"***Only as a warrior can one withstand the path of knowledge. A warrior cannot complain or regret anything. His life is an endless challenge, and challenges cannot possibly be good or bad. Challenges are simply challenges."***

Carlos Castaneda, Tales of Power

University of Alberta

The Role of Matrix Metalloproteinase-2 in the Pathophysiology of a Reduced Utero-Placental Perfusion Pressure Model of Preeclampsia

by

Ali Abdalvand

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Dedication

I lovingly dedicate this thesis to my family especially:

My wife **Mahsa** for living each and every moment of the way with me;

Shirin and **Taghi** for encouraging me to pursue my dreams;

Tayebeh and **Lotfy** for their endless support;

Pedarjoon for showing me that it is never too late to learn something new and

Madarjoon for her wholehearted prayers for me.

Abstract

Preeclampsia is a leading cause of maternal and fetal morbidity/mortality and induced preterm birth. Endothelin-1 (ET-1) is a potent vasoactive agent, shown to be involved in the vascular endothelial dysfunction of preeclampsia. Big endothelin (bigET) is cleaved to ET-1 by several enzymes including matrix metalloproteinase-2 (MMP-2). I hypothesized that increased levels and/or activity of MMP-2 may lead to enhanced production of ET-1 and thus increase vasoconstriction in preeclampsia. I used the reduced utero-placental perfusion pressure (RUPP) model of preeclampsia and studied vascular function using mesenteric arteries from Sham and RUPP to test my hypothesis. I showed that: 1) vascular contractility in response to bigET was greater in RUPP, 2) the contribution of MMP-2 to bigET to ET-1 cleavage was greater in RUPP, 3) nitric oxide can modulate the function of MMP-2 and several other bigET cleaving enzymes. These novel findings can provide avenues for new therapeutic approaches to preeclampsia.

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List of Abbreviations and symbols

° C: Degrees Celsius

µ: micro

µg/mL: micro gram per milliliter

µm: micro meter

2-ME: 2-Methoxyoesteradiol

kg: kilo gram

ELISA: Enzyme-linked Immunosorbent Assay

ml: milliliter

ANOVA: Analysis of Variance

AT1-AA: Angiotensin II type I receptor agonistic autoantibody

ATP: Adenosine Triphosphate

BigET-1: Big Endothelin-1

BP: Blood Pressure

cGMP: cyclic Guanosine Monophosphate

Ctl: Control

DHE: Dihydroethidium

DNA: Deoxyribonucleic Acid

DOCA: Deoxycorticosterone Acetate

ECE: Endothelin Converting Enzymes

EDHF: Endothelium-Derived Hyperpolarizing Factor

eNOS: endothelial Nitric Oxide Synthase

ET_A: ET receptor A

ET_B: ET receptor B

ET-1: Endothelin-1

ET-2: Endothelin-2

ET-3: Endothelin-3

FADH2: Flavin Adenine Dinucleotide Hydroquinone form

g: Relative Centrifugal Force (RCF)

H_2O_2: Hydrogen Peroxide

HBSS: Hanks Balanced Salt Solution

H&E: Hematoxylin and Eosin

HUVEC: Human Umbilical Vein Endothelial Cells

IL-6: Interleukin-6

iNOS :inducible Nitric Oxide Synthase

mm Hg: millimeter mercury

IP3: Inositol Triphosphate

K_i: Dissociation constant

kDa: Kilo Dalton

KPSS: High potassium physiological salt solution

L/min: Liter per minute

L-NAME: L-Nitro-Arginine Methyl Ester

mg: milligram

mm: millimeter

mL: milliliter

MMP: Matrix Metalloproteinase

mol/L: Mole per Litre

mRNA: messenger Ribonucleic Acid

n: nano

NADH: Nicotineamide Adenine Dinucleotide

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NEP: Neutral Endopeptidase

NHBPEP: National High Blood Pressure Education Program

nNOS: neuronal Nitric Oxide Synthase

NOS: Nitric Oxide Synthase

$O2^{\cdot -}$: superoxide anion radical

O_2: oxygen

O.C.T.: Optimum Cutting Temperature

$OH^{\cdot}$: Hydroxyl radical

$ONOO^{-}$: Peroxynitrite

PAS: Periodic Acid-Schiff

PDGF: Platelet Derived Growth Factor

PlGF: Placental Growth Factor

PKC: Protein Kinase C

PSS: Physiological Saline Solution

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

RUPP: Reduced Utero-placental Perfusion Pressure

sEng :Soluble Endoglin

sFlt-1: FMS-like Tyrosine Kinase

SOD: Superoxide Dismutase

TIMP: Tissue Inhibitors of Metalloproteinases

TNF-α: Tumor Necrosis Factor

VEGF: Vascular Endothelial Growth Factor

vs.: Versus

Chapter 1:

Introduction

1.1 Prologue

Preeclampsia is a pregnancy-specific syndrome that is a major cause of maternal and fetal morbidity and mortality. While understanding the etio-pathologic process of preeclampsia has been a goal for many researchers that has led to numerous valuable findings, the exact pathophysiology of preeclampsia has yet to be revealed. My general hypothesis of this thesis is that, in preeclampsia due to decreased perfusion to the placenta, the placenta releases factors into the maternal circulation causing increased oxidative stress and vascular endothelial dysfunction. This oxidative stress up-regulates matrix metalloproteinase-2 (MMP-2) activity to produce increased endothelin (ET-1)-dependent vasoconstriction through increased cleavage of big Endothelin-1 (bigET-1). Although multiple lines of evidence suggest an involvement of ET-1 in the development of preeclampsia, the pathophysiological process that leads to this syndrome is not completely determined yet.

The focus of my research project was to investigate mechanisms that can lead to increased vasoconstriction that is observed in preeclampsia. I used an animal model of preeclampsia and focused my studies on MMP-2 as well as several other enzymes with an involvement in the production process of ET-1.

1.2 Preeclampsia

1.2.1 Definition, Signs and Significance

Preeclampsia is a common pregnancy complication which affects 2-8% of the pregnancies worldwide (1). The "National High Blood Pressure Education Program's Working Group Classification (2000 Report)", describes eclampsia-

preeclampsia as de novo appearance of hypertension and proteinuria after mid pregnancy. Hypertension in pregnancy is defined as a blood pressure reading of >140 mm Hg systolic, and/or > 90 mm Hg diastolic. Proteinuria is defined as excretion of protein in the urine > 0.3 gram in 24 hours. Eclampsia is the occurrence of seizure in women with preeclampsia that cannot be attributed to other causes. Overall, there is involvement of several organ systems including kidneys, liver and brain which imposes a significant risk on maternal health (2). In addition, preeclampsia accounts for up to 12% of infants born small for gestational age and 20% of preterm births (3). Hence, preeclampsia is a complication of pregnancy that has deleterious outcomes for both the mother and child. A better understanding of the mechanisms for the maternal vascular complications is needed to develop effective treatment approaches.

1.2.2 History

The history of medicine shows that seizures in pregnancy have always been of great concern. There are still debates about the meaning of the word eclampsia. Castelli (1682) in his *Lexicon Medicum* described eclampsia as the translation of the word lightning from Greek. And, some authors have claimed that eclampsia has been mentioned in the ancient records of Chinese, Egyptian and Indian medical literature (4). One of the first descriptions of this condition is from ancient Greece. Hippocrates (460-370 BC), in his Aphorisms (Sec. VI, No. 30), wrote: “It proves fatal to a woman in a state of pregnancy, if she be seized with any of the acute diseases.”. Celsus, from the 2nd century, has referred to fatal

convulsions associated with the delivery of a dead fetus. The German physician, Rösslin, wrote a book in the 16th century which was the standard text book of midwifery for two centuries in Europe. Under maternal prognosis in labor, Rösslin discussed unconsciousness and convulsions as prognostic factors of fetal demise (5). Later in the 17th and 18th centuries, French physicians started taking over obstetrics from midwives and in their books they addressed eclampsia (4). In the 19th century, several physicians documented the association of proteinuria and hypertension with eclampsia. These observations led to the finding that hypertension and proteinuria are predictive factors for eclampsia and therefore, this syndrome of hypertension, edema and proteinuria was named preeclampsia.

1.2.3 Management

As it can be conveyed from its literal meaning, lightening, clampsia/preeclampsia has a stormy character. Therefore both American College of Obstetricians and Gynecologists and a National High Blood Pressure Education Program (NHBPEP) Working Group recommend close observation even if preeclampsia is only a distant possibility (6, 7). According to the latest version of "Chesley's Hypertensive Disorders in Pregnancy" (4), the basic management goals for preeclampsia can be summarized as: 1) termination of pregnancy with the least possible trauma to mother and fetus; 2) birth of an infant who subsequently thrives; 3) complete restoration of health to the mother. These considerations in the management of preeclampsia lead to birth of premature

infants in many cases and thus preeclampsia is one of the most important causes of premature birth.

After more than two thousand years that this condition has been known to humans, medicine is incapable of curing it and the treatment is limited to symptomatic therapy, for the most part. This is largely due to the fact that the specific etiology and pathology of preeclampsia is yet to be determined, thus making the preventive and therapeutic strategies hard to plan.

1.2.4 Pathophysiology of Preeclampsia

In the scientific literature there are numerous of publications on the pathophysiology of preeclampsia. Several factors including nutritional, genetic, immunologic and anti-angiogenic processes have been proposed as element in the pathophysiology of preeclampsia (8).

Pathophysiologic theories about preeclampsia are mostly based on the consideration of it being a two stage disease. The first stage is reduced perfusion of the placenta that leads to the second stage, of the maternal syndrome (9). The reduced perfusion is the result of shallow trophoblast invasion to the uterine vasculature (endovascular invasion) and the spiral arteries are not remodeled properly (10). The under perfused placenta is believed to release factors that cause the maternal syndrome. Cytokines (11), anti-angiogenic factors (12) and syncytiotrophoblast microparticles (13) are some of the factors which are produced by the placenta and have been proposed to be involved in the initiation of the pathophysiology of preeclampsia. Interestingly, all of these factors can lead

to oxidative stress which is an imbalance between the production and manifestation of reactive oxygen species (ROS) and the abilities of a biologic system to neutralize the processes and effects. Moreover, oxidative stress has been proposed as a linking process between the etiologic factors and the clinical syndrome of preeclampsia (14).

1.3 Oxidative Stress in Preeclampsia

1.3.1 Reactive Oxygen Species

Reactive oxygen species (ROS) include the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). Superoxide anion, which is produced by the addition of an electron to the molecule of oxygen (O_2), is the precursor for most of ROS and oxidative chain reactions (15). Dismutation of superoxide produces the more stable H_2O_2 that may be reduced to water and oxygen. In the presence of iron, hydrogen peroxide and superoxide can lead to the production of highly active hydroxyl radical (16).

The state of increased oxidative stress can also produce reactive nitrogen species (RNS). One of the important RNS is peroxynitrite ($ONOO^-$). This short-lived highly reactive molecule is the product of the reaction between superoxide and nitric oxide. This ultra-fast reaction is controlled at the rate of diffusion for both components (17-19) .

1.3.2 Source and Physiologic Role of Reactive Oxygen Species

The aerobic respiratory system in the mitochondria is one source of superoxide and hydrogen peroxide (20). Inside the mitochondria, adenosine triphosphate (ATP) is produced through oxidative phosphorylation. In this process, electrons move from nicotineamide adenine dinucleotide (NADH) or flavin adenine dinucleotide hydroquinone form ($FADH_2$) to O_2. This process generates superoxide (21). Mitochondria are also rich sources of intracellular nitric oxide and peroxynitrite (22, 23). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, cyclooxygenases, lipoxygenases, monooxygenase are enzymatic pathways that are believed to be extra mitochondrial sources of ROS (24).

ROS can also function as a second messenger in cells. It regulates signal transduction pathways that control gene expression and posttranslational changes of proteins in cell function, growth, differentiation and death, reviewed by Al-Gubory *et al.* (15). ROS are actively involved in developmental processes and cell survival though interactions with hypoxia-inducible factors, peroxisome proliferator-activated receptsor-γ coactivator, nuclear respiratory factor-1 and nuclear factor kappa B (25-27). They are also actively involved in programmed cell death (apoptosis) (28).

1.3.3 Reactive Oxygen Species and Oxidative Stress in Preeclampsia

Oxidants increase throughout gestation in a normal pregnancy, however, antioxidant mechanisms and systems become more predominant thus keeping the

balance (29-31). In contrast, in preeclampsia there exists a state of increased oxidative stress. Several studies have shown an increase in oxidative stress markers in the placenta, plasma, serum, erythrocytes and vasculature from preeclamptic women, reviewed in (15).Moreover the antioxidant capacity appears to be diminished in preeclamptic women. This has been shown in the case of enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase, glutathione-S-transferase (32, 33) and non-enzymatic antioxidants like vitamins C and E, carotenoids (vitamin A, β-carotene and lycopene) and total thiol (34-37). At odds, there are also reports of increased placental enzymatic antioxidants such as glutathione, glutathione peroxidase and catalase (35). Some other studies have also found either no decrease or increased levels of Vitamin E and carotenoid (37-39). The reasons for these contradictory findings can possibly be the result of differences in the severity of preeclampsia between different patient groups, different measurement techniques and even as an adaptive response to an increased oxidative stress (17).

Another source of oxidative stress is activated neutrophils and monocytes. These cells generate superoxide by NADPH oxidase activity and their role is prominent in preeclamptic women (40). Activated neutrophils can cause further oxidative stress by producing cytokines such as interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) and also increasing expression of vascular adhesion molecule VCAM-1(41).

As previously described, peroxynitrite is a high potency short-lived free radical. It causes cellular injury by interacting with lipids, deoxyribonucleic acid

(DNA) and proteins via either direct oxidative reaction or indirect radical mediated mechanisms reviewed extensively in (42). Nitrotyrosine is a foot print of peroxynitrite that has been shown to be increased in the placental villous tissue as well as vasculature of preeclamptic women (43, 44). Peroxynitrite may play such a prominent part in pregnancy complications since its production is directly affected by nitric oxide which is increased in pregnancy.

1.4 Nitric Oxide and Preeclampsia

Nitric oxide is a potent vasodilator and is produced in the process of transformation of L-arginine to L-citrulline by enzymatic activity of nitric oxide synthase (NOS) (45). There are three types of NOS: neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II) and endothelial NOS (eNOS or NOS III). Nitric oxide exerts its vasodilatory effects through cyclic guanosine monophosphate (cGMP) as the second messenger. During normal pregnancies, increased production of nitric oxide from vascular endothelial cells is, in part, responsible for the observed vascular changes (46, 47). The expression and activity of eNOS from uterine arteries are also increased during pregnancy (48). The data, however, from studies that have examined nitric oxide levels in preeclamptic women are conflicting. Since the biological half-life of nitric oxide *in vivo* is seconds, measurement of a relatively more stable by-product of nitric oxide, nitrite/nitrate, has been used to study nitric oxide levels. The studies probing nitrite/nitrate in preeclampsia showed decreased (49-52), increased (53-56) and unchanged (57-60) levels compared to normal pregnancies. Nonetheless,

the quantification of cGMP in urine, plasma and platelets, was consistent in showing lower levels in preeclamptic women compared to normal pregnant independent of nitrite/nitrate levels (60-63). While the paradoxical results of nitrite/nitrate levels could be attributed to different measurement techniques, small sample size or different bioavailability of nitric oxide in different tissues, the complementary results from cGMP measurement in preeclampsia is indicative of a general state of decreased bioavailability of nitric oxide in preeclampsia.

It is interesting that there is evidence for increased eNOS and decreased nitric oxide in preeclampsia. While decreased nitric oxide could be due to scavenging by superoxide, several mechanisms have been proposed for the role of NOS in decreased nitric oxide production as well as increased superoxide generation one of the most important of which being eNOS uncoupling. Some of the proposed mechanisms are briefly discussed here: 1) deficient L-arginine transport system was proposed by McCord *et al.* and Neri *et al.* after they showed this defect in polymorphonuclear leukocytes and platelets from preeclamptic women (64, 65); 2) deficiency in the co-factors necessary for normal activity of eNOS including ionic calcium (66, 67) and tetrahydrobiopterin (BH_4) (52, 68); 3) asymmetric dimethylarginine (ADMA), an endogenous inhibitor of eNOS, was shown to be increased in preeclamptic women (69-72); 4) decreased arginine, due to enhanced arginase activity is another proposed pathway. L-arginine is a common substrate for both NOS and arginase. Arginase catalyzes the transformation of L-arginine to L-ornithine and urea (73). Having a common substrate gives the ability to arginase to impose regulatory effects on NOS; therefore increased arginase

activity decreases the production of nitric oxide. Increased mitochondrial arginase (arginase II) expression has been shown in placenta and vascular endothelial cells from preeclamptic women (74, 75). Overall, decreased substrate for eNOS, not only decreased nitric oxide production, but also leads to superoxide production by uncoupling of eNOS (76, 77); 5) other possibility is lower enzymatic activity of NOS secondary to polymorphic variations (78). 6) In addition S-nitroso albumin acts as a stable reservoir of nitric oxide which releases this molecule when the concentration of low-molecular weight thiols is increased. It has been shown that in preeclampsia, plasma levels of S-nitroso albumin are increased (79). This could be due to decreased levels of vitamin C in preeclampsia which is an essential factor for decomposition of S-nitroso albumin and nitric oxide release (80).

While bioavailability of nitric oxide could well be affected by expression levels and activity of NOS, this decreased bioavailability could possibly have a positive feed-back thus a compensatory increase in NOS expression. Indeed, eNOS expression has been shown to be increased in women with preeclampsia. Myatt *et al.*, used an immunostaining technique and showed a significant increase in the expression of eNOS on the endothelium of terminal villous capillary and stem villous vessels of placentas from preeclamptic pregnancies (81). In addition, NOS activity was also reported to be increased when cultured endothelial cells were exposed to sera from preeclamptic women (82, 83). This increased activity of NOS however, would potentially do more harm than good. Because, as previously described, if NOS is left uncoupled due to insufficient substrate, it will produce more superoxide. And if it actually produces more nitric oxide, it will be

consumed in reaction with superoxide (already increased in the system) and produce peroxynitrite. Furthermore, peroxynitrite can disrupt the vascular function by inhibiting prostacyclin (84) and cyclooxygenase (85) in favor of vasoconstriction.

1.5 Vascular Endothelial Dysfunction and Oxidative Stress in Preeclamptic

While the placenta seems to be the site where everything starts in preeclampsia, the most important target is the maternal vascular endothelium (86). In preeclampsia the maternal inflammatory response is up regulated and the vascular endothelium becomes dysfunctional globally by factors released from the under perfused placenta (87). Several studies have shown endothelial activation using different markers including von Willebrand factor, cellular fibronectin, soluble tissue factor, soluble E-selectin, PDGF and endothelin (88, 89). *In vitro* experiments have also confirmed this finding by showing endothelial dysfunction in cells which were incubated with serum from preeclamptic women (90-94)**.**

Apart from the indirect measures of activated or inflamed endothelium that have been already discussed, there is evidence of actual vascular endothelial oxidative stress and damage in preeclampsia in the literature. Electron microscopy of arteries from myometrium and subcutaneous fat from preeclamptic women have revealed the intercellular junctions between endothelial cells to be disrupted (95, 96). Faster disappearance of intravascular albumin-bound Evans blue dye is another indicator of increased vascular permeability in the vasculature of preeclamptic women which is due to structural changes in the endothelial lining

of vessels (97). Zhang *et al.* described that the permeability of endothelial monolayer was increased in the presence of sera from preeclamptic women in the culture media. Moreover, they showed that this increased permeability could be inhibited by adding SOD to the media (98). These experiments suggest that circulating factors from preeclamptic sera disrupt endothelial function via a ROS-mediated mechanism. The evidence for oxidative stress has also been observed by showing increased nitrotyrosine staining in subcutaneous vascular tissue from preeclamptic women (44). All these studies indicate that in preeclampsia the endothelial cells are constantly exposed to increased levels of factors that cause oxidative stress. An example of the effect of oxidative stress on endothelial cells is lipid peroxidation of biological membranes and oxidative modification of lipoproteins (99). This process has been shown to be enhanced in preeclampsia by means of measuring some of the products of lipid peroxidation such as malondialdehyde and F2-isoprostane (100, 101).

Studies conducted on hemodynamic characteristics of women with preeclampsia have not only shown increased responsiveness to vasoactive factors (e.g. angiotensin II and norepinephrine) but also revealed impairment in endothelium-dependent vasorelaxation (102-104). These changes can even be seen before the syndrome manifests itself fully. In addition to decreased nitric oxide which was previously described, other factors have been shown to be involved in the impaired vasodilatory response of vasculature including prostacyclin (PGI_2) and Endothelium-Derived Hyperpolarizing Factor (EDHF).

These two vasodilatory factors have been shown to be decreased in preeclampsia (105-107).

On the other hand, other vasoconstrictors have been shown to be involved in preeclampsia. Gant *et al.* found that preeclamptic women, show increased angiotensin II sensitivity in their adrenal cortex and vascular system (103, 108). On top of that, Wallukat *et al.* found that preeclamptic women have an angiotensin II type I receptor agonistic autoantibody (AT1-AA) which can cause excessive angiotensin II type 1 receptor stimulation (109). From the same category, Endothelin-1 (ET-1) is a potent vasoconstrictor agent that appears to be increased in preeclampsia (51, 110-114), which is a focus of this thesis.

1.6 Endothelin

1.6.1 Discovery

The discovery of endothelin began in 1985 when Hickey *et al.* in a series of experiments exposed porcine, bovine, and canine coronary arteries to media obtained from bovine aortic endothelial cell culture and observed a dose dependent constriction. They proposed that endothelial cells produce a polypeptide that might have regulatory effects on the contractility of vascular smooth muscle cells (115). Yanagisawa *et al.* in 1988 identified this polypeptide and called it endothelin-1 or ET-1 (116).They figured out that ET-1 is a very potent vasoconstrictor with 21-amino-acid in its structure. Shortly after, Inoue *et al.*, discovered another two members of this family of peptides by further analyzing the ET-1 gene and named them endothelin-2 (ET-2) and endothelin-3

(ET-3) (117). They all have been shown to be vasopressors, with different pharmacologic characteristics. It is noteworthy that the peptide sequence for each of these agents is strictly preserved in most mammalian species.

ET-1 is the most extensively studied member of this family and is deemed to be the predominant isoform in the human cardiovascular system; hence in the following discussions the emphasis will be on ET-1.

1.6.2 Endothelin-1 and Big Endothelin-1

ET-1, like other members of its family, closely resembles sarafotoxin found in the venom of the Atractaspis engaddensis snake (118). The ET-1 gene is located on chromosome 6 (119) and encodes a 2,026-base pair messenger ribonucleic acid (mRNA) (120). A 212-amino acid preproendothelin-1 is encoded by the mRNA and then, after removal of a short sequence of amino acids, proET-1 is yielded. Production of ET-1 depends on a post-translation process. In this process, the proET-1 is cleaved by endopeptidases, including convertases, furin and PC7, to produce bigET-1 (116, 121, 122) (**Figure 1.1**).

1.6.3 Cleavage of Big Endothelin-1 to Endothelin-1

BigET-1 is a 38 amino acid peptide. And although bigET-1 has some vasoconstrictor properties itself, it needs is cleaved to produce a vasoactive 21 amino acids peptide with 140 times higher potency, called ET-$1_{(1-21)}$, which is fully physiologically functional (123). This cleavage process can be done by several enzymes (**Figure 1.2**).

1.6.3.1 Endothelin Converting Enzymes

Endothelin Converting Enzymes (ECEs) are members of the Neprilysin family, a thermolysin-like zinc metalloendopeptidase (124). Three isoforms have been discovered: ECE-1, -2 and -3 (125). ECE-1 and -2 catalyze the hydrolysis of Trp_{21}-Val_{22} in bigET-1 to cleave it to $ET\text{-}1_{(1\text{-}21)}$. ECE-1 is mainly localized to endothelium and has greatest affinity for bigET-1 (126). ECE-1a, -1b, -1c and -1d are the 4 isoforms of ECE-1 resulting from alternative splicing of ECE-1 gene which only differ in the N-terminus (127-130). This difference between the isoforms results in different localization of these enzymes. ECE-1a is present in intracellular vesicles and on the cell surface; ECE-1b is localized in endosomal compartment near the trans-Golgi network and ECE-1c and -1d are on the outside of the plasma membrane (125, 131). The physiologic importance of the different localization of ECE-1 isoforms is that bigET-1 can be cleaved into ET-1 both inside and outside of the cell. Identifying ECE-1 in the vesicles led to the hypothesis that ET-1 could be stored and secreted whenever required. This hypothesis has been proven to be true in endothelial cells where ET-1 can be stored in and released from Weibel-palade bodies (classically known to be reservoirs for von Willebrand factor and P-selectin) (132-134).

ECE-2 is 59% homologous with ECE-1 and can also cleave bigET-1 to ET-1. However, ECE-2, unlike ECE-1 which is optimally active in a neutral pH range, shows its biochemical properties in acidic pH (135). The previously mentioned ECE-3 has no known effect on bigET-1 but is able to cleave bigET-3 to ET-3 (136).

1.6.3.2 Chymase and Cathepsin G

Chymase is a member of the serine protease family of enzymes which is mainly found in mast cells. This enzyme also has been shown to be capable of cleaving bigET-1 at the Tyr_{31}-Gly_{32} bond to produce $ET\text{-}1_{(1\text{-}31)}$ (137-139). $ET\text{-}1_{(1\text{-}31)}$ can cause contraction in both vascular and nonvascular smooth muscle (140, 141). Polymorphonuclear leukocytes also contribute to cleavage of bigET-1 to $ET\text{-}1_{(1\text{-}31)}$ by production of Cathepsin G (142).

1.6.3.3 Neutral Endopeptidase

Also known as neprilysin, neutral endopeptidase (NEP) is a membrane-bound zinc dependent metalloproteinase. NEP is another enzyme with the capability to cleave bigET-1 to $ET\text{-}1_{(1\text{-}21)}$ (143, 144). Interestingly, owing to its capacity to cleave the amino side of hydrophobic amino acids, NEP can also cleave $ET\text{-}1_{(1\text{-}31)}$, produced by other enzymatic pathways, to $ET\text{-}1_{(1\text{-}21)}$ (145).

1.6.3.4 Matrix Metalloproteinase-2

Matrix metalloproteinase-2 (MMP-2) is another enzyme which was shown to be involved in cleavage of bigET-1 and due to its possible involvement in pathologic states generated a lot of excitement. This enzyme, its function and interaction with bigET-1 will be discussed more extensively later in its own dedicated section of this thesis.

1.6.3.5 Other Enzymes

There are other enzymes that have been shown to be able to cleave bigET-1 to ET-1. Endothelin converting enzyme-like-1, for example, is a putative zinc metalloproteinase which is similar to ECE-1 with a possible role in control of respiration in the nervous system (146). Kell blood group protein and soluble secreted endopeptidase can also potentially function similarly to ECE due to resemblances in their binding sequences (147, 148).

Similar to many other biologically active substances, ET-1 exerts its effects by binding to its specific receptors.

1.6.4 ET Receptors

There are two main receptors for ET, ET receptor A (ET_A) and ET receptor B (ET_B). ET_A is a 427 amino acid peptide which is coded by a gene on chromosome 4 (149). The human ET_B receptor has 442 amino acids and its coding gene is located on chromosome 13 (150). ET receptors are expressed ubiquitously in almost all human tissues, including the heart (151), lung (152), brain (153), kidney, adrenal, cerebellum, spleen (154) and the vasculature which is going to be the focus of this thesis. ET receptors are members of seven transmembrane segment G-protein-coupled receptor superfamily(155). Each receptor has an extracellular amino domain, seven membrane-spanning domains consisted of hydrophobic amino acid residues and an intracellular carboxy terminal. The endothelin receptors contain a highly conserved Asp-Arg-Tyr sequence of amino acids in the second intracellular loop, which is thought to be involved in g-protein

coupling(155, 156). As expected from the structure of the ET receptors, intracellular effects of ET involves coupling via G-proteins. Many of the functional responses to ET are mediated through pertussis toxin-sensitive and – insensitive pathways, indicating the involvement of multiple G-proteins (155). Almost all of the G-proteins that ET receptors couple with are from G_{α} subunit (123). However, there are also a few pathways that involve $G_{\beta\gamma}$ coupling(157).ET receptors couple with G_i, G_q, G_s, $G_{q/11,}$ $G\alpha_{12/13,}$ G_o etc. to activate different signaling cascades and enzymes, including adenylyl cyclases, cyclooxygenases, cytochrome P-450, NOS, serine/threonine kinase, tyrosin kinase, exert their biological effects (158164).

1.6.4.1 ET_A Receptors

ET_A has two subtypes: ET_{A1} and ET_{A2}. These subtypes are categorized based on their sensitivity to the ET_A receptor blocker BQ-123. ET_{A1} which is sensitive to BQ-123 is the main subtype and has been found on the smooth muscle cells of most arteries and the non-BQ-123 sensitive ET_{A2} has been found in the saphenous veins of humans (165, 166). When ET-1 binds to ET_A, it causes an unusually long lasting effect due to the almost irreversible nature of this binding (167, 168). As previously described the mechanism of action of the ET_A receptor involves binding to G_{α} proteins. As reviewed extensively by Brunner *et al.* (169) this receptor excitement process triggers a signaling cascade involving phospholipase C and inositol triphosphate (IP_3). IP3 increases the concentration of intracellular Ca^{2+} by activating voltage-gated Ca^{2+} channels and mediating Ca^{2+} release from

the sarcoplasmic reticulum. This increase in Ca^{2+} causes activation of calmodulin. IP3 can also activate diacylglycerol which by itself can activate protein kinase C (PKC). Calmodulin and PKC activate myosin light-chain kinase and cause vascular smooth cell contraction.

1.6.4.2 ET_B Receptors

ET_B has two subtypes: ET_{B1} and ET_{B2} (170). ET_{B1} is the major receptor on smooth muscle cell and ET_{B2} is mostly presented on the endothelial cell surface. Despite their minor structural differences, these two subtypes are involved in signaling pathways with opposite functional results.

Excitation of the ET_B receptor on vascular smooth muscles causes similar responses as previously described for the ET_A receptor after binding to G_α signaling protein. This ET_B-induced signaling pathway, however, comprise only a small portion of the total vasopressor effects of ET-1 (171). On the other hand, ET_B receptors on endothelial cells cause indirect vasodilatory effects by increasing the production of nitric oxide and PGI_2 (172, 173).

Clearance of ET-1 is another role proposed for ET_B receptors by Fukuroda *et al.* for the first time (174). This function was confirmed in several studies observing a rise of more than 20-fold in interstitial ET-1 levels after blocking ET_B receptors using a highly selective receptor antagonist, BQ-788 (175-177).

1.6.5 Vascular Effects of ET-1

One of the interesting properties of ET-1, as a potent vasoconstrictor, is that under physiological conditions it essentially contributes to basal vascular tone. This feature was first discovered after ET-1 antagonists were infused to normotensive human volunteers and decreased vascular resistance was consequently observed (178, 179). This effect was attributed to ET_A receptor actions since comparable results were seen in the presence of an $ET_{A/B}$ antagonist and ET_A specific antagonist (BQ-123).

Administration of ET-1, on the other hand, causes a biphasic response both *in vivo* and *ex vivo*. This response involves an initial transient decrease in arterial pressure (vasorelaxation) followed by a long-lasting vasoconstriction (180). The vasodilator effect depends mainly on ET_B signaling by an intact endothelium (181) while vasoconstriction mostly relies on the ET_A signaling pathway (182). ET-1 not only causes vasoconstriction itself, but is also capable of potentiating vascular contractile responses to other vasoconstrictors such as serotonin in coronary and cerebral arteries (183, 184).

There are also other effects attributed to ET-1 in the vascular system. For instance, ET-1 is involved in vascular remodeling (185, 186) and has also been shown to induce angiogenesis and endothelial cell proliferation in *ex vivo* and *in vivo* experiments (187, 188). The functional profile of ET-1 in the cardiovascular system is the product of its interaction with several other factors, among which, oxidative stress is of special interest in this project.

1.6.6 Endothelin-1 and Oxidative Stress

Evidence for the involvement of ET-1 in causing oxidative stress by stimulating superoxide production was reported in cultured pulmonary artery smooth muscle cells and isolated rat aorta (189, 190). Later, Dong *et al.* used human umbilical vein endothelial cells (HUVEC) to show that ET-1 increases oxidative stress by up-regulating NADPH oxidase, the main source of superoxide in the vasculature (191-193). This interaction between ET-1 and NADPH oxidase has been shown to be reproducible in carotid arteries of deoxycorticosterone acetate (DOCA)-salt rats too (194, 195).

Interestingly, in a recent study, it was found that angiotensin II-induced ET-1 expression in vascular adventitial fibroblasts from mouse aorta was also mediated by NADPH oxidase (196). Furthermore, Reuf *et al.* showed that stimulation of vascular smooth muscle cells of human aorta with H_2O_2, to mimic oxidative stress, resulted in increased expression of prepro-ET mRNA in as early as 1 hour (197). This process produced ET-1 which was functional and able to generate further oxidative stress in an autocrine fashion, confirmed by observing increased levels of 8-isoprostane (a marker of oxidative stress). While the exact interaction of ET-1 and oxidative stress in hypertension is yet to be identified, evidence supports an interaction between ET-1 and NADPH that can play a role in pathophysiology of hypertension.

1.6.7 Endothelin-1 and Preeclampsia

Due to its potent effects on the vascular system, the ET-1 system has always been an attractive target as an agent with a potential involvement in pathophysiology of preeclampsia. One of the easiest and most effective approaches to determine an association between ET-1 and preeclampsia would be the measurement of ET-1 in preeclamptic women and comparison of its levels to normal pregnant women. Although there are a few conflicting reports, several studies that have probed circulating levels of ET-1 in preeclamptic and normal pregnant women have found its levels to be significantly higher in women with preeclampsia (56, 61, 111, 113, 198, 199). In one of these studies, the level of immunoreactive ET-1 was followed post-partum and interestingly it went back to normal levels within 48 hour of delivery (113)**.** In another study, Barden *et al.* showed that women with essential hypertension do not have increased levels of ET-1 during pregnancy (200). This finding makes it possible to speculate that increased levels of ET-1 are probably not attributable solely to hypertension itself. In an experiment using HUVEC, ET-1 secretion was found to be significantly higher in endothelial cells collected from preeclamptic pregnancies compared to normal pregnancies (201). In line with the aforementioned studies, Napolitano *et al.* measured ET-1 mRNA expression in human trophoblast cell cultures obtained from placental villous tissues of preeclamptic and normotensive pregnancies (202). The mRNA expression levels were significantly higher in trophoblast cells from preeclamptic women compared to normal pregnant women. It is noteworthy though, that in these types of studies we are not dealing with maternal tissue,

therefore, interpretation and generalization of such studies needs to be done cautiously.

While greater mRNA levels could lead to this annotation that increased production is responsible for presence of greater levels of ET-1 in preeclampsia, this finding could well be the result of up regulated cleavage of bigET-1 to ET-1 by enzymes such as matrix metalloproteinase-2. This enzyme and its function will be discussed in the following section.

1.7 Matrix Metalloproteinases

1.7.1 History and Classification

Matrix Metalloproteinases (MMPs) were discovered by Gross and Lapiers in 1962 when they were studying the life cycle of amphibians (203-205). They cultured pieces of resorbing tadpole tails on collagen gels and recovered a gelatinolytic enzyme from the culture media. The same activity was found soon after, in several other tissues. In 1970, this collagenase was purified from human skin and rat uterus (206, 207). Since their discovery, MMPs have grown to 28 members with a wide variety of substrates and functions. These enzymes belong to a family of zinc-dependent proteases. Several methods have been used to classify MMPs. Functional nomenclature based on the substrate is the most commonly used system. In this system MMPs are classified as: collagenases (MMP-1, -8, -13 and -18), stromelysins (MMP-3, -10 and -11), membrane-type MMPs (MMP-14, -15, -16, -17, -24 and -25) and gelatinases (MMP-2 and -9).

There are other MMPs that do not fit in this classification which are covered in the evolutionary and gene classification systems.

My thesis has focused on the gelatinases and, specifically, MMP-2 due to its involvement in vascular function.

1.7.2 Matrix Metalloproteinase (Gelatinase A)

MMP-2 is synthesized and secreted in the form of a zymogen. In the nascent state, MMP-2 has an N-terminal signaling sequence. This domain directs the molecule to the endoplasmic reticulum. The signaling sequence is followed by propeptide sequence. This hydrophilic propeptide sequence acts as a shield for the adjacent catalytic region until it is cleaved or disrupted. The catalytic region has the catalytic domain as a back-bone structure. It also contains a zinc-binding region which is conserved and is involved in the activation of MMP-2. The catalytic region also has three cysteine-rich repeats which resemble collagen-binding type II repeats of fibronectin. These repeats play a crucial role in the binding and cleavage of collagen. The catalytic region is connected to a hemopexin/vitronectin-like domain by a hinged region. The hemopexin domain is involved in tissue inhibitors of metalloproteinases (TIMP) binding, the binding of certain substrates, membrane activation, and some proteolytic activities (**Figure1.3**).

MMP-2 is found in a variety of human cells and tissues including cardiomyocytes, endothelium and smooth muscle cells of vasculature and fibroblasts (208-211).

1.7.3 Functions of Matrix Metalloproteinase-2

Traditionally, MMP-2 has been known for its ability to degrade proteins in the extracellular matrix. MMP-2 is physiologically involved in tissue remodeling such as embryonic development, angiogenesis, ovulation, mammary gland involution and wound healing. More recent studies have revealed novel functions for MMP-2. Fernandez-Patron *et al.* in the Davidge lab for the first time described that MMP-2 can cleave the Gly_{32}-Leu_{33} bond of bigET-1 to produce $ET\text{-}1_{1\text{-}32}$ (212).This group also demonstrated that MMP-2 is able to decrease the vasodilatory potency of calcitonin gene-related peptide to a significant extent by cleaving it at the Gly_{14}-Leu_{15} bond (213). MMP-2 is also able to cleave cell membrane associated heparin binding-epidermal growth factor. The product of this cleavage can transactivate the epidermal growth factor receptor and ultimately produce vasoconstriction (214, 215).

Since these functions can potentially be involved in pathophysiologic processes, it is important to understand the regulation of MMP-2.

1.7.4 Regulation of Matrix Metalloproteinase-2

The regulation of all MMPs occurs at transcriptional, post-transcriptional, and post-translational levels and MMP-2 is no exception.

1.7.4.1 Transcriptional Regulation

Research in cardiomyocytes has revealed that many factors involved in the pathogenesis of cardiac diseases can increase the expression of MMP-2. A short

list of these factors includes hypoxia, angiotensin II, ET-1, interleukin-1b, IL-6, TNF-α, hormones (estrogen and melatonin), and growth factors (epidermal growth factor, PDGF, and basic fibroblast growth factor) (216-219). The promoter sequence of MMP-2 lacks the activated protein-1 binding site and TATA box found in some other MMPs such as MMP-9. MMP-2 instead, possesses a GC box which is the binding site for various transcription factors (220). Furthermore, MMP-2 has a functional activated protein-1 binding sequence which is involved in transcriptional regulation of MMP-2 (216, 221).

1.7.4.2 Post-Transcriptional Regulation

Several factors have been proposed to regulate MMPs at the post-transcriptional level. For example, Limaye *et al.* after conducting a set of experiments in rat prostate tissue proposed that increased levels of MMP-2 mRNA post castration was at least partly attributable to post-transcriptional stabilization, increasing steady state mRNA levels (222). Delany *et al.* demonstrated that platelet derived growth factor (PDGF) and glucocorticoids stabilize the MMP-13 transcripts in osteoblasts. They also showed that transforming growth factor beta (TGF-β) destabilizes the MMP-13 mRNA in the same cells (223). Vincenti has also proposed that epidermal growth factor stabilizes MMP-1and MMP-3 mRNA in fibroblasts (224). Although, neither of these mechanisms has been demonstrated for MMP-2, due to structural similarities in MMPs, it can be speculated that these growth factors might be involved in the post-transcriptional regulation of MMP-2 as well.

1.7.4.3 Post-Translational Regulation

1.7.4.3.1 Tissue Inhibitor of Metalloproteinases

This family of molecules has four members, TIMP-1 to -4. They are major cellular inhibitors of MMPs. TIMPs bind to MMPs in a 1 to 1 ratio involving the Zn^{+2} of the active catalytic domain of MMPs (225). Even though all four TIMPs block the active forms of all MMPs studied to date, Olson *et al.* reported that TIMP-2 can preferentially bind to the latent form of MMP-2 through its hemopexin domain (226). There are several other proteins including netrins, secreted frizzled-related proteins and type I collagen C-proteinase enhancer protein that have similarities in their amino acid sequences to the N-terminal of TIMPs; and might, therefore, be able to act as MMP inhibitors, reviewed in (227). Tissue factor pathway inhibitor 2 is also another MMP-2 inhibitor that shares similar sequences with TIMPs in the internal region (228). Thrombospondin-2 is an MMP inhibitor that can regulate MMP-2 by forming a complex that facilitates scavenger-receptor-mediated endocytosis. Thrombospondin-2 has also been shown to inhibit proMMP-2 activation and modulate MMP-2 production (229).

α2-macroglobulin, a protein mainly synthesized in the liver by hepatocytes, can inhibit almost all endoproteinases, including MMPs (230). A major difference between α2-macroglobulin and TIMPs is that the latter mainly inhibits MMPs in a reversible manner, while α2-macroglobulin forms a complex with MMPs that can only be removed by scavenger receptor-mediated endocytosis. Therefore, this mechanism is mainly involved in the irreversible clearance of MMPs (231).

TIMP-2 can also activate MMP-2 by forming a tri-molecular complex with membrane type 1-MMP (MT1-MMP) (232-234). The activated MT1-MMP on the surface of the cell functions as a receptor for TIMP-2 by binding its amino-terminal domain (235-237).

TIMP-2 serves as a receptor for proMMP-2 via interacting with its hemopexin C domain (238-240). Additional active MT1-MMP in the proximity of proMMP-2-TIMP-2-MT1-MMP complex initiates the activation of proMMP-2. Autocatalytic cleavage of proMMP-2 results in fully active MMP-2 (241, 242). This pathway was shown to be dependent on TIMP-2 both *in vivo* and *in vitro* using TIMP-2 -/- mice and TIMP-2 -/- cell line (243-246).

1.7.4.3.2 Phosphorylation

Sariahmetoglu *et al.* showed that MMP-2 activity is also regulated by its phosphorylation status (247). Using the fact that the phosphorylation status of proteins can be modulated by the balance of action between numerous protein kinases and protein phosphatases, they used MMP-2 to demonstrate that phosphorylation of this molecule by PKC diminishes its activity, whereas alkaline phosphatase treatment enhances its activity.

1.7.4.3.3 Caveolae

Caveolae are a special type of membrane glycolipoprotein microdomain termed lipid rafts found in many vertebrate cells including endothelial cells and adipocytes (248). Caveolae are known primarily for their ability to transport

molecules across endothelial cells but they can also form a unique endocytic and exocytic compartment at the surface of most cells. Caveolae are capable of importing molecules and delivering them to specific locations within the cell, exporting molecules to extracellular space, and compartmentalizing a variety of signaling activities (248, 249). Caveolins are a family of integral membrane proteins which are the principal components of caveolae membranes and three of them are found in vertebrates, Cav-1 to -3 (250-252) . Chow *et al.* showed in a series of experiments that MMP-2 co-localizes with caveolin-1 (Cav-1) in cardiomyocytes of mouse hearts. They also demonstrated that MMP-2 activity is increased in the heart of the Cav-1 knock-out mouse. In addition, they showed that the caveolin scaffolding domain of Cav-1, which regulates several proteins including those involved with signaling cascades, inhibits MMP-2 activity *in vitro* (253, 254). These reports indicate a possible role for Cav-1 in the regulation of MMP-2 activity.

1.7.4.3.4 Oxidative Stress

S-glutathiolation is a post-translational modification which was first reported to occur in MMP-1, -8 and -9 by Okamoto *et al.* (255, 256) and then in MMP-2 by Viappiani *et al.* (257). In this process, peroxynitrite causes S-glutathiolation of the cysteine containing sequence of the propeptide domain of MMP; thus increasing its proteolytic potential. Interestingly, this activation process for MMP-2 was shown to occur in the concentration range of 0.3–10 mmol/L (peak at approximately 1 mmol/L) of peroxynitrite and concentrations higher than 100

mmol/L inactivated it (257). These findings show that the classical notion about pro and active MMPs, based on their molecular weights as seen by sodium dodecyl sulphate-polyacrylamide gel electrophoresis zymography (72 kDa proMMP-2 and 64 kDa active MMP-2), could be confusing as, in the presence of peroxynitrite, even pro-MMP can exert proteolytic activities. The interaction between oxidative stress and peroxynitrite in particular, with MMP activity does not end here. Several studies have shown that peroxynitrite is capable of inactivating TIMP-1 and -4 hence increasing the activity of MMPs (258-261).

The effect of oxidative stress on MMP-2 activity, and the state of increased oxidative stress in preeclampsia, makes it possible to speculate that MMP-2 plays a role in the pathophysiology of this syndrome. (**Figure 1.4**)

1.7.5 Evidence of Involvement of Matrix Metalloproteinase-2 in Preeclampsia

Many cross-sectional and prospective studies have been performed by different researchers to find the footprint of MMP-2 in the pathophysiology of preeclampsia. The results of these studies have not been consistent and this could be attributed to several factors including; case selection process and inclusion criteria, experimental design and measurement methods.

One of the first studies in this regard was done in 2001 by Narumiya *et al.* (262). In this study plasma levels of MMP-2 were shown to be significantly higher in women with preeclampsia versus women with normal pregnancies using zymography. The same results were seen when serum MMP-2 levels were measured using multiplexed sandwich enzyme-linked immunosorbent assays in

preeclamptic women compared to normal and non-pregnant women (263). Contrary to these results, Palei *et al.* found no difference in plasma MMP-2 levels between preeclamptic and normotensive pregnancies (264). These studies represent the most direct attempts to find a relationship between MMP-2 and maternal syndrome of preeclampsia by using pregnant women as the subjects of their studies. However, several other studies have been done using other tissues such as placental tissue to probe MMP-2 levels in preeclamptic versus normal pregnancies. These studies have found no significant difference in MMP-2 expression in placental tissues collected from preeclamptic and normotensive pregnancies by using different techniques including zymography, immunohistochemistry and western blot (265, 266). When Merchant *et al.* used umbilical cords from preeclamptic and normal pregnancies to culture HUVECs; they showed a significant increase in MMP-2 release in the supernatant of cultures from preeclamptic pregnancies compared to normal pregnancies (267). However, when placental biopsies were used to develop a bilayer co-culture model of decidual endothelial cells, zymographic studies demonstrated decreased secretion of MMP-2 in samples prepared from pregnancies that were compromised by preeclampsia (268).In another prospective study Lavee *et al.* used amniotic fluid samples from the 2nd trimester of pregnancy and reported that MMP-2 levels were significantly higher in cases which subsequently progressed to preeclampsia (269). Finally, the only study that checked MMP-2 levels in umbilical cord arteries from preeclamptic pregnancies, found that MMP-2 levels were less in cases compared to normal controls (270).

Aside from the aforementioned studies in which attempts were made to find a direct association between MMP-2 and preeclampsia, there are other interesting findings linking them indirectly. An important factor that needs to be addressed in this area is hypoxia. Hypoxia, which is most probably an inevitable part of preeclampsia, can affect the production of MMPs indirectly through two different growth factors: transforming growth factor (TGF-β1) and vascular endothelial growth factor (VEGF). The expression of these growth factors is enhanced by hypoxia and they, in turn, increase the production of MMP-2 (271-274).

The results from these studies do not provide decisive evidence about the involvement of MMP-2 in the pathophysiology of preeclampsia. One possible reason for that is the fact that using placental tissue and HUVEC and even amniotic fluid cannot readily address the issue which is mainly a maternal syndrome. Moreover the MMP-2 in vascular tissue has not been assessed. Furthermore, none of these studies demonstrated evidence of cause and effect between MMP-2 and preeclampsia. Elevated MMP-2 could be a result or side effect of preeclampsia or just a coincidence of two independent processes. Nonetheless, they can be used to speculate and design further studies in order to probe the possibility of an involvement of MMP-2 as a key role player in the pathophysiology of preeclampsia.

One of the most important approaches in the design of a research project to further investigate pathophysiological theories is choosing an appropriate model for the study. Unfortunately, since preeclampsia is exclusively seen in humans, finding an animal model for studies in this field is a major obstacle that

researchers face. The next section is dedicated to further describing a few of animal models and specifically the animal model we used in this project.

1.8 Animal Models of Preeclampsia

Preeclampsia is a condition observed in humans. There have been reports of spontaneous development of a preeclampsia-like syndrome in other mammals (275, 276). However, these cases are rare and do not allow for the study of the pathophysiology of preeclampsia. Several attempts have been made to develop animal models of preeclampsia in order to study the etiologic factors, pathogenesis and treatment options. Different animal models of preeclampsia have been explored in a number of excellent reviews (277-279). Here in this section, I will briefly describe several of these models and then the model chosen for this thesis, the reduced ureto-placental perfusion pressure (RUPP) model, will be described in more detail.

1.8.1 Angiogenesis

In several of the animal models developed for studying preeclampsia, angiogenesis has been the main mechanism targeted and manipulated. FMS-like tyrosine kinase (sFlt-1), a splice variant of the VEGF receptor, is an anti-angiogenic factor and antagonist of VEGF and placental growth factor (PlGF). Recent findings have shown that levels of sFlt-1 are increased in preeclampsia (277-279). In addition to its angiogenic effects, VEGF promotes nitric oxide and vasodilatory PGI_2 production in endothelial cells. It also is involved in decreasing vascular tone and maintaining the integrity of glomerular filtration barriers (280,

281). Based on these findings, Maynard *et al.*, used an adenovirus vector to administer exogenous sFlt-1 to pregnant rats (282). This experiment resulted in hypertension, proteinuria and glomerular endotheliosis, all features of preeclampsia. These findings were even seen in non-pregnant rats, indicating an interaction between VEGF and the systemic endothelium regardless of pregnancy. Adenovirus-mediated sFlt-1 over-expression was also performed in pregnant mice with similar results (283).

Soluble endoglin (sEng), another anti-angiogenic factor found to be up-regulated in preeclampsia, was used to produce a model of preeclampsia in rats (284). Using adenoviral delivery, Venkatesha *et al.* administered sEng to pregnant rats. In this study, the rats developed increased mean arterial pressure and mild proteinuria. Interestingly, co-administration of sFlt-1 and sEng into the rats caused the development of several severe symptoms resembling the HELLP syndrome (hypertension, elevated liver enzymes and low platelets) in human pregnancy.

The last model of preeclampsia based on angiogenesis discussed here was developed by Nash *et al.* (285). In this model, they induced placental insufficiency by injecting an anti-angiogenic substance, Suramin, to Sprague Dawley rats (U and H sub-strains) on the 10^{th} day of gestation. The Suramin treated pregnant rats developed hypertension, placental dysfunction and decreased pup weight (285, 286).

1.8.2 Inflammation

A large body of evidence proposes that in preeclampsia the maternal inflammatory response is in excess of normal pregnancy. This has led to the development of several animal models of preeclampsia based on inflammation.

Faas *et al.* developed a model of preeclampsia in rats by infusing low doses of endotoxin to pregnant animals (287). They documented increased blood pressure and urinary protein excretion only in pregnant rats. The pregnant rats also showed decreased platelet counts.

In another model, when pregnant rats were treated with TNF-α from day 14 to 15 of gestation, they developed hypertension, and decreased renal plasma flow and glomerular filtration rates (288). These changes were associated with a reduction in nNOS and iNOS production in kidneys (289).

With the same principals in mind, Orshal *et al.* used IL-6 administration to produce a model of preeclampsia in rats (290). They successfully demonstrated increased blood pressure and urinary protein excretion in pregnant rats treated with IL-6 compared with saline-treated pregnant and IL-6-treated virgin rats.

Hayakawa *et al.* in 2000 developed a model of preeclampsia by transferring T helper-1-like and T helper-2-like cells from virgin to pregnant mice (291). This experiment resulted in fetal resorption and glomerular nephritis associated with hypertension and proteinuria. Four years later, Zenclussen *et al.* repeated this experiment with T helper-1-like cells and found similar results (292). Furthermore, they reported that the same treatment (T helper-1) does not cause preeclampsia-like syndrome in non-pregnant mice.

1.8.3 Renin Angiotensin System

Based on the finding that an agonistic autoantibody to the angiotensin type 1 receptor (AT_1-AA) can cause hypertension and is probably involved in pathophysiology of preeclampsia (293-295). Zhou *et al.* injected a purified form of this substance to pregnant rats (296). This experiment resulted in the production of a new animal model of preeclampsia mimicking several features of preeclampsia including: hypertension, proteinuria, glomerular endotheliosis and increased sFlt-1, sEng and TNF-α (296, 297).

Takimoto *et al.* developed a mouse model of preeclampsia by mating transgenic females expressing angiotensinogen with transgenic males expressing renin. (298). The pregnant females started showing hypertension and proteinuria late in pregnancy. This model was reproduced in rats with comparable results (299).

1.8.4 Volume Expansion

Alper *et al.* produced a model of preeclampsia by administering deoxycorticosterone acetate (DOCA) to pregnant rats and replacing their drinking water with 0.9% saline, thus inducing volume expansion (300). The preeclamptic changes were not observed when non-pregnant rats were treated with similar regimen. This model mimics a subtype of preeclampsia which is accompanied by intrauterine growth restriction and a lack of glomerular endotheliosis.

1.8.5 Vasoconstriction

Several animal models of preeclampsia have been produced by induction of vasoconstriction. Nitric oxide inhibition has been the mainstay of most of these models (301). In these models, chronic inhibition of NOS by L-Nitro-Arginine Methyl Ester (L-NAME) in pregnant, but not non-pregnant rats, causes hypertension, proteinuria, and reduced glomerular filtration rate (301). Using the same rationale, Shesely *et al.* tried to reproduce a model of preeclampsia by creating an eNOS knock-out; however, since these mice developed high blood pressure prior to pregnancy, it turned out to be a model of chronic hypertension rather than preeclampsia (302).

1.8.6 Chronic Hypertension

When Davisson *et al.* studied a mouse with mild chronic hypertension called the BPH/5, they found that these animals develop a preeclampsia-like syndrome late in gestation characterized by high blood pressure, proteinuria and fetal demise (303). These symptoms could be attributed to endothelial dysfunction and abnormal placental development (303, 304).

1.8.7 2-Methoxyoesteradiol (2-ME)

2-ME is a metabolite of estradiol generated by the placenta and elevated in the third trimester of pregnancy. Kanasaki *et al.* discovered that, pregnant mice deficient in catechol-O-methytransferase, an enzyme involved in production of 2-

ME, developed hypertension and proteinuria accompanied by glomerular endotheliosis (305).

1.8.8 Other Models

Other than the animal models of preeclampsia that were briefly described above, there are other models which are either less popular as a model of preeclampsia or not as well-characterized as the aforementioned models. These models include but are not limited to: adriamycin-induced model of preeclampsia (306), insulin-induced model of preeclampsia (307) and stress-induced model of preeclampsia (308).

With a host of animal models for preeclampsia available, choosing the model which is most suitable for the research question is important. We chose a model of preeclampsia called the reduced utero-placental perfusion pressure (RUPP) based on its characteristics that will be described in further detail in the following section.

1.9 Reduced Utero-placental Perfusion Pressure Model of Preeclampsia

1.9.1 History

In 1914, Young hypothesized that hypertension in pregnant animals could be due to utero-placental ischemia (309). Later in 1929, Beker theorized that the etiology of preeclampsia is a hemodynamic imbalance caused by increased vascular resistance, particularly at the site of placenta (310). Further, Page in 1939 proposed that uterine ischemia could be the cause for preeclampsia and suggested

that this process could happen in three different ways: 1) increased fetal demand for blood, 2) failure of the developing uteroplacental circulation to keep pace with fetal demand or 3) decrease in maternal blood supply. To test this hypothesis, hypertension was produced in pregnant dogs by partially clamping the aorta below the level of renal arteries (311). Interestingly, they demonstrated that in non-pregnant animals and after removal of the uterus, hypertension did not occur. Ten years later in 1949, Bastiaanse and Mastboom elicited the same result after repeating Page's experiment (312). They hypothesized that preeclampsia is the result of an insufficient adaptation of the uterine arteries. Berger and Cavanagh in 1963, after conducting a series of experiments on pregnant rabbits, concluded that placental ischemia rather than uterine ischemia is the key factor in the production of hypertension and further proposed that the cause of preeclampsia is probably a humoral factor released from the placenta. Although all of these experiments were in favour of a role for ischemia or decreased perfusion to the uterus or placenta as an etiologic factor in preeclampsia, all of these experiments used an acute insult while in preeclampsia there is a chronic pattern to ischemia. To address this issue, Hodari designed an experiment to mimic chronic reduction in uterine and placental blood flow (313-315). In this experiment a snug fitting band was placed around the uterine arteries of non-pregnant female dogs. Once these dogs became pregnant, urine protein and blood pressure were measured in different stages during pregnancy and one month post-partum. Banded dogs were found to develop hypertension and excrete increased amounts of protein in their urine compared to non-banded and non-pregnant groups. In addition, blood pressure

levels and proteinuria were found to go back to control levels when measured one month post-partum. These results were repeated by Cavanagh *et al.* and Abitol *et al.* in rabbits, dogs and primates (baboons) (316-318). A similar experiment was performed in Rhesus monkeys and again a controlled degree of decreased aortic blood flow caused progressive hypertension and proteinuria in pregnant animals (319). Interestingly, many of these studies also reported histopathological changes in the kidney of the affected animal, such as glomerular endotheliosis, similar to that seen in human preeclampsia. While these animal models looked to be ideal for preeclampsia research, they shared some limitations. They all used large animal species which are more expensive, have relatively long pregnancies and complex legal and ethical issues; especially in the case of primates.

Therefore, first Eder and Macdonald and later the Granger group utilized the rat to characterise a reduced uteroplacental perfusion pressure model of preeclampsia to be used as animal model in preeclampsia research (320, 321). On day 14 of gestation, they induced approximately 40% reduction in uteroplacental perfusion by placing restrictive clips on the aorta, below the renal arteries and above the aortic bifurcation, and also on the ovarian arteries to prevent compensatory blood flow (322).

It is worth mentioning that although reduction of uteroplacental perfusion has resulted in preeclampsia-like symptoms in some animals, there are others, the sheep for instance, that have failed to show any changes in maternal blood pressure (323). This difference between species underlines the attention that

should be directed towards species' specific anatomy and physiology when inferring data from these models to the human disease.

1.9.2 Characteristics of RUPP Model

The RUPP model has been used by several groups to study different aspects of preeclampsia. Interestingly, most of the features of this model have been successfully reproduced. When blood pressure measurement was performed on day 19 or 20 of gestation, mean arterial blood pressure was found to be increased by 20 to 30 mmHg compared to sham-operated animals, depending on the measurement technique and day (324-326). In a study focused on characterizing this model, Sholook *et al.* reported increased total peripheral resistance and diminished placental blood flow (327). These features are remarkably similar to human preeclampsia.

In contrast to significantly increased blood pressure, elevated urine protein excretion was a finding not uniformly observed by all researchers (321, 324, 328, 329) with only some groups reporting significant proteinuria (330-332). This inconsistency could probably be due to the short term of insult, six days, in this model which may not be severe enough to produce proteinuria. A possible proof for this speculation is that proteinuria has been reported in almost all other models of RUPP in other animal species with longer gestations and insult durations.

Smaller pup and litter size was another finding in RUPP animals (325, 333-335). The RUPP surgery causes an increase in serum TNF-α (336, 337) **,** IL-6

(338), sFlt-1 (339) and sEng (339) which is consistent with findings in preeclampsia.

Another noteworthy feature of the RUPP model is the possible role of reactive oxygen species. Sedeek *et al.* showed that 8-isoprostane and malondialdehyde (both markers of oxidative stress) were increased in placentas collected from RUPP animals compared to normal pregnant (340). They also demonstrated that total antioxidant status and renal cortical tissue SOD activity were significantly lower in RUPP animals. They even demonstrated that chronic treatment of these RUPP animals with tempol (an SOD mimetic) diminished their hypertension.

In regard to vascular reactivity, increased vasocontractility in response to vasoactive agents such as phenylephrine, potassium chloride and angiotensin II (341) and impaired vasorelaxation in response to acetylcholine and bradykinin are other vascular features of the RUPP model of preeclampsia (341). Impaired vasorelaxation was even observed when vessels from normal pregnant rats were incubated overnight with plasma from RUPP animals (341). Findings regarding vascular reactivity in RUPP model have not always been consistent and different results have been reported by different researchers and from different types of vessels. There are reports of increased vascular constriction vasoreactivity and decreased vasorelaxation in conduit arteries especially in aorta (324, 342). There is also one report of impaired endothelium dependent relaxation in uterine arteries from RUPP animals compared to sham (333). Mesenteric artery, as the representative of resistant arteries, has been used by several researchers to study the changes in vasoreactivity in RUPP model. While Anderson *et al.* and Giardina

et al. reported increase in active stress as well as maximal tension in mesenteric arteries in response to phenylephrine (343, 344), Chen *et al.* did not observe any differences in vasoconstriction of these vessels in response to phenylephrine, angiotensin-II or ET-1 compared to shams (345). Regarding the vasorelaxation of mesenteric arteries some researchers reported decreased endothelium dependent vascular relaxation capacity (329, 341), while Anderson *et al.* could not find any difference in vasorelaxation between mesenteric arteries of Sham and RUPP (343). These differences could be due to the strains of rat used as well as differences in the severity of the RUPP induced changes.

One of the significant features of RUPP model which made it suitable for our project is the role of endothelin in mediating hypertension in this model. Alexander *et al.* showed that preproendothelin levels were significantly higher in kidneys harvested from RUPP rats compared to controls (346). In addition, elevated blood pressure in RUPP animals was reduced almost to control levels by inhibiting the ET_A receptor. In a separate experiment, Roberts *et al.* exposed HUVEC cultures to serum from RUPP rats and observed increased endothelin production (347). While the mechanism through which endothelin is involved in the pathophysiology of preeclampsia is yet to be discovered, LaMarca *et al.* tried to approach this question by using TNF-α which is increased in both the preeclamptic women and RUPP rats, as the factor that starts the biological cascade leading to preeclampsia (348). To test their hypothesis, they infused TNF-α to pregnant rats to mimic the serum levels seen in preeclampsia. Interestingly, this treatment not only increased blood pressure in the pregnant rats but also led to

a significant elevation in endothelin production in the vasculature, kidney and placenta of the rats. Furthermore, pharmacologic inhibition of the ET_A receptor completely reversed the hypertensive effects of TNF-α.

These overall characteristics of RUPP made it a suitable candidate model for us to test our hypotheses and find answers to our questions.

1.10 Hypotheses

As mentioned in the previous sections of the introduction, in preeclampsia there is a state of increased oxidative stress. There are also increased levels of ET-1 in preeclampsia that can play a crucial role in the pathophysiology of the disorder. Based on available evidence, MMP-2 can cleave bigET-1 to produce ET-1. Furthermore, the activity of MMP-2 can be up-regulated by increased oxidative stress, in particular by peroxynitrite. Our general hypothesis is that, in preeclampsia due to decreased perfusion to the placenta, the placenta releases factors into the maternal circulation causing increased oxidative stress and endothelial dysfunction. This oxidative stress up-regulates MMP-2 activity to produce excess ET-1 through increased cleavage of bigET-1. The increase in ET-1 completes this vicious cycle by further decreasing placental perfusion and thus causing other pathophysiologic changes leading to the clinical symptom of preeclampsia. In order to test this hypothesis we used the RUPP model of preeclampsia.

1.11 Specific Aim of the Project

1.11.1 Aim 1

To establish the RUPP model of preeclampsia which has not been previously used in our laboratory. In order to accomplish this goal we performed RUPP surgery on rats and then measured several indices including: blood pressure, heart rate, kidney histopathology, urine protein and litter morphometry.

1.11.2 Aim 2

To compare the function of the resistance arteries of RUPP and sham animals in response to bigET-1 using the wire myography technique as a functional assay.

1.11.3 Aim 3

To determine the role of MMP-2, with its bigET cleaving capacity, in the differences in the vascular function of RUPP versus sham animals using the enzyme inhibitor and the wire myography.

1.11.4 Aim 4

To determine the role of other enzymes, capable of cleaving MMP-2, in the differences in the vascular function of RUPP versus sham animals using the enzyme inhibitor and the wire myography.

1.11.5 Aim 5

To use quantitative and semi-quantitative assays including: zymography, western blot and fluorescent staining to probe the biological differences that could be responsible for the observed differences in the vascular function of RUPP animals.

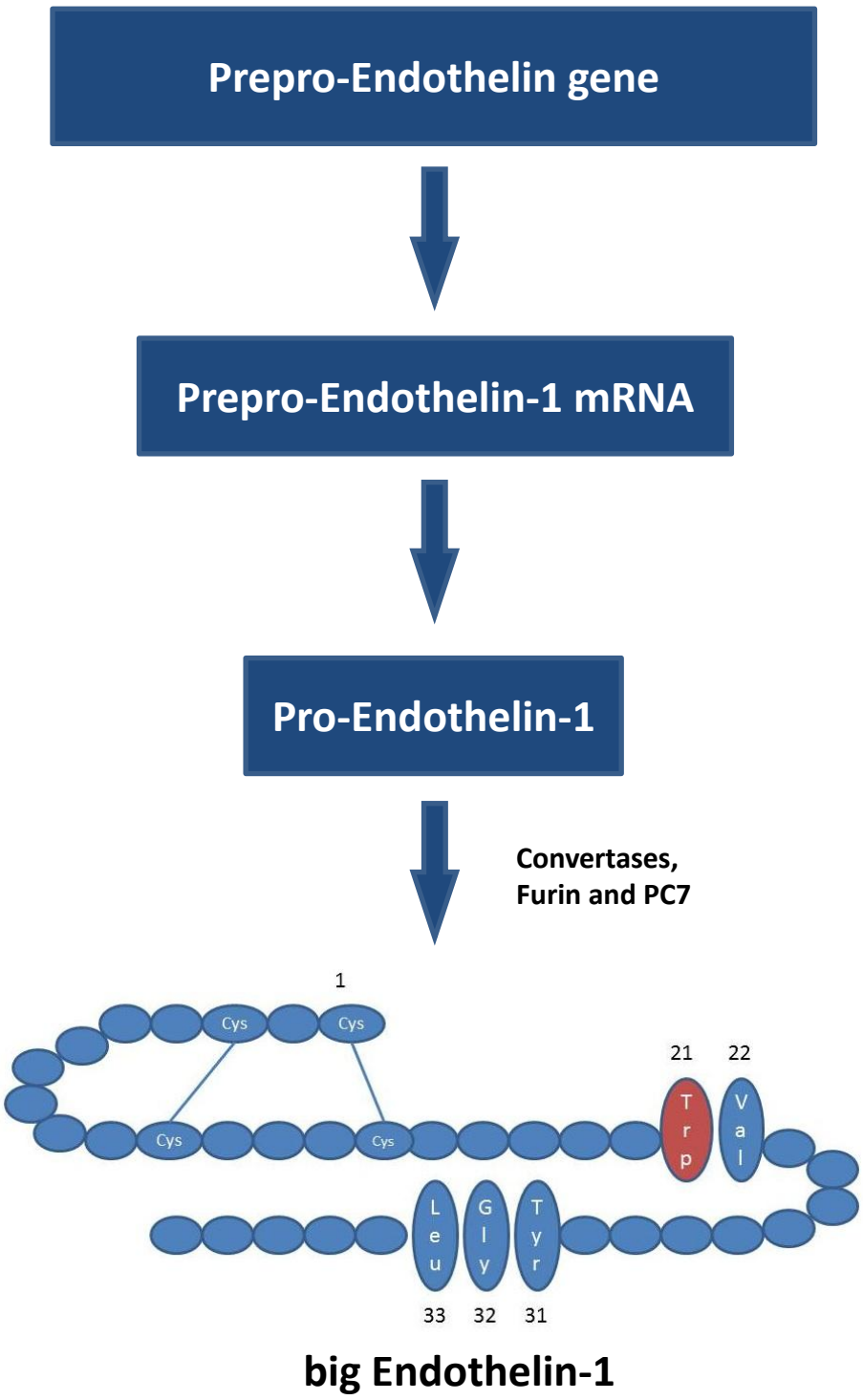

Figure 1.1- Production process of big Endothelin-1

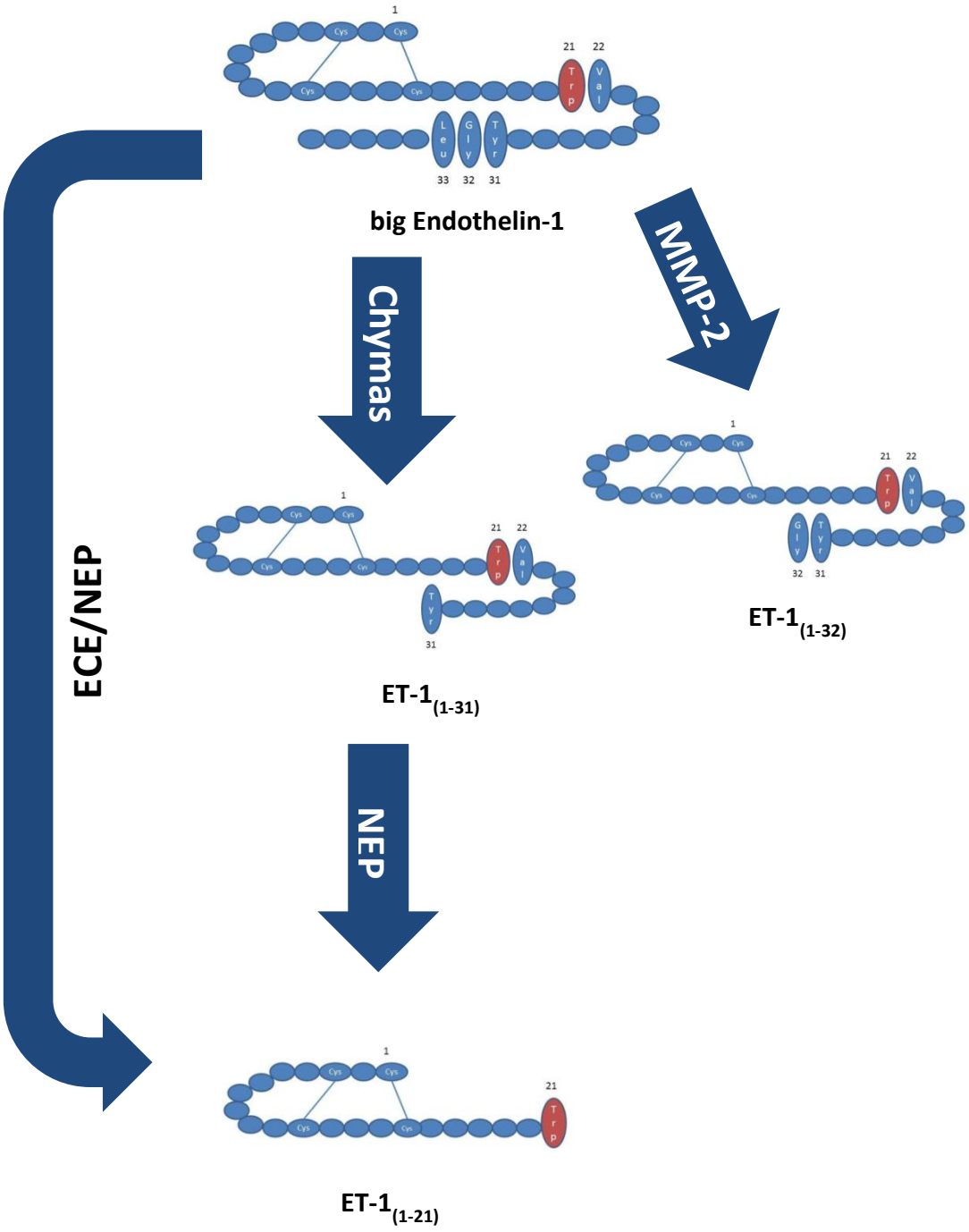

Figure 1.2- Cleavage process of big Endothelin-1 (bigET-1) to Endothelin-1 (ET-1). Matrix Metalloproteinase-2 (MMP-2) cleaves bigET-1 to **ET-1$_{(1-32)}$**. Chymase cleaves bigET-1 to **ET-1$_{(1-31)}$**. Endothelin converting enzyme (ECE) and Neutral endopeptidase (NEP) cleave bigET-1 to **ET-1$_{(1-21)}$**. NEP can further cleave **ET-1$_{(1-31)}$** to **ET-1$_{(1-21)}$**.

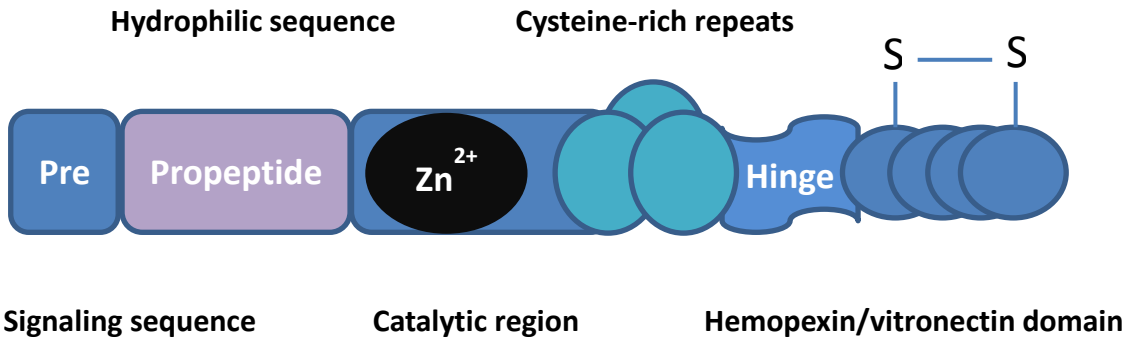

Figure 1.3- Molecular structure of Matrix Metalloproteinase -2 (MMP-2) enzyme.

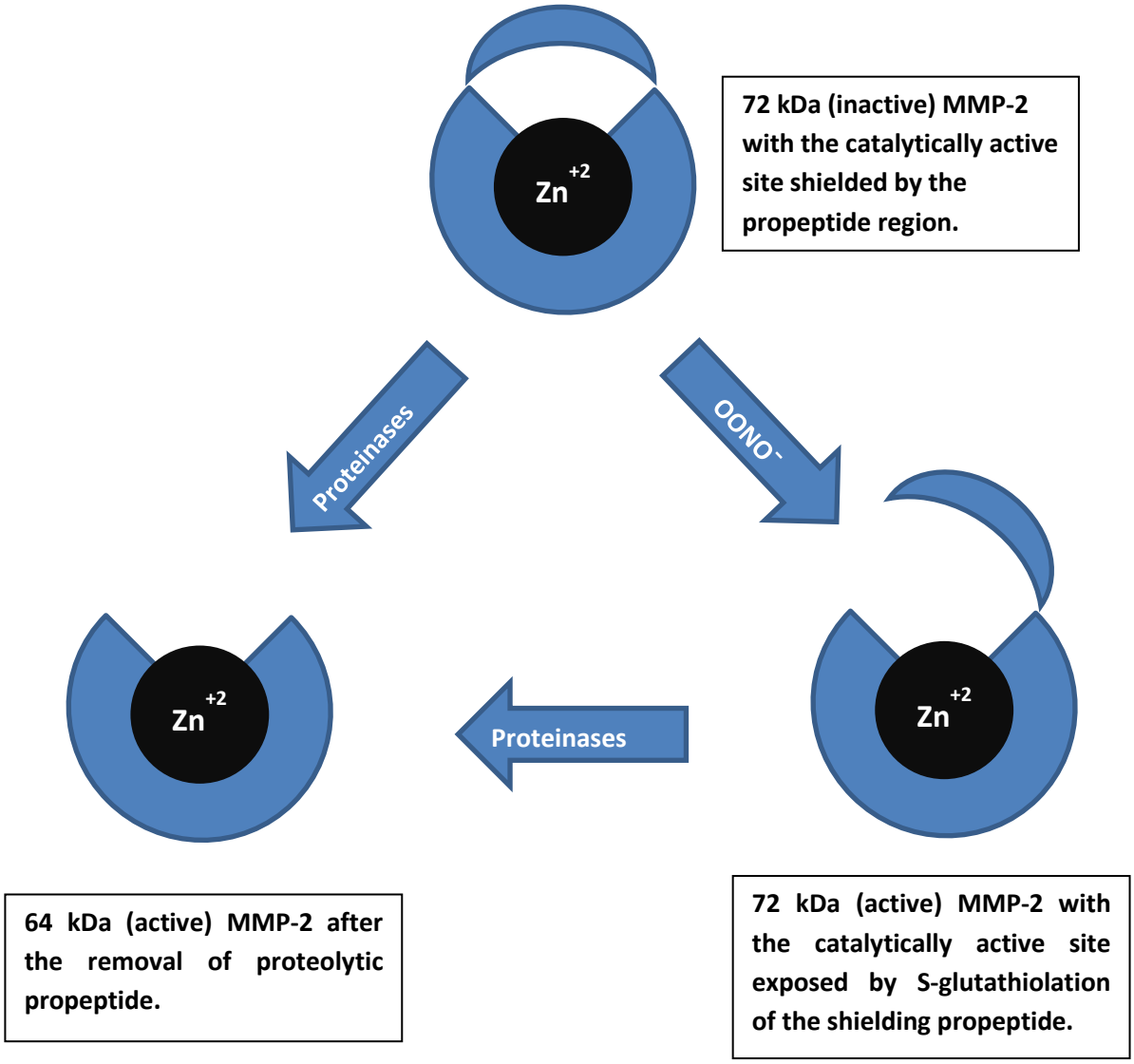

Figure 1.4- Activation of MMP-2 by proteinases (right) and oxidative stress (left).

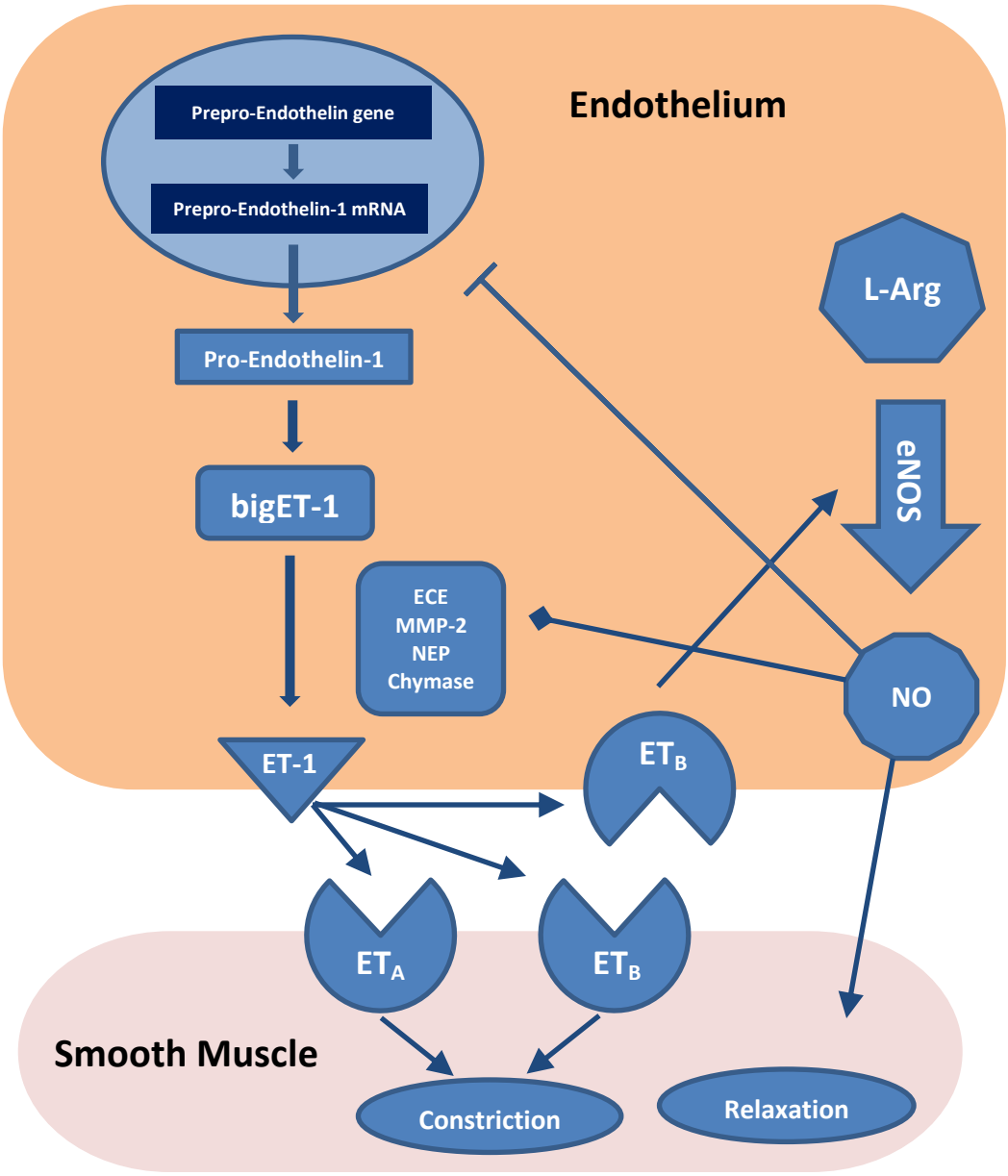

Figure 1.5- Schematic of the interaction between nitric oxide (NO) and ET-1 production/function.

NO has an inhibitory effect (⊣) on transcription and translation of ET-1. It also antagonizes the effect of ET-1 on smooth muscle cells. Furthermore, NO decrease the release and increase the clearance of ET-1 (not shown in the figure). On the other hand, NO has several modulatory effects (◆) on the cleaving enzymes of big endothelin-1. Activation of endothelial ET_B increases eNOS activity (→) leading to increased NO production.

Chapter 2:

Materials and Methods

2.1 Ethics

This study was approved by the University of Alberta Animal Welfare Committee and followed the Canadian Council on Animal Care guidelines and the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* (349-351) .

2.2 RUPP Model

As previously described in the introduction chapter, the RUPP model was chosen for our experiments. Three month old female Sprague Dawley rats (Charles River Inc.) were housed in the Health Sciences Laboratory Animal Services where they were kept in 20° C temperature and a 12/12 hour light/dark cycle. Rats were fed regular rat chow and water *ad libitum*. Female rats were bred by putting each in the same cage with a breeder male in the afternoon and pregnancies were confirmed by the presence of sperm in a vaginal smear the following morning. The day on which pregnancy was confirmed was considered as day zero (D0) of gestation. Pregnant rats were then housed in single occupancy cages to minimize environmental stressors.

Using the principles of a well-established method (322), on day 14 of pregnancy, pregnant dams were randomly assigned to receive either a Sham surgery or the RUPP surgical procedure that was performed in HSLAS surgery suites. Two types of restrictive clips, ovarian and aortic, were used during the surgery, both made out of silver plate (Goodfellow Cambridge Limited, thickness: 0.25 mm, purity: 99.95%). To make the ovarian clips; strips of silver were cut to

11 mm long and 2 mm wide. The strips were then filed with ultrafine sandpaper (600B) to make the corners round and remove all burrs. The strips were then bent to a 'J'-shaped clip with a 4 mm arm and a 7 mm arm. Next, using a filler, the distance between the two arms of the clip was reduced to 100 μm. Lastly, the end section of the shorter arm of the clip was bent outward slightly to make it easier to slide the vessels between the arms during surgery. The aortic clips were made using the same principles with exception of the width of the strips, which was 1 mm, and the filler used to adjust the distance between the arms, which was 203 μm thick.

General anesthesia was induced using a small animal non-rebreathing anesthesia machine and isoflurane as the induction agent. Using standard sterile techniques, a 2 cm midline incision was made in the abdominal wall of the rats; approximately extending between the level of the 2nd and 3rd nipples. The uterine horns were externalized and the number of fetuses and resorptions in each horn were recorded. After that, the ovarian artery and vein that form the proximal part of the uterine vascular arch were dissected from the surrounding fat tissue at a position between the branch supplying the ovary and the branch supplying the first fetus. Lastly, the ovarian artery and vein were slid between the arms of an ovarian silver clip. This process was repeated for the second ovarian artery. The second stage of the surgery consisted of dissecting the abdominal aorta from the inferior vena cava at a position below the renal arteries and above the bifurcation of the aorta to iliac arteries. When the aorta was cleanly dissected from the inferior vena cava, the aortic clip was placed around the dissected aorta (**Figure**

2.1). The surgery was completed by suturing the muscular layer of the abdominal wall using vicryl suture 4-0 (Ethicon Inc., Somerville, NJ, USA) and the skin with silk suture 4-0 (Ethicon Inc., Somerville, NJ, USA). Sham surgery was performed following the same steps as RUPP surgery. The only difference was that after dissecting the arteries from veins, the clips were attached to perivascular fat tissue. Throughout the surgery, animals were kept warm and their respiration and heart rate were checked frequently to make sure that the animals were hemodynamically stable. At the end of the surgery, animals were given a sub-cutaneous morphine injection (2 mg/kg) to ease post-operation pain. Since they tend not to drink water after the surgery for about 6 hours, 10 ml of normal saline was injected subcutaneously to protect them from possible hypovolemia.

The rats were closely observed for 4-6 hours after surgery for possible immediate complications of surgery (e.g. internal bleeding and shock). After this period the rats were housed back in the animal facility. They were then checked daily for more chronic complications of surgery (e.g. paraplegia, abortion, vaginal bleeding etc.) and effective wound healing process. Additional morphine was administered to animals exhibiting signs of pain and animals were euthanized in cases of excess pain and distress (e.g. paraplegia).

2.3 Urine Collection and Urine Protein Measurement

Urine collection was performed at two points during gestation: the day before surgery (D13) and the day before euthanasia (D19). To have a better representation of urine protein excretion, urine was collected for 24 hours,

aliquoted and stored in -80˚ C for subsequent analysis. On the experimental day, all samples were taken out of the freezer and thawed. Urine albumin and creatinine were measured using a rat albumin Enzyme-linked immunosorbent assay (ELISA) kit (Assaypro Inc., St. Charles, MO, USA) and a urinary creatinine assay kit (Cayman chemical Inc., Ann Arbor, MI, USA). Finally, the albumin to creatinine ratios were calculated.

2.4 Blood Pressure Measurement

We measured blood pressure on day 20 (D20) of the gestation which was the day of experiment. To measure blood pressure, general anesthesia was used using the same technique described for surgery. The carotid artery was cannulated with PTFE #30 catheter. The cannula was fixed in the carotid artery using a sling silk suture and tissue glue. Systolic and diastolic blood pressures were measured using a pressure transducer (Type 379, Hugo Sachs Elektronik, Harvard Apparatus, Holliston, MA, USA). The heart rate was also monitored during the procedure by an EKG tracer. After a stable blood pressure reading was recorded for at least 10 minutes, mean readings were calculated for systolic and diastolic blood pressure and heart rate.

2.5 Euthanasia and Tissue Collection

After the end of the blood pressure measurements, while the rat was still under general anesthesia, a longitudinal incision was made from supra-pubic area of the abdomen to mid-sternum. The heart was exposed and 7 to 10 ml of blood was

drawn directly from right atrium. The blood was then transferred to a plain tube with no additive (to collect serum) and an ethylenediaminetetraacetic acid (K_2 EDTA) coated tube (to collect plasma). Euthanasia was completed by exsanguination. In the next step the uterus was dissected and placed in a beaker with cold 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered physiological saline solution (PSS). After that a piece of small intestine, including its supporting vascular tissue, was excised and kept in a cold PSS filled petri dish. The rest of mesenteric vessels, heart, kidneys, thoracic and abdominal aorta and liver were harvested and snap frozen by immersing in liquid nitrogen and stored at -80˚ C. The collected blood was centrifuged at 1500-2000×g for 10-15 minutes in 4° C. The serum and plasma were collected and, after being snap frozen in liquid nitrogen, were stored at -80˚ C. Small pieces of mesenteric artery, abdominal and thoracic aorta were cleaned of adipose and connective tissue, and then fixed in optimum cutting temperature (O.C.T.,Tissue-Tek®, Sakura Finetek, Torrance, CA, USA) and snap frozen in liquid nitrogen to be used in immunofluorescent and fluorescent microphotography studies.

2.6 Morphometric Studies of the Pups

Pups and their supporting placentas were exposed after cutting the uterus open. The number of resorbed fetuses was recorded. The crown-rump length, abdominal girth and weight of viable pups were measured. Placental weights were also measured and recorded.

2.7 Kidney Preparation for Pathology Studies

One of the kidneys was cut in two halves longitudinally and fixed in Z-fix. The kidneys were then transferred to core pathology facility of University of Alberta where pathology blocks of the kidneys were prepared. The facility also prepared the hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) stained slides of kidney tissues to be reviewed by a veterinary pathologist.

2.8 Wire Myography

The wire myograph is a system which is capable of measuring and recording isometric tension forces generated by small vessels. The vascular experiments detailed in this thesis were performed using a DMT wire myograph (Danish Myo Technology A/S Inc., Aarhus, Denmark) and Chart 5 Pro software (ADInstruments Inc., Colorado Springs, CO, USA).

Perivascular fat and connective tissues were cleaned from 2nd order mesenteric arteries. The dissected mesenteric artery was cut into four smaller pieces (~2 mm long) and mounted on the wire myograph using 40 μm tungsten wires. Dissected vessels were bathed in PSS which was constantly oxygenated (bubbled with compressed dry air) and maintained at 37 ˚C. The length of each piece of vessel was measured and recorded. In the next step, vessel wall tension of mounted mesenteric arteries was normalized to 0.8 L_{100} (the internal circumference equivalent to a transmural pressure of 100 mmHg). After normalization, mesenteric arteries were equilibrated for 10 minutes. Then, vascular integrity was assessed using a single dose of phenylephrine (PE) (10 μmol/L, Sigma-Aldrich

Inc., St. Louis, MO, USA) twice with a wash and equilibration period between them followed by a single dose of methacholine (MCh) (3 μmol/L, Sigma-Aldrich Inc., St. Louis, MO, USA). At this point in time, vessels were ready to be incubated with inhibitors and exposed to vasoactive agents.

2.9 Big Endothelinin-1 and Endothelin-1 Cumulative Concentration Curves

To test for differences between bigET-induced contractile responses in Sham versus RUPP, a series of functional vascular studies were designed. The first set of experiments consisted of exposing mesenteric arteries from Sham and RUPP animals to cumulative concentrations of big endothelin (bigET-1, AnaSpec Inc., Fremont, Ca, USA; 3 nmol/L to 310 nmol/L), the precursor for ET. Since we provide the vascular tissues only with the precursor of ET with negligible activity, a conversion (cleavage) is necessary before we can observe the response curve to cumulative concentrations of bigET-1. Therefore, the results of this experiment protocol could be considered an activity bioassay of the cleaving enzymes in the tissue.

In the next set of experiments, mesenteric arteries were exposed to cumulative concentrations of endothelin (ET-1, Sigma-Aldrich Inc., St. Louis, MO, USA; 1 nmol/L to 200 nmol/L) with 5 minute intervals between doses. This step was planned to see if differences in bigET-induced contractile responses in Sham versus RUPP were the result of differences in the ET receptor responsiveness.

To further investigate the mechanisms involved in the differences between Sham and RUPP, we targeted enzymes and substances with a known or

hypothetical involvement in the cleavage process of bigET or net contractile response to bigET. In this process, specific enzymes were inhibited by incubating the vessels with respective inhibitors for 30 minutes in the bath before the concentration response curves to bigET.

GM6001 (30 μmol/L, Calbiochem Inc., San Diego, CA, USA), a potent broad-spectrum hydroxamic acid inhibitor of matrix metalloproteinases was used to investigate the involvement of MMP. GM6001 inhibits both MMP-2 (K_i = 0.5 nmol/L) and MMP-9 (K_i = 0.2 nmol/L) as well as MMP-1, -3 and -8. IC50 of GM6001 for inhibition of MMP-2 and MMP-9 are 7 nmol/L and 15 nmol/L respectively.

Phosphoramidon (30 μmol/L, Sigma-Aldrich Inc., St. Louis, MO, USA) which is a metallo-endopeptidase inhibitor was used as an inhibitor of ECE and NEP. To further differentiate between the role and contribution of ECE and NEP, we used **CGS 35066** (25 nmol/L, Tocris Bioscience Inc., Ellisville, MO, USA), a potent ECE inhibitor that displays more than 100-fold selectivity for ECE over NEP (IC50 values are 22 and 2300 nmol/L respectively), and **DL-Thiorphan** (thiorphan) (25 μmol/L, Calbiochem Inc., San Diego, CA, USA), a thiol-containing amino acid that selectively binds to NEP and inhibits its activity (IC50 = 2.1 nmol/L).

Another enzyme known to be directly involved in the cleavage process of bigET is Chymase. **Chymostatin** (100 μmol/L, Sigma-Aldrich Inc., St. Louis, MO, USA), a chymase inhibitor (IC50 value of 0.05 mmol/L), was used to explore the role of this enzyme. Chymostatin is a strong inhibitor of many

proteinases, including chymotrypsin, chymotrypsin-like serine proteinases, chymases and lysosomal cysteine proteinases.

Due to the known vasodilator effects of nitric oxide on the vasculature, as well as its possible interaction with upstream enzymes involved in the cleavage process of bigET-1, the role of nitric oxide was studied using a NOS inhibitor, **L-N^G-Nitroarginine methyl ester** (L-NAME**,** 100 μmol/L, Sigma-Aldrich Inc., St. Louis, MO, USA). L-NAME has K_i of 4-65 μmol/L with IC50 of 3.1 μmol/L.

As previously described in the introduction, the endothelial ET_B receptor is involved in nitric oxide production following excitation by ET. Therefore, we selectively blocked ET_B using **BQ788** (1 μmol/L, Calbiochem Inc., San Diego, CA, USA) with IC50 of 12 nmol/L to > 1 μmol/L, to decipher differences in the complex cleavage process of bigET in Sham versus RUPP.

Matched data from the same animals were used for comparison of curves. The only exception to this was data from bigET curves in the presence of L-NAME and CGS 35066, thiorphan and chymase in which case the comparisons were made with representative bigET-1 curves of Sham and RUPP in the presence of L-NAME (unmatched data).

2.10 Pressure Myography

To specifically confirm bigET cleavage occurs in the endothelium, we designed a set of experiments to denude the endothelium using pressure myography. The pressure myograph is a system that is capable of exposing the microvasculature to determined intraluminal pressure and flow. Since in this

apparatus it is possible to expose the intraluminal endothelium of arteries to drugs and chemicals of interest, we took advantage of this capacity to denude the endothelium of mesenteric arteries. Following the same principles of the wire myograph technique, 2nd order mesenteric arteries of rats were dissected and divided into 5 to 7 mm sections. The proximal end of each segment of mesenteric artery was tied on to one of two glass cannulas of a two chamber pressure myograph system (Living System Instrumentation, St. Albans, VT, USA). After it was flushed of any remaining blood using PSS, the distal end was tied to the second cannula. The artery was then pressurized to 60 mmHg. During the experiment, the temperature of the system was monitored and kept at 37˚C (Thermistor sensor and heating plate, Living System Instrumentation, St. Albans, VT, USA). The vessels were then allowed to equilibrate with the following protocol: 20 minutes at 60 mmHg, 10 minutes at 80 mmHg and 10 minutes at 60 mmHg. During equilibration the PSS was replaced every 10 minutes. The diameter of the mesenteric artery was measured with a digital micrometer (Lasico Los Angeles Scientific Instrument Co. Inc., Los Angeles, CA, USA). At this stage the arteries were exposed to cumulative concentrations of bigET (0.1 µmol/L to 0.3 µmol /L) and their contractile responses were recorded by measuring changes in diameter. Then the endothelial layer of the vessels was denuded using 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (352). Briefly, a 1% solution of CHAPS at a flow rate of 66 µL/min was passed intraluminally through the mounted artery for 5 minutes. Following this step, the mesenteric artery was washed through with PSS at the same flow for 45 minutes.

Denuding of the vessels was tested using a single dose of PE (10 µmol/L) which causes the vessels to constrict followed by a single dose of MCh (3 µmol/L). Failure to cause vasodilatation by MCh was indicative of successful denuding. After denuding the vessels, they were again exposed to the same cumulative concentrations of bigET (0.1 µmol/L to 0.3 µmol /L) that were used prior to denuding and the vascular responses were recorded.

2.11 Dihydroethidium Staining

In view of the fact that oxidative stress was hypothesized as the causative factor in the functional up-regulation of MMP-2, we studied dihydroethidium (DHE) and nitrotyrosine levels in the vessel walls of mesenteric arteries in order to see evidence of oxidative stress. DHE is cell permeable and reacts with intracellular and extracellular superoxide to yield ethidium, which binds to nuclear DNA and generates nuclear fluorescence (353).

Slides with 20 µm sections of mesenteric arteries were prepared from frozen tissues stored in O.C.T.. The O.C.T. was washed off the slides using Hanks Balanced Salt Solution (HBSS). The slides were then incubated in 37 °C with HBSS for 10 minutes. A 1:1000 (20 µmol/L) dilution of DHE (Molecular Probes Inc., Eugene, OR, USA) in HBSS was used and the slides were incubated for 30 minutes at 37 °C and covered to avoid light exposure. In the next step the slides were rinsed using HBSS and images were taken of mesenteric artery sections using an IX81 Olympus fluorescence microscope. The mean fluorescence

intensity of images was analyzed using the histograph function of Adobe Photoshop elements 2.0 (Adobe Systems Inc., Mountain View, CA, USA).

2.12 Nitrotyrosine Staining

This antibody staining technique is an immune-fluorescence method of visualizing nitrotyrosine; a product of tyrosine nitration mediated by reactive nitrogen species such as peroxynitrite. The staining protocol was performed on slides prepared with 10 µm sections of mesenteric arteries prepared from frozen tissues stored in O.C.T.. Slides were incubated for 1 hour in room temperature with 1:150 dilution of primary antibody (Upstate Biotechnology Inc., Lake Placid NY, USA). The slides were then incubated with a secondary antibody (Alexa Fluor 488, Invitrogen Inc., Burlington, ON, Canada) for 45 minutes. The images were taken using an IX81 Olympus fluorescence microscope (Olympus, Center Valley, PA, USA) and analyzed in the same manner as DHE staining.

2.13 Gelatin Zymography

Gelatin zymography detects both the pro-enzyme and mature forms of MMP-2 based on their molecular weight. While it is capable of measuring *ex vivo* gelatinase activity in a semi-quantitative manner, this indirect activity measurement is mostly utilized to detect the quantity of different types of gelatinases. In fact, since many factors which are involved in the gelatinase activation such as TIMP are not present in the experiment medium, the

measurements could not be indicative of actual gelatinase activity. This technique is capable of detecting both pro (72 kDa) and active (64 kDa) forms of MMP-2.

Protein homogenates were prepared for both Western blot and zymography. Frozen mesenteric arteries were thawed and dissected clean of adjacent veins, perivascular fat or blood clots. Homogenizing buffer and protease inhibitor cocktail (Dimethyl sulfoxide, 4-(2-Aminoethyl) benzenesulfonylfluoride hydrochloride, Trypsin inhibitor, pancreatic basic, Bestatin hydrochloride, N-(trans-Epoxysuccinyl)-L-leucine 4- guanidinobutylamide, Acetyl-leucine-leucine-arginal, hemisulfate, Pepstatin A) (P8340, Sigma-Aldrich Inc., St. Louis, MO, USA) were added to the dissected mesenteric arteries which were then homogenized. The homogenates were centrifuged and supernatants were collected. Lastly, protein concentration was measured using BCA Protein Assay Reagent (Thermo Fisher Scientific Inc., Rockford, IL, USA) in each sample.

Briefly, aliquots of the supernatant samples of mesenteric arteries, as mentioned above, with equal amount of protein were prepared using 6X loading buffer (7 mL of Tris buffer, 3.0 mL of glycerol, 1.0 gram of SDS and 1.2 mg bromophenol made up to 10 mL using double distilled water). The samples were loaded into lanes of a 30% acrylamide gel containing SDS copolymerized with 20 mg/mL of gelatine. After running the electrophoresis for 1.5 hours the gel was washed in TritonX-100 at room temperature for 1 hour (3 x 20 minutes). The gel was then incubated in development buffer (50 mmol/L Tris HCl, 0.15 M NaCl, 5 mmol/L $CaCl_2$) overnight in 37 ˚C. Subsequently, the gel was stained using Coomassie Brilliant Blue G-250 (0.05%) for 1.5 hours and then put in to

destaining solution for 2 hours. Lastly, the gel was scanned using Fluor-S-MAX MultiImager (Bio-Rad Laboratories (Canada) Ltd., Mississauga, On, Canada). Quantification was performed using Quantity One Software (Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON, Canada).

2.14 Western Blot

As a complement to our functional studies, Western blot experiments were designed to measure possible differences in enzyme expression of MMP-2, ECE-1 and eNOS.

Briefly, samples of homogenates from mesenteric arteries were loaded in to lanes of a 30% acrylamide gel. A molecular weight ladder was also loaded and electrophoresis was run until the dye front reached the bottom (approximately 1 hour). After that, the stacking gel was removed. The separating gel was then placed next to a nitrocellulose membrane in a transfer sandwich. The assembled sandwich was put into the electrophoresis apparatus filled with transfer buffer and run for 1 hour. After the end of transfer, the membrane was placed in blocking solution (25% Rockland commercial blocking buffer in phosphate buffered saline) for 1 hour at room temperature. In the next step, the membrane was incubated with a primary antibody against the protein of interest in phosphate buffered saline (PBS) (1 μg/mL) and β-actin antibody (1:1500) for a protein loading control for 2 hours at room temperature.

After rinsing with PBS for 5 minutes, the membrane was incubated with labeled secondary antibody for 1 hour at room temperature. Lastly, the membrane

was rinsed in double distilled water and images were taken and analyzed using the Li-Cor Odyssey v3.0 imager and software system (Mandel Scientific Company Inc., Guelph, ON, Canada). The primary antibodies and their dilutions used for Western blot protocols are as follows: eNOS (BD Bioscience Inc., Mississauga, ON, Canada) at 1:250, nNOS (BD Bioscience Inc., Mississauga, ON, Canada) at 1:250, MMP-2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:200 and ECE-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:200 dilutions. Goat anti-rabbit and donkey anti-mouse flourochrome conjugated (LI-COR Bioscience Inc., Lincoln, NE, USA; 1:10000 dilution) were used as the secondary antibodies.

2.15 Statistics

Data are presented as mean ± SEM. For bigET-1, dose-response curves in the absence or presence of L-NAME, was calculated as area under the curve and compared between groups. Analyses with two comparisons were conducted using Student's t test. Two-way analysis of variance (ANOVA) with Bonferroni's post-hoc analysis was used to compare vascular responses to bigET and ET-1 in Sham and RUPP with and without inhibitors. Data were analyzed using GraphPad Prism 5.02 Software (GraphPad Software Inc. California U.S.A.). A $p<0.05$ was considered significant.

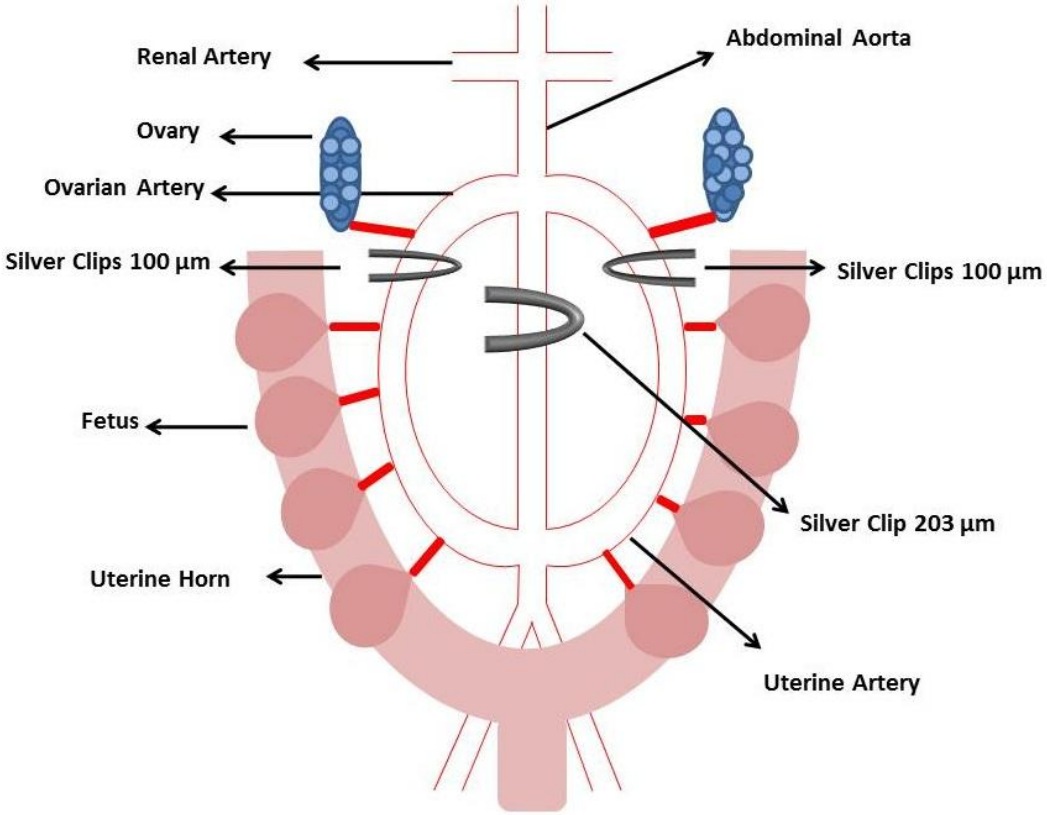

Figure 2.1- Schematic of the Reduced Utero-placental Perfusion Pressure (RUPP) surgery.

Chapter 3:

Results

3.1 Animal Model

3.1.1 Blood Pressure and Heart Rate

Blood pressure measurements on the experimental day (D20) revealed both systolic and diastolic blood pressures to be significantly higher in the animals who underwent RUPP surgery compared to Sham animals (systolic BP: 116.3 ± 2.6 mmHg vs. 103.8 ± 3.6 mmHg, $p<0.05$ and diastolic BP: 96.6 ± 2.8 mmHg vs. 84.3 ± 5 mmHg, $p<0.05$) (**Figure 3.1A and B**).

ECG heart rate readings at the time of blood pressure measurement did not show any difference between RUPP and Sham animals (**Figure 3.1C**).

3.1.2 Proteinuria and Kidney Morphology

Measurement and calculation of the albumin/creatinine ratio in a 24-hour urine sample showed an increase in the excreted protein on the day prior to euthanasia (D13) compared to the day before surgery (D19) in some of the RUPP animals (**Figure 3.2B**). However, this did not reach statistical significance between the groups (**Figure 3.2C**).

Pathology review of the H&E and PAS stained slides of prepared kidney tissues exhibited modest changes in glomerular morphology. In these rats, multifocal to segmental sections of renal cortex had glomeruli that were slightly enlarged compared to sham and displayed mild to moderate swelling of endothelial cells that appeared to reduce capillary space (glomerular endotheliosis) (**Figure 3.3**).

3.1.3 Conceptus Numbers and Measurements

Comparison of litter size at the time of surgery and euthanasia, revealed that viability was significantly reduced in RUPP compared to Sham animals (22.7% ± 3.2 vs. 84.5% ± 5.9, $p<0.0001$) (**Figure 3.4A**). Mean weight of pups at euthanasia demonstrated a trend ($p=0.058$) to be lower in RUPP versus Sham (**Figure 3.4B**). Crown-rump length was significantly shorter (39.2 ± 0.5 mm vs. 42.7 ± 0.4 mm, $p< 0.0001$) and length to girth ratio was significantly smaller (1.05 ± 0.01 vs. 1.11 ± 0.02, $p<0.05$) in RUPP compared to Sham (**Figures 3.4C and 3.4D**).

3.2 Functional Studies

The first step in the functional studies to test our hypothesis was a comparison of the contractile responses of mesenteric arteries to bigET. The forces generated by arteries from the RUPP group were significantly greater than Sham (**Figure 3.5**, $p<0.0001$, two-way ANOVA). While this finding supports our hypothesis of enhanced bigET responsiveness in a model of preeclampsia, it does not tell us whether the difference was due to increased processing and cleavage of bigET or altered smooth muscle responsiveness to ET-1. In the next set of experiments, we exposed mesenteric arteries to cumulative concentrations of ET (the product of the bigET cleavage process and the molecule that binds to receptors to elicit a vascular response). The contractile force generated by the vessels to ET-1 was not different between Sham and RUPP animals (**Figure 3.6**).

To confirm that the contractile capacities of the vessels are the same in Sham and RUPP, we exposed the mesenteric arteries to high potassium physiological

salt solution (KPSS, 123.70 mmol/L). Since the vascular contractions seen in response to KPSS are independent of bigET processing (cleavage) and receptors, this provides a reasonable mean to test the capacity of vessels to generate contractile forces. Vascular responses to KPSS did not show any difference between Sham and RUPP groups (**Figure 3.7**).

Since it has been previously shown that the endothelium-dependent relaxation pathway involving nitric oxide is reduced in systemic vessels of RUPP animals (324), an accurate interpretation of the findings in vascular function studies is not possible without knowing the contribution of nitric oxide to each of the contractile responses. After incubation of vessels with L-NAME (an inhibitor of NOS) mesenteric arteries were exposed to cumulative concentrations of bigET. The contractile responses of mesenteric arteries were significantly greater in both Sham and RUPP groups in the presence of L-NAME in comparison to their respective controls (**Figures 3.8A and B**, $p<0.0001$ and $p<0.05$ respectively, two-way ANOVA). Further analysis and comparison of the responses revealed that nitric oxide had a greater contribution to bigET-induced vascular tone in the Sham group compared to RUPP (**Figure 3.8C**, $p<0.05$). Interestingly, comparison of bigET response curves in the presence of L-NAME for both Sham and RUPP did not reveal any significant difference between the groups (**Figure 3.8D**). L-NAME also modulated response to ET-1 in both Sham and RUPP (**Figures 3.9A and B**, $p<0.5$ and $p<0.001$ respectively, two-way ANOVA). Contrary to our finding in bigET response curves, comparison of the responses did not show any significant difference between Sham and RUPP (**Figure 3.9C**). Moreover, comparison of the

contractile forces of mesenteric arteries in response to ET-1 between Sham and RUPP did not show any difference (**Figure 3.9D**). These findings show that nitric oxide modulates bigET and ET-1 responses in both Sham and RUPP. However, the observed differences between the contribution of nitric oxide in Sham and RUPP indicates that while the modulating effect of nitric oxide on bigET cleavage (upstream pathway) is more prominent in Sham (less prominent in RUPP), its contribution to vascular tone is not different between Sham and RUPP when the response to ET-1 (product) is measured.

The next step was to investigate the role of specific enzymes in the processing of bigET to ET-1 in Sham versus RUPP groups. Following our hypothesis that oxidative stress would increase MMP activity, leading to increased processing of bigET, mesenteric arteries from Sham and RUPP animals were pre-incubated with GM6001 (a gelatinase inhibitor with a high affinity for MMP-2) prior to bigET responses. In Sham animals, response curves in the absence or presence of GM6001 were not significantly different (**Figure 3.10A**). GM6001, however, significantly decreased bigET responses in RUPP (**Figure 3.10B**, $p<0.0001$, two-way ANOVA).

Although this finding suggests greater MMP processing of bigET in RUPP animals, proper attention should also be given to two factors that could have played a role in this finding. One factor is that the modest contractile forces generated by vessels from the Sham group in response to bigET could have been a confounding factor; limiting the power to detect differences between responses in the absence and presence of inhibitors in Shams. Moreover, since the responses to

bigET were shown to be significantly higher in RUPP versus Sham animals, the two groups have significantly different baselines from which the inhibitors were being compared. Since the contribution of nitric oxide is different in Sham and RUPP groups, and the baseline responses to bigET are normalized between Sham and RUPP groups in the presence of L-NAME, we incubated mesenteric arteries with L-NAME prior to investigating the effect of inhibitors on bigET responses. Since the presence of L-NAME also increased the forces generated in response to bigET in Sham animals, this approach also resolved the confounding factor of the modest contractile forces in this group. This approach, therefore, was repeated for all enzymatic pathways investigated.

Contrary to our finding in the absence of L-NAME, after inhibition of nitric oxide production in Sham animals, a small but significant decrease in bigET response forces were observed in the presence of GM6001 (**Figure 3.11A**, $p<0.05$, two-way ANOVA). In RUPP animals, however, after the incubation of mesenteric arteries with L-NAME, the responses to bigET did not show any significant difference in the presence of GM6001 (**Figure 3.11B**).

In order to further investigate the role of MMP-2 in the observed differences, we compared the quantity of MMP-2 in the mesenteric arteries from Sham and RUPP. Zymography, as a semi-quantitative method, was used to compare the levels of MMP-2 in mesenteric arteries (**Figure 3.12A**). While the results showed that the proMMP-2 (72kDa) had a significantly lower quantity in RUPP compared to Sham (**Figure 3.12B**, $p<0.05$, t-test), no significant difference was observed when active MMP-2 (64kDa) levels were compared (**Figure 3.12C**). The Western

blot analysis of MMP-2 was used to complement our quantitative data. Interestingly, expression levels of MMP-2 in mesenteric arteries were significantly higher in RUPP compared to Sham (**Figure 3.13**).

We further investigated whether other enzymes involved in the cleavage of bigET to ET-1. ECE is a well-characterized enzyme which is considered to be the most active enzyme involved in the cleavage of bigET to ET-1. Inhibition of ECE by phosphoramidon significantly decreased bigET-induced responses in both Sham and RUPP groups (**Figures 3.14A and B**, $p<0.0001$, two-way ANOVA). Similar results were seen in the presence of L-NAME (**Figures 3.15A and B**, $p<0.0001$, two-way ANOVA). Moreover there was no significant difference in ECE expression between Sham and RUPP (**Figure 3.16**).

Although phosphoramidon is primarily an ECE inhibitor, it also has actions to inhibit NEP. To better understand the role of these different enzymes we, therefore, chose to use inhibitors with more specific action. CGS35066, an ECE specific inhibitor, and thiorphan, an NEP specific inhibitor, were chosen to be used in our experimental protocols.

Similar to phosphoramidon, preincubation of vessels from Sham and RUPP with CGS35066 significantly decreased responses to bigET compared to controls in both groups (**Figures 3.17A and B,** $p<0.05$ and $p<0.001$, two-way ANOVA). A similar effect of CGS35066 was also observed in the presence of L-NAME in both Sham and RUPP groups (**Figures 3.18A and B**, $p<0.0001$, two-way ANOVA).

The results for the vessels pre-incubated with thiorphan followed a slightly different trend. Incubation with NEP inhibitor did not have any significant effect on the contractile responses to bigET either in Sham or RUPP (**Figures 3.19A and B**). However, interestingly, in the presence of L-NAME, the responses were significantly inhibited in both Sham and RUPP (**Figures 3.20A and B**, $p<0.05$ and $p<0.0001$, two-way ANOVA).

Another enzyme known to be able to cleave bigET to ET-1 is chymase. Since several studies have linked higher chymase activity with the pathophysiology of preeclampsia (354-356), we investigated the possibility of involvement of this enzyme in the observed differences between Sham and RUPP. In this set of experiments preincubation of vessels with the chymase inhibitor (chymostatin) did not alter responses to bigET in either Sham or RUPP (**Figures 3.21A and B).** However, when these experiments were performed in the presence of L-NAME, contractile responses to bigET were significantly decreased in RUPP (**Figure 3.22B**, $p<0.01$, two-way ANOVA) but not in Sham (**Figure 3.22A**).

Observation of the contribution of nitric oxide and its possible interaction with other enzymes involved in the cleavage process of bigET led us to investigate the role of ET_B for our last functional study. Knowing that ET_B receptors on the endothelium contribute to endothelial nitric oxide production, we pre-incubated the vessels with an ET_B specific receptor blocker (BQ788) before running the response curves of bigET. Inhibition of the ET_B receptor by pre-incubation of vessels with BQ788 did not cause any significant changes in the response curves of Sham and RUPP to bigET (**Figures 3.23A and B**). Moreover, the same results

were observed in the presence of L-NAME in both groups (**Figures 3.24A and B**).

Cellular localization of cleavage of bigET to ET was probed using pressure myography and endothelium denuding technique. The contractile response to bigET, observed in the intact mesenteric arteries, was absent after the vessels were denuded from their endothelium (**Figure 3.25**, $p<0.0001$, two-way ANOVA).

Further investigation in the role of nitric oxide in the enzymatic pathways as well as the potential contribution of NOS enzymes to oxidative and nitrative stress (as described earlier in the introduction) led us to perform Western blot assay of eNOS in the mesenteric arteries. The result of the experiment showed that eNOS was significantly more abundant in the mesenteric arteries from RUPP compared to Sham animals (**Figure 3.26**, $p<0.05$, t-test).

Lastly, as measures of oxidative and nitrative stress, DHE and nitrotyrosine staining techniques, respectively were performed on sections of mesenteric arteries from RUPP and Sham animals. Contrary to our expectation, the levels of DHE (**Figure 3.27**) or nitrotyrosine (**Figure 3.28**) were not different between Sham and RUPP.

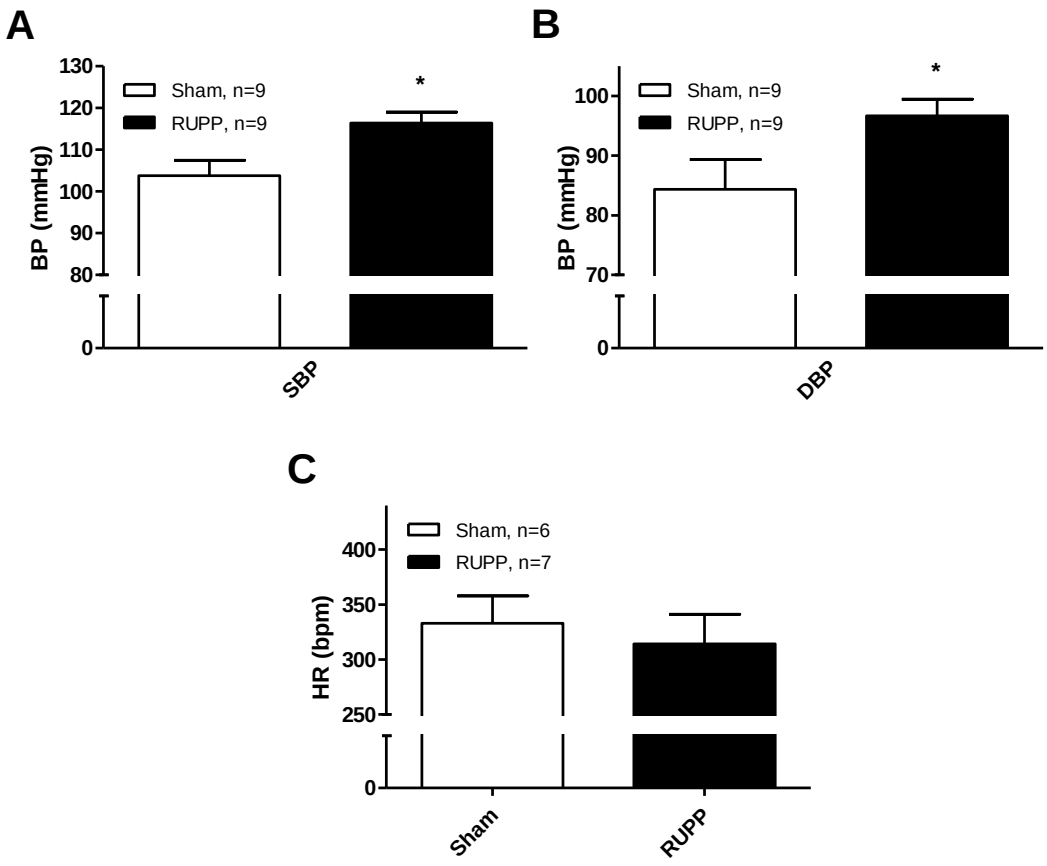

Figure 3.1- Systolic (SBP) and Diastolic (DBP) blood pressures and heart rate measurements from Sham and RUPP animals on day 20 of gestation.

Both systolic (A) and diastolic (B) blood pressures were significantly higher in RUPP compared to Sham (*: $p<0.05$, t-test). There was no significant difference in heart rates (C) between Sham and RUPP (ns, t-test). *Blood pressure and heart rate measurements were performed by Dr. Jude Morton.*

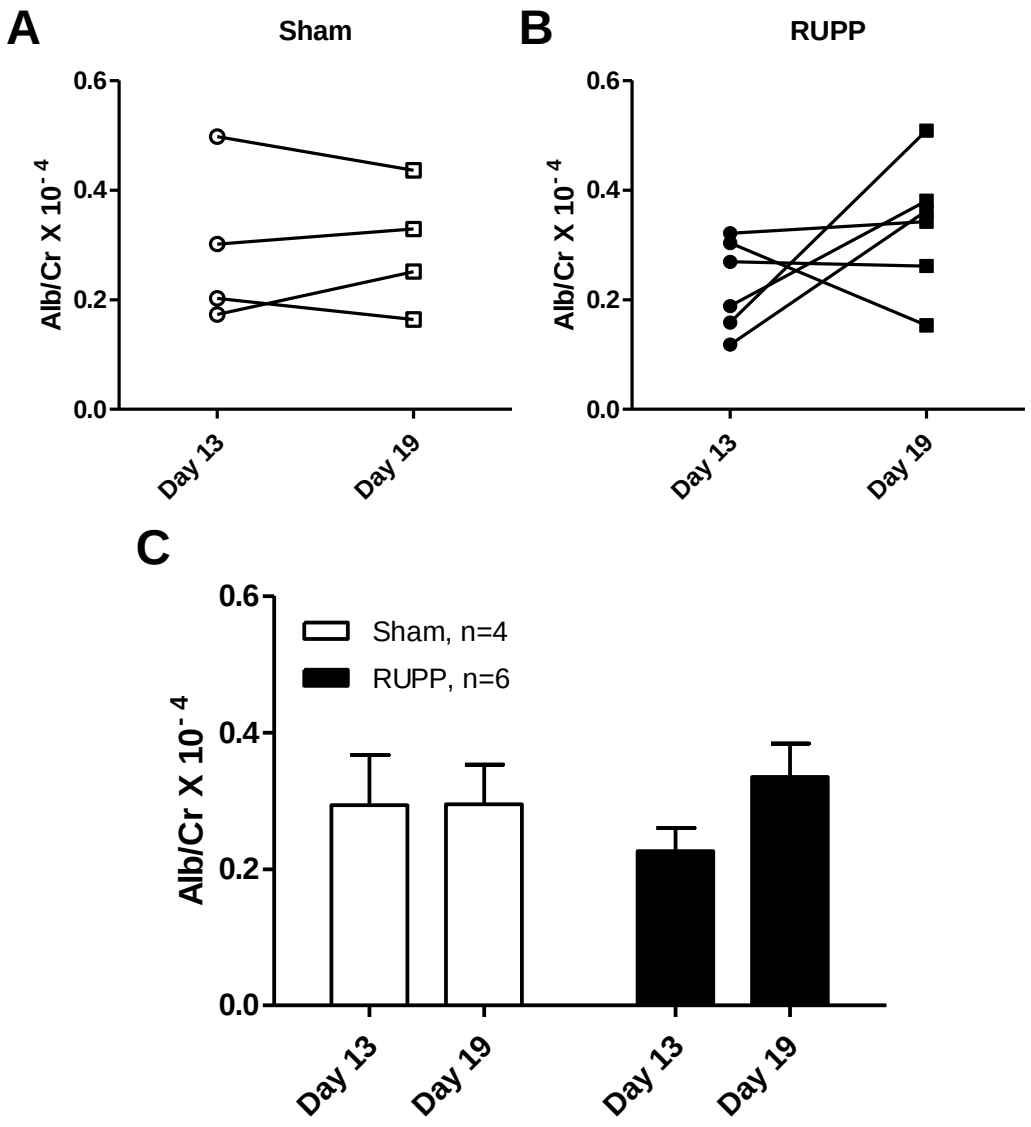

Figure 3.2- Comparison of albumin (Alb) to creatinine (Cr) ratio excreted in a 24-hour urine sample in Sham and RUPP animals, day 13 (pre-surgery) and day 19 (pre-euthanasia).

Albumin to creatinine ratios were not significantly different in either Sham (A) or RUPP (B), in the comparison of pre-surgery to pre-euthanasia values (ns, paired t-test). There was no significant difference when albumin to creatinine ratios were compared between Sham and RUPP, day 13 (pre-surgery) and day 19 (pre-euthanasia) (ns, two-way ANOVA).

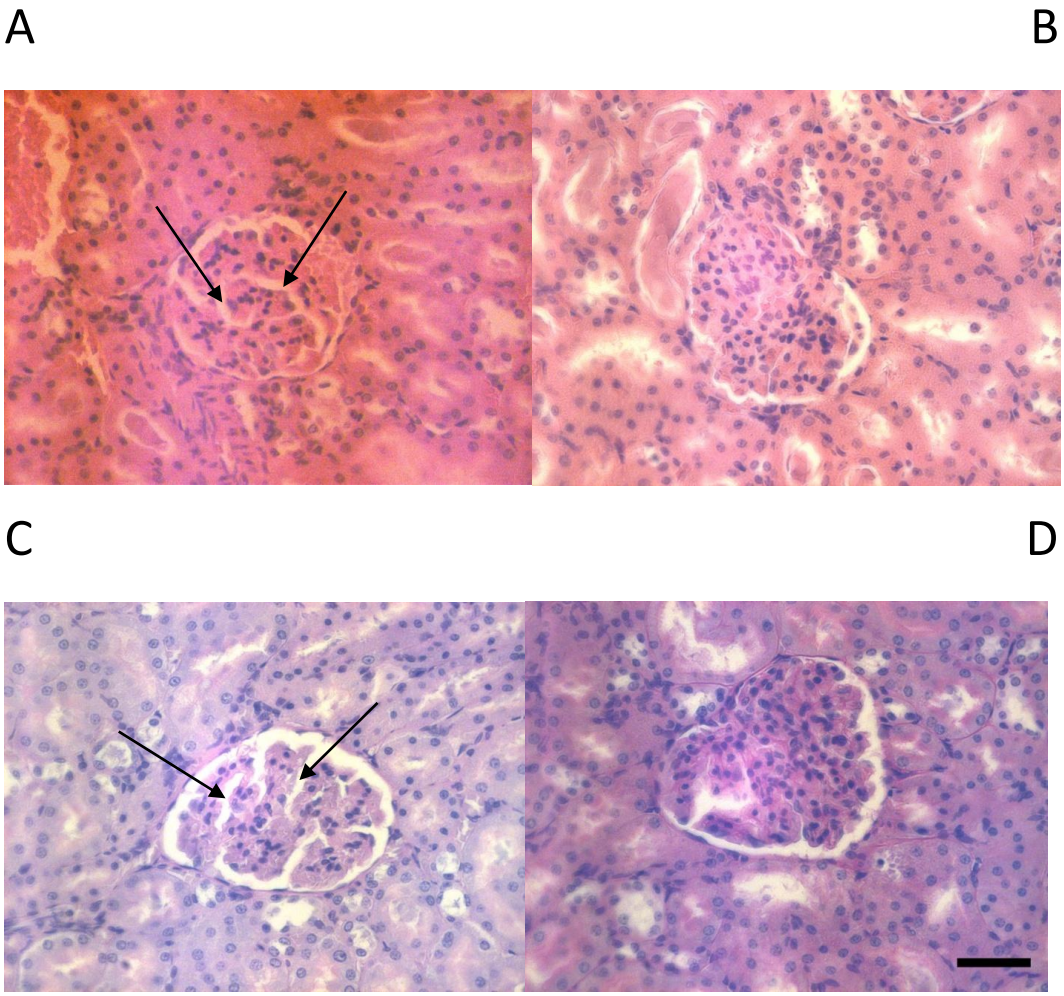

Figure 3.3- Hematoxylin and eosin (A and B) and periodic acid-Schiff (C and D) staining of kidney sections from Sham and RUPP animals.

The arrows in the slides from Sham (A and C) denote the spaces that we expect to see in a normal nephron. In the slides from RUPP animals (B and D) those aforementioned spaces are clearly diminished due to vascular endotheliosis. *Slides were reviewed by Dr. Richard Uwiera, Veterinary Pathologist.*

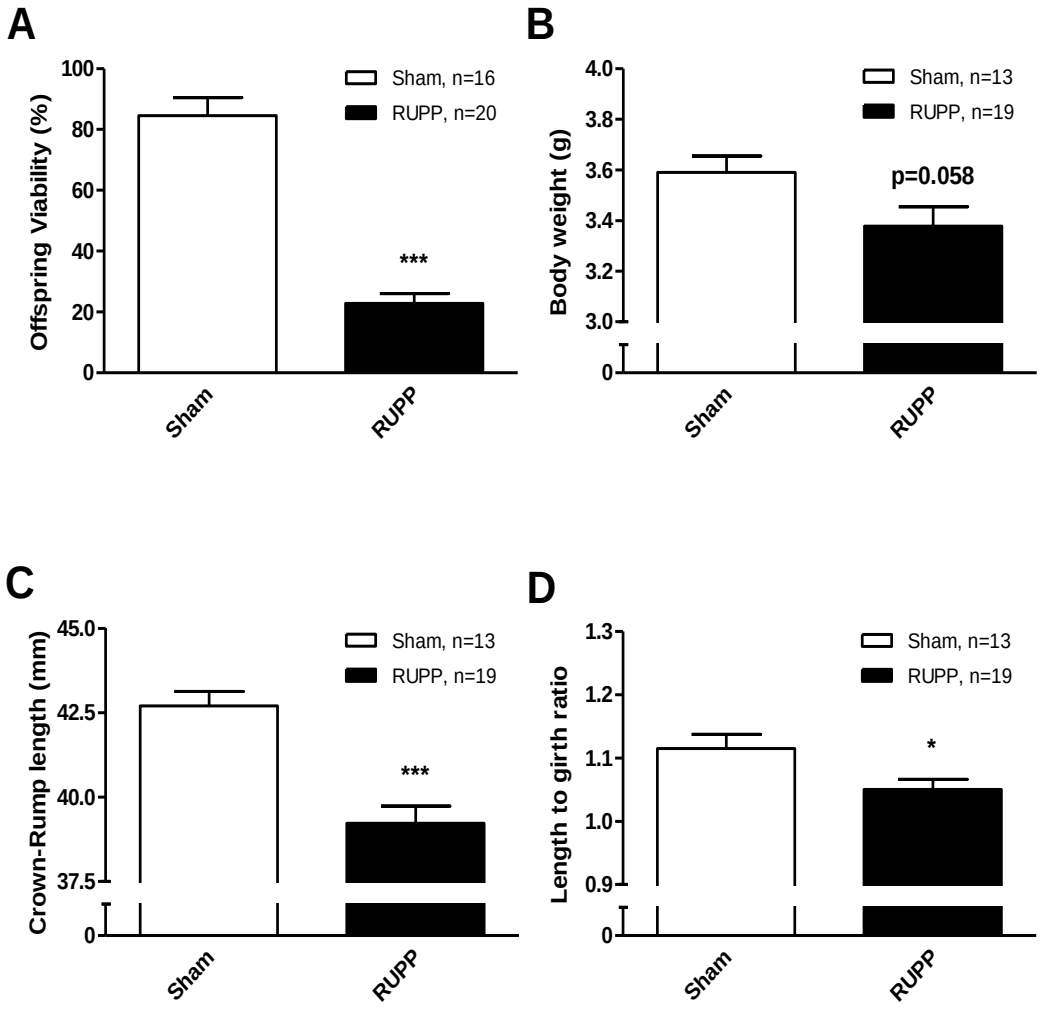

Figure 3.4- Pup viability and morphometric data from Sham and RUPP animals.
Viability of offspring (A) was significantly less in RUPP compared to Sham. The difference between pup weights (B) in Sham and RUPP did not reach significance. Crown-rump length (C) was significantly shorter and length to girth ratio (D) was significantly smaller in the pups from RUPP compared to Sham (ns,*: $p<0.05$, ***: $p<0.0001$, t-test).

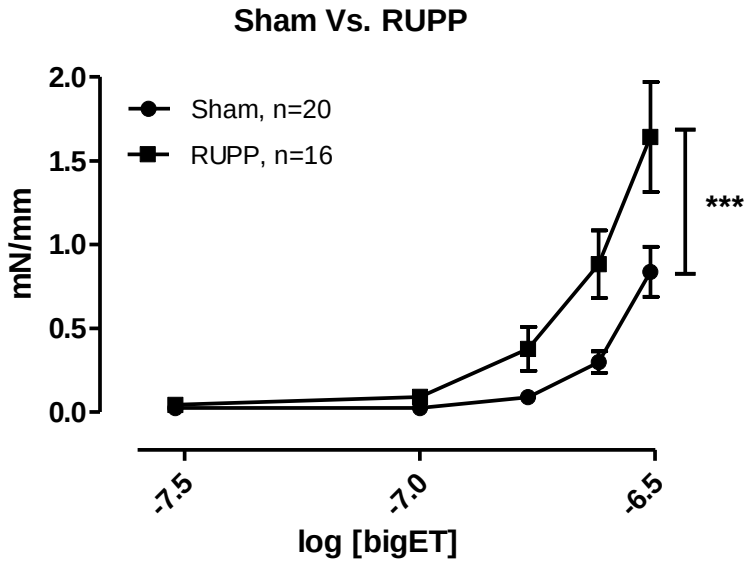

Figure 3.5- Response curves of mesenteric arteries from Sham and RUPP animals to cumulative concentrations of bigET.

Contractile responses to **bigET** were significantly greater in RUPP compared to Sham (***: $p<0.0001$, two-way ANOVA).

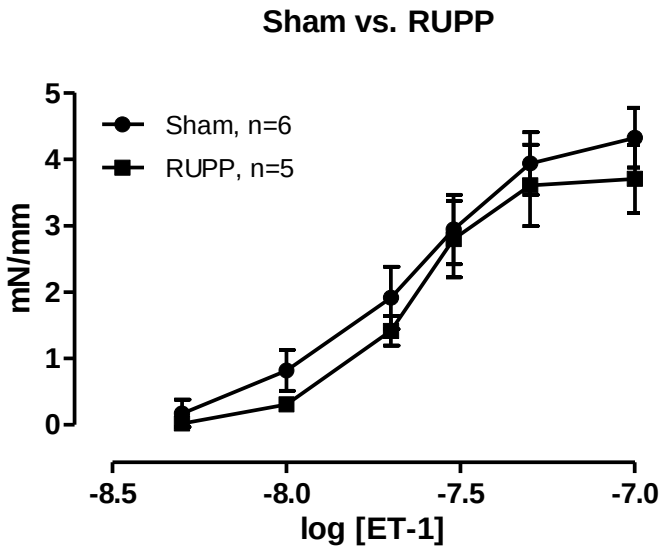

Figure 3.6- Response curves of mesenteric arteries from Sham and RUPP animals to cumulative concentrations of ET-1.

There was no significant difference between contractile responses to **ET-1** in Sham compared to RUPP (ns, two-way ANOVA).

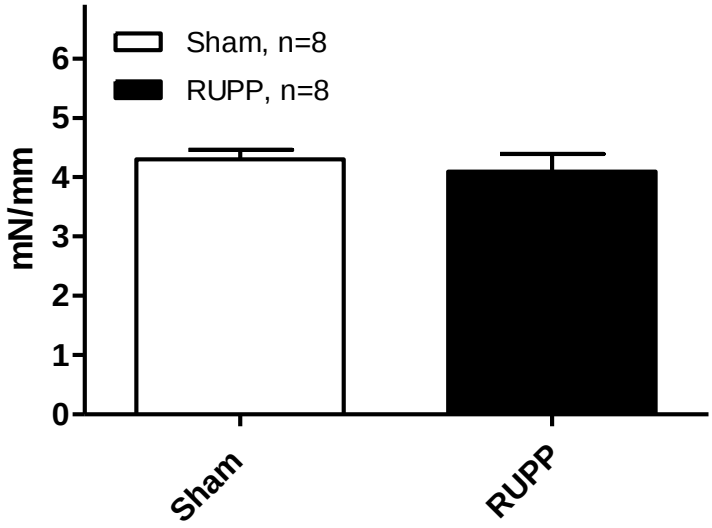

Figure 3.7- Contractile forces generated by mesenteric arteries from Sham and RUPP animals in response to KPSS.

There was no significant difference between the contractile forces generated by mesenteric arteries from Sham and RUPP.

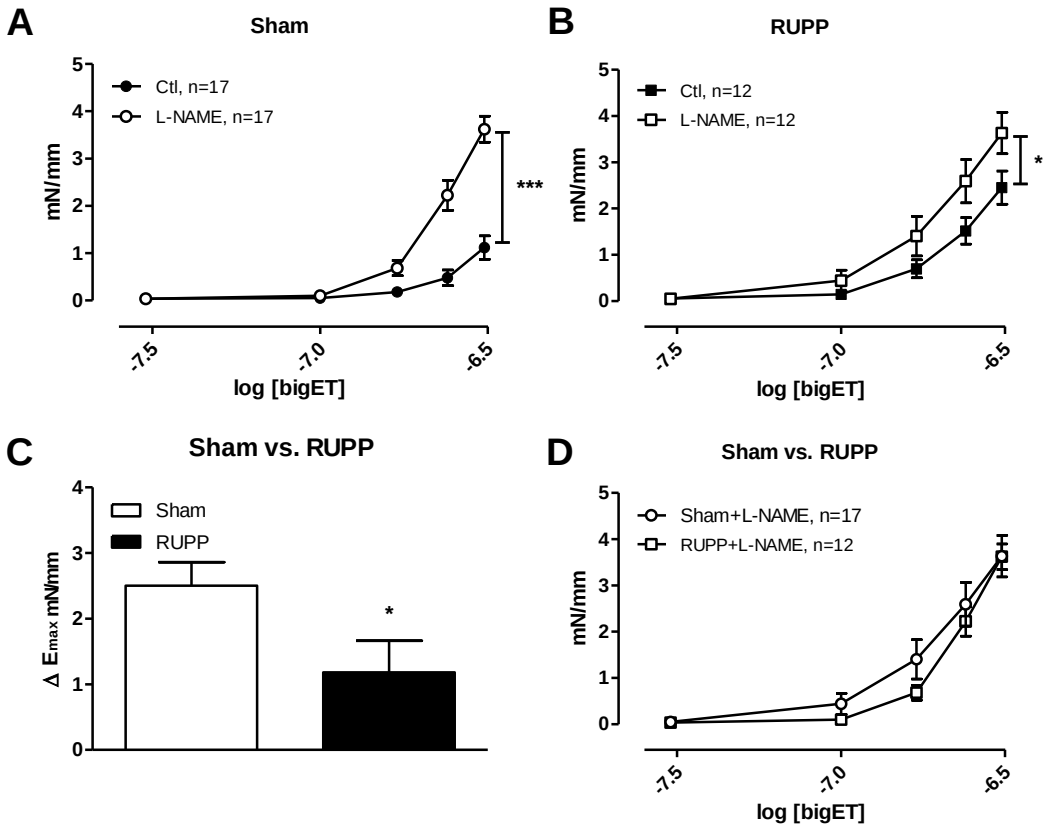

Figure 3.8- Response curves and response difference (Δ E_{max}) of mesenteric arteries from Sham and RUPP animals in response to cumulative concentrations of bigET in the absence (Control/Ctl) or presence of L-NAME (NOS inhibitor, 100 µmol/L).

Contractile responses to **bigET** were significantly increased in both Sham (A) and RUPP (B) in the presence of **L-NAME**. Δ E_{max} (C) was significantly smaller in the presence of **L-NAME** in RUPP compared to Sham. (D) There was no significant difference between **bigET** responses from Sham and RUPP in the presence of **L-NAME** (ns, *: $p<0.05$, **: $p<0.01$ and ***: $p<0.0001$, two-way ANOVA).

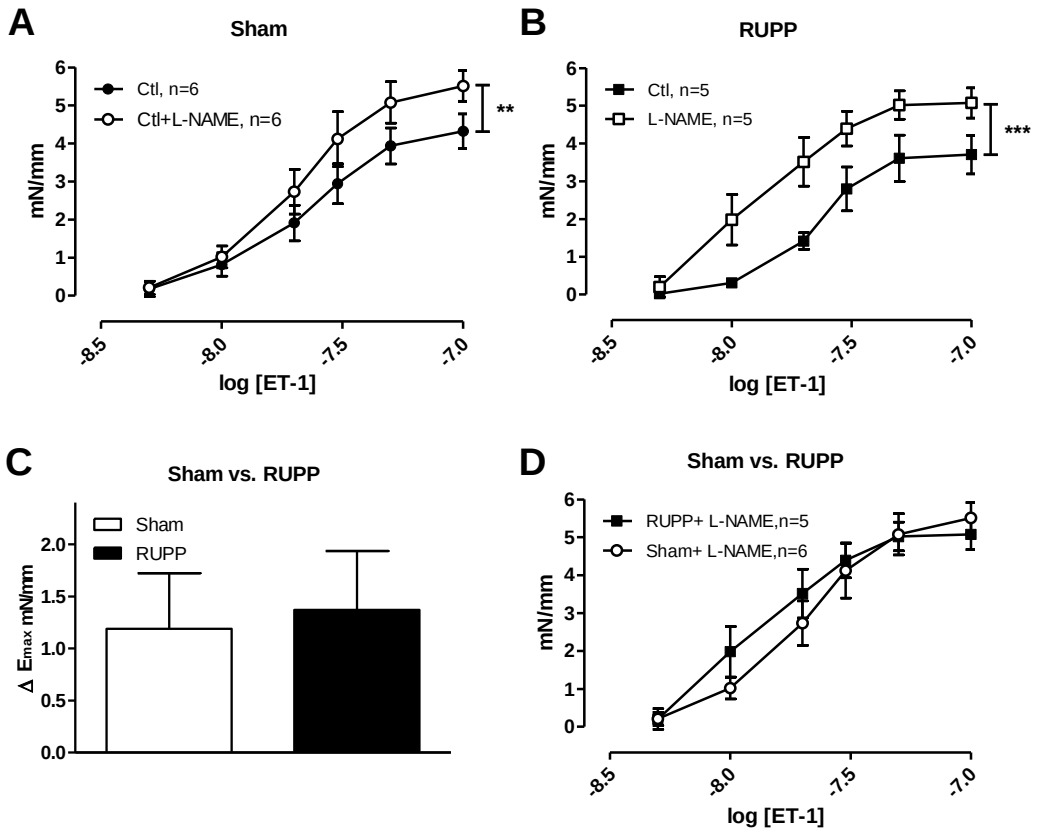

Figure 3.9- Response curves and response difference (Δ Emax) of mesenteric arteries from Sham and RUPP animals in response to cumulative concentrations of ET in the absence (Control/Ctl) or presence of L-NAME (NOS inhibitor, 100 µmol/L).

Contractile responses to **ET** were significantly increased in both Sham (A) and RUPP (B) in the presence of L-NAME. There was no significant between Δ E_{max} (C) in Sham and RUPP in the presence of **L-NAME**. There was no significant difference between **ET** responses from Sham and RUPP in the presence of **L-NAME** (ns, **: $p<0.01$ and ***: $p<0.0001$, two-way ANOVA).

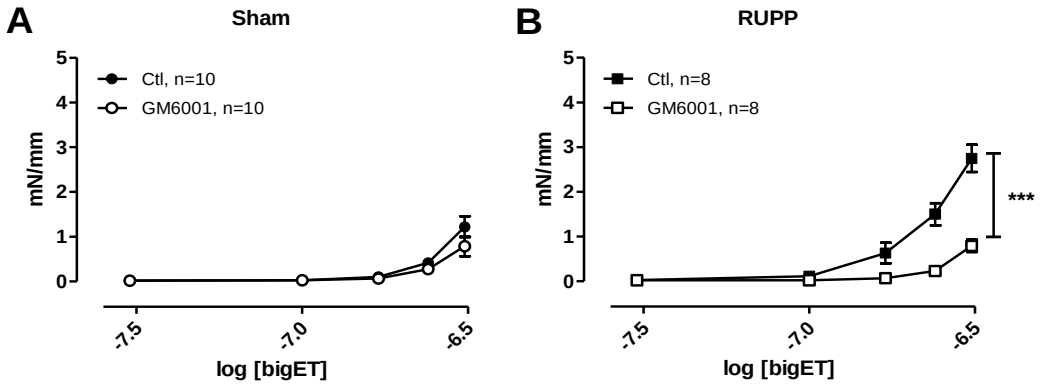

Figure 3.10- Response curves of mesenteric arteries from Sham and RUPP animals to cumulative concentrations of bigET in the absence (Control/Ctl) or presence of GM6001 (MMP-2 inhibitor, 30 µmol/L).

Contractile responses to **bigET** were not different in Sham (A) in the presence and absence of **GM6001**. The responses were significantly inhibited in RUPP (B) in the presence of **GM6001** (***: $p<0.0001$, two-way ANOVA).

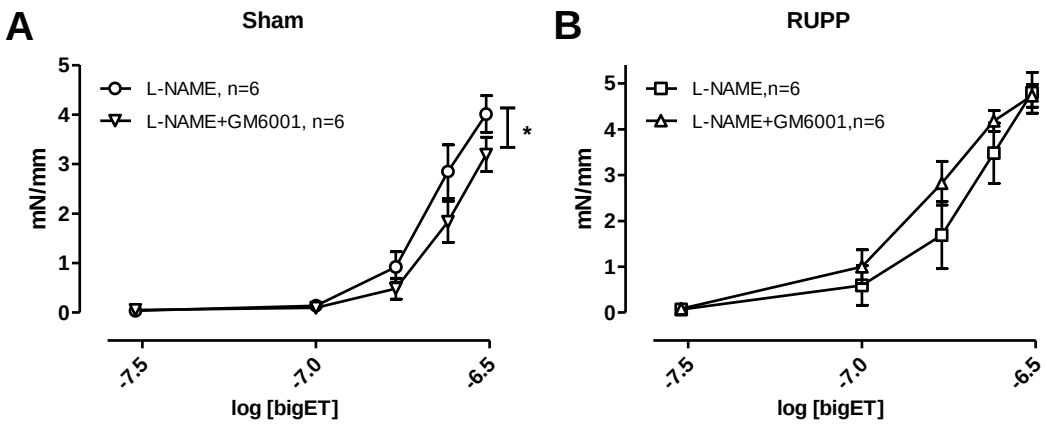

Figure 3.11- Response curves of mesenteric arteries from Sham and RUPP animals pre-incubated with L-NAME (NOS inhibitor, 100 µmol/L) in response to cumulative concentrations of bigET in the absence (Control/Ctl) or presence of GM6001 (MMP-2 inhibitor, 30 µmol/L).

In the presence of **GM6001**, contractile responses to **bigET** were significantly inhibited in Sham (A). However, the contractile responses were not different in RUPP (B) in the presence or absence of **GM6001** (*: $p<0.05$, two-way ANOVA).

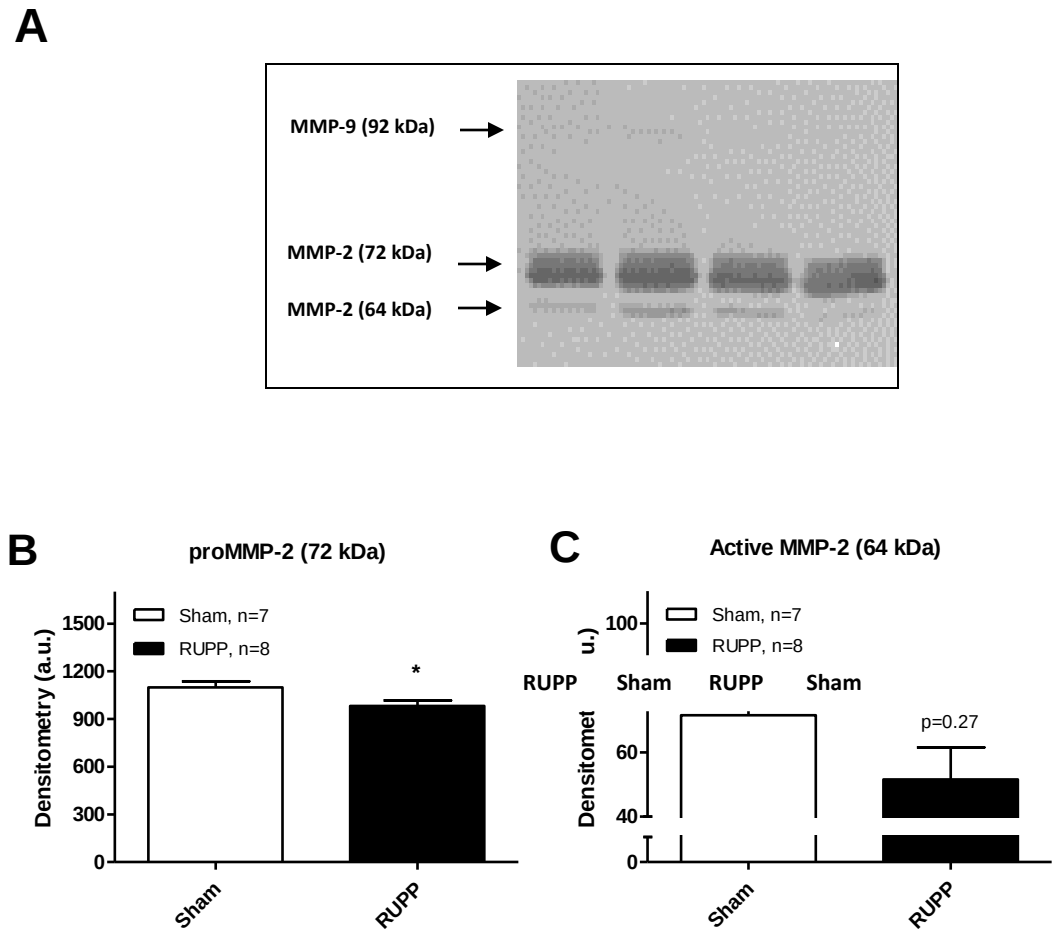

Figure 3.12- Representative zymogram and MMP-2 levels of mesenteric arteries from Sham and RUPP animals, analyzed by gelatine zymography.

Zymographic band densities from mesenteric artery samples were quantified by densitometry, representative image shown (A). MMP-9 (92 kDa) band is almost non-existant compared to MMP-2. The relative enzyme quantity of 72 kDa MMP-2 (B) were significantly lower in RUPP compared to sham. Nonetheless, in the comparison between the quantity of the active form of MMP-2 (64 kDa) (C), there were no difference between Sham and RUPP (ns and *: $p<0.05$, t-test) (a.u.: arbitrary units). *Zymography was performed with the help of Dr. Stephane Bourque.*

A

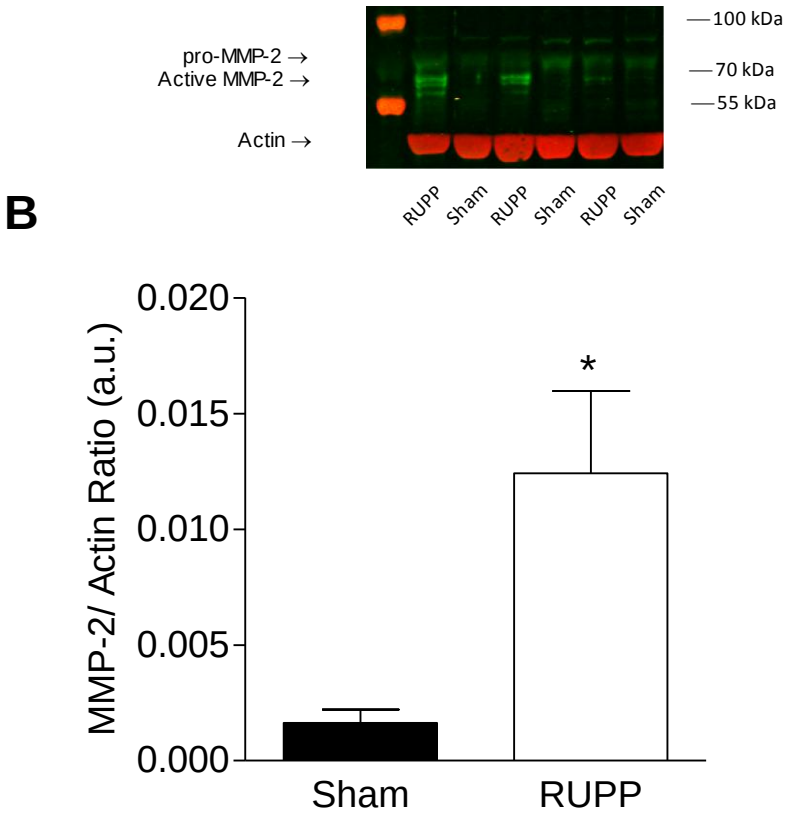

Figure 3.13- Representative Western blot image (A) and relative quantification of MMP-2 (B) in mesenteric arteries from Sham and RUPP analyzed by fluorescent intensity.

MMP-2 levels were significantly higher in RUPP compared to Sham (different in Sham and RUPP (*: $p<0.05$, t-test).

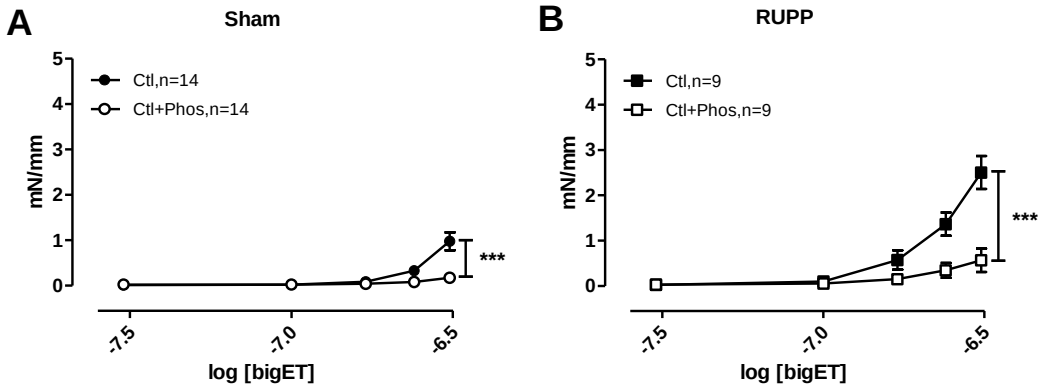

Figure 3.14- Response curves of mesenteric arteries from Sham and RUPP animals to cumulative concentrations of bigET in the absence (Control/Ctl) or presence of phosphoramidon (Phos, ECE inhibitor, 30 µmol/L).

Contractile responses to **bigET** were significantly inhibited in both Sham (A) and RUPP (B) in the presence of **phosphoramidon** (***: $p<0.0001$, two-way ANOVA).

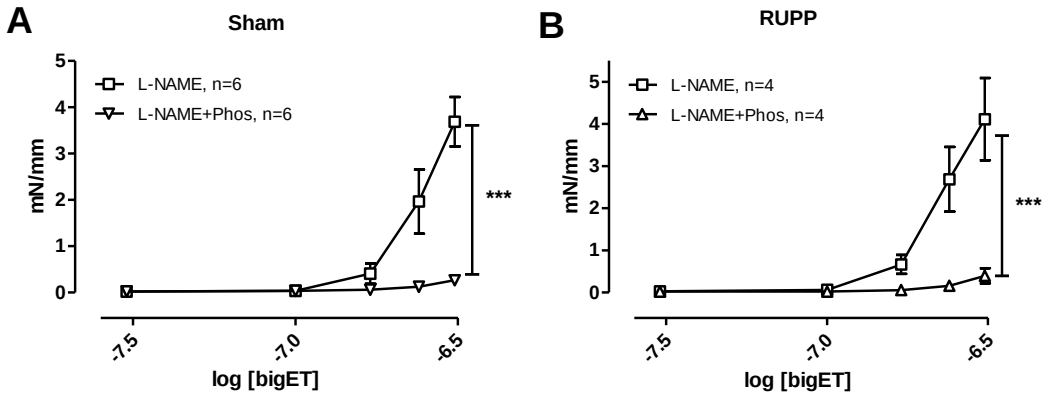

Figure 3.15- Response curves of mesenteric arteries from Sham and RUPP animals preincubated with L-NAME (NOS inhibitor, 100 µmol/L) to cumulative concentrations of bigET in the absence (Control/Ctl) or presence of phosphoramidon (Phos, ECE inhibitor, 30 µmol/L).

Contractile responses to **bigET** were significantly inhibited in both Sham (A) and RUPP (B) in the presence **phosphoramidon** (***: $p<0.0001$, two-way ANOVA).

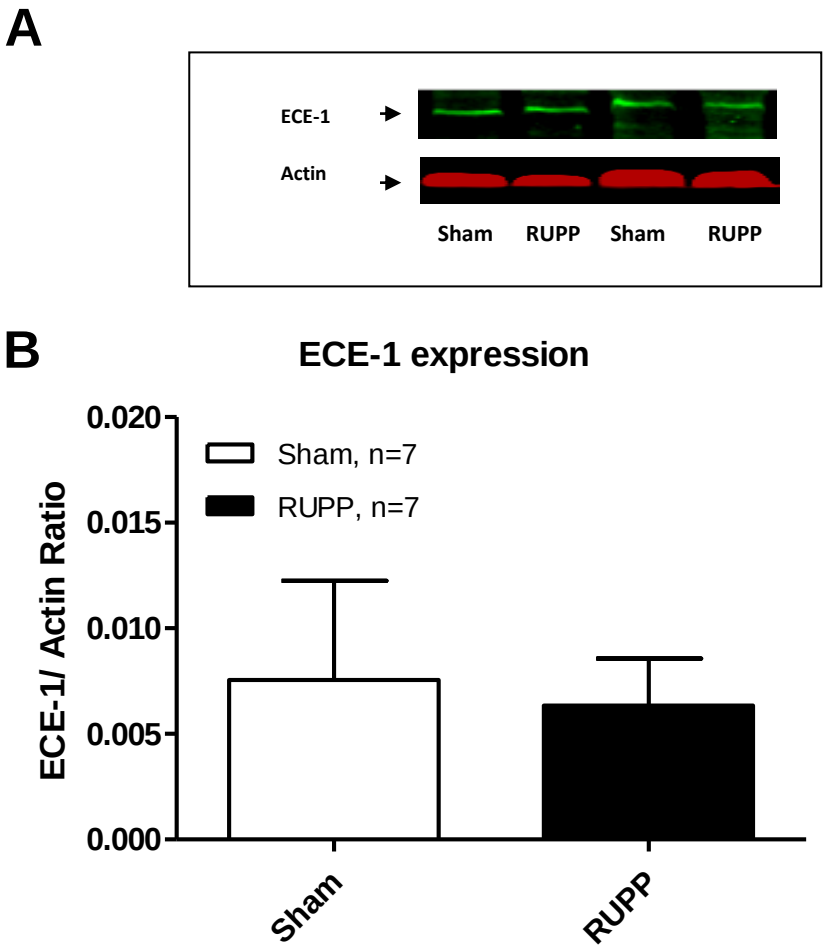

Figure 3.16- Representative Western blot images (A) and relative quantification of ECE-1 (B) in mesenteric arteries from Sham and RUPP analyzed by fluorescent intensity.

ECE-1 levels were not significantly different in Sham and RUPP (ns, t-test).

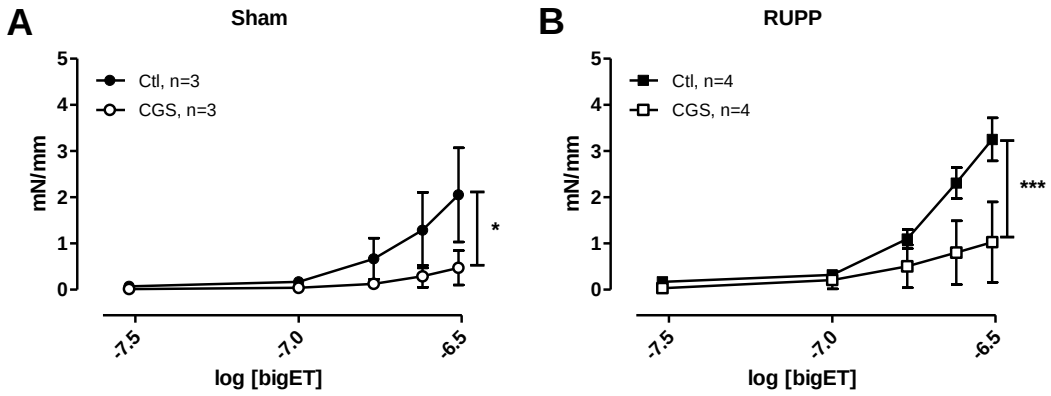

Figure 3.17- Response curves of mesenteric arteries from Sham and RUPP animals to cumulative concentrations of bigET in the absence (Control/Ctl) or presence of CGS35066 (CGS, ECE inhibitor, 25 nmol/L).

Contractile responses to **bigET** were significantly inhibited in both Sham (A) and RUPP (B) in the presence **CGS35066** (*: $p<0.05$ and ***: $p<0.001$, two-way ANOVA).

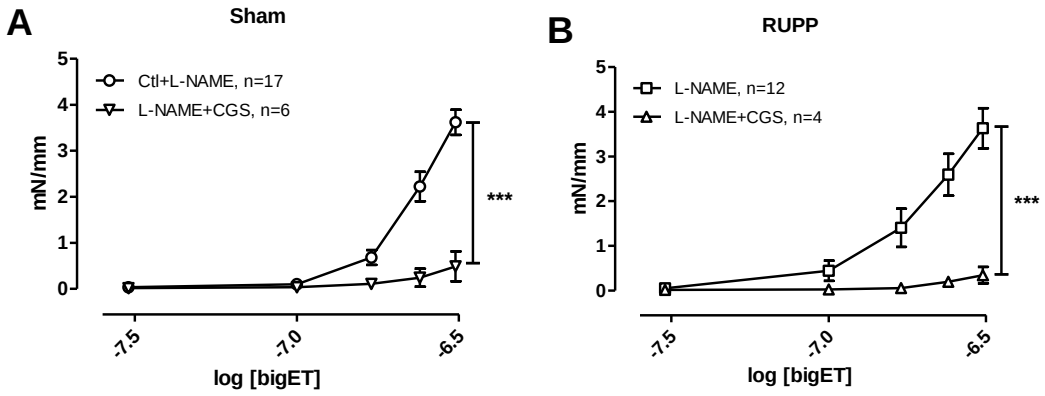

Figure 3.18- Response curves of mesenteric arteries from Sham and RUPP animals preincubated with L-NAME (NOS inhibitor, 100 µmol/L) to cumulative concentrations of bigET in the absence (Control/Ctl) or presence of CGS35066 (CGS, ECE inhibitor, 25 nmol/L).

Contractile responses to **bigET** were significantly inhibited in both Sham (A) and RUPP (B) in the presence of **CGS35066** (***: $p<0.0001$, two-way ANOVA).

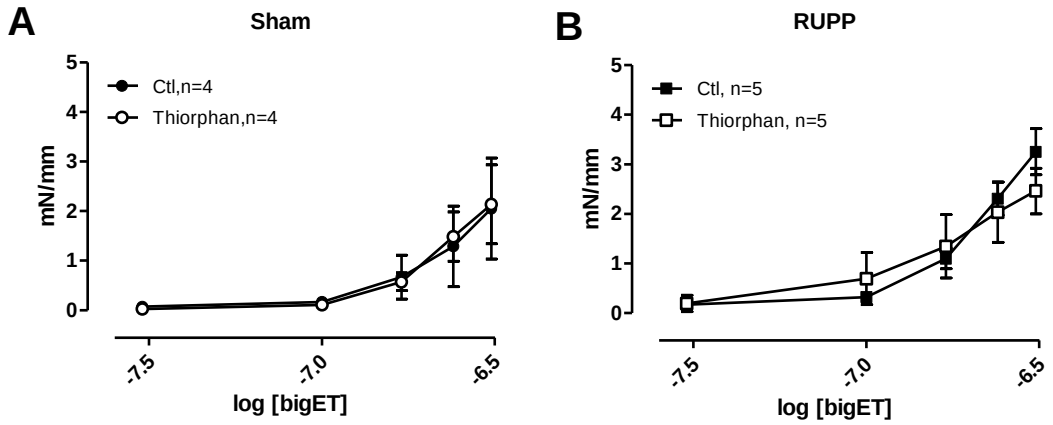

Figure 3.19- Response curves of mesenteric arteries from Sham and RUPP animals to cumulative concentrations of bigET in the absence (Control/Ctl) or presence of DL-Thiorphan (Thiorphan, NEP inhibitor, 25 µmol/L).

There was no significant difference in contractile responses to **bigET** in either Sham (A) or RUPP (B) in the presence and absence of **DL-Thiorphan** (ns, two-way ANOVA).

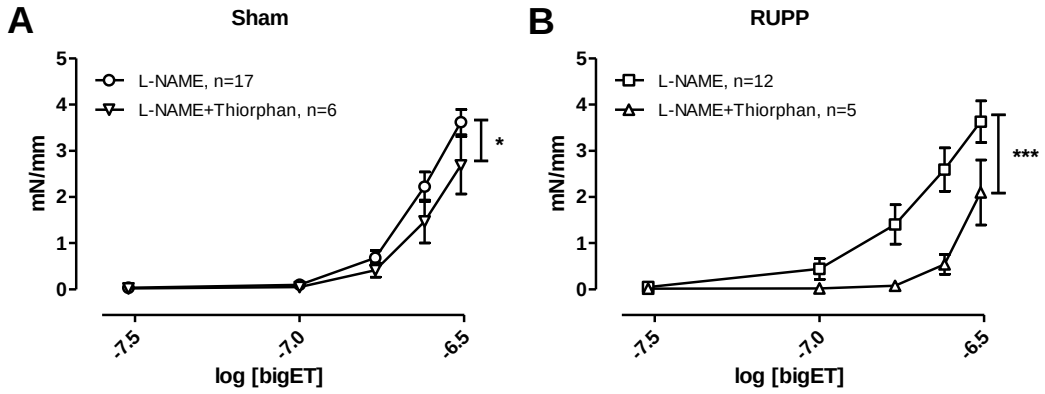

Figure 3.20- Response curves of mesenteric arteries from Sham and RUPP animals preincubated with L-NAME (NOS inhibitor, 100 µmol/L) to cumulative concentrations of bigET in the absence (Control/Ctl) or presence of DL-Thiorphan (Thiorphan, NEP inhibitor, 25 µmol/L).

Contractile responses to **bigET** were not different in Sham (A), in the presence or absence **DL-Thiorphan**. However, in the presence of **DL-Thiorphan**, contractile responses to **bigET** in RUPP (B) were significantly inhibited (*: $p<0.05$ and ***: $p<0.0001$, two-way ANOVA).

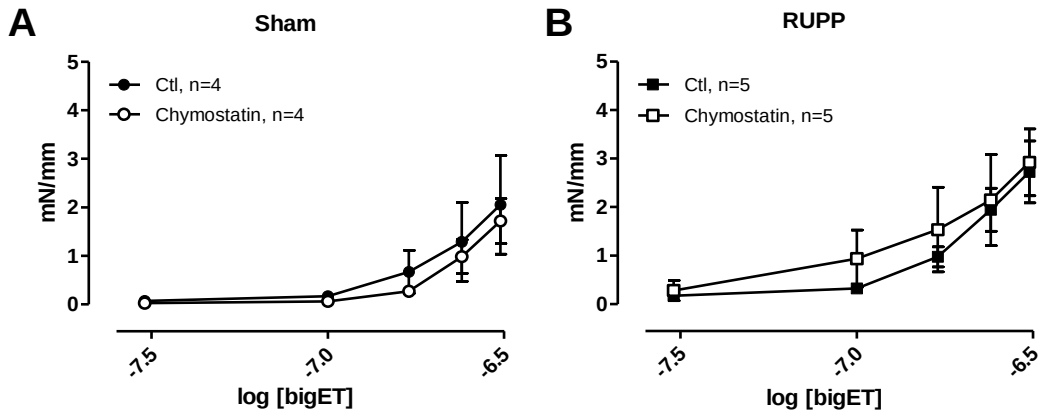

Figure 3.21- Response curves of mesenteric arteries from Sham and RUPP animals to cumulative concentrations of bigET in the absence (Control/Ctl) or presence of Chymostatin (Chymase inhibitor, 100 µmol/L).

There was no significant difference in contractile responses to **bigET** in either Sham (A) or RUPP (B) in the presence or absence of **chymostatin** (ns, two-way ANOVA).

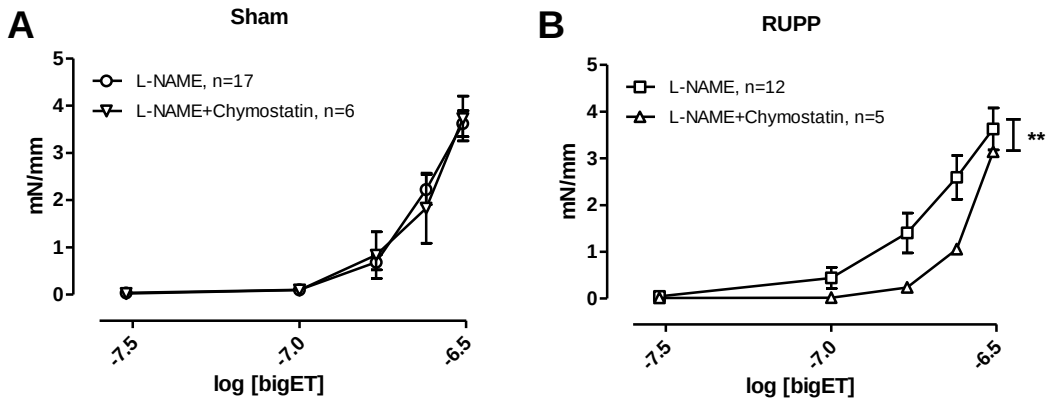

Figure 3.22- Response curves of mesenteric arteries from Sham and RUPP animals pre-incubated with L-NAME (NOS inhibitor, 100 µmol/L) to cumulative concentrations of bigET in the absence (Control/Ctl) or presence of chymostatin (chymase inhibitor, 100 µmol/L).

Contractile responses to **bigET** were not different in Sham (A), in the presence or absence of **chymostatin**. However, in RUPP (B), contractile responses to **bigET** were significantly inhibited in the presence of **chymostatin** (**: $p<0.01$, two-way ANOVA).

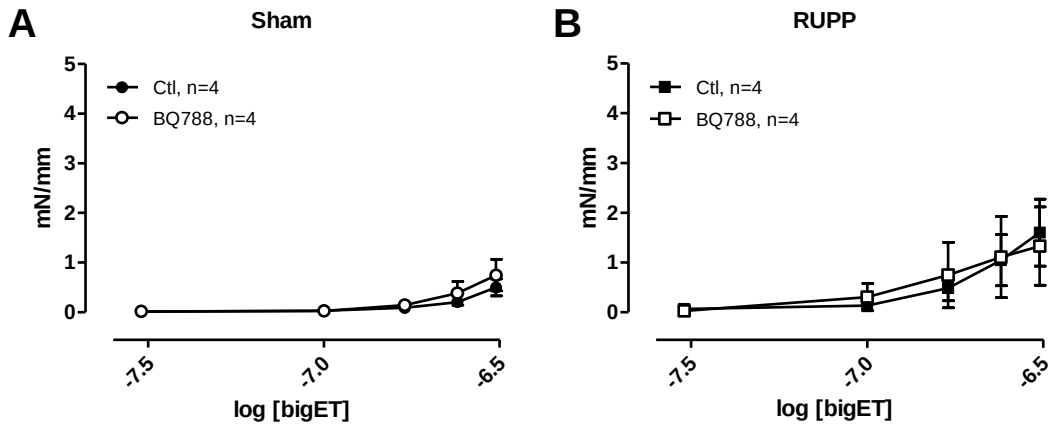

Figure 3.23- Response curves of mesenteric arteries from Sham and RUPP animals to cumulative concentrations of bigET in the absence (Control/Ctl) or presence of BQ788 (ET_B receptor inhibitor, 1 µmol/L).

There was no significant difference in contractile responses to **bigET** in either Sham (A) or RUPP (B) in the presence or absence of **BQ788** (ns, two-way ANOVA).

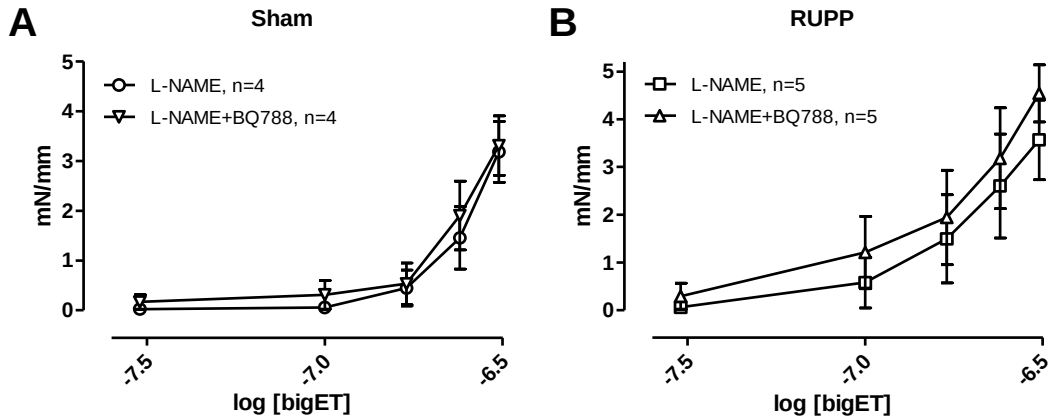

Figure 3.24- Response curves of mesenteric arteries from Sham and RUPP animals pre-incubated with L-NAME (NOS inhibitor, 100 µmol/L) to cumulative concentrations of bigET in the absence (Control/Ctl) or presence of BQ788 (ET_B receptor inhibitor, 1 µmol/L).

There was no significant difference in contractile responses to **bigET** in either Sham (A) or RUPP (B) in the presence or absence of **BQ788** (ns, two-way ANOVA).

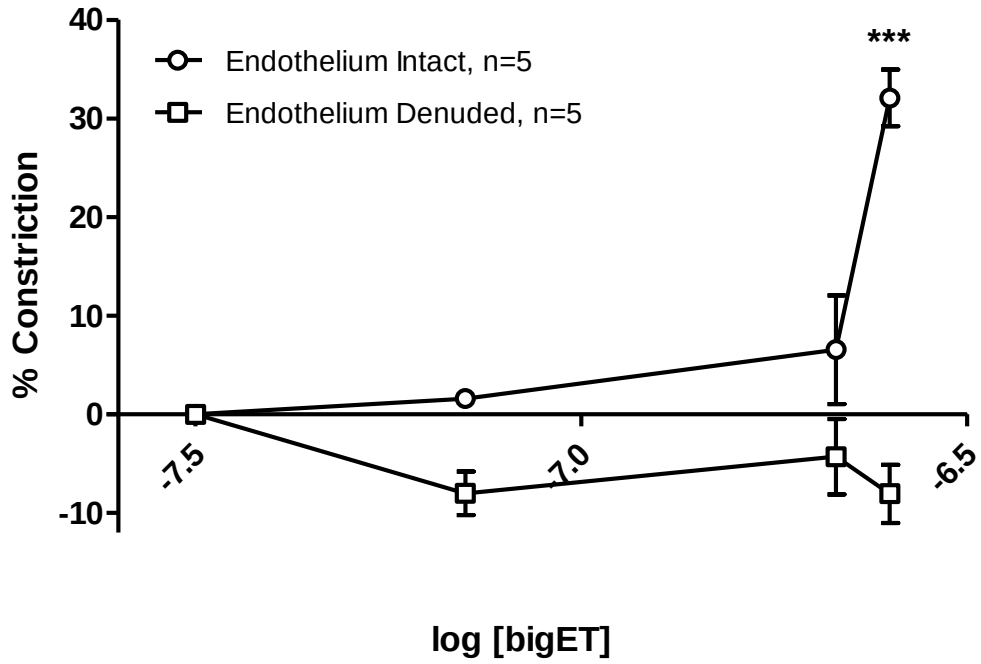

Figure 3.25- Response curves of mesenteric arteries from female rats to cumulative concentrations of bigET pre and post endothelium denuding, presented as % constriction of phenylephrine maximum concentration.

Contractile response to **bigET** absent after the endothelium was denuded (***, $p<0.0001$, two-way ANOVA).

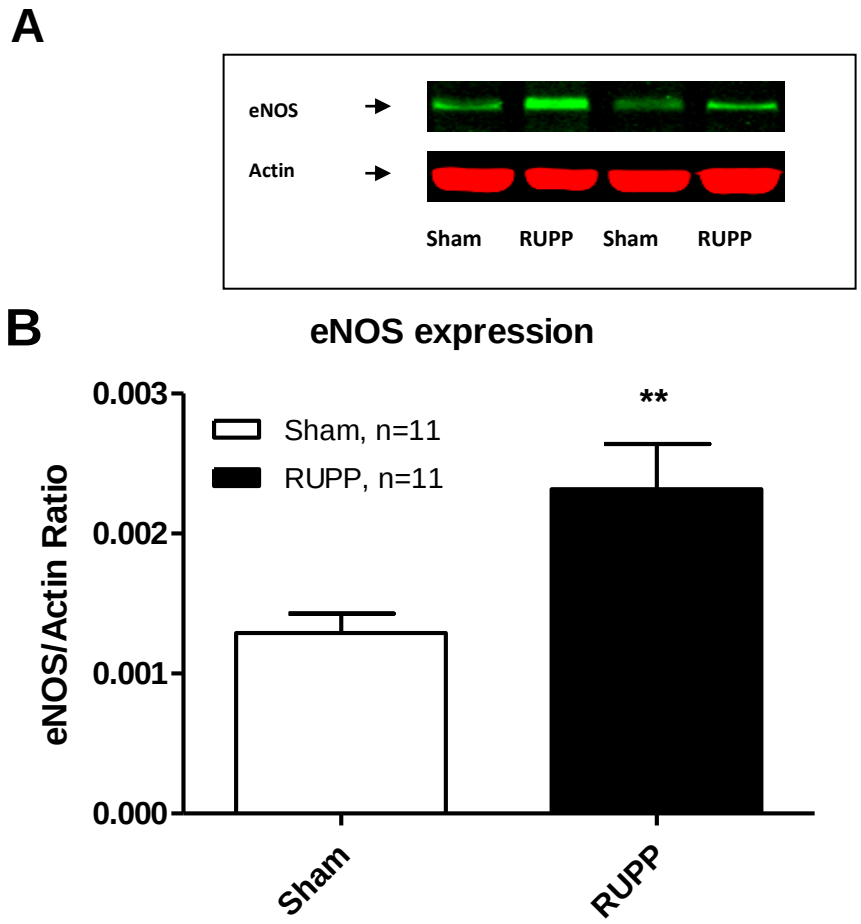

Figure 3.26- Representative Western blot and their respective controls (A) and relative quantification of eNOS (B) in mesenteric arteries from Sham and RUPP analyzed by fluorescent intensity.

eNOS levels were significantly higher in RUPP compared to Sham (**: $p<0.01$, t-test).

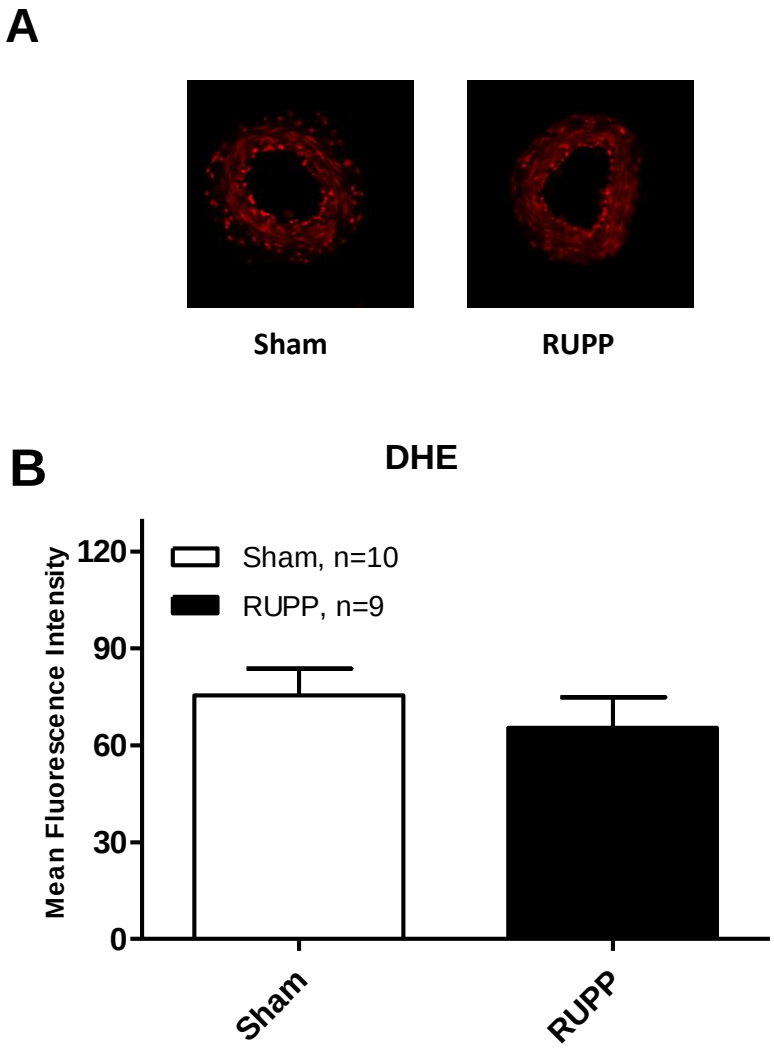

Figure 3.27- Representative DHE staining (A) and quantitative analysis of fluorescent markers (B) in mesenteric arteries from Sham and RUPP analyzed by mean fluorescence intensity.

The intensity of fluorescence was not different between Sham and RUPP (ns, t-test).

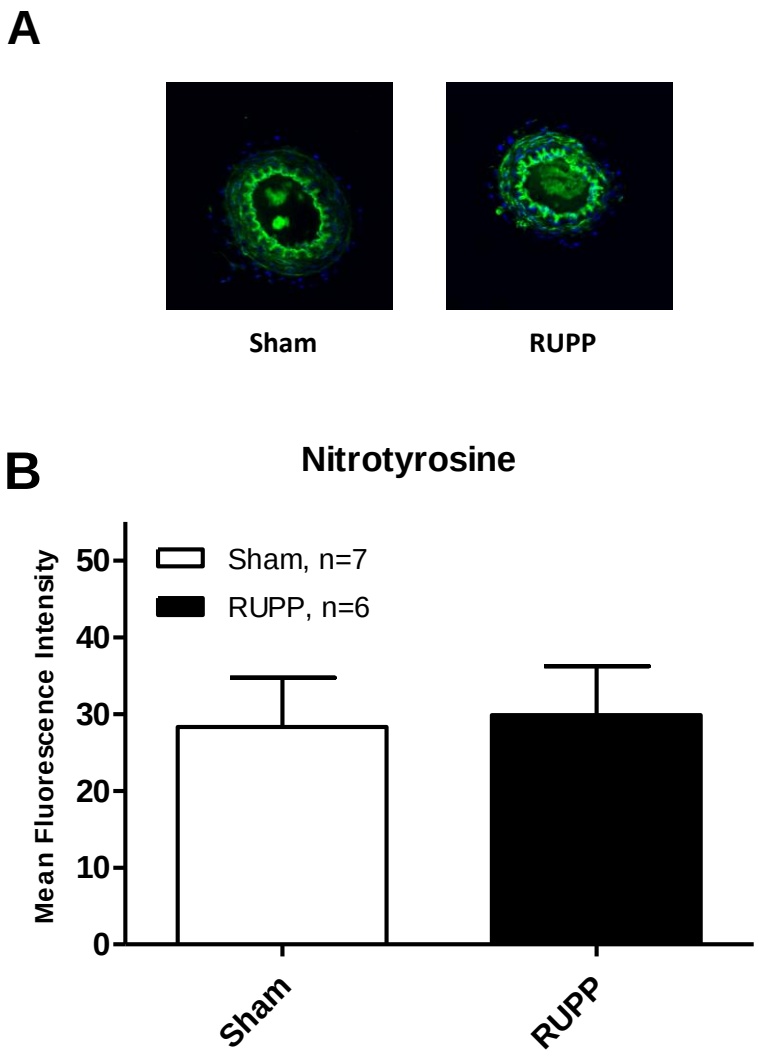

Figure 3.28- Representative nitrotyrosine staining (*A*) and quantitative analysis of the fluorescent markers (B) in mesenteric arteries from Sham and RUPP analyzed by mean fluorescence intensity.

The intensity of fluorescence was not different between Sham and RUPP (ns, t-test). Basement membrane auto-fluorescence is not included in the assessment.

Chapter 4:

Discussion

4.1 Summary

The primary objective of this thesis was to investigate the role the role of MMP-2 in bigET-induced vasoconstriction in the RUPP model of preeclampsia. Our study showed that the contractile response to bigET in mesenteric arteries from RUPP animals was significantly greater compared to that in Sham animals. We also showed that the contribution of MMP to the cleavage process of bigET to ET-1 was greater in RUPP compared to Sham. Another finding of our study was that the cleavage capacity of MMP alongside, other alterations in the enzymatic processing of bigET was primarily modulated by nitric oxide.

4.2 Animal Model

My first objective was to establish the RUPP model in our laboratory. As has been previously shown, there was increased systolic and diastolic blood pressure in RUPP compared to Sham animals. We also showed altered vascular function in RUPP animals, which has been recently published (357). The effect of RUPP surgery on proteinuria was more complex. While it appeared that RUPP surgery had a tendency to increase urinary protein excretion, the difference did not reach statistical significance. However, pathological review of the renal cortex demonstrated mild to moderate glomerular endotheliosis in RUPP animals. Glomerular endotheliosis is the result of swelling and vacuolization of the endothelial cells and loss of the capillary space(358). In this condition filtration surface area decreases secondary to subendothelial fibrin deposition(359). Furthermore, decrease in glomerular endothelial fenestrae leads to a significant

decline in glomerular filtration rate (359). These pathologic changes in the morphology and function of glomerule have been characterized in preeclampsia(360-362). It is worth mentioning here that among all the groups who have worked with the RUPP model in rat, only a few have demonstrated proteinuria in these animals (330-332). Several authors, including the Granger group who has done the most work in this area, indicated that the RUPP model of preeclampsia causes variable proteinuria (321, 324, 328, 329, 363). Studies using larger animals with longer gestational periods and, therefore, more chronic insult (rabbit, dog, non-human primates etc.) resulted in more consistent proteinuria (331, 364). Therefore, a possible reason for the variable proteinuria observed in the rat RUPP model is the relatively short length of gestation (21-22 days) which does not give the necessary time period for kidneys to become pathologically affected and start excreting larger than normal volumes of protein (albumin) into the urine.

In regards to the offspring, the number of viable pups in RUPP was significantly less than Sham, indicating increased rates of resorption. There was a trend for reduced mean pup weight in RUPP compared to Sham ($p=0.058$). The reported differences between mean fetal weight in Sham and RUPP have been variable. Some studies have reported the mean fetal weight to be significantly less in RUPP compared to Sham (329, 365, 366). However, others have reported changes in the weight of pups which were not consistent with the aforementioned findings. For instance, Isler *et al.* found the fetal weight to be only moderately less in RUPP compared to Sham (335). In addition, Balta *et al.*, did not find any

significant difference between mean fetal weight in Sham and RUPP which was in accordance with our findings (332). The reason for variability difference between fetal weight in Sham and RUPP could be due to the number of pups that are resorbed in the RUPP model. Therefore, the limited blood supply could be directed to a fewer number of pups so that they could survive and achieve greater than expected body weight. However, further morphometric studies of the pups showed asymmetrical growth restriction compatible with insults experienced during later stages of pregnancy. These findings regarding the characteristics of the RUPP model suggested the appropriateness of this model to study our research questions.

4.3 Functional Studies

4.3.1 Contractile Responses to Big Endothelin-1 and Endothelin-1

To test our hypothesis we chose mesenteric arteries which are well established as resistance arteries with a significant role in blood pressure control and hypertension pathophysiology (367-370). We, for the first time demonstrated that the contractile forces in response to bigET generated by mesenteric arteries from RUPP animals were significantly greater compared to Sham. As previously discussed in the methods section, the contractile response that we measure in response to cumulative concentrations of the precursor (bigET) allows for using the results as an activity bioassay for the enzymes which are involved in the cleavage process of bigET to ET-1. Therefore, this finding was consistent with our initial hypothesis regarding increased enzymatic cleavage of bigET to ET-1 in

RUPP compared to Sham. On the other hand, at least two other factors could have played a role in this finding. Firstly, a difference between the intrinsic contractile potential of the vessels could be the reason for greater contractile responses to bigET in vessels from RUPP. For instance, surgical manipulation and subsequent changes in hemodynamics might have contributed to changes in the structure of the vessels; causing vascular smooth muscle hypertrophy and a greater ability to generate contractile forces. This possibility was ruled out as we found similar contractile responses in Sham and RUPP to high concentrations of potassium (KPSS, 123.70 mmol/L). The contractile responses to high concentrations of potassium are secondary to membrane depolarization and influx of calcium (371, 372), which is independent of bigET cleavage and ET-1 receptors. Thus, in our study, the RUPP surgery did not change the contractile capacity of the vessels. Secondly, changes in receptor mediated signals could be the reason for greater responsiveness to bigET in vessels from RUPP compared to Sham. To investigate this, we exposed the vessels to ET-1, the end product of bigET cleavage. Using this approach, not only did we avoid the enzymatic cleavage step, but also made it possible to make a direct comparison between the vessels from Sham and RUPP when the end product (ET-1) exerted the contraction through its receptors. We observed no difference between the contractile responses to ET-1 in Sham and RUPP. Therefore, the differences between Sham and RUPP in their response to bigET are likely to have been in the endothelium and the upstream enzymatic pathways involved in bigET to ET-1 cleavage. Indeed, we showed that denuding the vessels of endothelium prevented constriction in response to bigET.

4.3.2 The Contribution of Nitric Oxide to the Contractile Responses to Big Endothelin-1 and Endothelin-1

Having known that endothelium-dependent relaxation involving nitric oxide is impaired in systemic vessels from RUPP (324), we pre-incubated the vessels from Sham and RUPP with L-NAME (a NOS inhibitor) before exposing them to cumulative concentrations of both bigET and ET-1. By this process we could find the relative contribution of nitric oxide to the contractile responses in each group. This step was also necessary for interpretation of later functional studies in this project.

Exposing the vessels to bigET after inhibiting nitric oxide production revealed that nitric oxide had a significant contribution to basal tone and net contractile responses in both Sham and RUPP. However, further analysis revealed that the L-NAME effect in increasing the contractile response was significantly greater in Sham compared to RUPP, indicating that the nitric oxide contribution is reduced in RUPP. For ET-1 however, our findings suggested that nitric oxide had a similar contribution to the contractile response in vessels from Sham and RUPP. It is important to note that the modulatory role of nitric oxide on the response to bigET and ET-1 occurs in different locations and through different mechanisms. The modulatory effect of nitric oxide on the response to bigET occurs at the level of endothelial enzymatic pathways upstream of the ET receptors, where bigET is cleaved enzymatically to ET-1 in the endothelium. Whereas, the nitric oxide modulation of the response to ET-1 occurs at the level of downstream pathways. This is where the end product exerts its contractile effects through its receptors on

the vascular smooth muscle cells. Further assessment of the relationships of both bigET and ET with nitric oxide would need to be considered to determine how these factors interact to produce the final vascular response.

The interaction between nitric oxide and ET-1 can be investigated by understanding the processing of ET-1. Upstream of the ET-1 function, the very first steps in its production are gene transcription and translation. Several studies have shown that nitric oxide decreases both the transcription and translation of the preproendothelin-1 gene (201, 373-375). The next step in which nitric oxide might have an effect is the cleaving of bigET to ET-1. Although this process is yet to be understood completely there are a few studies in the literature about the modulatory role of nitric oxide on enzymes such as MMP-2 and ECE-1. Tronc *et al.* showed that nitric oxide has a role in increasing the activity of MMP-2 in conduit arteries that undergo surgically-induced vascular remodeling (376). In addition, it has been shown that nitric oxide can increase the activity of gelatinases including MMP-2 in plasma and fibroblasts (259, 377). The modulatory role of nitric oxide on ECE-1 has only been reported by Roach *et al.* who showed that nitric oxide regulates ECE-1 expression through a cGMP/protein kinase G-dependent regulatory mechanism at the post-transcriptional level via the 3'-unsaturated region of ECE-1 gene (378). Therefore, we designed our study to investigate the possibility of acute modulatory interactions between nitric oxide and the upstream enzymes involved in the cleavage process of bigET to ET-1 by performing the functional studies in the presence or absence of L-NAME.

On the other hand, the interaction between nitric oxide and ET-1 in the downstream pathways has been extensively studied. One of the mechanism through which nitric oxide interacts with ET-1 is its inhibitory action on the release of ET-1 from endothelial cells through a cGMP-dependent mechanism (373, 379, 380). Moreover, Goligorsky *et al.* showed that nitric oxide can also shorten the duration of interaction between ET-1 and its receptors (381). They used an ET receptor mapping technique as well as fluorescence microscopy to show that nitric oxide donors (3-morpholino-sydnonimine HCl and sodium nitroprusside) can caused a rapid, concentration-dependent, and reversible dissociation of ET-1 from the ET_A receptor. Furthermore, at the calcium signalling level, there is an antagonistic interaction between nitric oxide and ET-1. This post-receptor interference between nitric oxide and ET-1 eventually inhibits the end response to ET-1. Decreased release, faster dissociation from receptors and the post-receptoral antagonistic effects of nitric oxide on ET-1 can act in a much faster way thus being responsible for acute modulatory effects of nitric oxide on ET-1. In our own study, the L-NAME specific effect on the contractile response to ET-1was similar between Sham and RUPP. While part of this effect was due to the loss of nitric oxide vasorelaxation, the loss of a direct interaction between nitric oxide and ET-1 could also play a role (**Figure 1.5**). While pre-incubation of vessels with L-NAME helped us make a more detailed observation of the role of nitric oxide in the contractile responses of the vessels, it also benefitted our study design by bringing the contraction curves to the same

baseline and making it possible to equally compare the role and contribution of each of the cleaving enzymes in Sham and RUPP.

4.3.3 Endothelial Nitric Oxide Synthase

Given the significant contribution of nitric oxide to contractile responses and the crucial role of eNOS in the production of nitric oxide as well as oxidative stress, we investigated the expression level of eNOS in mesenteric arteries from Sham and RUPP. Interestingly, we found the expression levels of eNOS to be significantly higher in vessels from RUPP compared to Sham. To the best of our knowledge this is the first time that an increase in eNOS in the systemic resistance arteries of the RUPP model has been shown. The significance of this finding lies in demonstrating the suitability of the RUPP model to study preeclampsia and also explaining some of the findings in this preeclampsia-like model. In the published literature, there are reports of increased eNOS expression in other animal models of preeclampsia and in tissues other than mesenteric arteries. Schmid *et al.* used an immunologic model of preeclampsia in the mouse, produced by inoculating the mice with activated Th1 cells, and showed that eNOS expression was augmented both in kidneys and placentas of the treated animals compared to controls (382). Mitchell *et al.* also showed increased aortic eNOS expression in DOCA rats (a model of preeclampsia previously described in the introduction) compared to normal pregnant rats (383). Several studies have also shown increased expression of eNOS protein and mRNA in human placental issue (384, 385), cultured trophoblast cells from placenta (202) , and myometrium (386)

in preeclampsia compared to normal pregnancies. There is also evidence of increased eNOS mRNA in trophoblastic cells cultured from preeclamptic placentas (202). Furthermore, Davidge *et al.* showed increased eNOS expression in cultured endothelial cells after exposure to plasma from women with preeclampsia (83). Later, the Davidge lab also found evidence of increased eNOS in micro-vessels in subcutaneous fat biopsies from women with preeclampsia compared to uncomplicated pregnancies or non-pregnant women (44).

Through functional studies previously discussed in this thesis, we have shown that nitric oxide modulation is decreased in the systemic vessels of RUPP animals compared to Sham, probably due to decreased production or bioavailability of nitric oxide. Coupled with increased eNOS expression in the same vessels, we can speculate that increased eNOS could be a compensatory response to decreased baseline nitric oxide levels. Alternatively, increased production and consumption of nitric oxide as a result of increased oxidative stress may have led to increased levels of reactive oxygen and nitrogen species, most importantly peroxynitrite. Even if the increased eNOS expression leads to increased nitric oxide production, due to increased oxidative stress this nitric oxide may be consumed to generate more peroxynitrite. Another mechanism that can be proposed for this increased expression of eNOS and its contribution to the pathophysiology of preeclampsia is the role of uncoupled eNOS. As described in greater detail in the introduction, increased eNOS in the absence of sufficient amounts of substrate generates superoxide in place of nitric oxide (76, 77). In the present study we did not find any significant difference between the levels of peroxynitrite or superoxide in the

mesenteric arteries from Sham and RUPP. However, our lab has shown that superoxide levels were increased in the aorta of RUPP animals compared to Shams (357).

4.3.4 The Contribution of Cleaving Enzymes to the Contractile Response to Big Endothelin-1

4.3.4.1 Matrix Metalloproteinase-2

We started the functional study of the enzymes by focusing on our enzyme of interest, MMP-2, which has been previously shown to be increased in preeclampsia (262, 387). In mesenteric arteries, MMP-9 was not detectable by zymography while MMP-2 was highly expressed. Notably, using Western blots MMP-2 levels were higher in the RUPP group compared to controls. Overall, we showed a significant contribution of MMP (likely MMP-2) to the cleavage of bigET to ET-1 in RUPP which was not present in Sham animals. However, in the presence of L-NAME, contractile levels were similar and the impact of the inhibition of MMP was only seen in Sham, suggesting a nitric oxide-sensitive MMP contribution to vasoconstriction in RUPP subjects.

In Sham animals it is possible that the vasorelaxant effect of nitric oxide masked any contribution of MMP in the cleavage process of bigET to ET-1. Moreover, in spite of having more MMP-2 in RUPP animals, inhibition of NO production resulted in the loss of the significant contribution of MMP-2 to contraction. This finding is also indicative of the possible modulatory role of NO on the MMP activity.

In the presence of L-NAME, vasoconstriction to bigET was greater, thus a significant contribution of MMP to bigET cleavage could be detected in Sham. The reason behind this finding could be the increased baseline contraction which makes it possible to detect more subtle differences before and after inhibiting MMP. It has been shown that oxidative stress (peroxynitrite) can increase the activity of MMP-2 by cysteine S-glutathiolation of the propeptide domain as well as by inactivating TIMP-1 and -4 (255-260). Although we did not find any changes in superoxide and peroxynitrite in the RUPP vessels, there might be more cell specific changes that we could not detect because of the technique we used and the small arteries that were being assessed. In addition, we observed decreased nitric oxide contribution in the presence of a significant increase in eNOS expression in the vessels from RUPP. Therefore, it is possible to speculate that uncoupled eNOS in RUPP may contribute to superoxide and peroxynitrite production and cause oxidative stress in vessels from RUPP, which could have further activated MMP-2. In the presence of L-NAME, however, peroxynitrite production was reduced through eNOS inhibition hence we observed a loss of MMP contribution to the contractile response. This explanation with its focus on the function of MMP-2 as well as our finding regarding increased expression levels of MMP-2 in RUPP compared to Sham can justify the significant contribution of this enzyme in the cleavage of bigET in RUPP.

4.3.4.2 Endothelin Converting Enzyme-1

In regard to the contribution of ECE to bigET responses, we did not find any significant difference between the relative contribution of ECE-1 in Sham and RUPP. In addition, we complemented our study by comparing the expression of ECE-1 in the mesenteric arteries from Sham and RUPP which did not show any significant difference. Our finding that the function and quantity of ECE-1 were not significantly different between Sham and RUPP was in line with a study by Lee *et al.* that showed that VEGF-induced ET-1 production in preeclampsia was not the result of an increase in the function of ECE-1 (388). On the contrary, Ajne *et al.* found that ECE-1 activity was higher in the sera from preeclamptic women (389). Furthermore, when Nishikawa *et al.* incubated HUVECs with sera from preeclamptic women, they observed an up-regulation in ECE-1 expression (390). These findings suggest that factors other than nitric oxide levels may affect ECE-1 expression in preeclampsia.

Since phosphoramidon, the ECE inhibitor that we used, can have inhibitory actions against NEP; we also used two specific enzyme inhibitors for ECE and NEP separately. Specific inhibition of ECE by CGS35066 gave similar results to those found with phosphoramidon (non-specific inhibitor of ECE). However, interesting results were observed when assessing a role for NEP.

4.3.4.3 Neutral Endopeptidase

Inhibition of NEP by its selective pharmacological inhibitor, thiorphan, did not show any difference in the contribution of this enzyme between Sham and RUPP

in the absence of L-NAME. However, when we pre-incubated the vessels with L-NAME, we found a significant contribution of NEP in both RUPP and Sham. Since the function of NEP was different in the presence or absence of nitric oxide, this finding suggests that nitric oxide can directly modulate the function of NEP. The interaction between nitric oxide and the function of NEP could also occur through another mechanism. In this project our focus was on the cleavage of bigET to ET-1 and that is one of the functions of NEP, however, as an endopeptidase this enzyme plays other important roles including breakdown of bradykinin (391-393). Bradykinin has long been known as a stimulator of nitric oxide production through increasing intracellular Ca^{2+} and activating eNOS (394, 395). Bradykinin can also cause vasodilation by stimulating the production of vasodilator prostanoids (PGI_2) and EDHF (396). Therefore, inhibition of NEP could have caused less contraction both by reducing ET-1 production and increasing the vasodilatory effects of bradykinin. Speculation on the presence of a regulatory effect between nitric oxide and NEP is justifiable, since on inhibiting nitric oxide production, the activity of NEP is up-regulated both by producing more ET-1 and degrading more bradykinin thus generating more contraction and less relaxation through PGI_2 and EDHF. In this situation, if NEP has a greater contribution, its inhibition should abolish the contractile forces, as was observed in both Sham and RUPP. While Li *et al.* showed increased expression of NEP in villi of placentas from preeclamptic pregnancies compared to normotensive pregnancies (397), our findings did not support the same pattern in the maternal resistance arteries from a preeclampsia-like animal model. Since in the literature

there is no report of a direct modulatory effect of nitric oxide on NEP, our result could serve as the first evidence for the possibility of such a relationship existing.

4.3.4.4 Chymase

Incubation of vessels with a chymase inhibitor (chymostatin) did not have any significant effect on the contractile forces in response to bigET in either Sham or RUPP. However, when vessels were pre-incubated with L-NAME, there was a significant contribution of chymase to the contractile forces generated in vessels from RUPP but not Sham animals. As described in the introduction, chymase cleaves bigET and produces ET-$1_{(1-31)}$. While ET-$1_{(1-31)}$ has some vasoconstrictor properties, its potency is significantly less than that of ET-$1_{(1-21)}$ (139, 398). This difference in potency may explain the difference in the results in the presence or absence of nitric oxide. When nitric oxide is in the system with its relaxing contribution to basal vascular tone, it can mask the modest but significant contribution of chymase to contraction through cleavage of bigET to ET-$1_{(1-31)}$. Inhibition of nitric oxide production could then unmask the subtle but significant contribution of chymase to the contractile forces generated in RUPP. This finding appears to indicate that nitric oxide modulates the function of chymase. Increased expression and activity of chymase in the maternal vascular endothelium as well as plasma and placenta of preeclamptic women has been shown previously (354-356). However, this increased expression and activity have been linked to an increased inflammatory response (through cytokine interleukin-8, the adhesion molecules; P-selectin and E-selectin etc.) and the renin-angiotensin system (as a

non-ACE angiotensin II producing enzyme). We, for the first time, have shown that increased chymase contribution in a preeclampsia-like animal model can directly contribute to increased ET-1 mediated contractile forces in the arteries. It is interesting to note that chymase and ET-1 are both stored in Weibel-Palade bodies of endothelial cells and activation of endothelial cells can release both from these endothelial cell-specific storage granules (399). Therefore, when the endothelial cells are stimulated to release their stored ET-1, simultaneous release of chymase could also increase the production of ET-1 through cleavage of bigET.

Another less studied function of chymase is its effect on the gelatinases. There are reports that chymase specific inhibitors can decrease the activity of gelatinase (MMP-9) (400, 401). It is speculated that this effect mainly happens through attenuation of inflammatory pathways (401, 402). So, as another possible function that can tie the function of chymase to preeclampsia, we can speculate that chymase increases the activity of gelatinases and one of their functions is the cleavage of bigET to ET-1.

4.3.5 Relative Potency of Different Endothelin-1 Subtypes

A factor that might have affected our ability to make proper comparisons and conclusions especially in the case of functional studies of the bigET cleaving enzymes was that we still do not have enough knowledge about the relative potencies of different products of the cleavage process of bigET to ET-1. We only know that ET-$1_{(1-31)}$, which is the product of cleavage of bigET-1 by chymase, is

less potent than ET-$1_{(1-21)}$ (139, 398). Niwa *et al.* showed that ET-$1_{(1-21)}$ is 10 times more potent than ET-$1_{(1-31)}$ in generating contraction in coronary artery rings from the porcine heart (398). Nakano *et al.* also compared the contractile potencies of different ET subtypes in the rat trachea and found ET-$1_{(1-21)}$ to be more potent than ET-$1_{(1-31)}$ (139). On the other hand, it is not yet known if the potency of ET-$1_{(1-32)}$ produced by MMP-2 is equal, less or more than the other two ET-1s. The finding that NEP is capable of further cleaving ET-$1_{(1-31)}$ to ET-$1_{(1-21)}$ (145), makes it even more complex to interpret the results of functional studies. Moreover, we can speculate that if NEP, with its capacity to cleave the amino side of hydrophobic amino acids, can cleave ET-$1_{(1-31)}$ to ET-$1_{(1-21)}$ then it should be able to cleave ET-$1_{(1-32)}$ in a similar manner and produce ET-$1_{(1-21)}$. These features in the cleavage process and potency of different isoforms of ET-1 are factors that need to be further characterized to properly interpret the findings from the functional studies.

4.3.6 The Role of the ET_B Receptor

The evidence from literature as well as the results of our experiments indicates that nitric oxide has an interesting role in the pathways involved in ET-1 production, function and metabolism. While the main effect of ET-1 on ET_A and ET_B receptors on the vascular smooth muscle cells is vasoconstriction, ET-1 increases the production of nitric oxide in endothelial cells through their ET_B receptors. As described in the introduction, the ET_B receptor has also been reported to have a role in the degradation of ET-1 in the vascular smooth muscle

thereby ending its contractile effects. Having more ET_B receptors could contribute to decreased vascular tone and responsiveness by both generating more nitric oxide and degrading ET-1 faster. We hypothesized that a difference between ET_B receptors in Sham and RUPP could be a contributing factor in the observed contractile differences. Inhibition of ET_B using a specific receptor inhibitor (BQ788) did not have any effect on the contractile response of vessels from Sham and RUPP, either in the presence or absence of L-NAME. These data indicate that the proportion of nitric oxide that is produced through stimulation of ET_B receptors on the endothelial cell, does not significantly contribute to differences in the tone of vessels from Sham and RUPP.

Overall, our study has shown that in a RUPP model of preeclampsia, alterations in bigET processing results in greater vasoconstriction. These results regarding maternal vascular reactivity and the interaction between nitric oxide and ET-1 production (processing)/function in this animal model of preeclampsia can be used to open the door for new therapeutic approaches to preeclampsia.

Like any other research, there were limitations in our project which will be discussed in more details in the following section.

4.4 Limitations

4.4.1 Animal Model

As described in the introduction, preeclampsia is a condition that occurs spontaneously in women; therefore, animal models of preeclampsia are not without their limitations. The rat has a hemochorial placentation with deep

intrauterine trophoblast cell invasion and trophoblast-directed uterine spiral artery remodeling; features shared with human placentation (403). These features make the rat a suitable animal to study pathologies related to placentation. The pathologies affecting the depth and quality of placentation and spiral artery formation in the early stages of pregnancy are deemed to be pivotal etiologic events in the pathophysiology of preeclampsia. Although in the manipulations that were performed in the RUPP model, the timing is such that the insult occurs after these structures are already formed, we were still able to mechanically reduce the uteroplacental perfusion to effectively model the endpoint hemodynamic changes observed in human preeclampsia syndrome. Another limitation in RUPP is the timing and the length of the vascular insult compared to women with preeclampsia. There is a consensus that a cumulative effect in the etio-pathologic factors lead to the development of preeclampsia; meaning the longer the system is exposed to etiologic factors the higher is the chance of developing symptoms and the more severe are the symptoms. But in the RUPP model, the vascular insult is abrupt and relatively short.

Meanwhile, some critics have pointed out that in the RUPP model there is a decrease in the cardiac output and cardiac index (unlike women with preeclampsia) as well as an increase in total peripheral resistance (327) in an already overloaded hemodynamic system and this insult could cause the hypertension and many other features that we observe in these animals. It is also believed that the reason why we do not observe the same changes in non-pregnant animals that undergo RUPP surgery is that they, unlike pregnant animals, do not

have an increased blood volume. It is possible that this model causes a pathologic process that mimics the changes that we see in the early stages of coacrtation of the aorta (404). Furthermore it is possible that the same changes occur by inducing relative ischemia or decreased perfusion to any organ in a pregnant animal not only the uterus. By this argument, the suitability of this model to address placental factors as the etio-pathologic factor to the maternal syndrome is debatable. There is no question that we need to further characterize the RUPP model in regards to the role of placental under-perfusion as the main etiologic role player. However, recent findings regarding changes in the factors produced by placenta and the close similarities between the change pattern in the RUPP model and human preeclampsia, further proves that this model is be a suitable model for studying preeclampsia. One of the factors that has been studied extensively is sFlt-1, an anti-angiogenic factor that has been shown to play an important role in disrupting the angiogenic balance and potentially involved in the pathophysiology of preeclampsia. sFlt-1 has been shown to be increased in plasma and amniotic fluid in preeclampsia (405, 406). The primary source of sFlt-1 in preeclamptic women is the hypoxic utero-placental unit (282, 331, 407-409). Interestingly, circulating sFlt-1 concentrations have also been reported to be increased in the plasma and amniotic fluid of RUPP compared with Sham controls rats (339). This indicates that the preeclampsia-like changes in the RUPP model are at least partially due to under-perfusion in the utero-placental unit.

In conclusion, we know that the RUPP model has contributed to increasing our understanding of the etiology and pathophysiology of preeclampsia and has been

one of the best animal models to study vascular pathophysiologies of this syndrome. However, we should bear in mind that the RUPP model, as well as any other animal model, is not the perfect model of preeclampsia and the extrapolation of experimental findings to the clinical situation should be done with caution.

4.4.2 Experimental Protocols

An important limitation in our study was that almost all the functional studies were done *ex vivo*. This design is applicable for mechanistic hypotheses addressed in this thesis. However, it is noteworthy that the hemodynamic features and characteristics *in vivo* including responses to different stimuli are affected by several factors: for example neural, humoral and local factors. These factors could play an even more important role in a system with this level of dependency on nitric oxide and endothelin; both of which are affected by multiple neuronal and humoral factors. Future studies using *in vivo* models could address this issue in more depth.

We used semi-quantitative methods in measuring the protein expression and activity of different enzymes in mesenteric vessels. However, almost all of the processes that we were trying to investigate occur in endothelial cells or the abluminal layer just between the endothelial layer and vascular smooth muscle cells of arteries (on the interface of endothelial cells and vascular smooth muscle cells). While the result of Western blot on mesenteric arteries showed an increase in MMP-2 expression in RUPP and zymography revealed an increase in proMMP-2 in Sham and no difference in active MMP-2 between Sham and

RUPP, we should keep in mind that these measurement were performed on the mesenteric vessels as a whole rather than endothelial layer alone. For example, it is known that MMP-2 has several functions and it is possible that proMMP-2 expression can be simultaneously up-regulated in vascular smooth muscle cells and connective tissues of the vessels but down-regulated in endothelial cells. Therefore, experiments assessing at whole endothelium/smooth muscle preparations may miss such localized changes.

4.5 Future Directions

Other than the modifications and new approaches that we propose to compensate for the limitations of the present study, there are a few other routes that we could consider for future directions in this field of research.

In this study we focused on MMP-2 as a gelatinase that has the capacity to cleave bigET to ET-1. Nevertheless, there is another MMP that is categorized as a gelatinase, MMP-9. MMP-9 shares several features with MMP-2 including the capacity to cleave bigET to ET-$1_{(1-31)}$ (410). Interestingly, changes in the levels of MMP-9 in preeclampsia have been reported to be comparable to those of MMP-2 by several researchers (264, 411-413). In our study we used GM6001 as a gelatinase inhibitor, which can inhibit both MMP-9 and MMP-2. In the rat mesenteric arteries, MMP-9 was not detectable whereas MMP-2 was highly expressed. Thus we speculate that MMP-2 was the primary enzyme inhibited by GM6001 in our studies. However, we note that since GM6001 also inhibits MMP-9 observed findings were possibly the combined result of inhibition of MMP-2

and MMP-9. As a future direction in this research, we could use different gelatinase inhibitors with higher specificity for MMP-2 and MMP-9. Performing such studies can help to determine the specific contribution of each of the gelatinases in functional studies. These studies can also be complemented by more sensitive activity and quantification studies that can differentiate MMP-2 and MMP-9 even when only minimal levels of each enzyme is present.

Our data regarding the significant role of chymase in the vasoconstriction of mesenteric vessels in RUPP in response to bigET, alongside other previous studies that have shown a correlation between levels of chymase and preeclampsia, makes this enzyme an attractive target for future studies. Future studies could further investigate the expression and function of chymase in the endothelium of resistance arteries in animal models of preeclampsia. Another aspect of the function of chymase that should be examined is its role in other mechanisms that can contribute to the pathophysiology of preeclampsia; for example, inflammatory responses and activation of the renin-angiotensin system.

Further, a subject that could be included in future studies is the factors that regulate the expression and function of MMP-2 (gelatinases). TIMPs and membrane-type MMPs (MT-MMPs) are examples of such MMP regulators. Interestingly, these factors have been reported to undergo alterations that disrupt the balance in expression and activity of MMP-2 (263, 414). A study of the role of MMP-2 in the pathophysiology of preeclampsia could benefit from gaining further insight into other factors such as TIMPs that can affect the function of MMPs.

4.6 Conclusions

The results of our study showed that resistance vessels from a RUPP model of preeclampsia generated significantly greater contractile forces in response to bigET compared to Sham-operated controls. We found that the expression as well as contributionof MMP-2 to the cleavage process of bigET to ET-1 was greater in RUPP compared to Sham. We also discovered that the function of MMP-2, as well as several other enzymes involved in the cleavage of bigET, can be modulated by nitric oxide. These interesting findings indicate that the role of nitric oxide goes beyond its direct contribution on vascular tone and affects the vasoreactive responses by interacting with enzymes involved in the cleavage of bigET to ET-1.

Another interesting finding of our project was that, for the first time, we showed that eNOS expression is significantly up-regulated in the resistance arteries of RUPP animals compared to Sham and this pattern of change is very similar to what has been previously shown in women with preeclampsia.

Finally, we found a possible novel role for chymase in the pathophysiology of preeclampsia regarding its capacity to cleave bigET to ET-1. Confirmation of its role and the extent of its involvement in the pathophysiologic process of preeclampsia would require more focused studies with experiments targeted at this enzyme and its functions.

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