normal pregnancy is indicative of a non-specific acute phase response, such as an increase in plasma fibrinogen, ceruloplasmin, and reduced plasma albumin [64-66]. In normal pregnancy, an increase in white blood cells, or leukocytosis, is seen throughout pregnancy, as well as increased activation of neutrophils and monocytes [67, 68]. Pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factors-alpha (TNF-α), are both also increased in normal pregnancy [69, 70].

In preeclampsia, this inflammation is further heightened. Several markers of inflammation are shown to be increased in preeclamptic pregnancy relative to normal pregnancy, including an increase in leukocytosis, leukocyte activation, complement activation, clotting factors, platelet activation, and pro-inflammatory cytokines such as IL-6 and TNF-α [63]. In preeclamptic placenta, an increased production of IL-6 shifts the differentiation of monocytes into macrophages, which increase the production of TNF-α resulting in a reduction of trophoblast invasion [71]. Interleukin-6 is also a pro-inflammatory cytokine which increases angiotensin receptor-1 autoantibodies (AT1-AAs; discussed in the next section) and infusion of IL-6 causes hypertension and proteinuria in healthy pregnant rats [72]. Plasma levels of TNF-α are also increased in preeclampsia by two-fold due to placental ischemia and local hypoxia [55, 73]. Furthermore, it has been shown that TNF-α modifies the expression of adhesion molecules in placental vessels, increasing the apoptosis of trophoblasts and resulting in a reduction in

trophoblast invasion [74, 75]. Other studies have also demonstrated that TNF-α may also stimulate placental production of sFlt-1 and sEng [73] and increase endothelin-1 (ET-1) levels causing hypertension in a rat model [76]. Etanercept, a TNF-α inhibitor, has been shown to decrease blood pressure, increase eNOS expression, and decrease vasoconstrictor endothelin-1 (ET-1) levels in a RUPP rat model of preeclampsia [76, 77], demonstrating the potential of cytokines as therapeutic targets in preeclampsia.

1.3.3 Angiotensin II and Angiotensin-1 Receptor Autoantibodies

In the vasculature, angiotensin II (Ang II) is an important regulator of electrolyte balance and vascular tone. In pregnancy, in addition to its production from angiotensin I by angiotensin converting enzyme (ACE) in the liver, about 40% of Ang II is produced locally in the placenta by chymase, which is a non-ACE serine protease mainly found in the syncytiotrophoblasts of placenta [55]. Physiologically, Ang II activates angiotensin type 1 receptor (AT_1R) which increases intracellular calcium levels in vascular smooth muscle cells (VSMCs), promotes vasoconstriction, and enhances inflammation [55]. Ang II also activates the endothelial angiotensin type 2 receptor (AT_2R) which activates eNOS, increases production of NO and prostacyclin (PGI_2), and opposes Ang II-induced vasoconstriction by the AT_1R [55]. In normal pregnancy, although there is an increase in plasma levels of renin (which converts angiotensinogen to angiotensin I, the precursor for Ang II) and Ang II, the vascular response to Ang II is decreased due to a down-regulation of AT_1R expression [55].

However, in preeclampsia, the AT_1R forms a heterodimer with the bradykinin B2 receptor and exhibits increased responsiveness to Ang II which contributes to increased peripheral resistance and the development of hypertension [55]. In animal models, it has also been shown that hypoxia induces a rapid increase in AT_1R expression and plasma Ang II levels

[78]. In preeclamptic placentas, there is an increase in Ang II levels, renin levels, and ACE mRNA levels when compared to placentas from normal pregnancy [79]. The concentration of Ang II, angiotensinogen, and AT_1R expression are also increased in chorionic villi from preeclamptic placenta, which may promote uteroplacental dysfunction via vasoconstriction [80].

In addition to enhanced expression of the AT_1R, there is also evidence that stimulatory autoantibodies to AT_1R (AT1-AA) are elevated in preeclampsia. Indeed, the levels of AT1-AA are elevated in 70-95% of women with preeclampsia and correlate with severity of the disease [81]. Siddiqui *et al.* have also shown that injection of immunoglobulins from women with preeclampsia induces hypertension and proteinuria in pregnant mice, which is prevented by a peptide that blocks antibody-mediated AT_1R activation [82]. Similarly, Zhou *et al.* have shown that injection of AT1-AAs from preeclamptic women into pregnant mice induces hypertension, proteinuria, glomerular changes in the kidneys, and abnormal placentation, which are all characteristics of preeclampsia [83]. In addition, binding of AT1-AAs to the AT_1R induces the production of sFlt-1 and sEng by human villous explants and the injection of AT1-AA into healthy rats induces an increase in sFlt-1 in a pregnancy-dependent manner [82, 84]. Other studies have also shown that AT1-AA reduces placental trophoblast invasion, increases placental reactive oxygen species (ROS) production, and increases blood pressure in animal models [85]. Therefore, there is strong evidence that an enhanced production of Ang II and AT1-AA is associated with the pathophysiology of preeclampsia.

1.3.4 Circulating Lipids and Lipoproteins

As previously eluded to, preeclampsia is associated with many risk factors of cardiovascular disease, including hyperlipidemia and metabolic syndrome. Indeed, the risk of preeclampsia is correlated with elevated maternal weight gain, BMI, and serum triglyceride

levels [21, 86]. It has also been shown that increased placenta oxidative stress in preeclampsia increases the production of oxidized lipids, such as oxidized low-density lipoprotein (oxLDL) [1]. Complicated pregnancies, such as preeclampsia and IUGR, have been associated with a significantly increase in circulating levels of oxLDL [87, 88]. Previous studies have also demonstrated that oxLDL can activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase leading to increased superoxide production via activation of the lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) [89, 90]. More importantly, Sankaralingam *et al.* have shown that oxLDL and LOX-1 expression are increased in women with preeclampsia, which is associated with an increase in NADPH oxidase activation and superoxide and peroxynitrite production [41]. Moreover, English *et al.* have shown that the impaired vasodilation in arteries treated with preeclamptic plasma is exacerbated by the exposure to oxLDL and prevented by incubation with the LOX-1 blocking antibody [91]. Thus, oxLDL, and more specifically LOX-1 activation, may play a role in the pathogenesis of preeclampsia contributing to systemic endothelial dysfunction in the maternal vasculature.

1.3.5 Placenta derived cell fragments

Another distinct population of placenta-released circulating factors is microparticles, which are cell fragments released from the cell membrane during cell activation and apoptosis. Microparticles are defined by size ranging from 100 to 1000 nm, but they can have a heterogeneous composition in terms of phospholipid and protein components depending on their origin [92]. Although first described as “dust” or cell debris in 1967 [93], microparticles are now recognized to play a role in cell-to-cell communication by displaying and presenting bioactive substances such as cytokines, signalling proteins, mRNA, and microRNA to target cells [94].

Microparticles are derived from endothelial cells, erythrocytes, platelets, leukocytes, and syncytiotrophoblast cells, although the majority of circulating microparticles are derived from platelets [93, 95]. While microparticles are found in the blood stream under normal physiological conditions, they are also found to be elevated in pathological conditions such as thrombosis, inflammation, and vascular dysfunction [92, 94]. Given that preeclampsia is recognized as a state of inflammation and endothelial dysfunction, it is unsurprising that microparticles are elevated in women with preeclampsia. Previous studies have shown that endothelium-, leukocyte-, platelet-, and syncytiotrophoblast-derived microparticles are all significantly increased in preeclampsia [96].

It is now recognized that the detachment of syncytial fragments from the placenta results in a release of a heterogeneous population of fragments and vesicles that are metabolically active and capable of transporting signals in the maternal circulation during pregnancy [27]. In the presence of placental hypoperfusion and hypoxia, syncytiotrophoblasts are activated and destabilized resulting in an increased release of microparticles containing oxidized lipids (Figure 1-7) [97]. However, instead of collectively referring to them as "microparticles" which by definition include particle sizes ranging from 100-1000 nm, the most recent literature suggests using a more accurate term of *extracellular vesicle*, which allows the inclusion of smaller bioactive exosomes ranging in size from 50-100 nm as well as the previously identified microparticles (personal communication with Dr. I. Sargent, University of Oxford). Therefore, for the rest of the thesis, I will refer to the placenta-released cell fragments as syncytiotrophoblast extracellular vesicles (STBEVs).

Syncytiotrophoblast extracellular vesicles are of particular interest because they have been shown to interact with both immune and endothelial cells which contribute to the systemic

inflammation and endothelial dysfunction seen in preeclamptic pregnancy [96]. Not only are STBEVs increased in the plasma from women with preeclampsia, but the composition and size of STBEVs are also found to be different from those with normal pregnancy [98], suggesting that

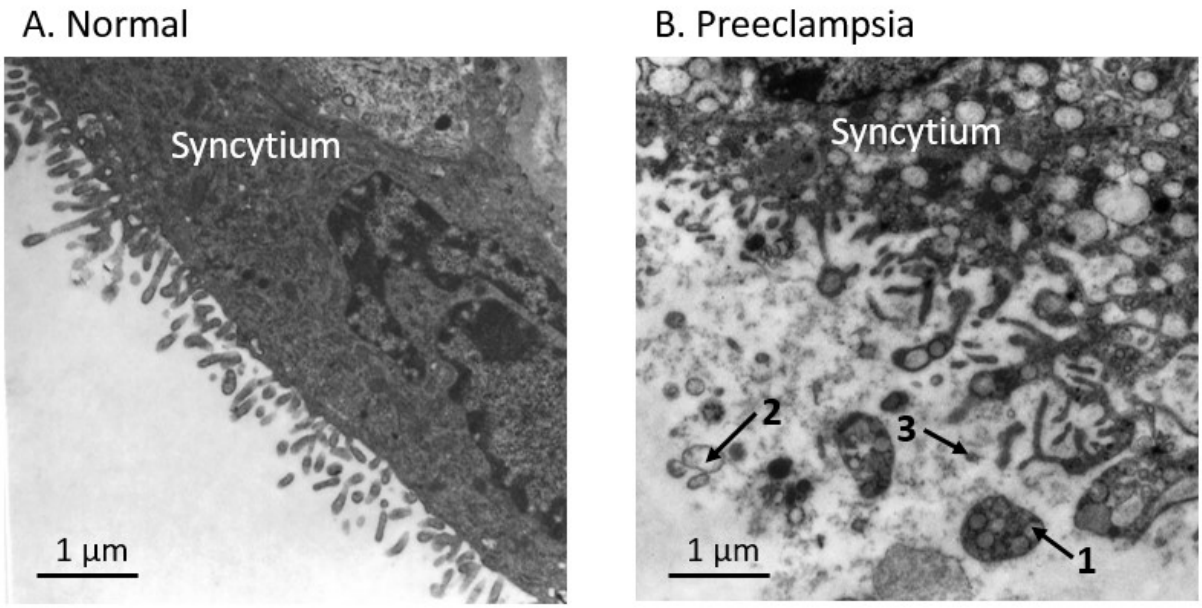

Figure 1-7: Electron micrograph of the syncytial surface of placenta.

(A) Placenta from healthy term pregnancy showing a regular microvilli arrangement on the apical surface. **(B)** Placenta from a severe preeclamptic patient delivered at 35 weeks of gestation due to uncontrolled hypertension and proteinuria demonstrates a loss of apical surface integrity, distorted microvilli, and shedding of placental debris of various sizes. 1: apoptotic bodies (1 µm); 2: microvesicles (100 nm – 1 µm); 3: exosome (70 – 120 nm). Reprinted with permission from Dr. Carolyn Jones, University of Manchester [97].

it is a unique entity that may be of diagnostic value or therapeutic target in preeclampsia. A number of *in vitro* studies have shown that STBEVs disrupt monolayer cell architecture, suppress proliferation, and increase apoptosis in HUVECs [99-101]. One of the first papers investigating the effect of STBEVs on endothelial function has shown that a 2-hour perfusion of human omental arteries with STBEVs significantly reduces acetylcholine (ACh)-induced vasodilation [71]. Although this finding has not been universal [102], others have demonstrated

similar findings using preeclamptic microparticles. Microparticles isolated from women with preeclampsia have been shown to impair endothelial function in human myometrial arteries [103] and generate vascular wall inflammation and blunt vascular contractility in mice aortas and mesenteric arteries [104, 105]. These latter studies have also shown that microparticles from preeclamptic women stimulate an increase in vasoconstrictor prostanoids and NO participation since the inhibition of PGHS-2 and NOS significantly reduced contractility [104, 105]. Moreover, increased STBEV levels have been found in women with early onset preeclampsia, but not in cases of normotensive IUGR, suggesting that although both pregnancy complications involve abnormal placentation, STBEVs may be unique to preeclampsia [106]. However, the specific mechanism(s) behind how STBEVs impair endothelial function remains unclear; thus, we are interested in investigating if and how STBEVs interact with the maternal vasculature.

1.4 The Endothelium

As previously discussed, preeclampsia is believed to be triggered by incomplete trophoblast invasion into the maternal spiral arterioles, resulting in placental hypoperfusion and local hypoxia. Consequently, a myriad of circulating factors are released in response into the maternal circulation and interact with the endothelium. In women predisposed to vascular dysfunction, this triggers several downstream effects and eventually the onset of preeclampsia.

Systemic endothelial dysfunction plays a central role in the pathophysiology of preeclampsia. Interestingly, the clinical symptoms of preeclampsia can be explained as a result of endothelial dysfunction and vascular permeability depending on the organ involved. For example, dysregulation of vascular tone results in hypertension while increased vascular permeability leads to generalized edema as a result of the extravasation of intravascular fluid into the interstitial tissues [40]. In addition, endothelial dysfunction in the central nervous system can

result in headaches, scotoma, seizures and stroke. Similarly, vascular dysfunction in the liver leads to right upper quadrant pain and elevated liver enzymes and in the kidney causes proteinuria and renal failure. Therefore, understanding how the endothelium is affected in the development of preeclampsia is crucial.

Vascular tone is tightly controlled by a balance of vasodilators and vasoconstrictors. In preeclampsia, it is believed that a decrease in the bioavailability of vasodilators and an increase in vasoconstrictors contribute to the imbalance in vascular control, which results in hypertension and abnormal blood flow to the end organs, such as the uterus, the kidneys, and the liver. In the endothelium, two of the most important vasodilators are NO and PGI_2, which will be further discussed in the following sections. Major vasoconstrictor pathways include TXA_2, ET-1, and Ang II. Thromboxane is a prostaglandin H synthase (PGHS) dependent vasoconstrictor which will be discussed in detail in the next section. Endothelin-1 is a potent peptidergic vasoconstrictor which activates the ET_A-receptor, but can also act as a vasodilator through activation of ET_B-receptor [107]. Overexpression of ET-1 is observed in women with preeclampsia. This is thought to contribute to increased peripheral resistance and maternal hypertension and decreased placental flow resulting in placental hypoperfusion and IUGR [107]. In addition, as previously discussed, there is an elevated levels of Ang II and AT1-AA which contribute to the endothelial dysfunction and hypertension observed in women with preeclampsia; therefore, an upregulation of the renin-angiotensin system is also implicated in the pathogenesis of preeclampsia. However, for the purpose of the thesis, we will focus on the NO pathways and the PGHS-dependent vasodilators and vasoconstrictors.

1.4.1 Nitric Oxide Pathway

One of the most important and potent vasodilators in the vasculature is NO, which is produced by nitric oxide synthase (NOS) from L-arginine. Nitric oxide diffuses into VSMCs and converts guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP) by activating soluble guanylyl cyclase (sGC) which results in vasodilation [108, 109]. Nitric oxide can also bind to oxygen-derived free radicals, such as superoxide, to reduce oxidative stress [108]. In addition, NO is especially important in the uterine artery vasculature as it regulates local tone to increase blood flow to the growing uterus, placenta, and fetus during pregnancy [110]. During normal pregnancy, increased NOS expression/activity in human uterine arteries increases NO levels [111]. Cyclic GMP levels are also increased in the plasma and urine of pregnant women, particularly during the first trimester [112]. Previous studies have shown that NO increases VEGF and PIGF and decreases sFlt-1 in hypoxic human trophoblast cells [113]. Pan NOS inhibition in pregnant rats further produces high blood pressure, proteinuria, thrombocytopenia, and IUGR [114]. However, when in excess, NO is also known as a "double-edged sword" as it can bind to superoxide to produce peroxynitrite (ONOO-) which leads to lipid peroxidation, cell apoptosis, and vasoconstriction [108]. Therefore, a precise balance of NO production is necessary to maintain appropriate vascular tone and function.

The majority of NO production in the vasculature is associated with eNOS, arguably the most important isoform of NOS in pregnancy. Previous studies have shown that both the stable metabolites of NO and eNOS expression are increased in healthy pregnancy [110]. However, studies comparing NO level and eNOS expression in preeclamptic women have shown conflicting results. Serum NO levels have been shown to decrease in women with preeclampsia [115], while eNOS expression has been found to be increased in placentas [116] and maternal

vasculature from preeclamptic pregnancies [117]. Interestingly, Bhavina *et al.* (2014) have found a decrease in eNOS expression in the fetal end of the umbilical cord from women with gestational hypertension (new onset hypertension after 20 weeks of gestation without proteinuria or other complications) and mild preeclampsia (new onset hypertension after 20 weeks of gestation with 1+ proteinuria), but an increase in eNOS expression from those with severe preeclampsia (new onset hypertension after 20 weeks of gestation with 3+ proteinuria). Therefore, we are interested in examining the functional contribution of eNOS to endothelial vasodilation and the effect of circulating factors on eNOS expression in pregnant uterine arteries.

In addition to the mild increase in oxidative stress, normal pregnancy itself is also considered a mild inflammatory state, which becomes apparent before implantation and develops as pregnancy progresses [63]. As previously eluded to, a similar inflammatory response occurs in preeclampsia but with greater intensity and heightened inflammation can stimulate an upregulation of inducible NOS (iNOS). Inducible NOS is known to contribute to excessive NO production, subsequent ONOO- formation, and endothelial dysfunction in pathological conditions, such as hypertension and cardiovascular disease [118]; however, its role in preeclampsia is still controversial. Conflicting studies have shown an increase in iNOS mRNA levels and iNOS protein expression in placentas from women with gestational hypertension and preeclampsia [119, 120] and increase in iNOS protein expression in HUVECs after exposure to serum from preeclamptic women [115]. In animal models of preeclampsia, the level of iNOS expression has also been shown to increase and the inhibition of iNOS attenuates hypertension and associated oxidative stress [121]. However, some studies have demonstrated no change in iNOS expression in platelets from preeclamptic women compared to those from healthy pregnant

women [122]. Thus, investigating how circulating factors interact with iNOS contribution to endothelial vasodilation would further contribute to understanding of its role in preeclampsia.

Lastly, neuronal NOS (nNOS) is a third isoform of NOS primarily expressed throughout the central and peripheral nervous systems. Neuronal NOS plays a role in blood pressure control by regulating the production of NO in the brain, autonomic inhibitory nerves and the heart [123, 124]. However, data on the role of nNOS in preeclampsia is very scarce. One previous study has shown that nNOS is expressed in vascular smooth muscle cells of the human umbilical veins, and is significantly down-regulated in umbilical cords obtained from preeclamptic pregnancy compared to those from normal pregnancy [125]. It would be interesting to examine how and if circulating factors in preeclampsia affect the expression of nNOS in the vasculature.

1.4.2 Prostaglandins Pathway

Derived from arachidonic acid by PGHS, which is also known as cyclooxygenase (COX), prostanoids are another important source of vasodilators in the maternal vasculature. Endothelium-derived PGI_2 are potent vasodilators and anti-platelet that contribute to reduced vascular resistance in pregnancy and are found in abundance in the endothelium of large blood vessels and microvascular vessels [126]. The production of PGI_2 has been shown to increase locally in the uterine circulation during pregnancy [126], as well as in the maternal circulation since urinary excretion of its major metabolites, 6-keto-prostaglandin F1α (PGF1α), is increased during early pregnancy and remains elevated throughout gestation [127]. On the other hand, PGHS-dependent vasoconstrictors, TXA_2 and its immediate precursor endoperoxide (PGH_2), are also important in the vasculature [128]. Although TXA_2 has a short half-life in the maternal circulation, measurement of its stable metabolites reveals that it is also increased 3- to 5-fold during pregnancy and remains elevated throughout gestation [128]. Furthermore, activation of

PGHS contributes to the production of superoxide by activating NADPH oxidase [129], which results in an increase in oxidative stress. Therefore, the activity of PGHS-dependent vasoconstriction is intricately associated with the NO pathway and ROS system as superoxide can initiate lipid membrane peroxidation as well as react with NO to produce ONOO- and thus reduce NO bioavailability.

Interestingly, although an increase in PGI_2 biosynthesis is observed in preeclampsia, the magnitude of increase is compromised when compared to the increase in PGI_2 production seen in healthy pregnancy [127, 130]. There is evidence that hypoxia downregulates PGHS which decreases endothelium-derived PGI_2 [55] and preeclamptic women have reduced levels of PGF1α, the main metabolite of PGI_2, when compared with normal pregnancy [131]. Thromboxane production is also significantly increased as evidenced by an increase in excretion of urinary metabolites and an increase in biosynthesis in trophoblast cells in placental tissue from women with preeclampsia [132-134]. Furthermore, there is also evidence of increased platelet activation and TXA_2 production in severe preeclampsia [55]. Taken altogether, a relatively compromised elevation in PGI_2 production combined with a significant increase in TXA_2 levels may contribute to the increase in TXA_2/PGI_2 ratio that has been consistently shown in preeclamptic women [135]. Furthermore, both the expression and activity of PGHS have been shown to be enhanced in placental trophoblasts from women with preeclampsia. Activation of PGHS may contribute to superoxide production, which in turns increases ONOO- formation and lipid peroxidation, leading to endothelial dysfunction seen in preeclampsia [129]. Therefore, we are interested in examining how circulating factors affect the balance of PGHS-dependent vasodilators and vasoconstrictors in the pathogenesis of preeclampsia.

1.4.3 Reactive Oxygen Species

As previously discussed, a major component in the control of vascular tone involves the regulation of oxidative stress and the interaction between vasodilators and vasoconstrictors with ROS. In normal pregnancy, there is evidence of a mild increase in oxidative stress and a mild decrease in antioxidants [136-138]. However, as mentioned, a number of circulating factors induce a further increase in placental and endothelial cellular oxidative stress and an excess production of ROS is believed to be central to the pathogenesis of preeclampsia.

Reactive oxygen species, such as superoxide, hydrogen peroxide (H_2O_2) and ONOO-, contain highly reactive oxygen radicals which are known to cause cellular damage. Endogenous antioxidants, such as superoxide dismutase (SOD) and catalase, break down superoxide and H_2O_2 into water and oxygen. In preeclampsia, there is an increase in ROS production and a decrease in antioxidant levels, which contribute to increased lipid peroxidation, endothelial cell damage, and vascular dysfunction [139]. Previous studies have shown that there is an increase in lipid peroxidation products and ROS, such as malondialdehyde, H_2O_2 and ONOO-, in erythrocytes from women with preeclampsia [140, 141]. There is also an associated reduction in the activity of antioxidants, such as SOD, catalase, and glutathione peroxidase in women with preeclampsia [140]. Furthermore, a prospective study has demonstrated that an increase in urinary excretion of ROS markers is correlated with the development of preeclampsia, shorter duration of gestation, and a lower birth weight [142].

An excess production of ROS damages the endoplasmic reticulum and triggers apoptosis in the syncytium [143]. This leads to a release of placental cell fragments entering the maternal circulation and stimulates the release of cytokines and free radicals further damaging the endothelium [144]. In preeclampsia, an increase of ROS has also been shown to contribute to the

degradation of polyunsaturated fatty acids in the cell membrane, causing alteration in membrane fluidity, permeability, and eventually cell damage [145, 146]. In addition, a number of factors that are increased in preeclampsia, such as TNF-α, Ang II, and oxidized lipoproteins, are also shown to increase the production of ROS in the uterine vasculature by activating NADPH oxidase and is reviewed elsewhere [147]. However, the functional consequence of how circulating factors affect endothelial function is still unknown and will be the focus of our studies.

1.4.4 Animal Models of Preeclampsia

Understanding the complex pathogenesis of preeclampsia requires several different experimental approaches, including various animal models. As previously mentioned, the RUPP model of preeclampsia is well studied in several types of animals, including rat, dog, rabbit, non-human primate, guinea pig, and sheep. Most commonly, mechanical reduction in uterine blood flow in rats can be accomplished in placing clips on the abdominal aorta and bilateral uterine arteries on day 14 of a 21-day-gestation pregnancy, which results in a 40-50% reduction in uterine blood flow [44]. Rat model of RUPP has been shown to demonstrate hypertension, decreased GFR, renal endotheliosis, fetal growth restriction and increase in biomarkers such as sFlt-1, sEng, TNF-α, IL-6, TXA2, ET-1, and AT1-AA, to name a few [44]. Another approach is the use of genetic modification of eNOS knock-out mice or chronic infusion of pan NOS inhibitor, L-NAME, in pregnant mice, both resulting in the hallmarks of hypertension, proteinuria, and endothelial dysfunction [148]. A variety of therapeutic strategies to increase NO bioavailability also exist, including the administration of NO precursor (L-arginine), NO donor (glyceryl trinitrate), inhibition of endogenous NOS inhibitor (ADMA), and inhibition

phosphodiesterase which increases the clearance of cGMP (sildenafil) – all of which are still under studies as potential treatment for preeclampsia [148].

The infusion of various types of circulating factors implicated in preeclampsia further enhance the understanding of its pathophysiology. This includes the overexpression of sFlt-1 in rodents, resulting in the development of hypertension, proteinuria, and renal endotheliosis which is exacerbated by the addition of sEng [52, 53]. Chronic infusion of inflammatory cytokines such as TNF-α or endotoxin in pregnant rodents also lead to hypertension and proteinuria with preservation of fetal biometric growth, suggesting a role for inflammation as one of the inciting events in the pathogenesis [148]. Similarly, the injection of AT1-AAs from women with preeclampsia into pregnant mice also induces hypertension, proteinuria, and abnormal placentation [83]. These models allow for the advantage of studying of specific factor(s) in the maternal endothelium; however, the disadvantage of which is the preclusion of the heterogeneous "big picture" that is seen in preeclampsia.

1.5 Lectin-like Oxidized Low Density Lipoprotein Receptor-1 (LOX-1)

In search of a common pathway between circulating factors, generation of oxidative stress, endothelial dysfunction, and atherosis of pregnancy as seen in preeclampsia, we are interested in examining the possible role of the lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) as the potential point of convergence in the pathogenesis. Initially discovered as a receptor for oxLDL nearly 20 years ago, LOX-1 is now recognized as a multi-ligand scavenger receptor that has been implicated in a number of cardiovascular diseases with an underlying pathophysiology involving vascular inflammation, atherosclerosis, and endothelial dysfunction. Specifically, LOX-1 has been shown to be elevated in hypertension, hyperlipidemia, diabetes mellitus, atherosclerosis, and more recently in preeclampsia [41, 149].

In this section, we review the structure and function of LOX-1 as well as its role in preeclampsia and the potential clinical application.

1.5.1 Structure and Function of LOX-1

The LOX-1 receptor is a scavenger receptor found on the plasma membranes of macrophages, endothelial cells, and smooth muscle cells [150-152]. By definition, scavenger receptors constitute a large family of cell membrane proteins that are capable of binding multiple ligands and function by endocytosis and activation of signalling pathways that lead to the elimination of the modified substances [152]. A recent review in the classification and nomenclature of scavenger receptors is available elsewhere [152]. Briefly, LOX-1 is a transmembrane scavenger receptor with four domains: 1) cytosolic domain, 2) transmembrane domain, 3) neck domain, and 4) C-type lectin-like domain (Figure 1-8) [153]. Initially

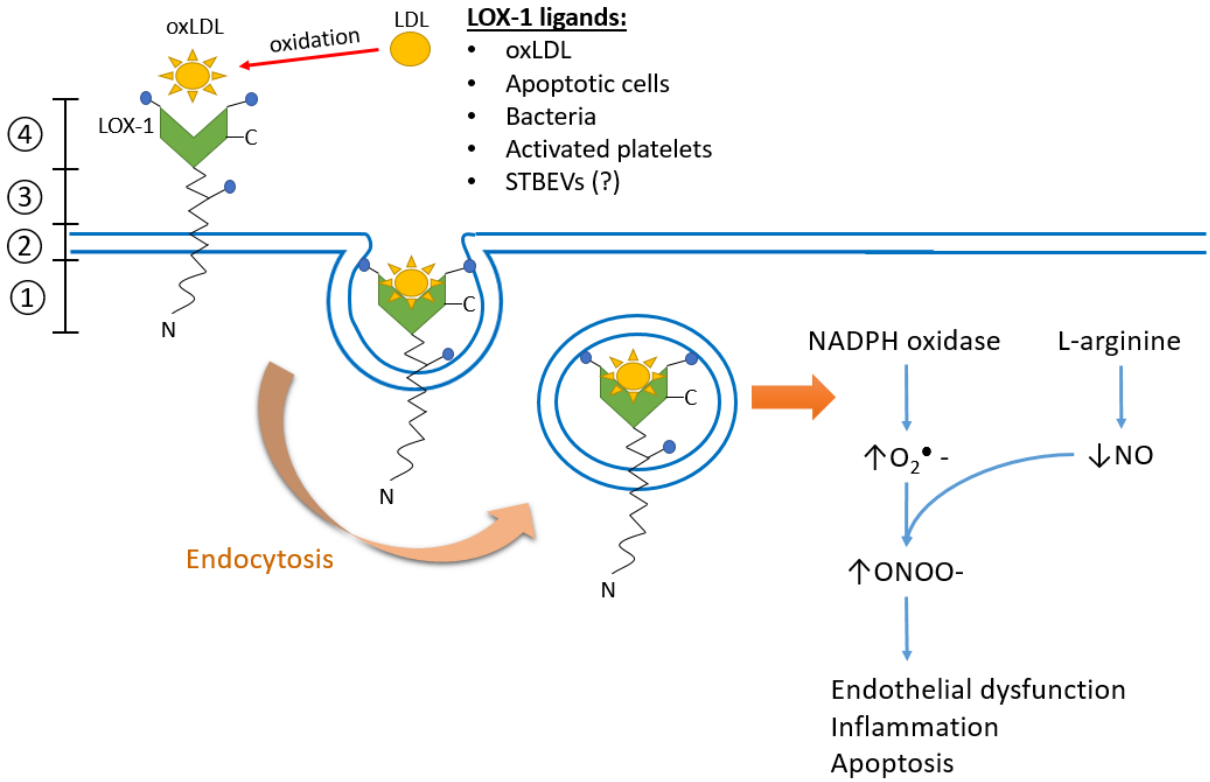

Figure 1-8: Activation of the LOX-1 receptor and downstream pathways.

The scavenger receptor, LOX-1, is a transmembrane protein consisted of four domains: (1) cytosolic domain, (2) transmembrane domain, (3) neck domain, and (4) C-type lectin-like domain. Multiple ligands can bind to LOX-1, which triggers endocytosis of the ligand-bound receptor and activates downstream pathways, including NADPH oxidase which increases superoxide production and decreases NO bioavailability. Modified from Zuniga FA (2014) *Biomed Res Int*:353616 Epub 2014/Jul/6.

Abbreviation: oxLDL, oxidized low density lipoprotein; LOX-1, lectin-like oxidized low density lipoprotein receptor-1; NADPH, nicotinamide adenine dinucleotide phosphate; $O_2^{\bullet}$-, superoxide; ONOO-, peroxynitrite; NO, nitric oxide.

discovered as a receptor for oxLDL, LOX-1 is now known to bind to several ligands, including charged phospholipids such as phosphatidylserine [154], apoptotic bodies [155], activated platelets [156], and Gram positive and Gram negative bacteria [157].

Although the basal expression is low, LOX-1 is induced by a number of factors that are also known to be increased in preeclampsia, namely ROS, TNF-α, CRP, Ang II and ET-1 [158]. Binding of oxLDL induces the transcription of its own receptor, which undergoes oligodimerization forming a homodimer, a critical step for its function [158]. Upon binding of the ligand, the oxLDL/LOX-1 complex undergoes endocytosis and becomes internalized. Although the exact protein facilitating the intracellular trafficking of oxLDL/LOX-1 complex remains unknown, it has been shown that LOX-1 separates from oxLDL in endosomes which becomes degraded via lysosomes while LOX-1 is recycled back to the plasma membrane [158].

Much of the earlier literature focuses on the function of LOX-1 and its affinity for oxLDL. In atherosclerosis, low density lipoproteins (LDL) in the circulation passes into the subendothelial layer and undergoes oxidation to become oxLDL. Oxidized LDL then binds to LOX-1 on the endothelial cell surface which leads to the secretion of chemokines and pro-

inflammatory molecules encouraging the recruitment and translocation of monocytes into the subendothelial layer [159]. The recruited monocytes migrate across into the intimal space and form macrophages and begin to express LOX-1 [151]. Oxidized LDL also binds to LOX-1 expressed on macrophages, which is then internalized and triggers the transformation of macrophages into foam cells contributing to the formation of atherosclerotic plaques [151]. Interestingly, the binding of oxLDL to LOX-1 as well as its activation of ROS production triggers an up-regulation of LOX-1 expression, thereby inducing a vicious cycle of increased inflammation [160]. Oxidized LDL further binds LOX-1 on VSMCs and triggers the migration and proliferation of VSMCs into the intima and results in narrowing of the arterial lumen [159].

More recently, the role of LOX-1 in endothelial function and the pathogenesis of cardiovascular diseases has been increasingly recognized. Activation of LOX-1 is known to increase the activity of NADPH oxidase which increases ROS generation. The increased ROS production, as previously eluded, not only increases self-induced up-regulation of LOX-1 expression, but also contributes to a decrease in NO bioavailability, resulting in endothelial dysfunction and subsequent vasoconstriction, apoptosis, and inflammation (Figure 1-8) [153]. Given the shared characteristics of heightened inflammation, increased oxidative stress, and acute atherosis seen in preeclampsia, we are interested in examining how circulating factors could interact with LOX-1 in the pathogenesis of endothelial dysfunction.

1.5.2 The Role of LOX-1 in Preeclampsia

Preeclampsia is increasingly recognized as a risk factor for cardiovascular disease, and *vice versa*, women with a predisposition to endothelial dysfunction and metabolic syndrome are at increased risk of developing preeclampsia. As previously discussed, enhanced inflammation, oxidative stress, abnormal lipid handling, and acute atherosis are essentially hallmarks of

preeclampsia; thus, it is unsurprising that LOX-1, which is independently implicated in these disease processes, is also found to be increased in the maternal vasculature of women with preeclampsia [41].

In addition, the placenta has been found to exhibit high expression levels of LOX-1 mRNA [161]. It has been suggested that LOX-1 may be involved in trophoblast invasion in early pregnancy and that accelerated trophoblast apoptosis and endothelial dysfunction seen in preeclampsia is mediated via LOX-1 [91, 161, 162]. Previously the Davidge laboratory has demonstrated that preeclamptic women exhibit an increased level of oxLDL and arterial LOX-1 expression and circulating factors in preeclamptic plasma induce up-regulation of LOX-1 and superoxide production in isolated endothelial cells [41]. Circulating factors in preeclamptic plasma have also been shown to impair endothelial function, which is exacerbated by the addition of oxLDL and obliterated by the inhibition of LOX-1 [91]. Furthermore, in a RUPP rat model of preeclampsia, arterial LOX-1 expression and superoxide production are increased compared to normal pregnant rats [163]. Altogether, this suggests that circulating factors in preeclamptic plasma induce endothelial dysfunction which is mediated by oxidative stress via activation of the LOX-1 pathway. As previously discussed, STBEVs are one of the circulating factors released from the placental syncytiotrophoblast cell membranes during activation and apoptosis; therefore, we are interested to investigate whether STBEVs act as a ligand for LOX-1 which can contribute to endothelial dysfunction seen in preeclampsia.

1.5.3 Therapeutic and Diagnostic Potential of LOX-1 in Preeclampsia

While basal expression of LOX-1 is low, the expression is upregulated by inflammatory stimuli and oxidative stress, such as TNF-α, IL-6 [164] and oxLDL [89], as well as in several cardiovascular diseases such as diabetes, hyperlipidemia, atherosclerosis, and preeclampsia [41,

149]. Despite the fact that LOX-1 is expressed in the placenta, previous studies have shown that LOX-1 is not a lethal gene since LOX-1 knock-out mice are still fertile and do not carry detectable abnormalities during pregnancy [165]. This adds to the therapeutic consideration of potentially targeting LOX-1 in the treatment of preeclampsia.

Interestingly, the extracellular domain of LOX-1 can be cleaved at the neck domain to generate a soluble molecule (sLOX-1) [166]. Although the specific role of sLOX-1 remains unknown, it is associated with several cardiovascular diseases and thus increasingly recognized as a potential biomarker for risk factors such as diabetes, hypertension, and hyperlipidemia [159, 167]. Therefore, understanding the role of LOX-1 in preeclampsia may also contribute to the potential use of sLOX-1 as a diagnostic marker in high risk pregnancy.

1.6 Summary

The cost of the morbidity and mortality associated with preeclampsia in terms of the immediate and long term health of the mothers, health of the offspring, and the utilization of our health care system, is enormous. We are intrigued by the complicated, and yet exciting, underlying pathophysiology of preeclampsia. We hope to contribute to the understanding of this disease and to finding a preventative or therapeutic strategy that will ultimately lead to a potential cure. The overall objective of this thesis is to investigate the potential roles and mechanisms behind circulating factors, and specifically STBEVs, as eliciting events in the development of endothelial dysfunction seen in preeclampsia. Given that STBEVs are packages of oxidized lipids and LOX-1 is known to bind to several ligands, including lipids, platelets, cell debris, inflammatory cells, and even bacteria [168, 169], we are intrigued by the possibility that STBEVs may impair endothelial function by acting as a ligand for the LOX-1 receptor (Figure 1-9).

For the first part of the thesis, we will focus on the study of circulating factors from women with preeclampsia and whether they interact with NO and prostaglandin pathways as potential mechanisms of vascular dysfunction. Although there is a wealth of literature on the effects of circulating factors (i.e. preeclamptic plasma) on isolated endothelial cells, there is a surprising paucity of data for mechanisms leading to the functional consequences of circulating factors on maternal vasculature. We hypothesize that circulating factors in preeclamptic plasma impair endothelial vasodilation in uterine and mesenteric arteries via increasing oxidative stress which decreases NO participation and prostaglandin vasodilators and contribute to vascular dysfunction. For the second part of the thesis, we will investigate the role of STBEVs in maternal endothelial function as well as its interaction with the LOX-1 pathway. We hypothesize that STBEVs contribute to impaired endothelial vasodilation by increasing oxidative stress and alteration of NO bioavailability via the activation of the LOX-1 pathway.

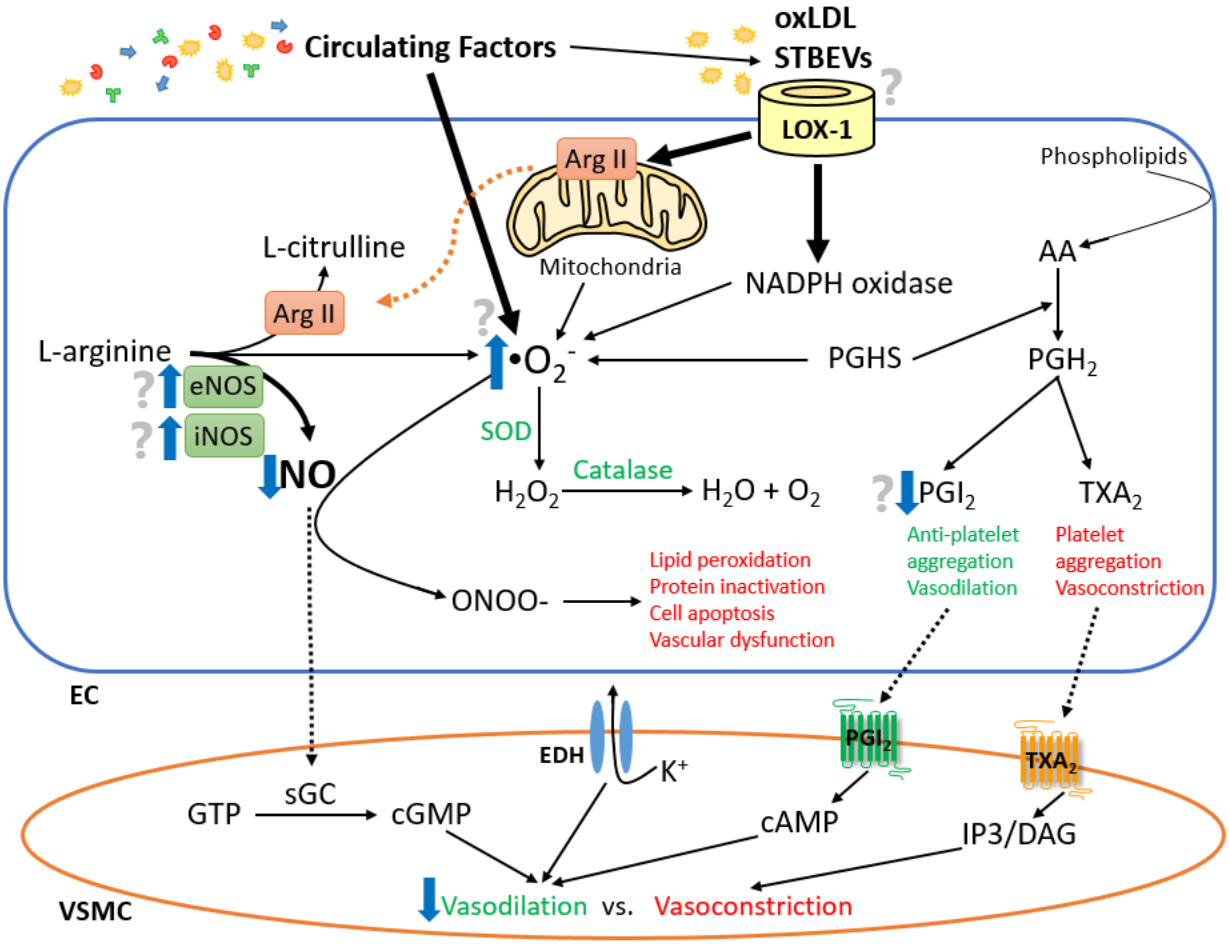

Figure 1-9: Summary diagram of the hypotheses.

Abbreviations: oxLDL, oxidized low density lipoprotein; STBEVs, syncytiotrophoblast extracellular vesicles; LOX-1, lectin-like oxidized low density lipoprotein receptor-1; Arg II, arginase II; NADPH, nicotinamide adenine dinucleotide phosphate; ●O_2-, superoxide; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; H_2O, water; O_2, oxygen; ONOO-, peroxynitrite; sGC; soluble guanylyl cyclase; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; EDH; endothelium derived hyperpolarization; K^+, potassium ion; AA, arachidonic acid; PGHS, prostaglandin H synthase; PGH_2, prostaglandin H_2; PGI_2, prostacyclin; TXA_2, thromboxane; cAMP, cyclic adenosine monophosphate; IP3, inositol triphosphate; DAG, diacylglycerol; EC, endothelial cells; VSMC, vascular smooth muscle cell.

CHAPTER 2

MECHANISM OF VASCULAR DYSFUNCTION DUE TO CIRCULATING FACTORS IN WOMEN WITH PREECLAMPSIA

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A version of this chapter has been submitted for publication in a peer reviewed journal.

Contributions: C. K. designed and performed the experiments, collected the tissue samples for DHE staining and Western blots, analyzed the data, and wrote the manuscript. A. Q. performed the DHE staining and Western blots. L. R. and P. L. J. collected the plasma samples. S. D. supervised and assisted in interpretation of the data and editing of the manuscript.

2.1 Introduction

As discussed in Chapter 1, systemic endothelial dysfunction is thought to play a central role in the development of preeclampsia. Placental hypoperfusion resulting from a lack of spiral artery remodelling in early placental development leads to local oxidative stress and the release of circulating factors into the maternal vasculature [40]. Previous studies have shown that plasma from preeclamptic women reduces endothelium-dependent vasodilation in healthy resistance vessels [45-47, 91], although this finding is not universal [103]. However, the mechanisms by which circulating factors lead to endothelial dysfunction remain unclear. Two key regulatory mechanisms in vascular function during pregnancy are the NO and prostaglandin pathways. Nitric oxide, a potent vasodilator, is produced from L-arginine by NOS, which exists in different isoforms: eNOS, iNOS, and nNOS. In the endothelium, the majority of NO production is associated with eNOS, while in diseased states such as hypertension and ageing, iNOS and nNOS have been shown to contribute to NO bioavailability in the cardiovascular system [121, 170]. Nitric oxide is the major vasodilator in the uterine artery vasculature and it, therefore, functions to support the increase in blood flow and fetal growth during pregnancy. Indeed, studies have shown that stable metabolites of NO and eNOS expression are both increased in pregnancy [110]. Nitric oxide is also essential for embryo development, placenta implantation, and trophoblast invasion [108] and regulates placental angiogenesis and maturation throughout pregnancy [120]. However, the bioavailability of NO is intricately affected by levels of ROS, such as superoxide, which scavenges NO to produce ONOO- and decreases NO-mediated relaxation of vascular smooth muscle [108]. Therefore, it is of interest to determine how circulating factors in women with preeclampsia interact with ROS and the NO system in the maternal vasculature.

Prostaglandins, produced from arachidonic acid by PGHS, are also intrinsically related to endothelial dysfunction in preeclampsia. Prostacyclin, an important vasodilator, is an abundant metabolite of PGHS in the endothelium and its biosynthesis is increased locally in the uterine and renal circulations during pregnancy. Thromboxane, a potent vasoconstrictor mainly derived from platelets but also produced in endothelium and VSMC, is also increased 3-5 fold during pregnancy [126]. Although both PGHS-dependent vasodilators and vasoconstrictors have both been shown to increase in pregnancy, a delicate balance between them is required to properly maintain vascular tone. A number of studies have reported a decrease in the PGI_2/TXA_2 ratio in preeclampsia as early as the first trimester and continues throughout pregnancy [171]. Given the ubiquitous nature of NO and prostaglandins in the vasculature, we are interested in examining how circulating factors in plasma from women with preeclampsia could interact with these pathways.

We hypothesize that circulating factors in preeclamptic plasma contribute to an increase in oxidative stress and result in an impairment of endothelial vasodilation in uterine (responsible for supplying the uterus and the fetus) and mesenteric (responsible for peripheral resistance and blood pressure control) arteries. Specifically, we hypothesize that circulating factors increase superoxide production in the endothelium, which scavenges and decreases the bioavailability of NO, resulting in endothelium-dependent vascular dysfunction. To compensate for the reduction in NO-mediated vasodilation, we hypothesize there would be an increase in NOS isoforms in arteries exposed to preeclamptic plasma We further hypothesize that circulating factors will lead to reduced PGI_2 production resulting in impaired vasodilation.

2.2 Materials and Methods

2.2.1 Ethics Approval

The animal use protocols were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee, in accordance with the Canadian Council on Animal Care guidelines. The study protocol for human blood sample collection was approved by the Foundación Cardiovascular de Colombia Institutional Ethics Review Board and conducted in Colombia. Written informed consent was obtained from all study participants. The blood sample collection protocols were also approved by the University of Alberta Ethics Committee to be used for studies in our laboratory.

2.2.2 Pooled Plasma Preparation

The plasma samples were obtained from the authors of a previously published multi-centre case-control study conducted in Colombia [172]. Briefly, subjects with preeclampsia (PE) were identified upon admission to the obstetrical services and recruited if blood pressure was ≥ 140/90 mmHg after the 20^{th} week of gestation with concurrent proteinuria (24-hour urinary protein ≥ 300 mg or dipstick protein ≥ 1+ in at least two random urine samples at least 4-6 hours apart). The control normotensive pregnant (NP) group was recruited consecutively from the same hospital after matching by age and ethnicity. Women with a history of chronic hypertension, cardiovascular disease, endocrine disease, autoimmune disease, renal disease, mental illness, human immunodeficiency virus and cancer were excluded. Plasma samples were collected in EDTA-coated tubes and transferred to Canada while stored at -80 °C. Prior to the experiments, plasma samples were randomly selected and pooled (NP group n = 10; PE group n = 12) and stored at -80 °C until use. The pooled samples were matched by maternal age and maternal BMI in both groups and none of the subjects smoked during pregnancy (Table 2-1).

Table 2-1: Characteristics of subjects from whom plasma samples were collected and pooled

Characteristics	Normotensive (NP; n=10)	Preeclampsia (PE; n=12)	P-value
Maternal age (years)	27.5 ± 2.5	23.7 ± 1.4	0.17
Gestational age (weeks)	38 (30 – 40)[1]	36 (25 – 38)[1]	0.01
Systolic blood pressure (mmHg)	113.2 ± 2.1	150.3 ± 4.3	<0.0001
Diastolic blood pressure (mmHg)	68.9 ± 1.9	95.1 ± 2.9	<0.0001
24h proteinuria (mg/24h)	-	360 (305-1184) [1]	-
BMI (kg/m^2)	26.3 ± 1.5	28.0 ± 1.6	0.45
Smoking during pregnancy (%)[2]	0	0	-

[1]Gestational age and proteinuria were not normally distributed. They were presented as median (range). A non-parametric Mann-Whitney test was used for statistical comparison.

[2]None of the subjects smoked during pregnancy. All 12 subjects in the PE group and all but one subject in the NP group were lifelong non-smokers. One subject in the NP group was a casual smoker who quit at least one year prior to conception.

2.2.3 Animal and Vessel Preparation

Three-month-old female Sprague Dawley rats were bred after acclimatization in 10:14-hour light:dark cycle cages with free access to standard rat chow and tap water. A total of 26 animals were used for experiments (NP: n = 14, PE: n = 12). Day 0 of pregnancy was confirmed by the presence of sperm in a vaginal smear following an overnight mating. On day 20 of gestation, the animals were sacrificed by exsanguination while under anesthesia by isoflurane and a laparotomy was performed to harvest the mesenteric and uterine arteries. The arteries were isolated under a microscope to ensure all connective tissues and adipocytes were removed. The arteries were then incubated overnight with 3% plasma from either normotensive pregnant or preeclamptic women with 1 U/mL heparin in physiologic saline solution at 4 °C. The

concentration of plasma incubation was chosen based on previous studies [46, 48, 91]. The arteries were mounted for myography experiments on the following day after 22-24 hours of incubation.

2.2.4 Superoxide detection

Superoxide level was measured by staining with dihydroethidium (DHE; n=5). DHE reacts with superoxide to yield ethidium, which binds to nuclear DNA and generates red fluorescence. After overnight incubation in plasma, segments of uterine arteries were embedded in optimal cutting medium (OCT) and snap-frozen in liquid nitrogen for storage. Sections of the arteries were cut at 10 μm, mounted on glass slides at -20 °C, and stored at -80 °C until use. On the day of analysis, the slides were thawed, washed with Hank's Balanced Salt Solution (HBSS) 3 times at 2 minutes each, and incubated with fresh HBSS for 10 minutes at 37 °C in a humid chamber. The slides were then incubated with DHE for 30 minutes at 37 °C, washed with HBSS 3 times at 2 minutes each, cover slipped, and visualized under an IX81 Olympus fluorescence microscope.

2.2.5 Western blot analysis for NOS expression

Following an overnight incubation in plasma, uterine arteries (n=5-8) were snap frozen in liquid nitrogen and stored at -80 °C until use. On the day of analysis, uterine arteries were thawed and homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Triton-100; 0.5% sodium deoxycholate; 0.1% SDS; 1 mM EDTA; 10 mM NaF; 1 mM PMSF; 1X protease inhibitor cocktail tablet [SIGMAI*FAST* Protease Inhibitor Cocktail Tablets, EDTA-free]; 2 mM sodium orthovanadate). Total protein was determined using bicinochoninic assay (BCA; Pierce-Thermo Scientific) and 50 μg of total protein was loaded onto 7.5% SDS-acrylamide gels. After electrophoresis, the proteins were transferred onto 0.2 μm nitrocellulose membrane (Biorad) and blocked with 50% blocking reagent (Rockland Inc.). The membranes were then incubated

overnight with primary antibodies: iNOS (mouse monoclonal; 1:500; BD Biosciences cat # 610432), eNOS (mouse monoclonal; 1:500; BD Biosciences cat # 610297), nNOS (mouse monoclonal; 1:500; BD Biosciences cat # 610309), and β-actin (rabbit polyclonal; 1:1000; Abcam cat # ab75186). On the following day, the membranes were incubated with secondary antibodies for one hour: donkey anti-mouse IgG (IRDYe 800CW; 1:10,000; Li-Cor cat # 926-32212) or goat anti-rabbit IgG (IRDYe 680RD; 1:10,000; Li-Cor cat # 926-68071). Blots were imaged using Odyssey infrared imaging system and densitometry was measured with Odyssey software v 3.0.021 (Li-Cor Biosciences). Results were normalized to β-actin as a loading control.

2.2.6 Myography

Following an overnight incubation in 3% plasma and 1 mU/mL of heparin, mesenteric and uterine arteries were mounted on two 40-µm wires and attached to a wire myograph (DMT, Copenhagen, Denmark) for isometric tension recordings. The vessels were normalized and equilibrated for a minimum of 30 minutes before they were exposed to two wake-up doses of phenylephrine (10 µmol/L) and a single dose of methacholine (3 µmol/L) to ensure endothelial function and smooth muscle integrity. A cumulative concentration response curve to phenylephrine (0.001 to 100 µmol/L) was performed to determine the concentration that produces 80% of maximal constriction. This concentration was then used to preconstrict the vessels for endothelium-dependent relaxation curves using cumulative doses of methacholine (MCh; 0.0001 to 10 µmol/L). The changes in vessel wall tension were measured and translated into dose-response curves as a representative measure of endothelium-dependent vasodilation for comparison.

2.2.7 Pharmacological Agents

To study the involvement of different vascular function pathways, specific inhibitors were added to the vessel bath 30 minutes prior to MCh concentration response curves. We used a pan NOS inhibitor, N-Nitro-L-arginine methyl ester hydrochloride (L-NAME; 100 μmol/L; Sigma N5751), to study the contribution of NO to endothelial vasodilation. We also used a highly selective inhibitor for iNOS, 1400W (10 μmol/L; Sigma W4262), which is at least 5,000-fold more potent against iNOS ($K_i \leq 7$ nM) versus eNOS ($K_i = 50$ μM) [173]. To study the effect of NO scavenging by superoxide, we used superoxide dismutase-polyethylene glycol (pegSOD; 50 U/mL; Sigma S9549) and catalase (500 U/mL; Sigma C1345). To study the involvement of prostaglandins, we used a non-selective PGHS inhibitor, meclofenamate (meclo; 1 μmol/L; VWR J60484).

2.2.8 Statistics

Statistical analyses were performed using GraphPad Prism software. Kolmogorov-Smirnov normality test was used for testing of the characteristics of human subjects (Table 2-1): normally distributed data were compared using a two-tailed Student's t-test and non-normally distributed data were compared using a Mann-Whitney test. Myography data was summarised using maximal response (E_{max}), the effective concentration required to achieve 50% of the maximal response (pEC_{50}) or area under the curve (AUC) values. Specifically, AUC is the quantification of the area between the dose-response curve and the x-axis defined at numerical value of zero in arbitrary unit. The effect of plasma (NP vs. PE) or inhibitors within a group were compared using a 2-way ANOVA and a Bonferroni *post hoc* test. A P-value < 0.05 was considered statistically significant.

2.3 Results

2.3.1 The effect of circulating factors on nitric oxide bioavailability in the uterine artery

Endothelium-dependent vasodilation of uterine arteries was impaired after overnight incubation with PE plasma when compared with those in NP plasma (Figure 2-1). Superoxide dismutase restored endothelium-dependent vasodilation in arteries exposed to PE plasma to a level comparable with those treated with NP plasma; however, SOD had no effect on vasodilation in arteries exposed to NP plasma (Figure 2-2). Similarly, catalase restored vasodilator responses in PE-plasma treated uterine arteries and had no effect on NP-plasma-treated vessels (Figure 2-2). Uterine vasodilation was largely abolished in the presence of L-NAME, a pan NOS inhibitor, in both NP- and PE-plasma treated vessels (Figure 2-3). However, the delta AUC was significantly reduced in PE-plasma treated arteries, suggesting that the contribution of NO to endothelial vasodilation was decreased in vessels exposed to PE plasma. Interestingly, inhibition of iNOS with 1400W significantly impaired endothelial function in uterine arteries exposed to NP plasma, but not in those exposed to PE plasma (Figure 2-4). Of note, similar findings were demonstrated comparing E_{max}, pEC50, and AUC, which were summarized in Table 2-2.

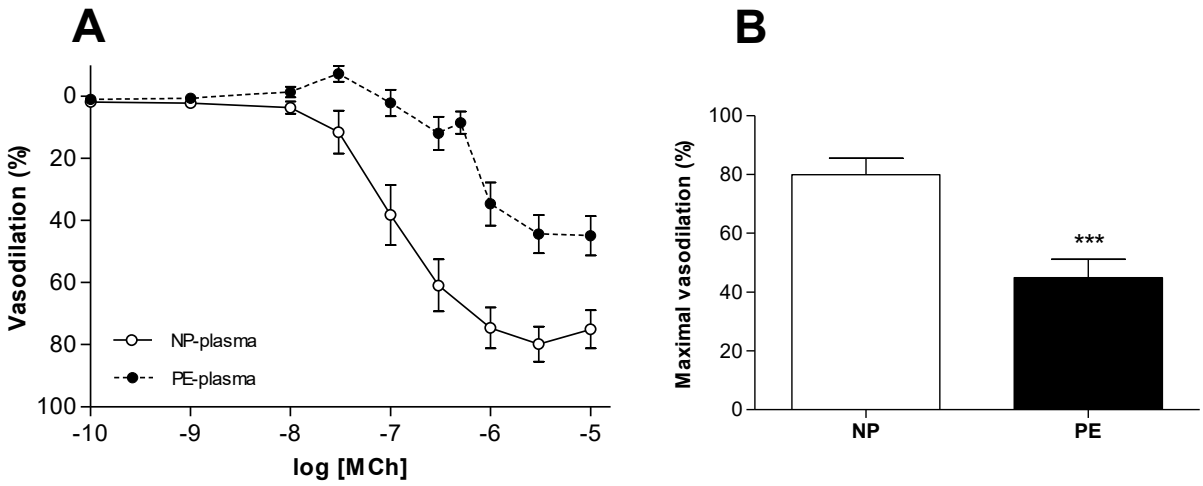

Figure 2-1: Vascular responses in plasma treated uterine arteries.

(A) Uterine vascular responses after overnight incubation in normotensive pregnant (NP) plasma vs. preeclamptic (PE) plasma to cumulative doses of methacholine (MCh), an endothelium-dependent vasodilator (NP: n = 14; PE: n = 12). Uterine endothelial vasodilation was significantly impaired by exposure to circulating factors in PE plasma (closed circles) vs. NP plasma (open circles). **(B)** Bar graphs showed percent maximal vasodilation. By Student's t-test: ***$P < 0.001$.

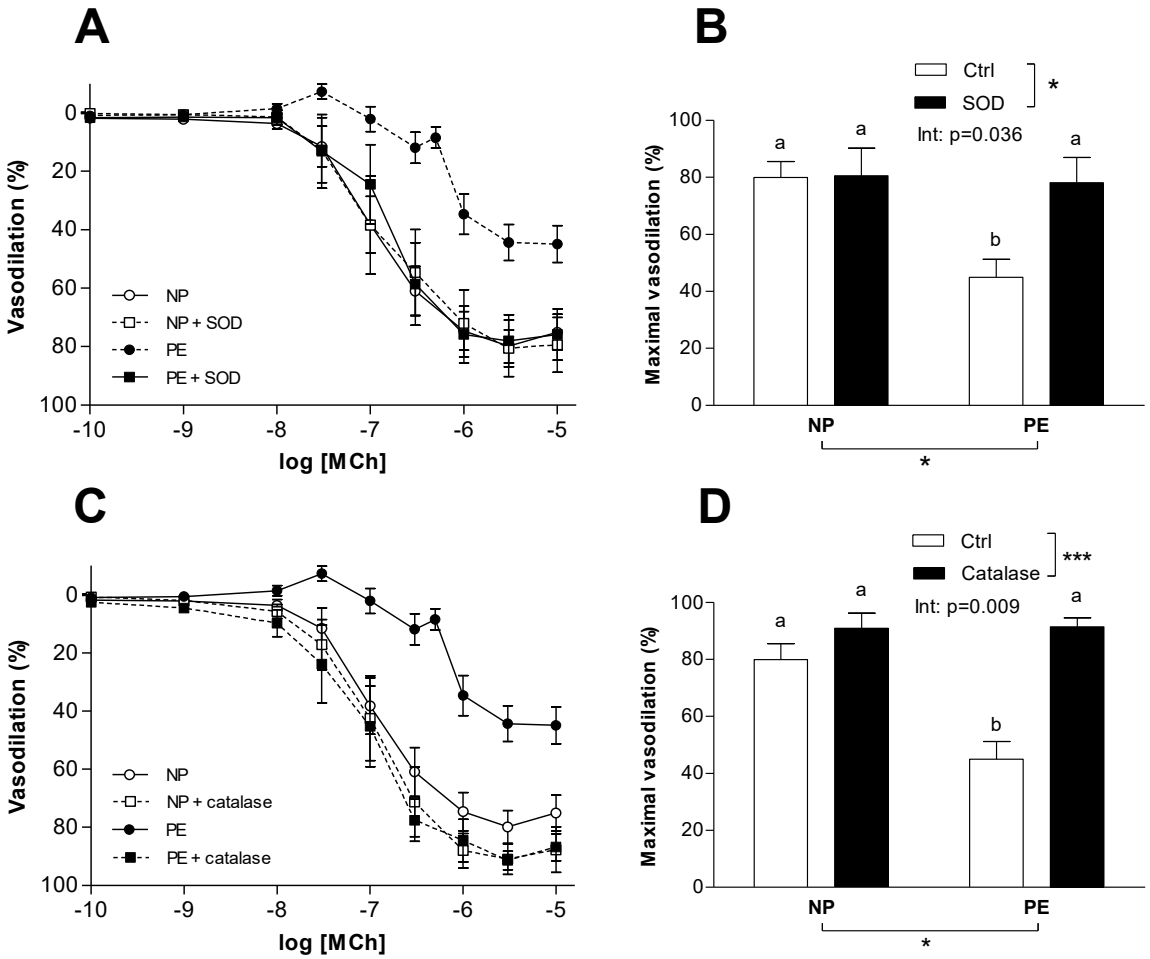

Figure 2-2: Vascular responses in plasma treated uterine arteries in the absence or presence of oxidant scavengers.

Uterine vascular responses after overnight incubation in normotensive pregnant (NP) plasma vs. preeclamptic (PE) plasma to cumulative doses of methacholine (MCh), an endothelium-dependent vasodilator, in the absence or presence of oxidant scavengers (n = 7 per group). **(A)** Scavenging of superoxide with superoxide dismutase (SOD) significantly restored vasodilation in arteries exposed to PE plasma, but had no effect in arteries exposed to NP plasma. **(B)** Bar graphs showed percent maximal vasodilation. **(C)** Catalase restored impaired vasodilation in PE-plasma exposed arteries to a level comparable with control arteries. **(D)** Bar graphs showed percent maximal vasodilation. Using two-way ANOVA: * $P < 0.05$, *** $P < 0.001$ and "a" denoted a statistical significance from "b" with $P < 0.05$.

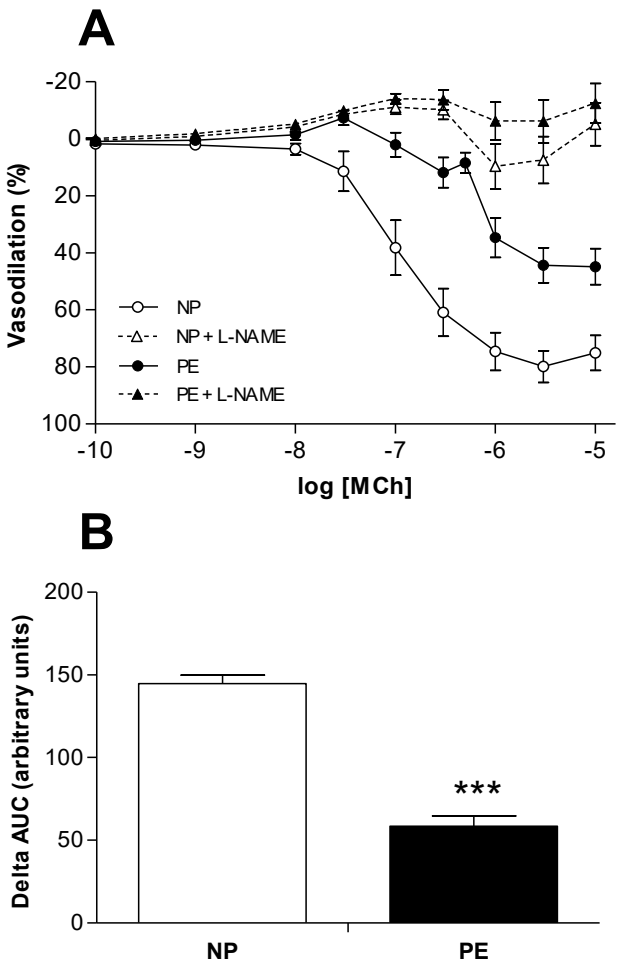

Figure 2-3: Vascular responses in plasma treated uterine arteries in the absence or presence of pan nitric oxide synthase inhibitors.

(A) Endothelial vasodilation was completely abolished in the presence of L-NAME, a pan nitric oxide synthase inhibitor, in both NP- and PE-plasma treated arteries (n = 8 per group). **(B)** The contribution of nitric oxide to vasodilation was decreased in arteries exposed to PE plasma, as summarized in the bar graphs with delta AUC in arbitrary units. Using two-way ANOVA: *** $P < 0.001$.

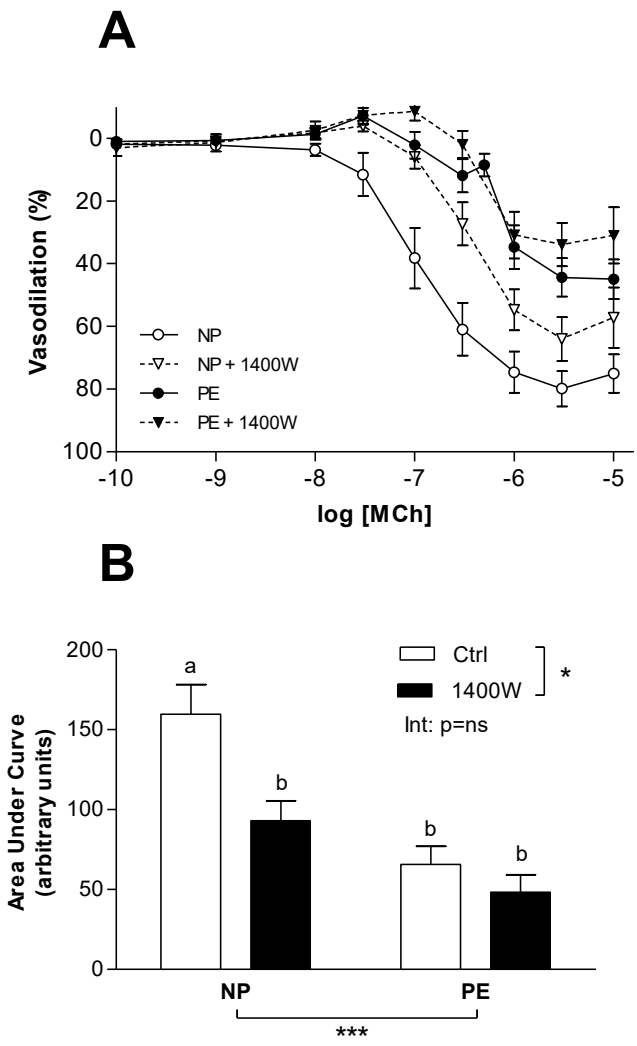

Figure 2-4: Vascular responses in plasma treated uterine arteries in the absence or presence of inducible nitric oxide synthase inhibitor.

(A) Inhibition of iNOS with 1400W resulted in impaired vasodilation in NP-plasma treated arteries but not in PE-plasma treated arteries (n = 8 per group). **(B)** Bar graphs showed AUC in arbitrary units. Using two-way ANOVA: * $P < 0.05$, *** $P < 0.001$ and "a" denoted a statistical significance from "b" with $P < 0.05$.

Table 2-2: Summary table of uterine vascular responses after exposure to normotensive pregnant plasma vs. preeclamptic plasma in the absence or presence of different inhibitors.

A. Maximal vasodilation (E_{max}) in percentage

	NP-plasma treated	PE-plasma treated			
			t-test		
Control	79.9 ± 5.6	44.9 ± 6.3	***		
			2-way ANOVA		
			Plasma	Inhibitor	Int
SOD	80.6 ± 9.7	78.1 ± 8.9	*	*	*
Catalase	91.0 ± 5.2	91.4 ± 3.2	*	***	**
L-NAME	9.8 ± 8.0	-6.0 ± 7.6	***	***	ns
1400W	64.0 ± 7.0	34.0 ± 7.5	***	ns	ns
Meclofenamate	74.6 ± 6.4	80.4 ± 7.2	*	*	**

B. Log EC50

	NP-plasma treated	PE-plasma treated			
			t-test		
Control	6.96 ± 0.10	6.2 ± 0.2	***		
			2-way ANOVA		
			Plasma	Inhibitor	Int
SOD	6.89 ± 0.2	6.8 ± 0.19	*	ns	*
Catalase	7.0 ± 0.1	7.0 ± 0.1	*	**	**
L-NAME	9.2 ± 2.7	--[1]	--[1]	--[1]	--[1]
1400W	6.49 ± 0.08	6.1 ± 0.3	**	ns	ns
Meclofenamate	6.6 ± 0.1	6.7 ± 0.1	*	ns	*

C. Area under curve (AUC)

	NP-treated	PE-treated			
			t-test		
Control	159.6 ± 18.5	65.6 ± 11.3	***		
			2-way ANOVA		
			Plasma	Inhibitor	Int
SOD	156.5 ± 29.8	153.0 ± 27.5	*	*	ns
Catalase	185.6 ± 21.9	197.0 ± 24.3	*	***	*
L-NAME	13.0 ± 4.7	7.0 ± 6.1	**	***	**
1400W	93.2 ± 12.2	48.3 ± 11.0	***	*	ns
Meclofenamate	130.3 ± 17.7	141.0 ± 10.2	*	ns	**

The effect of plasma was compared using a Student's t-test. The effect of plasma and the effect of inhibitors were compared using 2-way ANOVA: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. "Int" denoted interaction between the factors by 2-way ANOVA and "ns" denoted non-significance with $P > 0.05$. Each group consists of n = 12-14 for the plasma treated arteries in the absence of pharmacological agents, and n = 6-9 for those in the presence of pharmacological agents.

[1]Log EC50 was not applicable because the concentration response curves did not follow a sigmoidal pattern; therefore, statistical comparisons were not possible.

The level of DHE staining, which reacts with superoxide to produce fluorescent ethidium, was increased two-fold in PE-plasma treated arteries when compared with NP-plasma treated arteries (Figure 2-5), suggesting an increase in oxidative stress in arteries exposed to preeclamptic circulating factors. In addition, the uterine arterial eNOS expression was significantly increased in arteries exposed to PE plasma when compared to NP controls (Figure 2-6A). However, the expression of iNOS was decreased in PE-treated arteries (Figure 2-6B). The expression of nNOS in uterine arteries was negligible and below the level of detection by Western blot (data not shown).

2.3.2 The effect of circulating factors on prostaglandin bioavailability in the uterine artery

In uterine arteries, inhibition of prostaglandin synthesis with meclofenamate restored endothelium-dependent vasodilation in PE-plasma treated vessels to a level comparable with the controls, while meclofenamate had no effect on NP-plasma treated arteries (Figure 2-7). This suggests that circulating factors in PE plasma contribute to an increase in PGHS-dependent vasoconstrictors.

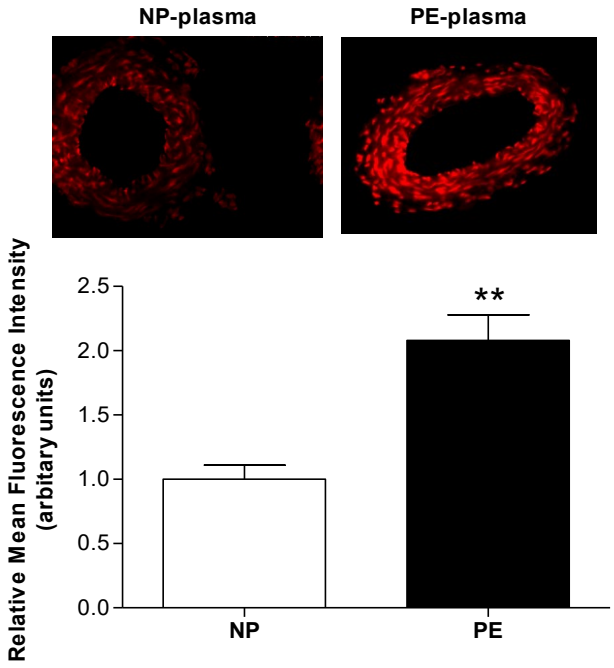

Figure 2-5: Vascular superoxide level in uterine arteries after exposure to normotensive pregnant (NP) vs. preeclamptic (PE) plasma.

Dihydroethidium (DHE) staining, as a marker of superoxide levels, was increased in PE-plasma treated arteries when compared to NP-plasma treated arteries (n = 5 per group). Representative images were shown. Red fluorescence indicated nuclear fluorescence generated by the reaction of DHE with superoxide to produce ethidium. All of the data were expressed as relative mean fluorescence intensity and analyzed using Student's t-test. ** $P < 0.01$.

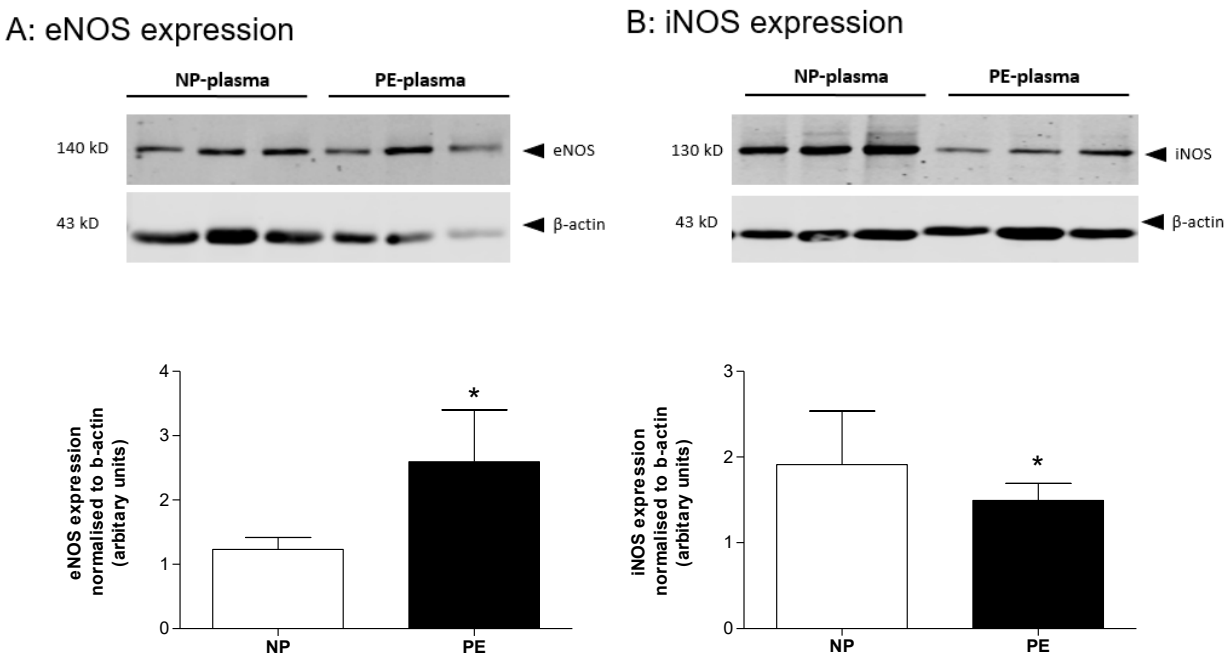

Figure 2-6: Nitric oxide synthase expression in uterine arteries after exposure to normotensive pregnant (NP) vs. preeclamptic (PE) plasma.

(A) Endothelial NOS (eNOS) expression was increased in PE-plasma treated arteries when compared to NP-plasma treated arteries (n = 5-8 per group). **(B)** Inducible NOS (iNOS) expression was decreased in PE-plasma treated arteries compared to NP-plasma treated arteries. Representative Western blots were shown. All of the data were expressed in arbitrary units relative to the actin protein expression and compared using Student's t-test: * $P < 0.05$.

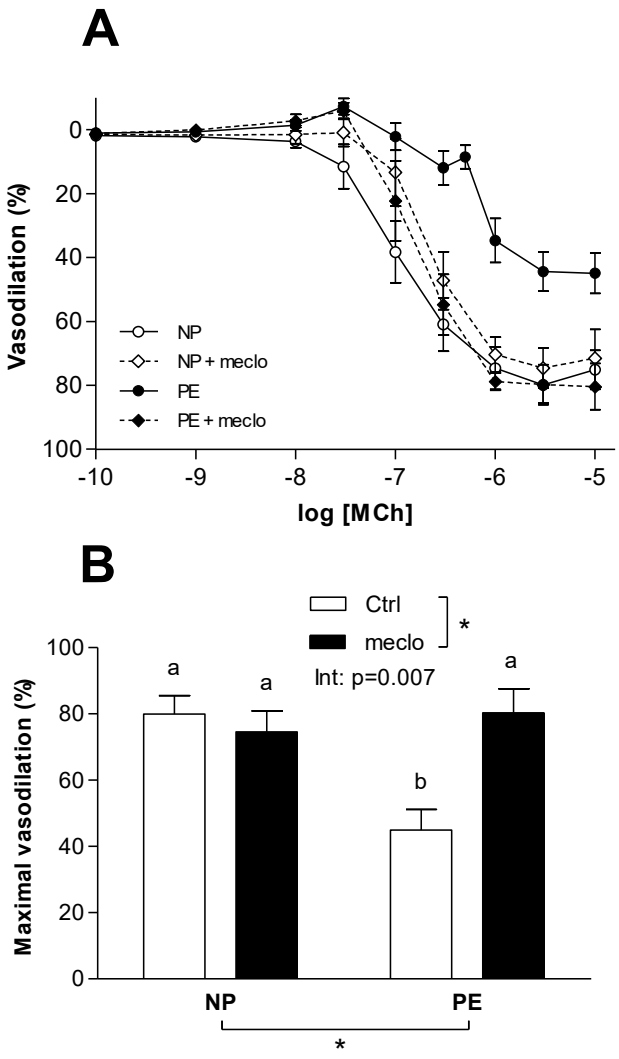

Figure 2-7: Vascular responses in plasma treated uterine arteries in the absence or presence of prostaglandin synthesis inhibitor.

(A) Uterine vascular responses after overnight incubation in normotensive pregnant (NP) plasma vs. preeclamptic (PE) plasma to cumulative doses of methacholine (MCh), an endothelium-dependent vasodilator, in the absence or presence of meclofenamate (n = 7 per group). Treating PE-plasma exposed uterine arteries with meclofenamate restored endothelial vasodilation but had no effect in NP-plasma exposed arteries. **(B)** Bar graphs showed percent maximal vasodilation. Using two-way ANOVA: *P < 0.05 and "a" denoted a statistical significance from "b" with P < 0.05.

2.3.3 The effect of circulating factors in the mesenteric artery

In contrast to the uterine arteries, endothelial vasodilation was not significantly different in mesenteric arteries exposed to either NP or PE plasma (Table 2-2). Superoxide dismutase and catalase increased vasodilation in both NP and PE plasma treated mesenteric arteries compared with their respective controls, but the effect appears to be independent of exposure to either NP or PE circulating factors. Inhibition of NOS with L-NAME significantly impaired sensitivity but not maximal vasodilation in both NP-and PE-plasma treated mesenteric arteries. Inhibition of iNOS also did not alter vasodilation responses in either NP- or PE-plasma treated mesenteric arteries. Furthermore, inhibition of prostaglandin synthesis with meclofenamate did not affect vascular responses in either NP- or PE-plasma treated mesenteric arteries. These data are summarized in Table 2-3 for succinct presentation.

Table 2-3: Summary table of mesenteric vascular response after exposure to normotensive pregnant plasma vs. preeclamptic plasma in the absence or presence of different inhibitors.

A. Maximal vasodilation (E_{max}) in percentage

	NP-plasma treated	PE-plasma treated			
			t-test		
Control	96.2 ± 2.2	94.4 ± 3.4			
			2-way ANOVA		
			Plasma	Inhibitor	Int
SOD	99.8 ± 0.2	100.0 ± 0.2	ns	ns	ns
Catalase	99.5 ± 0.3	99.9 ± 0.9	ns	ns	ns
L-NAME	93.6 ± 2.6	94.4 ± 2.7	ns	ns	ns
1400W	99.2 ± 0.2	99.2 ± 0.2	ns	ns	ns
Meclofenamate	99.1 ± 0.2	98.2 ± 1.3	ns	ns	ns

B. Log EC50

	NP-plasma treated	PE-plasma treated			
			t-test		
Control	7.34 ± 0.86	7.23 ± 0.10	ns		
			2-way ANOVA		
			Plasma	Inhibitor	Int
SOD	8.12 ± 0.07	8.25 ± 0.11	ns	***	ns
Catalase	7.82 ± 0.08	8.06 ± 0.12	ns	**	ns
L-NAME	6.68 ± 0.15	6.93 ± 0.11	ns	***	ns
1400W	7.47 ± 0.10	7.32 ± 0.04	ns	ns	ns
Meclofenamate	7.42 ± 0.09	7.27 ± 0.10	ns	ns	ns

C. Area under curve (AUC)

	NP-treated	PE-treated			
			t-test		
Control	262.8 ± 15.4	250.8 ± 19.2	ns		
			2-way ANOVA		
			Plasma	Inhibitor	Int
SOD	329.8 ± 14.6	338.3 ± 21.9	ns	**	ns
Catalase	296.9 ± 16.9	324.0 ± 24.6	ns	*	ns
L-NAME	176.0 ± 26.9	203.1 ± 23.0	ns	**	ns
1400W	268.2 ± 22.2	251.3 ± 14.0	ns	ns	ns
Meclofenamate	254.7 ± 19.1	239.2 ± 20.9	ns	ns	ns

The effect of plasma was compared using a Student's t-test. The effect of plasma and the effect of inhibitors were compared using 2-way ANOVA: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. "Int" denoted interaction between the factors by 2-way ANOVA and "ns" denoted non-significance with $P > 0.05$. Each group consists of n = 10-14 for the plasma treated arteries in the absence of pharmacological agents and, n = 6-9 for those in the presence of pharmacological agents.

2.4 Discussion

In this study, we have shown that circulating factors in preeclamptic plasma contribute to an increase in superoxide production and impairment of endothelium-dependent vasodilation in uterine arteries, but not in mesenteric arteries. Scavenging of superoxide, which increases the bioavailability of NO, results in restoration of endothelial vasodilation in arteries exposed to preeclamptic plasma. Circulating factors in preeclampsia also increase PGHS-dependent vasoconstrictors since meclofenamate significantly improves endothelial function in the exposed uterine arteries. Not only does this study highlight vascular bed specific mechanisms through which circulating factors interact with vasodilator pathways, but it also enhances the understanding of how circulating factors contribute to the endothelial dysfunction seen in the pathophysiology of preeclampsia.

Previous studies have demonstrated that preeclamptic plasma impairs endothelial vasodilation in arteries [45-47]; however, there is a paucity of data on the underlying mechanism. A recent study has shown that activation of the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), which is a scavenger receptor frequently implicated in cardiovascular disease and, more recently, preeclampsia, contributes to impaired vasodilation in omental arteries exposed to preeclamptic plasma [91]. Our group has also previously demonstrated that

preeclamptic plasma increases superoxide levels in isolated endothelial cells through activation of NADPH oxidase by LOX-1 [41]. However, the functional consequence has not been investigated. In the current study, we have shown that circulating factors in preeclamptic plasma contribute to an increase in superoxide production in uterine arteries and does indeed impair endothelial-dependent relaxation. We have also shown that scavenging superoxide with SOD, which would be expected to increase NO bioavailability, improves endothelial vasodilation in uterine arteries exposed to preeclamptic circulating factors. This supports our hypothesis that circulating factors in preeclamptic plasma impair endothelial function by contributing to an increase in the production of oxidative stress. Interestingly, the presence of catalase, which breaks down H_2O_2 into water and oxygen, also improved endothelial vasodilation. This suggests that H_2O_2 acts as a vasoconstrictor in uterine arteries exposed to circulating factors. Although H_2O_2 is known to act as an endothelium-dependent hyperpolarization factor (EDHF) which causes vasodilation in vascular smooth muscle by activating potassium channels [174, 175], it can also act as a vasoconstrictor [176]. Previous literature has shown that the level of H_2O_2 is increased in maternal circulation and placenta in term pregnancies [177] as well as in preeclamptic pregnancies [178]. Hydrogen peroxide also decreases the production of NO by increasing the release of arginase which breaks down L-arginine [179], contributing to an inverse relationship between the levels of H_2O_2 and NO in pregnancy [177]. This provides an explanation for our findings that catalase increases the breakdown of H_2O_2 which may contribute to an increase in NO production via activating arginase and improved endothelial vasodilation in arteries exposed to preeclamptic plasma.

Uterine arteries are highly dynamic and adaptive in order to accommodate a 40% increase in maternal blood volume during pregnancy which supplies the enlarging uterus,

placenta, and fetus [180]. Our finding that the inhibition of NOS completely abolished endothelial vasodilation supports that uterine arteries rely heavily on NO bioavailability. This is in agreement with studies demonstrating that pregnancy is associated with an increase in NO metabolites in the maternal vasculature and an increase in endothelial eNOS protein and mRNA levels in uterine arteries [110]. Our study shows an increase in oxidative stress in PE-plasma exposed vessels which contributes to a reduction in NO bioavailability for endothelium-dependent vasodilation. This is also associated with an increased uterine eNOS expression, which, by speculation, would compensate for reduced NO availability in order to maintain uterine blood flow to supply the feto-placental unit.

In pathological conditions, an up-regulation of iNOS has been reported in both experimental and clinical hypertension [121]. Pro-inflammatory cytokines that up-regulate iNOS have been associated with preeclampsia [147, 181] and the inhibition of iNOS attenuates hypertension and oxidative stress seen in an animal model of preeclampsia [121]. Contrary to our hypothesis, NP-plasma exposed arteries exhibited iNOS-dependent vasodilation that was absent in PE-plasma exposed arteries. We speculate that prolonged exposure to circulating factors in preeclamptic plasma may result in a down-regulation of iNOS to protect the vessels from the deleterious effects of the metabolites, such as peroxynitrite, which can be formed in the presence of abnormally high NO and superoxide levels [118]. Our finding that uterine arteries exposed to circulating factors in preeclamptic plasma exhibit a decreased iNOS expression supports this and is in line with previous studies that show a decrease in placental iNOS mRNA levels in women with preeclampsia [182]. This offers an interesting perspective to the current paradigm of preeclampsia that, instead of a state of heightened inflammation, we speculate preeclampsia may represent a state of failed compensation for the reduced NO bioavailability to maintain proper

vascular function. However, other conflicting evidence also exists. Previous studies have shown an increase in iNOS protein expression in HUVECs exposed to serum from preeclamptic women [115], an increase in iNOS protein expression in human omental arteries after exposure to microparticles from preeclamptic women [183], and no change in iNOS mRNA levels in HUVECs exposed to plasma from women with umbilical placental vascular disease defined by abnormal umbilical artery Doppler [184]. Whether this discrepancy is due to variations in species or different circulating factors present in different compartments of the blood sample (i.e. serum vs. plasma vs isolated microparticles) from preeclamptic women remains to be further elucidated.

In this study, we have also demonstrated that the inhibition of prostaglandin synthesis significantly restored endothelium-dependent vasodilation in uterine arteries exposed to preeclamptic plasma, suggesting that circulating factors contribute to an increase in PGHS-dependent vasoconstrictors rather than a reduction in PGI_2, as we had originally hypothesized. Although meclofenamate non-selectively inhibits PGHS, it is also known to inhibit TXA_2 synthase activity [185]. Many studies have shown that preeclampsia is associated with a decrease in the ratio of PGI_2/TXA_2 [171]. Our study supports the theory that circulating factors contribute to an increase in PGHS-dependent vasoconstrictors, presumably TXA_2 and/or its immediate precursor PGH_2, which reduce endothelial vasodilation and which are inhibited in the presence of meclofenamate. This is also in accordance with clinical trials that support the use of aspirin, a non-reversible PGHS-1 inhibitor which decreases TXA_2, in high risk patients during early pregnancy to reduce the risk of developing preeclampsia [2].

Unlike the uterine arteries, our data show that circulating factors in preeclamptic plasma do not impair endothelial vasodilation in mesenteric arteries. In both NP- and PE-plasma

exposed arteries, mesenteric vasodilation is increased in the presence of superoxide scavengers and reduced in the presence of NOS inhibition. This suggests that mesenteric arteries rely partially on NO vasodilator pathways and this is independent of the effect of circulating factors in preeclamptic plasma. Inhibition of prostaglandin synthesis also did not affect endothelial vasodilation in mesenteric arteries in the presence of circulating factors in preeclampsia. This is in agreement with previous literature that other vasodilators, such as EDHF, might play a more important role in mesenteric arteries which contribute to peripheral vascular resistance and blood pressure control [186-188]. This study highlights that the mechanism of action of the circulating factors present in preeclamptic plasma are vascular bed-specific.

In conclusion, our studies have shown that circulating factors contribute to endothelial dysfunction in preeclampsia by increasing superoxide production and decreasing NO bioavailability. Circulating factors also contribute to an increase in PGHS-dependent vasoconstrictors which are associated with impaired vascular responses. Further, we have shown that mechanism of action of circulating factors is dependent on the type of vascular bed. This study contributes to understanding of the pathophysiology and the identification of a potential target of intervention in improving the vascular dysfunction associated with preeclampsia.

CHAPTER 3

SYNCYTIOTROPHOBLAST EXTRACELLULAR VESICLES IMPAIR VASCULAR FUNCTION VIA ACTIVATION OF LECTIN-LIKE OXIDIZED LOW DENSITY LIPOPROTEIN RECEPTOR-1 (LOX-1)

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A version of this chapter is submitted in a peer-reviewed journal.

Contribution: C. K. designed and performed the experiments, analyzed the data, and wrote the manuscript. F. S. performed parts of the myography experiments using SOD and the DHE staining. T. S. provided the anti-LOX-1 antibody. I. L. provided the STBEVs. S. D. supervised and assisted in interpretation of the data and editing of the manuscript.

3.1 Introduction

As previously discussed in Chapter 1, the development of preeclampsia is thought to originate during early placentation when the spiral arteries fail to invade the myometrium and remodel into low resistance vessels, leading to placental hypoperfusion and local oxidative stress [40]. As a result, circulating factors are released into the maternal vasculature and a cascade of vascular responses is elicited causing systemic endothelial dysfunction. Previous studies have shown that plasma from preeclamptic women reduces endothelial vasodilation in healthy arteries [45, 46, 91]. As discussed in Chapter 2, we have also shown that circulating factors in preeclamptic plasma impair endothelial vasodilation via a decrease in NO bioavailability and an increase in oxidative stress as well as an increase in PGHS-dependent vasoconstrictors.

Within the plasma, microparticles have been identified as a unique population of bioactive cell fragments released during cell activation and apoptosis that are increased in women with preeclampsia [92]. *Ex vivo* experiments have shown that microparticles from women with preeclampsia contribute to vascular wall inflammation, blunted vascular contractility, and impaired endothelial vasodilation [104, 105]. Specifically, microparticles derived from placental cells, or syncytiotrophoblast extracellular vesicles (STBEVs), are increased in women with preeclampsia [96], especially in severe early onset preeclampsia [100]. STBEVs have also been shown to reduce ACh-induced vasodilation and inhibit endothelial cell proliferation [71]. However, the mechanism(s) by which STBEVs cause altered vascular reactivity remains unclear.

As previously discussed, the Davidge group has shown that activation of LOX-1 leads to impaired vasodilation in human omental arteries exposed to preeclamptic plasma [91]. Initially discovered as a scavenger receptor that binds oxLDL, LOX-1 is implicated in vascular

dysfunction and inflammation seen in cardiovascular diseases and known to be a multi-ligand receptor that binds lipids, platelets, inflammatory cells, cell debris, and bacteria [168, 169]. LOX-1 is also known to activate NADPH oxidase and contributes to increased production of superoxide, which binds and decreases NO bioavailability in the endothelium and leads to subsequent endothelial dysfunction [90]. Our group has further demonstrated that preeclamptic plasma increases oxidative stress in endothelial cells via activation of NADPH oxidase by LOX-1 [41]; however, whether STBEVs induce endothelial dysfunction via the same mechanism is unknown. Given that STBEVs are essentially packages of lipids and proteins released from the placental cell membrane, it is of interest to investigate whether STBEVs impair vascular function via activation of LOX-1.

We hypothesize that STBEVs contribute to endothelial dysfunction via the activation of LOX-1. Specifically, we hypothesize that exposing arteries to STBEVs would result in an increase in superoxide production and reduction in NO contribution to endothelial vasodilation.

3.2 Materials and Methods

3.2.1 Ethics Approval

The animal use protocols were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee, in accordance with the Canadian Council on Animal Care guidelines. The study protocol for human placenta and STBEV collection was approved by the Oxfordshire Research Ethics Committee C and conducted in Professor Ian Sargent's laboratory at the Oxford University in the U.K. Written informed consent was obtained from all study participants. The STBEV collection protocols were also approved by the University of Alberta Ethics Committee to be used for studies in our laboratory.

3.2.2 STBEV Collection and Preparation

The placenta and STBEV collections were performed at the University of Oxford under the supervision of Prof. Ian Sargent and shipped to our laboratory. Based on previous literature, there is a variety of methods for STBEV derivation; however, the method chosen for our study was based on Southcombe *et al.*'s study showing that STBEVs collected from placental perfusion represent more closely the pro-inflammatory characteristics seen in preeclampsia than those derived from other method [189]. They have also shown that this method consistently produces 80-90% of STBEVs stained positive for placental alkaline phosphate representing their origin. The received samples were collected in the manners as outlined here.

The placentas were collected from healthy pregnant women undergoing elective caesarean sections, without labor, with singleton pregnancies, and at term gestations. Women with any significant medical history or recent illnesses were excluded. The STBEVs were derived from placenta perfusion as detailed in Southcombe *et al.*'s paper [189]. Briefly, the placentas were collected at the time of delivery and processed immediately using a modified dual placental perfusion system as described by Eaton *et al.* [190]. An individual lobule was isolated and the fetal circulation was perfused with filtered tissue culture medium containing a 20-mL bolus of 100,000U streptokinase to promote clot removal. The placenta was turned upside down, placed inside a Perspex water jacket at 37 °C, and the maternal circulation was perfused with medium oxygenated at 95% O_2 and 5% CO_2. The placental lobule was perfused for 20 minutes to equilibrate the system and then the maternal circuit was closed with a total volume of 600 mL perfusion media. At the end of a 3 hour perfusion period, the maternal perfusate was collected and centrifuged at 600 x *g* for 10 minutes at 4 °C. The supernatant was then centrifuged at 150,000 x *g* for 1 hour at 4 °C. The pellets were pooled, washed in PBS, and suspended in PBS

to give a final protein concentration of 5 mg/ml. Protein content was measured using Pierce BCA protein assay kit (Thermo Scientific, Illinois, USA). The STBEVs were snap frozen and stored at -80 °C during shipment to our laboratory. Prior to experiments, the STBEVs were thawed on ice, aliquoted into physiological saline solution (PSS) for a final concentration of 200 µg/mL, and stored in -80°C until use. The concentration of STBEVs chosen for our studies was based on previous literature, which demonstrated perfusion of omental arteries with STBEVs at 200 µg/mL impaired ACh-mediated vasodilation and induced endothelial disruption under transmission electron microscopy [71].

3.2.3 Animal and Vessel Preparation

Three-month-old female Sprague Dawley rats were bred after acclimatization in 10:14-hour light:dark cycle cages with free access to standard rat chow and tap water. Day 0 of pregnancy was confirmed by the presence of sperm in a vaginal smear following an overnight mating. On day 20 of gestation, the animals were sacrificed by exsanguination while under isoflurane-induced anesthesia and a laparotomy was performed to harvest the uterine arteries. Given the findings that circulating factors in preeclamptic plasma impaired endothelial function in a vascular bed-specific manner as we have shown in Chapter 2, we chose to use only uterine arteries for the studies presented in this chapter. The uterine arteries were isolated under a microscope to ensure all connective tissues and adipocytes were removed. To investigate the effect of STBEVs as well as the involvement of the LOX-1 pathway, the arteries were incubated overnight at 4 °C in one of the following four groups: 1) control PSS; 2) control PSS + anti-LOX-1 antibody at 10 µg/mL; 3) STBEV at 200 µg/mL in PSS; 4) STBEV 200 µg/mL + anti-LOX-1 antibody at 10 µg/mL in PSS. The arteries were mounted for myography experiments on the following day after 22-24 hours of incubation (n = 9 per group).

3.2.4 Myography

Following an overnight incubation, uterine arteries were mounted on two 40-micrometer wires and attached to a wire myograph (DMT, Copenhagen, Denmark) for isometric tension recordings. The vessels were normalized and equilibrated for a minimum of 30 minutes before they were exposed to two wake-up doses of phenylephrine (10 μmol/L) and a single dose of methacholine (3 μmol/L) to ensure endothelial function and smooth muscle integrity. A cumulative concentration response curve to phenylephrine (0.001 to 100 μmol/L) was performed to determine the concentration that produces 80% of maximal constriction. This concentration was then used to preconstrict the vessels for endothelium-dependent relaxation curves using cumulative doses of methacholine (MCh; 0.0001 to 10 μmol/L). To study the contribution of NO to endothelial vasodilation, we used a pan NOS inhibitor, L-NAME (100 μmol/L; Sigma N5751), which was added to the vessel bath 30 minutes prior to the MCh concentration response curves (n = 9 per group). Similarly, to study the effect of NO scavenging by superoxide, we added superoxide dismutase-polyethylene glycol (pegSOD; 50 U/mL; Sigma S9549), which breaks down superoxide into hydrogen peroxide, to the vessel bath for 30 minutes of incubation prior the concentration response curves (n = 6 per group).

3.2.5 Superoxide detection

Superoxide production was measured by staining sections of uterine arteries with dihydroethidium (DHE; n=6-8). Dihydroethidium reacts with superoxide to yield ethidium, which binds to nuclear DNA and generates red fluorescence. After overnight incubation in either PSS or STBEVs, segments of uterine arteries were embedded in optimal cutting medium (OCT) and snap-frozen in liquid nitrogen for storage. Sections of the arteries were cut at 10 μm thickness, mounted on glass slides at -20 °C, and stored at -80 °C until use. On the day of

analysis, the slides were thawed, washed with HBSS 3 times at 2 minutes each, and incubated with fresh HBSS for 10 minutes at 37 °C in a humid chamber. The slides were then incubated with DHE for 30 minutes at 37 °C, washed with HBSS 3 times at 2 minutes each, cover slipped, and visualized under an IX81 Olympus fluorescence microscope.

3.2.6 Statistics

Statistical analyses were performed using GraphPad Prism software. Myography data was summarised using maximal response (E_{max}), the effective concentration required to achieve 50% of the maximal response (pEC_{50}) or area under the curve (AUC) values. The delta AUC between vascular responses in the absence or presence of L-NAME was compared between the groups using Student's t-test to summarize the contribution of NO to endothelial vasodilation. The effects of STBEVs between the groups and inhibitors within the group were compared using a two-way ANOVA and a Bonferroni *post hoc* test. The vascular function experiments using SOD and the DHE staining were compared using a one-way ANOVA and Dunnet's *post hoc* test. A P-value < 0.05 was considered statistically significant.

3.3 Results

3.3.1 The effect of STBEVs on vascular function in the uterine artery

Endothelium-dependent vasodilation was impaired in uterine arteries exposed to STBEVs as compared to the control vessels (Figure 3-1). We found that this effect was mediated via activation of the LOX-1 receptor as inhibition with an anti-LOX-1 antibody restored the endothelial vasodilation to a level comparable with the control arteries (Figure 3-1). Using a pan NOS inhibitor, we showed that the contribution of NO to endothelial vasodilation was significantly reduced in arteries exposed to STBEVs (Figure 3-2). As a proof of principle, we showed that inhibition of LOX-1 alone in the control arteries did not alter vascular responses nor

NO contribution (Figure 3-3). Conversely, in arteries exposed to STBEVs, the inhibition of LOX-1 significantly increased NO contribution to vasodilation; strongly suggesting that activation of the LOX-1 pathway by STBEVs results in a reduction of NO bioavailability (Figure 3-3).

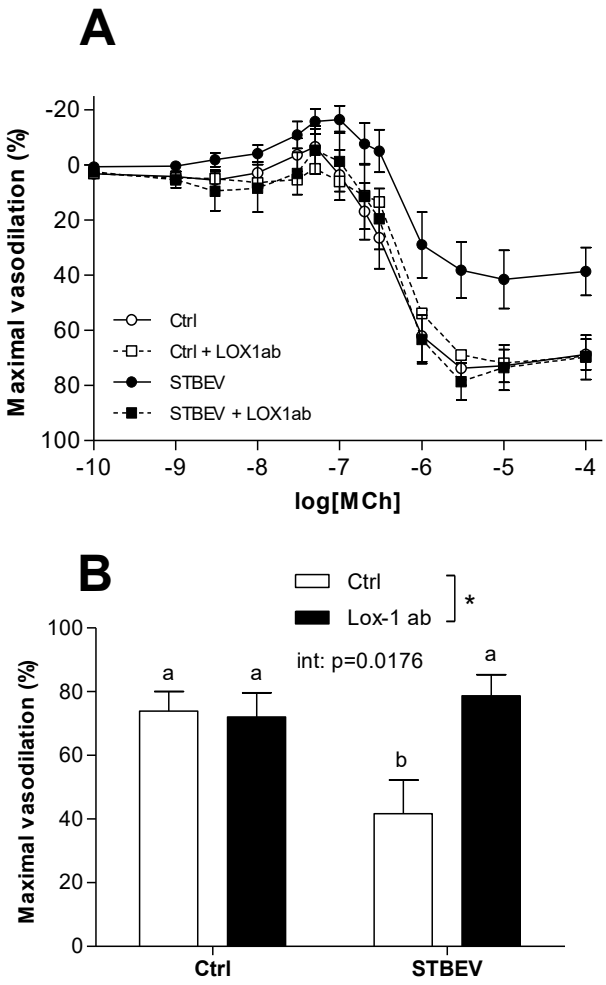

Figure 3-1: The effect of LOX-1 inhibition on vascular responses in uterine arteries exposed to control vs. STBEVs.

(A) Endothelial vasodilation was impaired by exposure to STBEVs and the inhibition of LOX-1 restored the vascular responses to a level comparable with the control vessels (n = 9 per group). **(B)** Bar graphs showed percent maximal vasodilation. By two-way ANOVA: * $P < 0.05$ and "a" denoted statistical difference from "b".

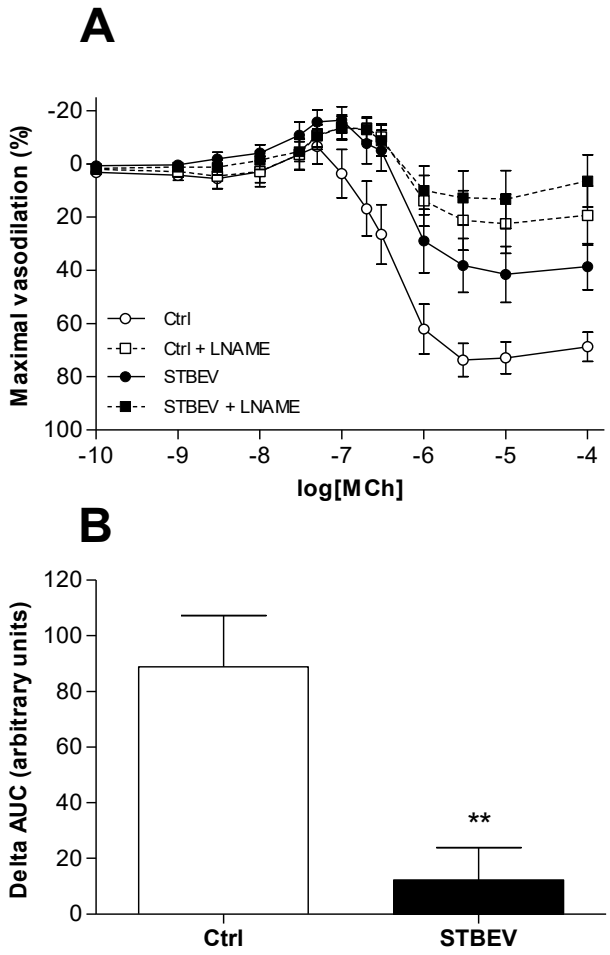

Figure 3-2: The effect of pan nitric oxide synthase inhibition on vascular responses in uterine arteries exposed to control vs. STBEVs.

(A) The contribution of NO to endothelial vasodilation was reduced in uterine arteries exposed to STBEVs compared to the control arteries (n = 9 per group). **(B)** Bar graphs showed delta AUC in arbitrary units. By Student's t-test: ** $P < 0.01$. L-NAME was a pan nitric oxide synthase inhibitor.

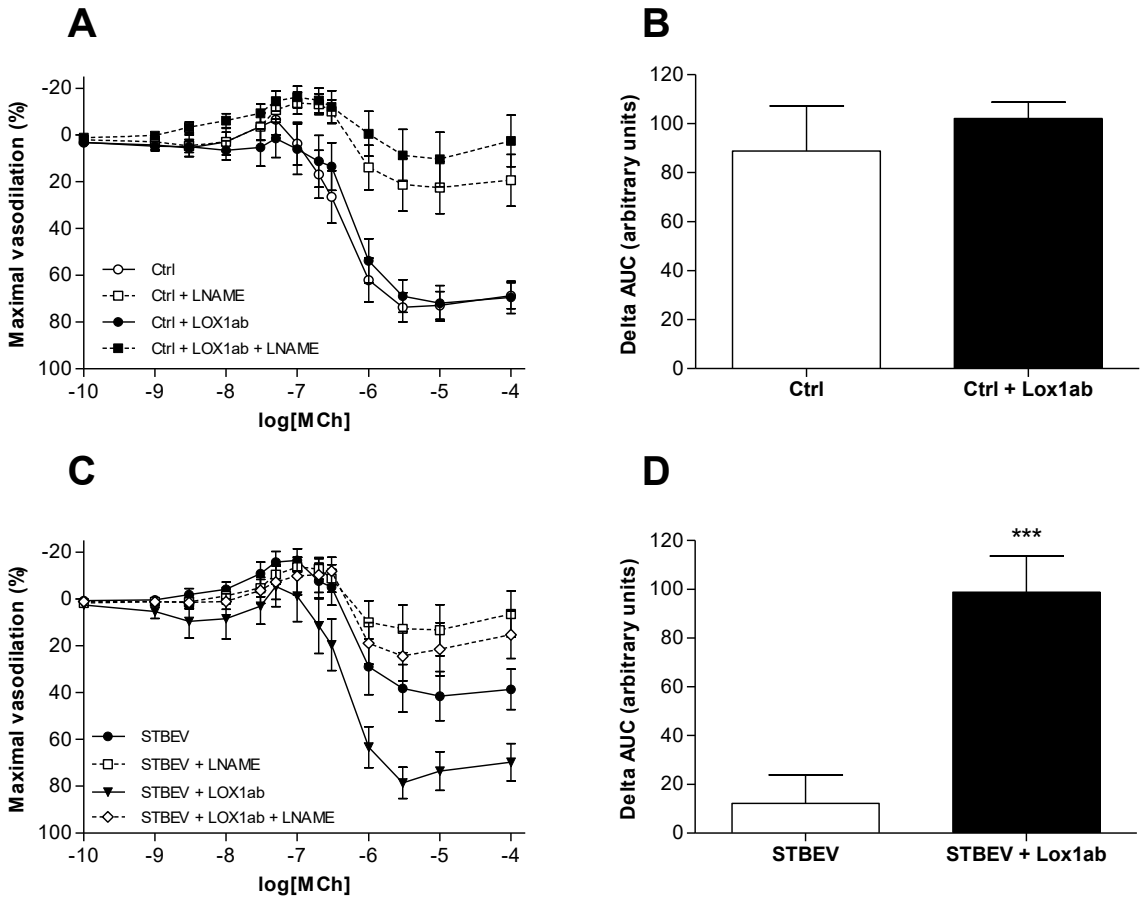

Figure 3-3: The effect of LOX-1 inhibition and nitric oxide synthase inhibition on vascular responses in uterine arteries exposed to control vs. STBEVs.

(A) The inhibition of LOX-1 had no effect on the contribution of nitric oxide to endothelial vasodilation in control arteries (n = 9 per group). **(B)** Bar graphs showed delta AUC in arbitrary units. **(C)** With the inhibition of LOX-1, the contribution of nitric oxide to endothelial vasodilation was increased in arteries exposed to STBEVs (n = 9 per group). **(D)** Bar graphs showed delta AUC in arbitrary units. By Student's t-test: *** $P < 0.001$. L-NAME was a pan nitric oxide synthase inhibitor.

3.3.2 The effect of STBEVs on superoxide production in the uterine artery

To investigate the role of superoxide in STBEV-induced vascular dysfunction, we utilized SOD which scavenges superoxide and converts it to H_2O_2. Contrary to our hypothesis, the addition of SOD did not affect STBEV-induced endothelial impairment (Figure 3-4 A-B). Furthermore, DHE staining in uterine arteries exposed to control, STBEVs, or STBEVs plus anti-LOX-1 antibody were not significantly different (Figure 3-4 C-D), suggesting that STBEVs did not affect superoxide production after a 24-hour exposure to the arteries.

3.4 Discussion

In this study, we have shown that STBEVs impair endothelium-dependent vasodilation via activation of the LOX-1 receptor. We have also demonstrated that arteries exposed to STBEVs exhibit reduced NO bioavailability that is associated with endothelial impairment. However, contrary to our hypothesis, exposure to STBEVs did not alter superoxide production in the uterine arteries. Regardless, our data support that STBEVs plays a role in vascular dysfunction through the activation of LOX-1 which is a potential target for intervention in preeclampsia.

Previous studies have shown that STBEVs impair endothelial vasodilation; however, to the best of our knowledge, we are the first to show that STBEVs act as ligand for LOX-1, which is known to play a role in endothelial dysfunction commonly seen in cardiovascular disease and preeclampsia [41, 168]. We have previously shown in Chapter 2 that arteries exposed to preeclamptic plasma exhibit impaired vasodilation via mechanisms of increased superoxide production and decreased NO bioavailability. Our group has also demonstrated that preeclamptic plasma increases superoxide levels in endothelial cells through the activation of NADPH by

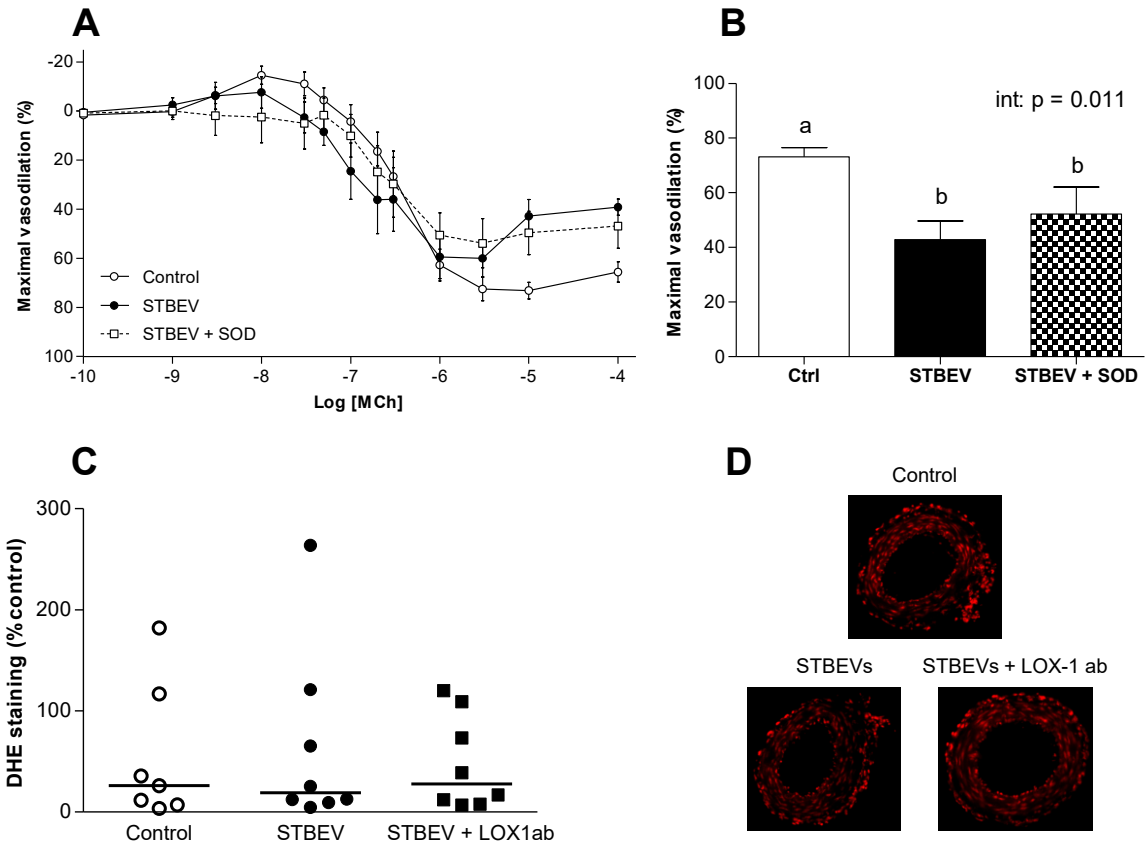

Figure 3-4[1]: The effect of STBEVs on superoxide production in uterine arteries.

(A) Endothelial vasodilation was significantly reduced in arteries exposed to STBEVs, but scavenging of superoxide with superoxide dismutase (SOD) did not alter the vascular responses (n = 6 per group). **(B)** Bar graphs showed percent maximal vasodilation. By one-way ANOVA: the interaction was significant and "a" denoted statistical difference from "b" at $P < 0.05$. (**C)** Dihydroethidium (DHE) staining, as a marker of superoxide level, after 24-hour exposure to either control or STBEVs was not significantly different between the three groups (n = 6-8). The inhibition of LOX-1 using the LOX-1 antibody did not affect vascular superoxide production. **(D)** Representative images of DHE staining were shown.

[1]The myography experiments and DHE staining presented in Figure 3-4 were performed and analyzed by Dr. F. Spaans.

LOX-1 [41]. Our current study supports the theory that STBEVs could alter endothelium-dependent vasodilation via activation of the LOX-1 receptor. LOX-1 is known to activate NAPDH oxidase which contributes to increased superoxide production and, when in excess, superoxide binds to NO to produce peroxynitrite which leads to impaired vascular function [90]. Although our study has shown that arteries exposed to STBEVs have significantly reduced NO modulation of vasodilation, which is restored in the presence of LOX-1 inhibition, we did not observe an effect on the vascular production of superoxide. One possible explanation for a decreased NO bioavailability in STBEV-exposed arteries is a reduced production by NOS rather than increased sequestration by superoxide. Previous studies have demonstrated that activation of LOX-1 by oxLDL can lead to an increase in the liberation of arginase from the mitochondria which increases catabolism of L-arginine, the primary substrate for NOS to produce NO [191]. We have also shown that isolated endothelial cells exposed to preeclamptic plasma exhibit increased arginase expression; however, this also induced uncoupling of eNOS leading to increased superoxide levels [192]. Alternatively, activation of the LOX-1 receptor and its downstream effects has been shown to be dependent on the concentration of its agonists. A recent study by Lin *et al.* has shown that a high concentration of oxLDL increases NADPH oxidase via LOX-1 activation, but a low concentration of oxLDL potentiates endothelial progenitor cell growth and increases eNOS activation [193]. We speculate that the LOX-1 mediated effect of STBEVs on endothelial function could also be concentration-dependent.

The role of STBEVs in vascular dysfunction is not well known based on the current literature. Previous studies using microparticles from preeclamptic women have demonstrated that microparticles do not alter ACh-induced endothelial vasodilation in virgin aorta and mesenteric arteries; instead, they have shown that microparticles reduced serotonin (5-HT)-

induced contraction via enhanced NO production [104, 105]. In contrast, our study has found that STBEVs impair endothelial vasodilation which is associated with reduced NO-mediated vasodilation. The difference in these findings might be due to several factors. We used pregnant rat uterine arteries, instead of virgin arteries, which arguably resemble the pathophysiology in preeclampsia more accurately because of the effect of numerous hormonal, immune, and vascular changes incurred during the pregnancy state. We also use pooled STBEVs collected from perfusion of the placentas, instead of microparticles derived from the plasma samples from women. Furthermore, microparticles come from a number of different sources, including, but limited to, those derived from leukocytes, platelets, and monocytes; therefore, their combined effect on vascular function might differ from those isolated from one single source, the placental syncytiotrophoblast, which is the type of STBEVs used in our experiments. Lastly, the vascular response between different animal species might also be variable.

Our finding that STBEVs impair endothelial function agrees with previous studies [71, 103], although the finding is not universal [102]. In the latter study, the arteries were perfused for three hours with STBEVs at a lower concentration of 20-2000 ng/ml and the authors found that STBEVs did not affect maximal endothelial vasodilation [102]. We acknowledge that the levels of STBEVs in pregnant women are found at a lower concentration than what has been utilized in our studies (i.e. 71.2 ± 20.7 ng/ml in early-onset preeclampsia compared to 26.3 ± 11.2 ng/ml in normal pregnancy, according to one study) [100]. However, we have designed our *ex vivo* experiments as a bioassay tool to examine the potential mechanisms behind the vascular pathophysiology in preeclampsia. In order to preserve arterial integrity for functional studies using myography, we have to limit the incubation time to 24 hours whereas, *in vivo*, the maternal vasculature would be constantly exposed to STBEVs throughout the duration of gestation.

Therefore, we used higher concentrations with a short exposure in order to establish a feasible tool that allows us to study the functional consequences of STBEVs on the endothelium.

In this study, we have provided evidence that STBEVs plays a role in the vascular dysfunction commonly seen in preeclampsia via activation of LOX-1. Not only does this further the collective understanding of the pathophysiology, but it also provides insight into the potential treatment or prevention of preeclampsia by targeting the LOX-1 pathway. From a clinical perspective, endothelial dysfunction is a key point of convergence underlying the pathophysiology of preeclampsia; however, the exact mechanism of how placental circulating factors affect the maternal vasculature is still under investigation. Our group has demonstrated that STBEVs, a specific type of extracellular microparticles and vesicles derived from placental syncytiotrophoblasts, impair endothelial vasodilation associated with reduced NO bioavailability via activation of LOX-1. This supports that LOX-1 could be a potential target for the treatment or prevention of preeclampsia. STBEVs could also potentially be used as a diagnostic or prognostic biomarker for diagnosing preeclampsia early in pregnancy to allow more appropriate surveillance and management.

CHAPTER 4: GENERAL DISCUSSION

4.1 Summary of the Most Significant Findings

In the first part of the thesis, we have demonstrated that circulating factors in preeclamptic plasma impair endothelial vasodilation in rat uterine arteries, but not in mesenteric arteries, highlighting important differences in the vascular responses among different vascular beds. We have further demonstrated that the mechanisms behind impaired vasodilation in preeclamptic plasma treated arteries included reduced NO-mediated vasodilation due to superoxide scavenging. Interestingly, and contrary to our hypothesis, the plasma-induced impairment was also associated with an increase in PGHS-dependent vasoconstrictors, rather than a reduction in prostaglandin vasodilators, since inhibition of PGHS led to an improved vasodilatory response.

In the second part of the thesis, we have investigated the role of pregnancy-specific STBEVs in endothelial function. We have shown that arteries exposed to STBEVs exhibit reduced endothelium-dependent vasodilation, which was associated with the activation of LOX-1 since the inhibition of LOX-1 improved the impaired response. We have further shown that this vascular impairment in STBEV-exposed arteries is associated with decreased NO contribution. However, we did not find a difference in superoxide production nor modulation of endothelial vasodilation in arteries incubated with STBEVs. Altogether, our studies highlight the role of placenta-derived circulating factors in the pathophysiology of endothelial dysfunction seen in preeclampsia.

4.2 The Effect of Circulating Factors in Endothelial Function

4.2.1 Healthy Endothelium in Normal Pregnancy

We have provided evidence that the endothelium in the uterine artery relies heavily on NO production during normal pregnancy. The majority of the NO present in the endothelium is produced from L-arginine by eNOS, some by iNOS, while nNOS plays a negligible role. We have confirmed that nNOS expression is undetectable in uterine arteries exposed to either normotensive pregnant or preeclamptic plasma. Furthermore, superoxide is one of the major sources of oxidative stress in the endothelial cell: it scavenges NO, decreases NO bioavailability, increases production of ONOO- and leads to oxidative cell damage. Of note, superoxide can be produced from various sources, such as mitochondria, PGHS enzymes, and NADPH oxidase [109]. Under normal physiological conditions, NO diffuses to the VSMCs and induces vasodilation by activating sGC which increases cGMP and decreases intracellular calcium levels. The endothelial cell can self-regulate by balancing excess superoxide production with antioxidants, such as SOD, which break down superoxide to H_2O_2 and further into water and oxygen by catalase [108]. Hydrogen peroxide is also known to act as an EDHF and causes vasodilation via activation of potassium channels on VSMCs [174, 175]. All in all, the endothelial cell contains many built-in mechanisms that regulate cellular functions and protect it from excessive oxidative stress in order to maintain homeostasis.

4.2.2 Alteration in Nitric Oxide Pathway and Reactive Oxygen Species in Preeclampsia

In women with preeclampsia, the presence of circulating factors alters these vasoactive pathways, which may contribute to the pathophysiology of endothelial dysfunction (summarized in Figure 4-1). First of all, we have demonstrated that circulating factors contribute to an increase

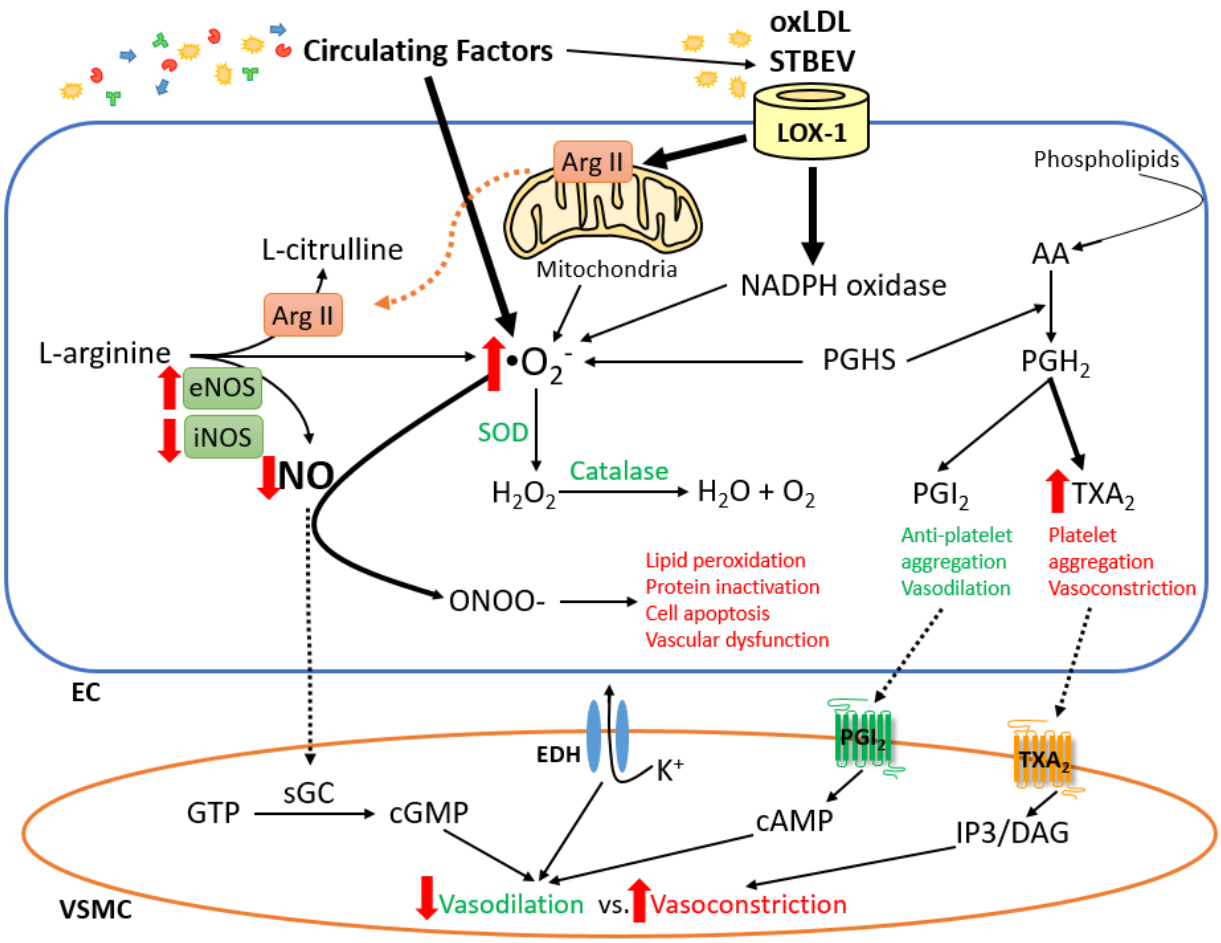

Figure 4-1: Summary of the major findings.

Abbreviations: oxLDL, oxidized low density lipoprotein; STBEVs, syncytiotrophoblast extracellular vesicles; LOX-1, lectin-like oxidized low density lipoprotein receptor-1; Arg II, arginase II; NADPH, nicotinamide adenine dinucleotide phosphate; ●O_2-, superoxide; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; H_2O, water; O_2, oxygen; ONOO-, peroxynitrite; sGC; soluble guanylyl cyclase; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; EDH; endothelium derived hyperpolarization; K^+, potassium ion; AA, arachidonic acid; PGHS, prostaglandin H synthase; PGH_2, prostaglandin H_2; PGI_2, prostacyclin; TXA_2, thromboxane; cAMP, cyclic adenosine monophosphate; IP3, inositol triphosphate; DAG, diacylglycerol; EC, endothelial cells; VSMC, vascular smooth muscle cell.

in superoxide production in arteries exposed to plasma from preeclamptic women. The Davidge laboratory has previously shown that preeclamptic plasma upregulates LOX-1 and NADPH oxidase in isolated HUVECs which contribute to an increase in superoxide and ONOO- production [41]. These ROS contain oxygen radicals and are known to cause several adverse effects such as lipid peroxidation, protein inactivation, cell apoptosis, and endothelial dysfunction [108]. Our studies support the hypothesis that preeclamptic circulating factors contribute to an increase in oxidative stress. We have also further shown the functional consequences of circulating factors, resulting in a reduction in NO bioavailability and impairment in endothelium-dependent vasodilation.

Moreover, we have found that circulating factors in preeclamptic plasma increase vascular eNOS expression and decreases iNOS expression. Our finding is in accordance with previous studies that have shown an up-regulation of eNOS in the preeclamptic placenta [116] and subcutaneous fat arteries from preeclamptic women [117]. We speculate that this may be a compensatory increase in NO production by eNOS in response to the reduced NO bioavailability observed in arteries exposed to preeclamptic plasma. However, contrary to our expectation, we have found that there is a down-regulation of iNOS in uterine arteries exposed to circulating factors. Preeclampsia is known to be a state of exaggerated inflammation and oxidative stress; therefore, one would expect an increase in circulating cytokines and ROS lead to an up-regulation of iNOS expression to further compensate for the enhanced vasoconstriction and reduced NO levels in the preeclamptic vasculature. However, our findings suggest that preeclampsia may be characterized by a lack of compensatory responses, which predispose the vasculature to the effect of increased contractility, thus developing hypertension and reduced perfusion to the end organs. This is supported by previous studies that have shown that chronic

infusion of TNF-α in pregnant rats induces a reduction in renal iNOS expression [194]. In addition, other groups have demonstrated an increase in iNOS expression in peripheral blood mononuclear cells (PBMCs) from normal pregnant women, but no change in PBMCs from preeclamptic women [195] and that there is a reduction in iNOS expression in preeclamptic syncytiotrophoblasts [196]. Therefore, our findings suggest that a lack of compensatory up-regulation in iNOS expression may be an important feature leading to endothelial dysfunction in preeclampsia.

Furthermore, both EDHF and NO have been shown to contribute to the physiologic decrease in peripheral resistance and blood pressure to accommodate an increase in blood volume and uterine blood flow to supply the growing fetus in a normal pregnancy [197]. Briefly, endothelium-derived hyperpolarization (EDH) is a collective term including endothelium-derived factors or chemical signals that act on the VSMCs to cause hyperpolarization and vasodilation which is not accounted for by NO and PGI_2 contribution [198]. In general, EDH is considered a feature of "healthy" endothelium and previous studies have shown that EDH contributes significantly to the control of vascular tone in small resistance arteries (i.e. diameter ≤ 300 μm) [198]. The physiologic contribution of EDH is also thought to be increased in pregnancy [199]. Indeed, it has been shown that NOS inhibition with L-NAME significantly impaired bradykinin-induced vasodilation in non-pregnant myometrial arteries, whereas vasodilation in pregnant myometrial arteries was maintained in the presence of NOS and PGHS inhibition [200]. Previous literature has also shown that H_2O_2, a metabolite of superoxide when broken down by SOD, can act as an EDHF in small resistance arteries by activating potassium channels in the VSMCs [175, 201]. On the contrary, studies have also shown that the level of H_2O_2 is inversely related to the level of NO in pregnancy [177] and that H_2O_2 stimulates arginase

metabolism and decreases the substrate availability of L-arginine [179]. In accordance with these findings, our data suggest that H_2O_2 acts as a vasoconstrictor in rat uterine arteries exposed to preeclamptic circulating factors since the addition of catalase restored the plasma induced endothelial impairment. We speculate that, in the presence of circulating factors, H_2O_2 may contribute to arginase metabolism of L-arginine, thereby causing a decrease in NO production and endothelial dysfunction. However, further studies are needed to elucidate the exact mechanism.

4.2.3 Alteration in Prostaglandin Pathway in Preeclampsia

Normal pregnancy is associated with an increase in prostaglandin vasodilators as demonstrated by an increase in PGI_2 levels in the placenta and maternal circulation as well as an increase in maternal urinary excretion of its major metabolite, PGF1α [126, 127]. However, in preeclampsia, there is evidence that the up-regulation of PGI_2 is compromised while an enhanced production of PGHS-dependent vasoconstrictors, TXA_2 and/or PGH_2, lead to increased peripheral resistance in the maternal vasculature [127, 130]. Our studies have shown that, in the presence of circulating factors, PGHS-dependent vasoconstrictors predominate and contribute to impaired endothelial vasodilation. This is supported by the fact that meclofenamate, which inhibits PGHS, restores endothelial function in arteries exposed to preeclamptic plasma. Since PGHS is also a known source of superoxide production in the endothelium [129], the increase in prostaglandin vasoconstrictors associated with exposures to preeclamptic plasma ties in with the proposed mechanism of circulating factors resulting in increased oxidative stress. Therefore, we speculate that circulating factors not only activate PGHS dependent production of TXA_2/PGH_2, but also contributes to an increase production of ROS, which scavenges NO and increase ONOO- production, both of which contribute to endothelial impairment. An increase of PGHS-

dependent vasoconstrictors has also been observed in other vascular pathologies. For instance, in humans with essential hypertension, it has been shown that indomethacin, a PGHS inhibitor, increases ACh-mediated vasodilation in human with essential hypertension, suggesting the predominance of PGHS-dependent vasoconstriction in adults with primary hypertension [202]. Similarly, in the ageing vasculature, it has been shown that PGHS-dependent vasoconstrictors contribute to impaired ACh-mediated vasodilation that is detectable in hypertensive adults beginning at 31-45 years of age and progressively increases with advancing age in adults aged 46 to 60 and those > 60 year of age [203]. Therefore, we speculate that an up-regulation of PGHS-dependent vasoconstriction may be the common underlying mechanism behind vascular diseases and may explain the reason why women with essential hypertension and/or advancing age are at a significantly elevated risk of developing preeclampsia in their pregnancies.

4.3 The Role of STBEV and LOX-1 in Endothelial Function

Our findings on the effect of circulating factors on endothelium has led us to the investigation of STBEVs, a pregnancy-specific circulating factor derived from syncytiotrophoblast cells. The concentration of STBEVs has been shown to be increased and associated with the heighted inflammatory responses and endothelial dysfunction seen in preeclampsia [98, 100]. As previously summarized in Figure 4-1, we have provided data that support STBEVs indeed contribute to endothelial dysfunction by activation of LOX-1, which is a multi-ligand scavenger receptor implicated in cardiovascular disease and preeclampsia [41]. Although LOX-1 is known to activate NADPH oxidase and contribute to superoxide production [153], our studies have shown that STBEVs do not increase superoxide production in uterine arteries and scavenging superoxide with SOD has no significant effect on the STBEV-induced endothelial impairment. One possible explanation for this discrepancy is that previous studies

have shown LOX-1 stimulation by oxLDL leads to the release of arginase from the mitochondria [191], which decreases the level of L-arginine as the rate-limiting substrate for NO production. Interestingly, the activation of arginase leading to L-arginine catabolism has also been shown to contribute to the uncoupling of eNOS, which in turns decreases NO and increases superoxide production [192]. Other studies have demonstrated that the activation of LOX-1 leads to a bi-phasic effect: low oxLDL stimulation results in endothelial progenitor cell growth and eNOS activation, while high oxLDL stimulation results in the activation of NADPH oxidase [193]. We speculate that there may also be an intricate regulation of LOX-1-dependent arginase metabolism and a delicate balance between 1) low stimulation which increases arginase, decreases the level of substrate L-arginine, and decreases NO bioavailability, and 2) high stimulation which leads to the uncoupling of eNOS and increases ROS production. In our studies, we speculate that the use of STBEVs from healthy placentas might provide a low level of stimulation of LOX-1 which contributes to arginase metabolism without inducing uncoupling of eNOS. This could provide an explanation for our finding that STBEV-treated arteries did not exhibit increased superoxide production nor did scavenging superoxide affect STBEV-induced endothelial impairment. This further leads to our directions of future studies which will be covered in a later section.

4.4 Limitations of the Studies

An important limitation of our studies using normal pregnant and preeclamptic plasma in Chapter 2 is the consideration of the heterogeneity among different blood samples from different women. Preeclampsia consists of a wide spectrum of disease that can vary in presentation from those with mild hypertension with concomitant proteinuria to those with severe preeclampsia presenting with hypertensive emergencies and/or serious complications, such as HELLP syndrome, renal failure, stroke, eclampsia, severe IUGR, or intrauterine fetal demise. The

multifactorial nature of the disease implies that the blood samples collected from individual women might vary in composition; thus, we have decided to pool plasma samples for our experiments in order to account for this variability. We acknowledge that, by combining the plasma from women with potentially different entity of the disease, we risk masking the most serious spectrum of the disease or, *vice versa*, exaggerating the disease pathophysiology of the mild spectrum. However, pooled plasma treatment also gives us distinct advantages in *ex vivo* and *in vitro* studies: 1) to enable us to interpret the effect of the pooled plasma to represent an "average" effect of the plasma combined from 10-12 randomly selected women, and 2) to allow us to perform several different experiments using the same pool of plasma in order to compare and contrast the results and ensure continuity of sample consistency.

Another limitation on the interpretation of our results is the lack of data on fetal outcome from whom the plasma samples were collected. It is well documented that the role of placental insufficiency is significant in the pathophysiology of preeclampsia and it is often associated with IUGR in those with severe disease. Notably, not all women with preeclampsia have IUGR, thus it is likely representative of a subset of patients with different pathophysiology involved. Therefore, it would have been of valuable information to know the birth weight and neonatal outcome of the infants born to women from which our plasma samples have been collected and pooled.

In addition, the plasma samples were collected from the Colombian population in a developing country. Although, traditionally, there has not been any distinction made between the pathogenesis of preeclampsia in developing versus developed country, the WHO does recognize the fact the there is a larger proportion of maternal mortality in South America due to hypertensive disorders of pregnancy than those in the developed countries (25.7% in Latin

America vs. 16.1% in North America) [17]. However, recent data have been published from the same population of Colombian women demonstrating that they share similar risk factors as previous studies, such as nulliparity, use of barrier contraception, and metabolic syndrome [172]. Biochemical analyses of the plasma samples from Colombian population have also shown a decrease in PIGF and an increase in sEng and oxLDL among women with severe preeclampsia compared to those without [204]. Therefore, we speculate that the underlying mechanism of action of the circulating factors would be similar regardless of ethnicity.

As previously discussed in Chapter 3, we acknowledge the concentration of STBEVs utilized in our study (200 μg/ml) is higher than those found at (patho)physiological levels (71.2 ± 20.7 ng/ml in early onset preeclampsia vs. 26.3 ± 11.2 ng/ml in normal pregnancy) [100]. However, previous studies using 20-2000 ng/ml of STBEVs in 3-hour perfusion with human myometrial arteries have shown no significant difference in endothelial function [102]. In contrast, Cockell *et al.* have found that perfusion with 200 μg/ml of STBEV impairs ACh-induced endothelial vasodilation in human subcutaneous fat arteries [71]. We argue that using STBEVs in *ex vivo* vascular experiments is a bioassay tool to examine the mechanism behind associated endothelial dysfunction rather than to replicate the exact pathophysiological conditions. It would be infeasible to mimic the *in vivo* environment using the (patho)physiologic concentrations and a prolonged exposure over months of gestation. In order to preserve the integrity of the vessels for *ex vivo* vascular experiments, we have utilized a maximum of 24 hour incubation; therefore, we anticipate that a higher concentration of STBEVs over a short duration of exposure is justified in order to study the mechanism behind endothelial dysfunction.

4.5 Future Directions

Our studies in Chapter 3 using STBEVs from healthy placentas provides strong evidence as a pilot study that STBEVs contribute to endothelial impairment via activation of LOX-1. Given these positive findings, we are now in the process of acquiring STBEVs from preeclamptic placentas (PE-STBEV). It has been shown that STBEVs from women with preeclampsia differ from those with normal pregnancies not only in concentration, but also in composition and size [98]. PE-STBEVs are found to be larger in size and contain distinct immune-regulatory proteins, which might contribute to their pro-inflammatory properties [98]. Therefore, it would be of interest to examine how they interact with the vasculature and whether or not they would also act as a ligand for LOX-1.

As previously discussed, our *ex vivo* myography experiment utilizes STBEVs at a higher concentration than physiologically observed. One possible way to overcome this would be to design an *in vivo* experiment using PE-STBEVs in animal models. It would of interest to see if continuous parenteral exposure to PE-STBEVs at a lower concentration via intravenous, intramuscular, or subcutaneous injections would induce endothelial dysfunction and possibly hypertension and inflammation in pregnant rats, thus further supporting the role of STBEVs in the pathogenesis of preeclampsia.

We also hope to further dissect the pathway downstream of LOX-1 receptor activation by STBEVs. As previously shown by our group, preeclamptic plasma activates LOX-1 and NADPH oxidase which results in increased superoxide production in isolated endothelial cells [41]. Other studies have also shown that LOX-1 activation may induce the release of arginase from mitochondria resulting in a reduction in NO production [191]. Therefore, it would be interesting to examine whether PE-STBEVs act via this pathway, which would explain our data that

STBEVs did not alter vascular superoxide production in uterine arteries after 24 hours of exposure. To further decipher the impact of STBEVs on NO bioavailability, we also propose to study the mRNA levels, protein expression, and phosphorylation levels of different NOS isoforms to understand their contributions to the mechanisms behind endothelial dysfunction.

4.6 Clinical Relevance and Application

With increasing maternal age and BMI in our society, both of which are recognized as major risk factors for preeclampsia, the search for knowledge and cure of preeclampsia is arguably more critical than it has ever been. This thesis highlights the importance of understanding the mechanism of action behind how placental circulating factors and, specifically, STBEVs, interact with maternal vasculature as implicated in the endothelial dysfunction seen in preeclampsia. This contributes to two important aspects of clinical care in high risk obstetrical patients.

First of all, STBEVs released from preeclamptic placenta are now recognized as a separate entity from those derived from normal placenta. Preeclamptic STBEVs have been found to be larger in size and carry different types of immuno-regulatory, complement, pro-inflammatory, and anti-angiogenic molecules [98]. Currently, the management and surveillance of preeclamptic patients are often challenging due to the variable onset of the disease, ranging from an insidious development over months to a sudden flash of serious complications over days. Identifying the function and composition of PE-STBEVs may one day shed light into finding a diagnostic or prognostic biomarker that allows early identification and prompt triaging of patients at risk of preeclampsia.

Secondly, STBEVs have been shown to interact with immune cells as well as endothelial cells which arguably are the two most important underlying mechanisms of the pathophysiology in preeclampsia. We have shown that STBEVs directly contribute to endothelial dysfunction in pregnant vasculature which is prevented by the inhibition of LOX-1; thus, this provides a potential therapeutic target for the development of drug to antagonize LOX-1 as a treatment for preeclampsia. In addition, a very recent clinical study has shown that the removal by apheresis of sFlt-1, an anti-angiogenic protein implicated in the pathophysiology, increases the duration of gestation and improves proteinuria in women with early onset preeclampsia [205]. Since PE-STBEV is also a circulating factor that is found to be increased in the maternal circulation, the removal of STBEV by plasma apheresis may provide an alternate method for treatment in preeclampsia.

As previously discussed, women with preeclampsia are at an elevated risk of cardiovascular morbidity and mortality in later life. Therefore, treating preeclampsia during pregnancy would not only improve the immediate health outcome of the mother and the infant(s), but it would also potentially further reduce the disease burden of women at risk in the latter period of their life span.

4.7 Conclusion

Altogether, our studies have contributed to the understanding of how circulating factors interact with endothelium in maternal vasculature in the development of preeclampsia. Our data support three important findings: 1) the effect of circulating factors in preeclamptic plasma is dependent on the type of vascular bed, 2) circulating factors in preeclamptic plasma impair endothelial vasodilation by increasing superoxide production, decreasing NO bioavailability, as well as increasing PGHS-dependent vasoconstrictors, 3) STBEVs are potentially one of the

bioactive factors found in preeclamptic plasma that contribute to endothelial dysfunction in maternal vasculature by activating LOX-1. By the means of this thesis, we hope that we have illustrated the importance of understanding placental circulating factors and STBEVs and the potential of this research leading to the development of a diagnostic test or treatment modality for preeclampsia, a disease which currently has no cure.

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