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REACTIVITY OF ISOLATED CEREBRAL PARENCHYMAL ARTERIOLES TO
THROMBIN DURING PREGNANCY AND PREECLAMPSIA IN RATS

A Thesis Presented

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ABSTRACT

Stroke in pregnancy is a global leading cause of maternal morbidity and mortality. Pregnancy is a hypercoagulable state characterized by changes in the levels of hemostatic factors, including prothrombin and thrombin, starting at the beginning of gestation. While this hypercoagulability evolved to help the body face the hemostatic challenge of delivery, it can increase the risk of prothrombotic complications and cardiovascular conditions, such as stroke. Pregnant women have a 3-fold greater risk of stroke compared to nonpregnant age-matched people. In the presence of preeclampsia (PE), stroke risk increases to 6-fold. PE is a serious hypertensive disorder of pregnancy associated with new-onset hypertension and extensive multi-organ system dysfunction. PE complicates 6 - 8% of pregnancies worldwide and is the second leading cause of maternal morbidity and mortality in the United States. This disorder further heightens the already hypercoagulable state of pregnancy. Thrombin is a key coagulatory protein that works to arrest bleeding by cleaving fibrinogen into fibrin. However, thrombin is also known to be vasoactive via protease-activated receptors (PARs). PAR types 1 and 2 are found in vasculature throughout the body and can have impacts on vascular tone. In physiological conditions, endothelial cells primarily mediate the vascular effects of PARs. Yet PARs in vascular smooth muscle cells can be induced under pathological conditions and elicit vasoconstriction. In stroke, additional vasoconstriction could increase cerebrovascular resistance and ultimately increase hypoxia and ischemia, worsening neuronal injury in critical brain regions. To investigate thrombin in the brain and cerebrovasculature in an experimental model of preeclampsia (ePE), this study sought to determine the reactivity of brain parenchymal arterioles (PAs), the expression of PARs in those blood vessels, and the levels of prothrombin and thrombin in circulating plasma and cerebrospinal fluid (CSF). PAs from nonpregnant (NP), healthy late-pregnant (LP), and ePE rats were isolated and pressurized within an arteriograph system to study their reactivity to thrombin. PAR expression in the smooth muscle cells of PAs were examined via Western blot and immunohistochemistry. Prothrombin and thrombin levels in plasma and CSF were assessed via enzyme-linked immunoassays. This study found that thrombin was elevated 12- and 14-fold in plasma from rats with ePE compared to NP and LP rats, respectively. Prothrombin was found in ePE plasma, whereas NP and LP prothrombin levels were undetectable. In CSF, thrombin was found in all groups and was elevated significantly in the LP CSF (NP: 0.137 ± 0.014 ng/mL; LP: 0.241 ± 0.015 ng/mL, $p < 0.05$; ePE: 0.192 ± 0.028 ng/mL). In isolated PAs, thrombin produced modest vasoconstriction that was similar between groups and appeared endothelium-independent. PARs 1 and 2 were found expressed on PAs, which could mediate thrombin's vasoactivity. This study demonstrates the presence of thrombin in brains from NP, LP, and ePE animals, as well as thrombin receptors on cerebral intraparenchymal vessels that were modestly reactive to thrombin. The presence of thrombin in CSF suggests its expression and secretion by cells within the brain, the role of which requires further investigation. Together, the results of this study highlight that thrombin plays not only a coagulatory but a vasoactive role in cerebrovasculature during pregnancy and PE, and may contribute to maternal stroke outcome.

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“A strong support system has helped this girl feel like she has moved those green mountains” – Brooke Bednarke

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CHAPTER 1:
INTRODUCTORY COMPREHENSIVE
LITERATURE REVIEW

1.1 Hemodynamic and Cerebrovascular Changes During Pregnancy

During pregnancy, the body experiences widespread, significant adaptations in nearly every physiological system throughout the body. The adaptations in the cardiovascular, hemostatic, and endocrine systems that begin early in gestation have impacts throughout the maternal body and have evolved to facilitate both fetal-placental growth and maternal survival [1 - 4]. The maternal brain and cerebral circulation, specifically, undergo especially unique changes compared to the rest of the body to maintain cerebral homeostasis [5]. From changes in the structure and function of cerebral arteries, to the blood brain barrier, and the hemodynamics of cerebral blood flow; the brain goes through extensive change to maintain the delicate microenvironment amongst the global hormonal and cardiovascular changes throughout the rest of the body [4, 5].

The brain is a particularly unique organ with specified physiological needs that enable it to carry out its complex, critical processes. Separated physically and biochemically by the skull and blood-brain barrier, the brain is a distinct microenvironment that closely maintains hemostasis, intracranial and intravascular pressure, and cerebral blood flow [2, 6]. In pregnancy, this precise maintenance of the cerebral circulation must adapt in response to the substantial hemodynamic changes seen in other organ systems [1, 2, 6]. Gestation is associated with changes in all aspects of hemostasis. For example, there is an average increase of plasma volume by 40 - 50%, increased cardiac output, and decreased peripheral vascular resistance [2, 6]. In particular, pregnancy is a hypercoagulable state characterized by a marked increase in most pro-coagulation factors and decrease in anti-coagulation factors [1].

There is a significant rise in the concentrations of clotting factors V, VII, VIII, IX, X, XII and von Willebrand factor during pregnancy [1]. Specifically, factor VIII and von Willebrand factor are elevated late in gestation and reach maximal concentrations around delivery [1]. Fibrinogen concentration prominently increases and can reach levels twice that of non-pregnant levels [1, 7]. Factors XI and XIII are pro-coagulant factors that both decrease during pregnancy [1, 8]. Normal pregnancy is also associated with an increase in platelet levels compared to nonpregnant healthy women, particularly in the third trimester [8, 9]. Notably, there is known to be a significant increase in prothrombin fragments and thrombin-antithrombin complexes [7 - 11]. When longitudinally evaluating thrombin generation from preconception to postpartum, McLean et. al found that the majority of women generate increasing levels of thrombin as pregnancy continues and thrombin generation, specifically the activation of coagulation by tissue-factor, increases during pregnancy even though individuals vary considerably in their baseline thrombin generation levels [12].

Conversely, there are key changes in physiological anticoagulants and fibrinolytic activity in gestation. Fibrinolysis is the enzymatic cascade that when activated deploys the trypsin-like protease plasmin to split the insoluble fibrin of a clot into soluble degradation products [13]. The fibrinolytic system works to breakdown and dissolve thrombi, thus helping to protect against thrombotic occlusion of vessels. Plasma fibrinolytic activity is reduced during gestation, beginning within the first weeks of the first trimester and remaining low during labor followed by a return to normal activity shortly after delivery

[1, 13]. Overall, it is believed that pregnancy-induced hormonal changes, especially the rise in estrogen levels, drive the transformation into a prothrombotic state [8, 12, 14].

Together, these widespread hemodynamic changes elevate the potential thrombotic activity throughout the body and establish the hypercoagulable state of pregnancy. This hypercoagulability presumably evolved to help the body face the significant hemostatic challenge of delivery [1]. Because maternal and fetal-placental blood flow are intimately intertwined, these hemodynamic adaptations also contribute to the maintenance of blood supply necessary for placental function and fetal health [1, 4, 15]. Complications of the maternal circulation's ability to clot and arrest bleeding can be life-threatening. In fact, maternal hemorrhage, which includes bleeding from the antepartum through postpartum periods, is the leading cause maternal morbidity and mortality worldwide [12, 16]. However, this state can also increase the risk of prothrombotic complications and cardiovascular conditions, such as stroke [17].

1.2 Stroke in Pregnancy and Preeclampsia

Stroke is the second leading cause of death worldwide and the third leading cause of disability [60, 61]. The American Heart Association (AHA) and the American Stroke Association (ASA) broadly defines stroke to include ischemic stroke (IS) due to central nervous system arterial or venous infarction or hemorrhagic stroke (HS) including intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (SAH) [17]. In adults, within ages corresponding to commonly defined child-bearing years between 15 - 44, the incidence of stroke is 10 per 100,000 [18, 20]. Variability in the causes of stroke in young adults is higher than seen in older populations [18]. Comorbidities such as hypertension, hyperlipidemia, and diabetes mellitus; and altered coagulation states such as pregnancy and preeclampsia increase stroke risk, particularly in adult IS [18, 19].

In pregnancy specifically, stroke is a leading cause of maternal morbidity and mortality throughout the world [17, 18]. Pregnancy-related stroke can additionally lead to disability that can impact a woman's ability to care for herself and children, as well as have broader personal and professional effects [17]. While estimates of pregnancy-related stroke incidence vary, a meta-analysis study by Swartz et. al reported that stroke affects 30.0 per 100,000 pregnancies with the highest risk in the peripartum and postpartum periods [18]. They additionally report that central venous sinus thrombosis, IS, and HS impact roughly equal number of pregnancies at 9.1, 12.2, and 12.2 per 100,000 [18]. When ultimately compared to age-matched people, pregnant women have a 3-fold increase in the risk of stroke [18]. The upregulation of coagulation factors and overall hypercoagulable state of pregnancy is a key contributor to that increased stroke risk.

Pregnancy-related stroke risk rises to 6-fold in the presence of preeclampsia (PE) [18]. The American College of Obstetricians and Gynecologists (ACOG) defines PE as a disorder of pregnancy associated with new-onset hypertension that often occurs after 20 weeks of gestation and frequently near-term [21]. Traditionally, the definition of PE included the presence of new-onset proteinuria. However recent guidelines, including those issued by ACOG, now state that while PE can often be accompanied by proteinuria, the disorder can present with de novo hypertension and symptoms of maternal multi-organ system dysfunction and abnormal protein levels in urine [21, 22]. Diagnostic markers of PE outside of hypertension include thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema, new-onset headache that is resistant to medication, and/or visual symptoms [21].

Hypertensive disorders of pregnancy occur in women all over the world and contribute to maternal mortality in both developed and developing regions. PE is estimated to complicate between 2 - 8% of pregnancies globally [21, 23]. Hypertensive disorders are responsible for nearly 26% of maternal deaths in Latin America and the Caribbean. Across Africa and Asia, it is reported that about 9% of maternal deaths are attributed to hypertensive disorders during gestation [23, 24]. In the United States, not only has the incidence of PE increased, but the risk of severe PE has increased as well. From 1987 to 2004, the US rate of PE increased by 25% [25]. Additionally, the risk of PE in patients giving birth in 2003 was 6.7-fold greater compared to those in 1980 [25, 26]. From Stevens et. al, PE reportedly cost \$2.18 billion in the US in 2012 due to patient complications within

the first 12 months of delivery. \$1.03 billion of which was due to healthcare costs of the mothers, while \$1.15 billion came from care for the infants [26, 27].

While PE can only occur in pregnancy and early postpartum, its effects have been associated with health risks and conditions later in life. Meta-analysis-based reviews, such as Wu et al., have demonstrated that PE was associated with increased risk of future heart failure, coronary heart disease, cardiovascular disease death, and stroke even when adjusting for age, body mass index, and diabetes mellitus [28]. PE can also have direct pathological impacts on the maternal brain and cerebrovasculature, including blood-brain barrier disruption, cerebral blood flow autoregulation, stroke, and long-term cognitive outcomes [28 - 30]. Importantly, by introducing dysregulation of coagulation, platelets, adhesion ligands, and hematologic factors, PE further increases the hypercoagulable state of pregnancy [31].

1.2.1 The Middle Cerebral Artery and Parenchymal Arterioles in Stroke

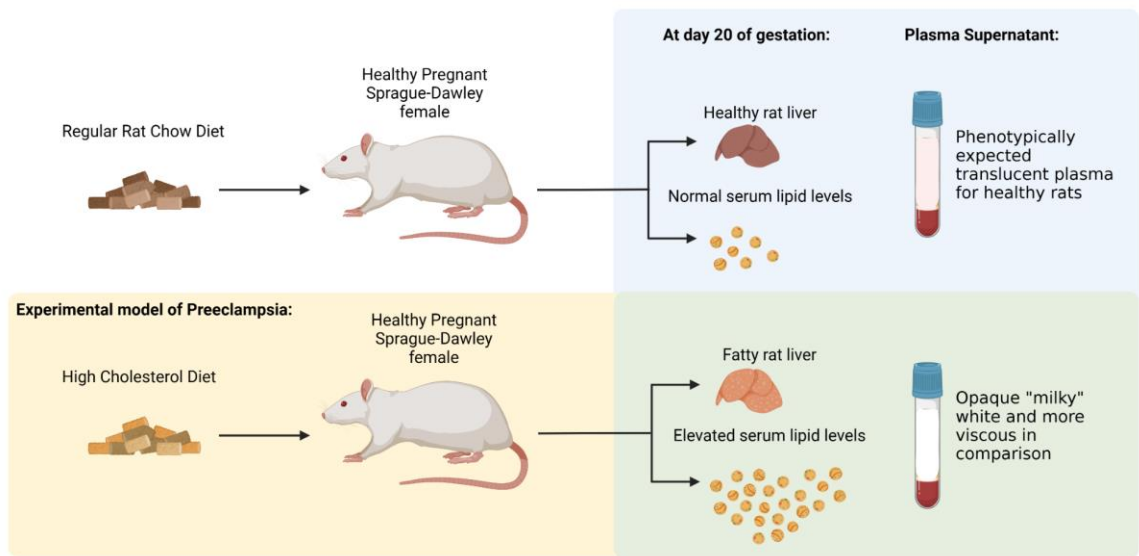
While several pathophysiology mechanisms are thought to lead to PE, the associated endothelial dysfunction, oxidative stress, increased vascular stiffness, and super-hypercoagulability likely drive that increased stroke-risk [17, 32 - 34]. Although stroke of all subtypes can occur anywhere in the brain, the middle cerebral artery (MCA) is the most common artery involved in acute stroke [17, 34]. Branching from the internal carotid artery, the MCA and its four main branches provides blood supply in part to the frontal, temporal, and parietal lobes of the brain. Beyond that, the MCA also supplies blood

to deeper regions such as the thalamus, internal capsule, and caudate nucleus via smaller intracerebral vessels that branch from it [34 - 37].

Parenchymal arterioles (PAs) are high resistance blood vessels that dive into the brain parenchyma. PAs regulate cerebral blood flow to critical brain structures including the frontoparietal white matter tracts (WMTs) of the sensorimotor cortex and basal ganglia [30]. PAs are a part of the cerebral microcirculation that can be affected by pregnancy and PE. A study by Cipolla et al. reported that isolated PAs from late-pregnant animals had larger lumen diameters than those from nonpregnant animals, both when myogenic tone was present and when passive, due to pregnancy-induced outward remodeling. That outward remodeling was specifically reported to be hypotrophic in nature and at the expense of the vascular wall where wall thickness was decreased during pregnancy yet outer diameter remained unchanged [38, 39]. Using an experimental model of preeclampsia (ePE), which was utilized in the research within the body of this thesis, Johnson and Cipolla reported that impaired function was present in PAs from animals with ePE by way of impaired conducted vasodilation, reversal of the structural remodeling associated with

normal pregnancy, and elevated circulating proinflammatory cytokines leading to a state of oxidative stress [30].

Figure 1.1: Graphical illustration of the experimental model of PE accomplished by feeding pregnant rats a high cholesterol diet.



PAs and the MCA notably contribute to cerebral perfusion and cerebrovascular resistance, making them crucial regulators of cerebral blood flow during ischemia and reperfusion [40]. In fact, it has been reported that in response to ischemia and reperfusion, the cerebral endothelium of PAs from rats compensates to prevent the loss of basal myogenic tone [40]. Given their direct relationship with the MCA and their unique role in cerebral blood flow during and after stroke, parenchymal arterioles present an important element of cerebral microcirculation to be studied when investigating the complex relationship and impacts of stroke pregnancy and preeclampsia [30, 40].

1.3 Thrombin

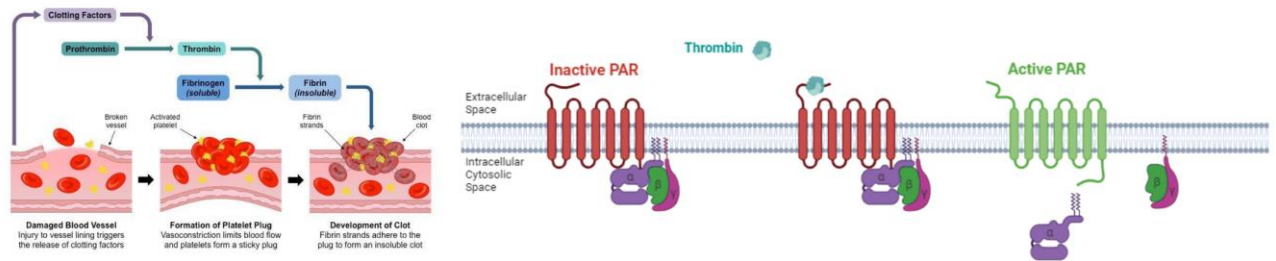
One of the procoagulation factors that is increased in healthy gestation and further heightened in PE is prothrombin and its activated form, thrombin. Thrombin is a multifunctional blood-derived serine protease that plays a major role in the coagulation cascade [41 - 43]. At the intersection of the intrinsic and extrinsic coagulation pathways, thrombin works to arrest bleeding by cleaving soluble fibrinogen into insoluble fibrin, which in turn stabilizes clot formation [41]. Once activated by Factor XIII, thrombin also influences the cross-linking of the fibrin monomers to produce a firm fibrin clot [41, 42].

1.3.2 Vasoactive Effects of Thrombin and Protease-Activated Receptors

While thrombin is widely recognized as a coagulatory protein responsible for clot formation during wound healing, thrombin can also have non-hemostatic effects and is known to be vasoactive [43, 44]. Thrombin stimulates a broad range of cellular responses, including immune reactions and inflammation, in a variety of cell types [45]. Notably, thrombin can have various cellular impacts on endothelial and smooth muscle cells of blood vessels via protease-activated receptors (PARs) [44, 45]. PARs are G-protein coupled receptors characterized by seven transmembrane domains that span the cell membrane and the family consists of four members, PAR-1, -2, -3, and -4. Proteases activate PARs by cleaving the extracellular N-terminus to create a tethered ligand, which triggers a conformational change within the receptor that initiates various intracellular signaling pathways (Figure 1.2) [44, 45]. Among their potential protease agonists, thrombin is a major agonist for PAR-1, -3, and -4, while PAR-2 is activated by trypsin

[46]. All four PARs are widely expressed in the brain cell types, including neurons, microglia, astrocytes, and oligodendrocytes [46]. Studies have shown that PARs can contribute to neuroprotection and neurodegeneration, particularly through their pro-inflammatory effects [41, 46].

Figure 1.2: *Thrombin's role in clot formation as well as its ability to initiate intracellular processes by activating PARs.*



Takahashi et. al showed that in isolated and pressurized porcine retinal arterioles, thrombin caused biphasic vasoactivity in a concentration-dependent manner. That is, vasoconstriction at low concentrations and vasodilation at high concentrations [44]. Thrombin was additionally shown to cause endothelium-dependent biphasic response in porcine renal interlobar arteries mainly by activating PAR-1, where vasorelaxation was mediated by nitric oxide and hyperpolarizing factors and vasoconstriction was mediated by thromboxane A2 and prostaglandin H2 [47]. In porcine pulmonary and canine cerebral arteries, thrombin caused vasoconstriction [48 - 50]. Thrombin caused vasodilation in newborn porcine pulmonary arteries and in the coronary arteries of pigs, dogs, and humans [51 - 55].

PAR-1 and -2 are found within the vascular system and contribute to the mediation of various vascular effects, including vascular tone [56, 57]. In physiological conditions, endothelial cells primarily mediate the vascular effects of PARs. However, PARs in smooth muscle cells can be induced under pathological conditions and elicit vasoconstriction. For example, Kuwabara et al. reported that thrombin causes more pronounced vasoconstriction in pulmonary vessels from rats with experimental pulmonary arterial hypertension than in vessels from normotensive rats [57]. Thrombin may therefore have vasoactive effects on the rat cerebral circulation via PARs in the vascular cell walls. Furthermore, those vasoactive effects could vary in pregnancy and PE, a hypertensive pathology that impacts cerebrovasculature structure and function. In stroke, additional vasoconstriction could increase cerebrovascular resistance and ultimately increase hypoxia and ischemia, worsening neuronal injury in critical brain regions.

1.4 Project Goals and Hypotheses

The research encompassed by this thesis was an investigation into the vasoactive role of thrombin in pregnancy and experimental preeclampsia in the cerebrovasculature of rats, specifically in cerebral PAs. We investigated the vasoactive effects of thrombin in isolated and pressurized PAs from rats with ePE compared to those from nonpregnant (NP) and normal late pregnant (LP) rats. The PAs that supply the frontoparietal white matter tracts and the basal ganglia were specifically targeted, isolated, pressurized, and studied within an arteriograph system. This study sought to determine the reactivity of brain parenchymal arterioles (PAs), the expression of PARs in those blood vessels, and the levels of prothrombin and thrombin in circulating plasma and cerebrospinal fluid (CSF). It was hypothesized that prothrombin and thrombin levels would be elevated in plasma and CSF in LP rats compared to NP, and further elevated in ePE. Finally, we hypothesized that thrombin would cause vasoconstriction in PAs and that PAR expression would vary in pregnancy and PE, potentially in correspondence with the observed vasoactive effects of thrombin.

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CHAPTER 2: MATERIALS AND METHODS

The following chapter is intended to provide additional information on some of the experimental techniques utilized in this research presented in Chapter 3.

2.1 Isolated Vessel Experiment

To investigate the potential vasoactive effects of thrombin in PAs, an isolated vessel system was utilized to observe the changes in lumen diameter of individual PAs in real-time. Isolated vessel experiments allow researchers to study vessels and record their reactivity to different variables outside of the brain while still under physiological conditions of intravascular pressure. Changes in lumen diameter and wall thicknesses are recorded in order to obtain calculated measures such as percent change in lumen diameter, percent constriction or dilation, as well as reactivity or sensitivity to specific stimuli introduced into the system.

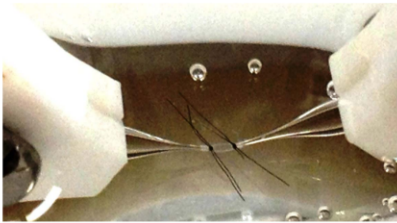
After the brain is dissected from the skull, it is placed in cold, oxygenated artificial cerebral spinal fluid (aCSF). To isolate and dissect PAs, a 2 mm thick section of cerebral cortex that contains the MCA is microsurgically cut from the brain. Under a microscope, the section of cerebral cortex was then separated using forceps to uncover the PAs branching off of the MCA into the white matter tract contained in the cross-section. PAs branching off of the MCA that were > 1.0 mm in length and entering the frontoparietal white matter tracts of cerebral cortex were dissected along with the portion of MCA the vessels traced back to. This dissection yielded a “T-shaped” vessel section consisting of the PA intended to be studied and the upstream portion of MCA. This geometry allowed for the PA to be mounted onto glass cannulas in the arteriograph

chamber, as shown in Figure 2.1, by threading the glass cannula through the wider MCA and then into the PA lumen while decreasing how much the targeted PA was handled by forceps. Surgical threads were then tied around the vessel to secure the vessel walls to the cannulas and close the intraluminal space. The arteriograph chamber ensures that the PA is bathed in circulating aCSF at physiological temperature and pH, $37^{\circ}\text{C} \pm 0.02$ and 7.4 ± 0.02 . This aCSF is oxygenated using a physiologically relevant blood gas mixture, which controls the pH of the bath.

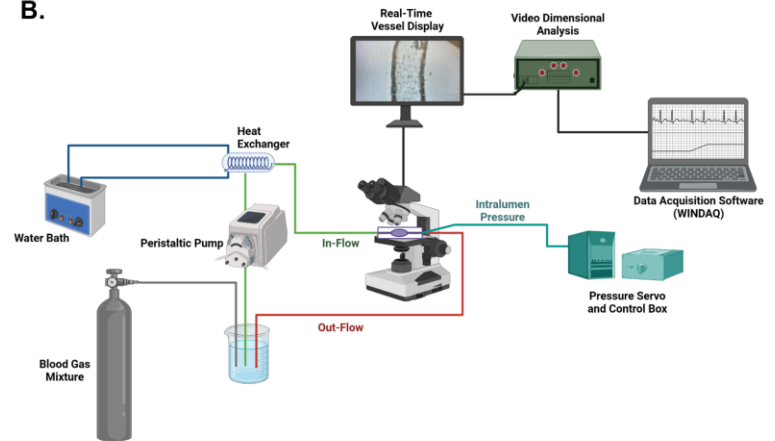
Intravascular pressure can then be controlled with a pressure servo, to stimulate the vessel and assess myogenic tone and reactivity. Changes in intravascular pressure can stimulate the development of myogenic tone, or the physiological, dynamic state of pre-constriction that blood vessels tightly regulate *in vivo* to control vascular resistance and blood flow. The vessels undergo leakage tests to ensure that the intraluminal space is closed, and shear stress is not damaging the endothelium. After demonstrating that they can hold pressure throughout the leakage test, PAs are equilibrated at an intravascular pressure of 20 mmHg for 1 hour. Pressure is then increased stepwise to 120 mmHg. Lumen diameters are recorded at each pressure to determine if the vessels develop spontaneous myogenic tone and to measure myogenic reactivity. For the remainder of the experimental protocol utilized in this research, intravascular pressure is set to 40 mmHg. The remaining sections of experimental protocol are further explained in Chapter 3.

Figure 2.1: A graphic representation of the isolated vessel experiment system and an isolated cerebral artery in an arteriograph. Part B of this sub-system schematic includes the arteriograph, shown in purple, on the microscope stage.

A.



B.



**CHAPTER 3: REACTIVITY OF ISOLATED
CEREBRAL PARENCHYMAL ARTERIOLES TO
THROMBIN DURING PREGNANCY AND
PREECLAMPSIA IN RATS**

Olivia M O'Brien, Sarah M Tremble, Marilyn J Cipolla

3.1 Abstract

Objectives

To investigate the vasoactive potential of thrombin in brain parenchymal arterioles (PAs) in nonpregnant (NP), healthy late pregnant (LP) rats and rats with experimental preeclampsia (ePE).

Study Design

This was an investigative study utilizing isolated, pressurized PAs from female rats that were bathed in increasing doses of thrombin in an arteriograph system (NP n = 13, LP n = 12, ePE n = 13). Prothrombin and thrombin-antithrombin levels in plasma and cerebrospinal fluid (CSF) were analyzed via enzyme-linked immunosorbent assay (ELISA). Expression of protease-activated receptor (PAR) types 1 and 2 in PAs were investigated via Western blot (WB) and immunohistochemistry (IHC) images.

Main Outcome Measures

Changes in lumen diameter and wall thicknesses were recorded to assess vasoconstriction or vasodilation as well as structural properties of the PAs. Constriction to thrombin with and without inhibition of cyclooxygenase (COX) and nitric oxide synthase (NOS) was a principal outcome measure. Relative levels of prothrombin fragments 1+2 and thrombin-antithrombin complex in plasma and CSF were compared. Using IHC, sections of PAs were stained for PAR-1 and -2 and localized using α -smooth muscle actin for smooth muscle. Protein levels of PAR-1 and -2 detected via WB were normalized to NP levels.

Results

Thrombin and prothrombin were elevated in plasma in rats with ePE compared to NP and LP plasma. Thrombin was found in CSF in all groups and was significantly elevated in LP CSF (NP: 0.137 ± 0.014 ng/mL, LP: 0.241 ± 0.015 ng/mL, ePE: 0.192 ± 0.028 ng/mL). COX and NOS inhibition caused greater constriction in PAs from healthy pregnant rats than in PAs from NP rats, however PAs from ePE rats did not exhibit that same constriction. Thrombin was a modest vasoconstrictor in parenchymal arterioles when COX and NOS were both uninhibited and inhibited. Thrombin decreased myogenic reactivity in PAs from rats with ePE, but not in NP and LP PAs.

Conclusions

These findings suggest that thrombin has modest constrictive effects on cerebrovasculature in pregnancy and PE, which may occur via activation of PARs in the smooth muscle of the vasculature.

3.2 Introduction

Pregnancy is a hypercoagulable state associated with widespread hemodynamic changes encompassing increases in pro-coagulation factors, such as fibrinogen and prothrombin, and decreases in anti-coagulation factors and fibrinolytic activity [1, 2]. The physiological changes that lead to this state begin early in gestation and can last more than 8 weeks postpartum [3]. This prothrombotic state can be further heightened by preeclampsia (PE), a common yet serious disorder of pregnancy characterized by hypertension and extensive maternal multi-system dysfunction [4, 5]. PE complicates 2-8% of pregnancies worldwide and is the second leading cause of maternal morbidity and mortality [6, 7]. While the pronounced hemodynamic changes that occur during pregnancy promote healthy placental function and help face the hemostatic challenge presented during delivery, these changes can predispose women to thrombotic complications and cardiovascular conditions, such as stroke [8].

Prothrombin, the inactive precursor of the pivotal coagulation enzyme thrombin, is among the pro-coagulation factors that increase during gestation [3, 9]. Thrombin is a platelet-activated serine protease that drives the coagulation cascade by cleaving fibrinogen into fibrin, which crosslinks to stabilize platelet plug formation and arrest bleeding [9, 10]. Thrombin can also play a non-hemostatic role in arresting bleeding as a vasoactive circulatory protein. It has been shown to be a vasoconstrictor via protease-activated receptors (PAR) in the circulation of multiple organ systems, including cerebral arteries [10, 11, 12].

The hypercoagulable state of pregnancy contributes to the increased risk of maternal stroke, which affects 30.0 out of every 100,000 pregnancies [2]. Compared to age-matched people, pregnant women have a 3-fold increased risk of stroke [2]. PE further increases that risk to 6-fold and while the pathophysiology that leads to PE is not definitively characterized, the associated endothelial dysfunction, oxidative stress, increased vascular stiffness, hypertension, and super-hypercoagulability likely drive that increased stroke-risk [2, 5, 13, 14].

Stroke can be a devastating complication of pregnancy and occur anywhere in the brain; however, the middle cerebral artery (MCA) is the most common artery involved in acute stroke [8, 15]. The MCA supplies blood to the temporal, frontal, and parietal lobes of the brain as well as deeper brain regions via vessels branching from it, including parenchymal arterioles (PAs). PAs are high resistance blood vessels that dive into the brain parenchyma and regulate cerebral blood flow to deeper brain structures including the frontoparietal white matter (WM) tracts of the sensorimotor cortex and basal ganglia [16]. The experimental model of preeclampsia used in this study has also been shown to cause dysfunction of PAs and these vessels are a growing focus for understanding PE's effects on cerebrovasculature [16].

In the present study, we investigated the vasoactive role of thrombin in pregnancy and experimental preeclampsia in the cerebrovasculature of rats, specifically in cerebral parenchymal arterioles (PAs). Using an experimental model of preeclampsia, we investigated the vasoactive effects of thrombin in isolated and pressurized PAs from rats with experimental preeclampsia (ePE) compared to those from nonpregnant (NP) and

normal late pregnant (LP) rats. Specifically, we studied the PAs that supplied the frontoparietal WM tracts and the basal ganglia. We investigated prothrombin and thrombin-antithrombin complex levels in plasma and cerebrospinal fluid (CSF) of NP, LP, and ePE rats. Finally, we investigated expression levels of PARs 1 and 2 via Western Blot, paired with immunohistochemistry images of the PAs to localize PAR expression.

3.3 Materials and Methods

3.3.1 Animals

All experiments were conducted using virgin, NP or LP Sprague-Dawley female rats between 10 and 18 weeks of age (Charles River, Canada). Rats were housed singly with enrichment in the University of Vermont Animal Care Facility, an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility. Rats were maintained on a 12-hour light/dark cycle and allowed access to food and water ad libitum. Pregnant rats were used late in gestation on day 20 of a 22 day gestation. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The investigator was not blinded to animal group during experiments, as this was not possible due to the visual difference in body size during pregnancy. The order of experiments, however, was randomized by animal group (randomizer.org). All euthanasia was under isoflurane anesthesia according to NIH guidelines.

3.3.2 Rat Model of experimental PE (ePE)

PE is a known state of dyslipidemia that has been associated with maternal endothelial dysfunction, inflammatory responses, and peroxynitrite generation in cerebral arteries [12 – 15]. It is thought that dyslipidemia could play a contributing role in the pathogenesis of PE and could be a risk factor for the disease [16, 17]. Pregnant rats were maintained on a high cholesterol diet (Prolab rat chow with 2% cholesterol and 0.5%

sodium cholate; Scotts Distributing Inc., Hudson, NH, USA) from days 7 – 20 of gestation to induce dyslipidemia. This model has additionally shown to induce symptoms congruent with PE such as elevated blood pressure, maternal endothelial dysfunction, and increased inflammatory markers such as TNF- α and 8-isoprostane [13, 14].

3.3.3 Measurement of circulating factors via enzyme-linked immunosorbent assays (ELISAs)

To investigate if ePE affected prothrombin and thrombin levels on both the circulatory side and neuronal side of the blood brain barrier, plasma levels of prothrombin fragments 1+2 and thrombin-antithrombin (TAT) complex as well as CSF levels of TAT complex were measured using commercially available ELISA kits for Prothrombin Fragment 1+2 (NBP3-06906, Novus Biologicals, Centennial, CO, USA) and TAT (NBP2-68132, Novus Biologicals, Centennial, CO, USA). Plasma was collected from NP, LP, and ePE rats via cardiac puncture into lithium heparin blood collection tubes and spun at 2500 G for 10 minutes. CSF was collected microsurgically from the cisterna magna from rats under isoflurane anesthesia and centrifuged at 1000 G for 20 min. Plasma and CSF samples were measured undiluted and in duplicate. A portion of the CSF samples were pooled to make up the required volumes for the ELISA.

3.3.4 *In-vitro* isolated vessel experiments

NP, LP, or ePE rats were decapitated under deep isoflurane anesthesia (3% oxygen) and brains immediately removed and placed in cold, oxygenated artificial cerebrospinal

fluid (aCSF). In accordance with the dissection protocol of Johnson and Cipolla, a section of cerebral cortex that was 2 mm thick containing the MCA were dissected from the brain, and a PA was isolated and pressurized [16]. PAs were equilibrated at an intravascular pressure of 20 mmHg for 1 hour. After which, pressure was increased stepwise to 120 mmHg. Lumen diameter and wall thickness were recorded at each pressure to determine if the vessels developed spontaneous myogenic tone and to measure myogenic reactivity. Intravascular pressure was set to 40 mmHg for the remainder of the experiment.

Reactivity to NS309, a small- and intermediate-conductance calcium-activated potassium (SK/IK) channel agonist, was used to test viability. To investigate the response of PAs to thrombin, cumulative doses were added to the bath and lumen diameters were recorded at each dose. A separate group of PAs from each group were given single doses of indomethacin (10^{-5} M) and L-NAME (10^{-3} M) prior to receiving thrombin to inhibit the two major endothelium-dependent dilatory pathways, COX and NOS, respectively [17, 18]. In the presence of thrombin, intravascular pressure was increased in a stepwise manner to 120 mmHg to measure myogenic reactivity. At the end of each isolated vessel experiment, aCSF was replaced with aCSF containing zero calcium and 0.5 mM EGTA. Diltiazem (10^{-5} M) and Papaverine (10^{-4} M) were added to obtain fully relaxed measurements of passive lumen diameter and biomechanical properties.

3.3.5 Measurement of PAR expression via Western Blot

After dissection, remaining PAs branching from the MCA were dissected, collected, and suspended in aCSF, and snap-frozen with liquid nitrogen to be stored at -80° C. PAs from NP, LP, and ePE rats were pooled for protein expression levels of PARs 1

and 2 via WB. For protein extraction, samples were pooled by group and homogenized in a glass Dounce tissue grinder 3 mL lysis buffer comprising: 50 mM Trizma hydrochloride, 150 mM NaCl, 10 mM EDTA, 0.25% deoxycholate, 1% nonylphenol polyethylene glycol detergent, 10% glycerol, 1% sodium dodecyl sulphate, 1MM dithiothreitol and 1% protease inhibitor at 1:200 (Sigma P8340). The homogenate was transferred and centrifuged at max speed for 10 min at 4° C. 12% gel membranes were blocked for 1 hour at room temperature, and subsequently incubated overnight at 4° C with two primary antibodies: an affinity purified rabbit polyclonal antibody raised against synthesized peptide derived from human PAR1, 1:500 (Invitrogen, ThermoFischer, Waltham, MA, USA), and a mouse monoclonal antibody raised against amino acids 37-50 of human PAR2 (Santa Cruz Biotechnology Inc., Dallas, TX, USA). Membranes underwent repeated washing in TBST and incubation in secondary antibodies for 1 hour at room temperature, specifically a goat anti-rabbit (LI-COR, Lincoln, NE, USA) and donkey anti-mouse (LI-COR, Lincoln, NE, USA) were used. Densitometry of PAR1 and PAR2 bands was determined with Odyssey Imaging software (5th generation). All experiments were done in triplicate. The ratio of PAR1/beta-actin or PAR2/beta-actin intensity was calculated for each sample in each group and normalized to the NP group.

3.3.6 Localization of PARs via IHC

To investigate the localization of PARs 1 and 2 within the vessel walls of PAs, immunohistochemical staining was performed for PAR1, PAR2, and α -smooth muscle actin. PAs were fixed while pressurized at 40 mmHg in 10% formalin for 30 minutes and

stored in phosphate-buffered saline at 4° C until being paraffin embedded for immunohistochemistry. According to standard procedures, 3 µm sections were cut and prepared for immunofluorescence. PAR1 and PAR2 were stained for on separate sections using rabbit anti-PAR-1 (ThermoFisher PA5-116040) or rabbit anti-PAR-2 (Abcam ab180953), respectively, at 1:50, 1:100, and 1:200. Goat anti-rabbit IgG (Invitrogen A21429) was used as a secondary antibody. Both PARs were captured at a wavelength of 647 nm. α -Smooth Muscle Actin (α SMA) was stained with anti-SMA to identify the smooth muscle medial layer and captured at 555 nm. Sections were imaged on a Nikon A1R HD Confocal Microscope. Images were captured at x20 magnification for each PA using NIS-Elements Ar (version 4.30.02; Nikon United States, Tokyo, Japan).

3.3.7 Drugs and solutions

NS309, indomethacin, L-NAME, diltiazem, and papaverine were purchased from Sigma-Aldrich (St. Louis, MO, USA). The bovine thrombin used was sourced from BioPharm Laboratories LLC (Bluffdale, UT, USA). Stock solutions of indomethacin, L-NAME, diltiazem, and papaverine were made weekly and stored at 4 °C until use. NS309 was diluted into dimethyl sulfoxide, and thrombin into double-distilled H₂O, and stock solutions were aliquoted and stored until use at - 20 °C and - 80 °C, respectively. Isolated PA experiments were performed using aCSF containing (mM): NaCl 122.0, NaHCO₃ 26.0, NaH₃PO₄ 1.25, KCl 3.0, MgCl₂ 1.0, CaCl₂ 2.0, and glucose 4.0. Buffer solutions were made each week and stored without glucose at 4 °C. Glucose was added immediately before each experiment. aCSF was aerated with 5% CO₂, 10% O₂, and 85% N₂ to maintain

pH at 7.40 ± 0.05 and the temperature within the arteriograph chamber bath was maintained at 37.0 ± 0.2 °C throughout the experiments.

3.3.8 Data calculations

At each intravascular pressure, percent tone was calculated by the equation $\% \text{ Tone} = [1 - (\varphi_{\text{active}} / \varphi_{\text{passive}})] * 100\%$; φ_{active} is the lumen diameter under physiological conditions and φ_{passive} is lumen diameter under fully relaxed conditions at that same pressure. Reactivity to NS309 was calculated from the equation: $\% \text{ Reactivity} = [(\varphi_{\text{dose}} - \varphi_{\text{baseline}}) / (\varphi_{\text{passive}} - \varphi_{\text{baseline}})] * 100\%$; where the φ_{dose} is the lumen diameter after treatment with a specific concentration of drug and $\varphi_{\text{baseline}}$ is the starting diameter of the vessel before any drug treatment. Percent change in diameter from baseline to indomethacin was calculated using the equation: $\% \text{ Change} = [(\varphi_{\text{dose}} - \varphi_{\text{baseline}}) / (\varphi_{\text{baseline}})] * 100\%$. Percent change to L-NAME and to thrombin were also calculated using the aforementioned equation.

Distensibility of the PAs was calculated using the equation: $[(\varphi_{\text{passive}} - \varphi_{\text{passive at 5 mmHg}}) / (\varphi_{\text{passive at 5 mmHg}})] * 100\%$; where $\varphi_{\text{passive at 5 mmHg}}$ is the passive diameter at an intravascular pressure of 5 mmHg. Outer diameter (φ_{outer}) was calculated at each pressure using the equation: $\varphi_{\text{outer}} = \varphi_{\text{inner}} + 2WT$; where φ_{inner} is the lumen diameter of the fully relaxed PA and WT is the measured wall thickness. Cross-sectional area was calculated using the equation: $(\frac{\varphi_{\text{outer}}}{2})^2 - \pi(\frac{\varphi_{\text{inner}}}{2})^2$ at pressures within the range 5 - 200 mmHg. All diameter and WT measurements were converted to centimeters to calculate wall tension, wall stress, and wall strain. Wall tension was calculated by converting each pressure across the range from mmHg into dynes/cm² x $(\frac{\varphi_{\text{inner}}}{2})^2$. Wall stress was

calculated at each pressure using the equation: Wall Stress = wall tension / WT. Wall strain was calculated using the equation: Wall Strain = $[(\varphi_{\text{passive}} - \varphi_{\text{passive at 5 mmHg}}) / \varphi_{\text{passive at 5 mmHg}}]$. The stiffness parameter β was calculated using the following regression equation [19, 20]

$$\ln\left(\frac{p}{p_s}\right) = \beta \left(\frac{\varphi_{\text{outer}}}{\varphi_s} - 1\right)$$

where p is the intravascular pressure, p_s is a reference pressure chosen in the physiological pressure range, φ_{outer} is the outer diameter, and φ_s is the outer diameter of the vessel at the reference pressure. The reference pressure was chosen to be 40 mmHg because it lies within the physiological pressure range.

3.3.9 Statistical analyses

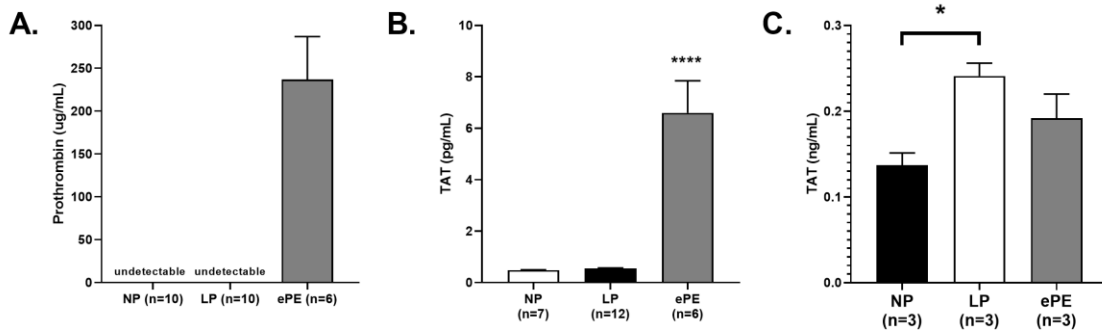
Results are presented as mean \pm SEM. After running Shapiro-Wilk normality tests, one-way ANOVAs were used to determine differences between the three groups. For percent change to indomethacin and L-NAME, two-way ANOVAs were utilized to compare the effects of drug and pregnancy-state. Differences were considered significant at $p < 0.05$. All ANOVAs had *post-hoc* Tukey's to correct for multiple comparisons and were performed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA).

3.4 Results

3.4.1 Coagulation factor levels in plasma and CSF

Prothrombin fragments 1+2 were found in the plasma from rats with ePE but was undetectable in NP and LP plasma (Fig. 3.1A). Plasma levels of thrombin-antithrombin (TAT) were similar in plasma from NP and LP rats. However, TAT levels in plasma from rats with ePE were significantly greater than both NP and LP groups (Fig. 3.1B). CSF levels of TAT were detected in all three groups (Fig. 3.1C). A nonparametric Kruskal-Wallis test reported that the median CSF thrombin levels CSF varied significantly ($p < 0.05$). Thus, circulating levels of prothrombin and thrombin-antithrombin levels were elevated in ePE compared to NP and LP rats, but CSF levels of thrombin-antithrombin in ePE were not.

Figure 3.1: Hemostatic Factors in Pregnancy and Preeclampsia. (A) Prothrombin fragments 1 + 2 levels from plasma from NP, LP, and ePE rats collected via cardiac puncture and analyzed via ELISA. (B) TAT levels from plasma from NP, LP, and ePE rats collected via cardiac puncture and analyzed via ELISA. (C) TAT levels from plasma from NP, LP, and ePE rats collected via cardiac puncture and analyzed via ELISA. **** $p < 0.0001$ vs. NP and LP one-way ANOVA with Tukey's multiple comparisons post-hoc tests. (C) TAT levels from CSF from NP, LP, and ePE collected from the cisterna magna and analyzed via ELISA. One-way ANOVA with multiple comparisons post-hoc tests revealed that LP CSF thrombin concentration was significantly greater than NP CSF thrombin ($p < 0.05$).

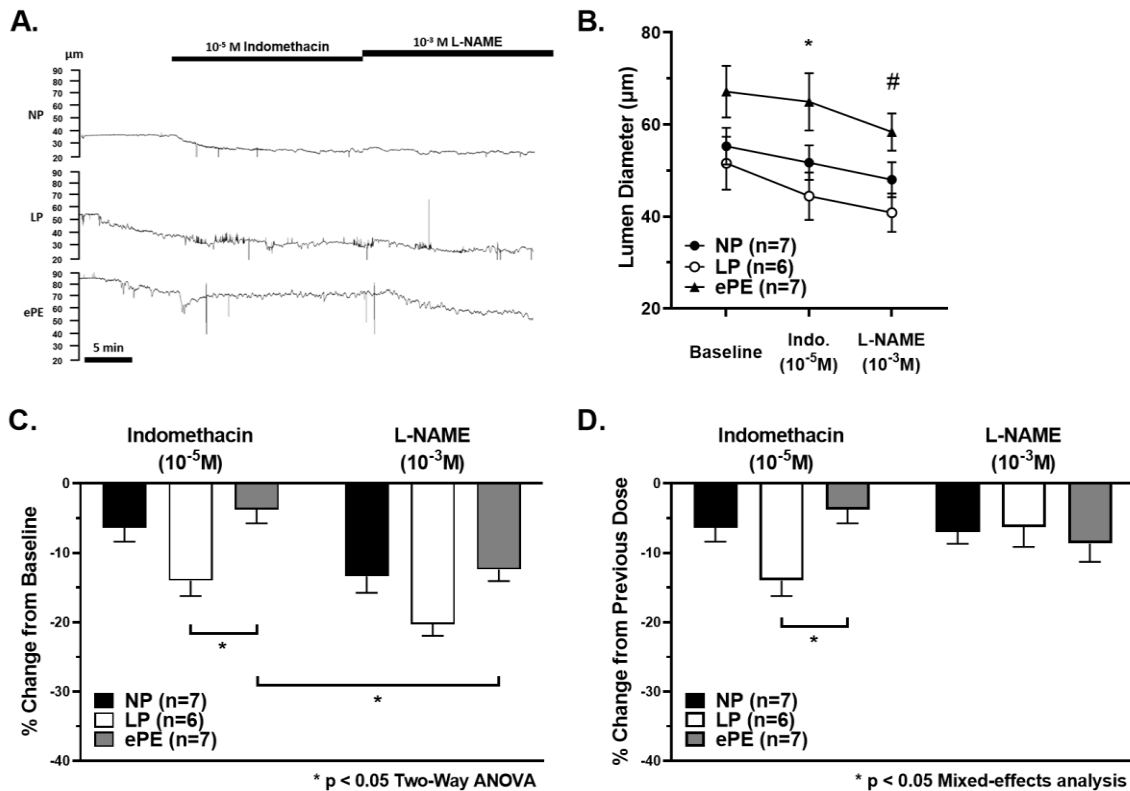


3.4.2 Reactivity of isolated PAs

NS309 was administered to assess vessel viability and caused vasodilation of PAs in all groups, with the maximum concentration (10^{-5} M) eliciting similar dilations between groups ($92 \pm 2\%$ in NP, $86\% \pm 4\%$ in LP, and $87 \pm 2\%$ in ePE). Fig. 3.2A shows representative tracings of lumen diameters of PAs from NP, LP, and ePE rats in response to indomethacin and L-NAME. When comparing percent change from baseline (Fig. 3.2C), there was a significant effect of drug ($F_{(1,34)} = 19.53$; $p < 0.01$ by 2way ANOVA) and effect of pregnancy status ($F_{(2,34)} = 10.70$; $p < 0.01$ by 2way ANOVA), with Indomethacin causing greater constriction in PAs from LP rats compared to PAs from NP and ePE rats. PAs from LP rats exhibited significantly more change from baseline to indomethacin than the ePE

group. NOS-inhibition by L-NAME caused vasoconstriction in all three groups as each grouped exhibited negative percent change from baseline. PAs from ePE rats exhibited significantly greater constriction in response to L-NAME than to Indomethacin (Fig. 3.2C).

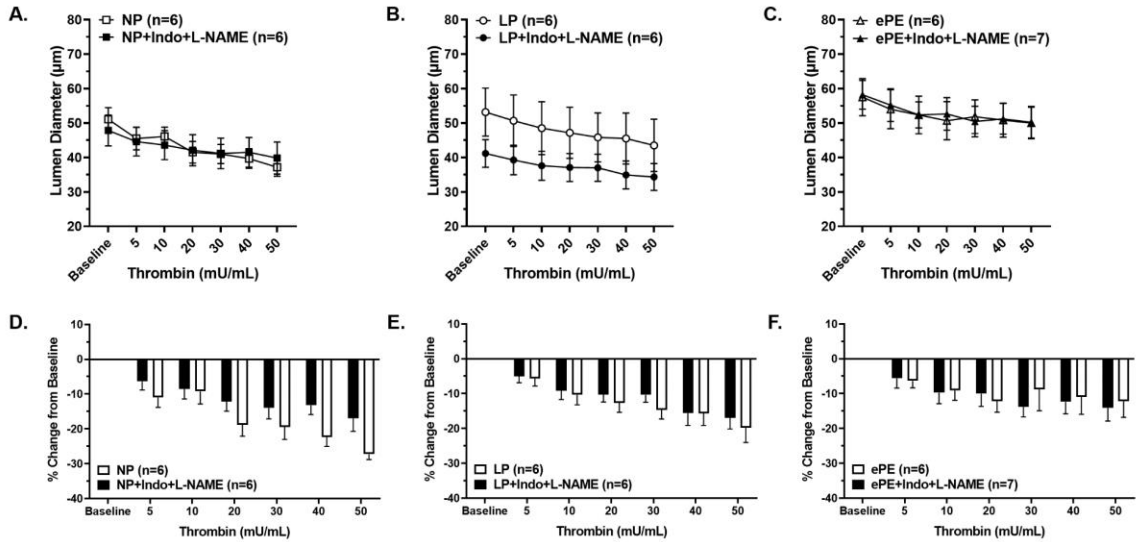
Figure 3.2: The role of COX- and NOS-pathway inhibition in vasoconstriction. (A) Representative lumen diameter tracings of PAs from nonpregnant (NP; top panel), late pregnant (LP; middle panel), and experimental preeclamptic (ePE, bottom panel) rats in response to cumulative treatment with Indomethacin and L-NAME. (B) Lumen diameters of PAs from NP, LP, and ePE rats in consecutive dose of Indomethacin and L-NAME. (C) Percent change from baseline in lumen diameter of PAs in response to COX and NOS pathway inhibition. (D) Percent change from previous dose in lumen diameter of PAs in response to COX and NOS pathway inhibition. * $p < 0.05$ by two-way ANOVA with post-hoc Tukey test.



Cumulative doses of thrombin were administered and changes in lumen diameter were measured at each concentration from baseline at 0 mU/mL to 50 mU/mL. Thrombin was administered extraluminally both alone (NP, LP, and ePE) and after the PAs were administered indomethacin and L-NAME (NP+Indo+L-NAME, LP+Indo+L-NAME, and

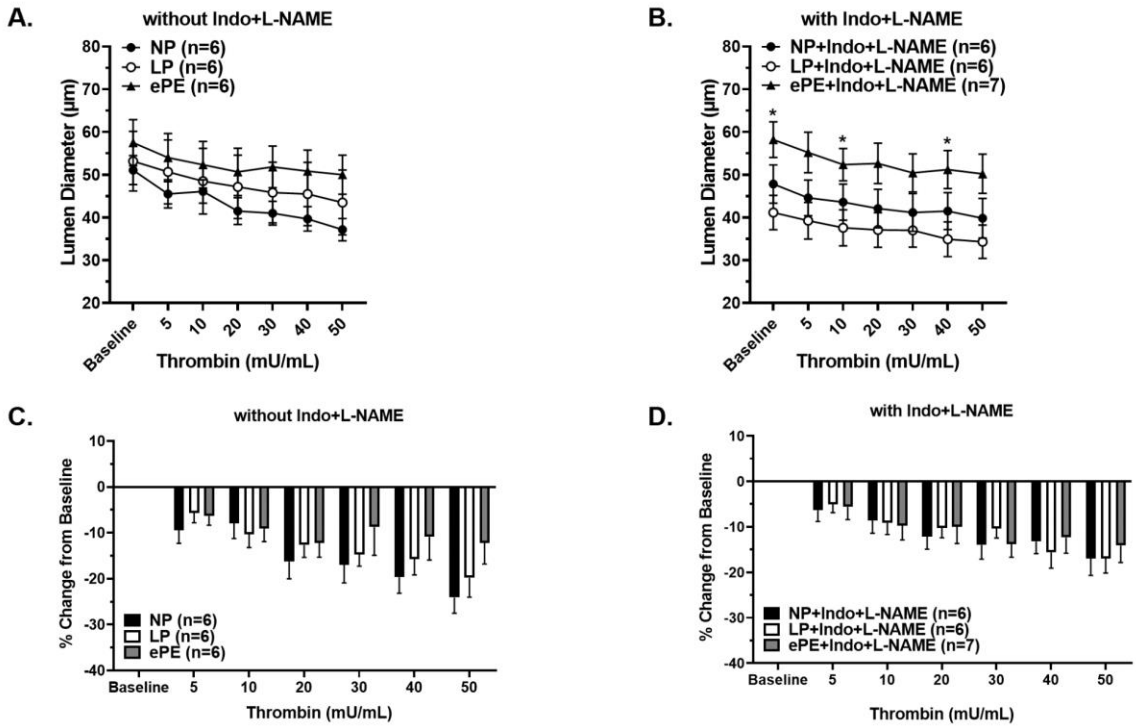
ePE+Indo+L-NAME). Thrombin elicited modest vasoconstriction in PAs from NP, LP, and ePE rats (Fig. 3.3). The highest concentration of thrombin caused similar constriction in PAs from NP, LP, and ePE rats both with and without COX and NOS inhibition (Fig. 3.4).

Figure 3.3: Reactivity of PAs from nonpregnant (NP), late-pregnant (LP), and experimental preeclamptic (ePE) rats to thrombin. Graphs showing lumen diameter responses of PAs from (A) NP rats, (B) LP rats, and (C) ePE rats; and percent change in diameter of PAs from (D) NP rats, (E) LP rats, and (F) ePE rats.



To determine the effect of thrombin on myogenic reactivity and tone in parenchymal arterioles, intravascular pressure was increased stepwise from 20 mmHg to 120 mmHg while the PAs were bathed in 50 mU/mL thrombin and changes in lumen diameter were measured. Percent myogenic tone was calculated to compare to the percent tone exhibited by the PAs prior to the administration of thrombin. Before thrombin, vessels from all 3 groups maintained similar lumen diameters across the pressure range and exhibited similar percent tone at the highest intravascular pressure ($33 \pm 2\%$ in NP, $32 \pm 2\%$ in LP, and $35 \pm 3\%$ in ePE). Once in 50 mU/mL thrombin, the NP and LP group exhibited more tone at 120 mmHg ($41 \pm 9\%$ in NP and $42 \pm 8\%$ in LP); whereas PAs from ePE rats exhibited less tone at $26 \pm 5\%$.

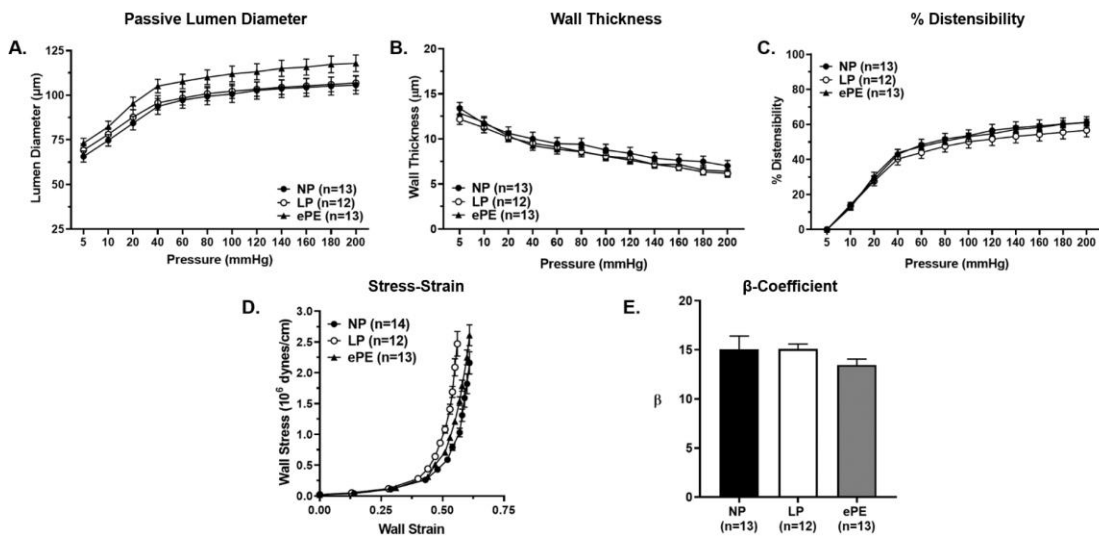
Figure 3.4: Reactivity of PAs from NP, LP, and ePE rats to thrombin with and without COX and NOS inhibition. Graphs showing lumen diameter responses of PAs from (A) NP, LP, and ePE rats; and (B) lumen diameter responses of PAs from NP, LP, and ePE rats with indomethacin and L-NAME present (NP+Indo+L-NAME, LP+Indo+L-NAME, ePE+Indo+L-NAME); (C) percent change in diameter of PAs from NP, LP, ePE rats; and (D) percent change in diameter of PAs from NP, LP, and ePE rats with indomethacin and L-NAME present. (* LP+Indo+L-NAME vs. ePE+Indo+L-NAME; $p < 0.05$ by one-way ANOVA with multiple comparisons).



3.4.3 Structural and biomechanical properties of PAs

Passive lumen diameters (Fig. 3.5A), wall thickness (Fig. 3.5B), and percent distensibility (Fig. 3.5C) of PAs from NP, LP, and ePE rats were similar. To investigate vascular stiffness in pregnancy and ePE, wall-stress strain curves were calculated and were similar between groups (Fig. 3.5D). In addition, the stiffness parameter β was also calculated and stiffnesses were similar between groups (Fig. 3.5E).

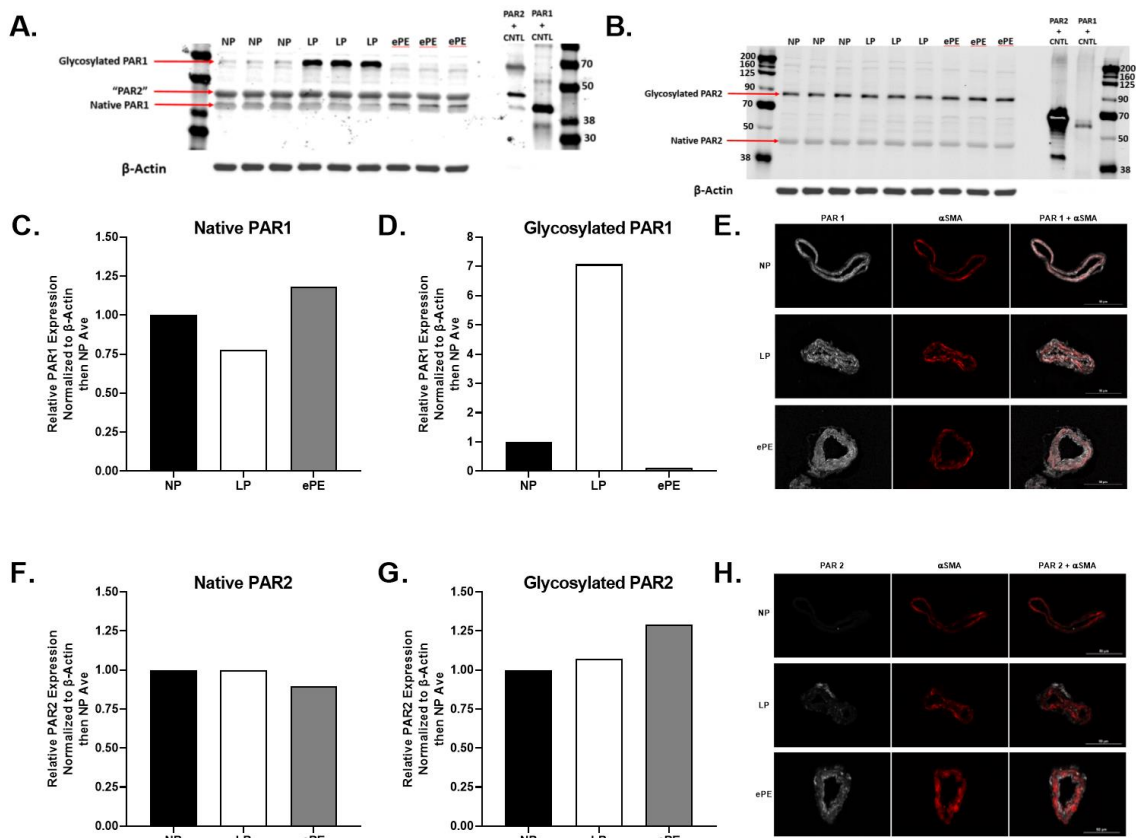
Figure 3.5: Structural and biomechanical properties of PAs. Graphs showing (A) passive lumen diameter, (B) vascular wall thickness, (C) percent distensibility, (D) stress-strain curves, and (E) mean values of the stiffness parameter β of PAs from NP, LP, and ePE rats.



3.4.4 Expression of PARs 1 and 2 in PAs

Protein bands of PARs 1 and 2 were analyzed relative to β -actin levels and then normalized to NP levels as a control (Fig. 3.6A – B). Based on the PAR1 control from mouse brain tissue lysates, similar PAR1 protein levels were observed across the groups at 44 kDa. A second band at 70 kDa was observed and indicated greater protein levels in the LP group (Fig. 3.6A). Two protein bands were analyzed for PAR2 expression, both indicated similar protein levels across groups (Fig. 3.6B). Comparative photomicrographs of NP, LP, and ePE PAs stained for PAR1 or -2 showed localization of PAR1 in the smooth muscle of PA vessel walls of all three groups, and PAR2 in the ePE group (Fig. 3.6E and 3.6H).

Figure 3.6: Expression of PARs 1 & 2 in PAs. (A) WB gels of PAR1 and (B) PAR2. Calculated expression levels of (C) native PAR1, (D) glycosylated PAR1, (F) native PAR2, and (G) glycosylated PAR2. Photomicrographs of PAs stained for (E) PAR1 (white) or (H) PAR2 (white), α SMA (red), or both channels overlaid.



3.5 Discussion

As a leading cause of maternal morbidity and mortality, the increasing incidence of stroke in pregnancy is a pressing concern of patients and clinicians alike. The increasing rate of PE is suggested to be a driving factor for the rising incidence of stroke [2, 8]. PE puts increased stress on the cardiovascular system in the form of increased arterial stiffness, increase in vascular resistance, vascular remodeling, endothelial dysfunction, and oxidative stress [2, 8, 13, 14]. PE-associated cardiovascular dysfunction can negatively impact maternal health during pregnancy and the postpartum period and has been suggested to have lasting impacts on cardiovascular and brain health later in life [18, 28, 29]. Furthering our understanding of the effects of PE on cerebrovascular health could strengthen support for pregnant and preeclamptic women in the short- and long-term.

Considering PE increases the already hypercoagulable state of pregnancy, it was expected that prothrombin and thrombin levels in LP rats would be elevated from NP rats and more elevated still in ePE. During normal pregnancy in humans, prothrombin increases as gestation continues and PE further increases prothrombin levels during pregnancy and postpartum [30, 32]. The current study showed that TAT levels in LP rats were similar to NP, and that prothrombin levels were undetectable in both LP and NP. When comparing LP and NP TAT levels only, a one-way ANOVA revealed that LP levels were significantly greater than NP levels yet that significance was masked by the significantly greater TAT levels in ePE plasma. It has been measured that prothrombin time, or the time required for prothrombin to be activated and contribute to clot formation, decreases in pregnant rats as gestation continues [39]. Where plasma was collected late in gestation, thrombin may be

circulating more in its active form than inactive prothrombin due to the decrease in activation time, which may account for the undetectable LP levels when measured using ELISAs. That prothrombin levels were elevated in ePE suggests that ePE may disrupt this decrease in prothrombin time in rats. Similarly, PE alters prothrombin time in humans as mean prothrombin time in patients with PE was found to be 15.27 seconds compared to a mean prothrombin time of 13.72 seconds in normal pregnancy [55].

In CSF, TAT was found in all three groups and was significantly elevated in LP CSF compared to NP. However, the ePE TAT levels were not significantly increased. That thrombin was found in CSF gives relevance to studying thrombin's vasoactive properties in PAs that are surrounding by CSF as opposed to pial arteries because the intraparenchymal vessels may experience those vasoactive effects from CSF thrombin in addition to the thrombin circulating in plasma. Prothrombin, thrombin, and PARs have been localized to astrocytes and neurons in the brain [40 - 42]. Specifically, prothrombin mRNA expression is greatest in the cerebral cortex [43]. It has been suggested that high concentrations of thrombin in the brain have damaging cellular effects, while low concentrations are neuroprotective. By activating PAR-1, thrombin has been seen to have anti-inflammatory effects and contribute to endothelial barrier stabilization through signaling of activated protein C, thus serving a cellular protective role [40, 44]. Thrombin can be additionally protective against cellular insults including glucose deprivation and reactive oxygen species [40, 44]. However, Krenzlin et al. reported that high CSF thrombin concentrations were associated with poorer functional neuronal outcome following intracerebral hemorrhage (IHC) in humans

[44]. The concentrations observed in the current study were similar to the healthy human control levels in the associative IHC study by Krenzlin et al. [44]. Mechanisms by which thrombin can damage cerebral cells include TNF-alpha upregulation, neuronal cell apoptosis, and degradation of basal lamina matrix fibers such as collagen IV and laminin [44]. Importantly, pregnancy has been shown to be protective against stroke in rats due to robust collateral flow and greater ischemic tolerance, but ePE was associated with greater infarction due to poor collateral flow, endothelial dysfunction, and oxidative stress [45]. In the current study, the increased thrombin concentration in healthy LP CSF but not ePE CSF may indicate a protective role in healthy pregnancy that is disrupted in ePE. However, further investigation is needed to identify a specific cellular mechanism. The current study also indicates that thrombin may be expressed by cerebral cells, such as neurons and astrocytes, in rats and that expression may increase in pregnancy.

Inhibition of COX and NOS caused greater constriction in PAs from healthy pregnant rats than in NP but was not seen in ePE PAs. Pregnancy is a high nitric oxide state, and the vasodilatory effects of NOS are believed to contribute to decreased peripheral vascular resistance in response to the increase in blood volume [33 - 35]. COX activity contributes to a healthy pregnancy on the maternal and fetal sides by influencing uterine contraction and fetal development [36]. The results of the current study indicate a greater role of COX, likely along COX-1 and prostacyclin, and NOS in cerebrovasculature during pregnancy compared to in NP rats [53, 54]. That vessels from ePE constricted less than the LP group suggests that ePE disrupts or dampens the increased COX and NOS activity seen

in healthy pregnancy. Furthermore, the ePE group showed significantly greater constriction to L-NAME than to indomethacin, indicating a potentially greater role of NOS in regulating vascular tone of PAs than COX during ePE albeit still decreased compared to healthy pregnancy.

WB analysis showed expression of PAR-1 and PAR-2 in PAs in pregnant and ePE rats and IHC photomicrographs showed localized PARs in the smooth muscle layer. Despite each PAR type being activated at a unique extracellular N-terminus, the PAR family share structural and sequential similarity in their transmembrane domains [47, 48]. Based on the PAR-2 control band of mouse brain tissue lysate in the PAR-1 blot, it is believed that the PAR-1 antibody was cross-reactive and attached to PAR-2 proteins as well. In blots for PAR-1 and PAR-2, two distinct bands were observed, one that corresponded with the control bands of each receptor, and additional bands at higher molecular weights. The higher molecular weight band could be due to a posttranslational modification. N-linked glycosylation is a common post-translational process in G-coupled protein receptors in which oligosaccharides attach to extracellular domains of the receptor [49, 50, 51]. Glycosylation causes structural and function modification that are important for receptor maturation and can increase the molecular weight of the receptor [49, 50]. PAR-2 possesses 2 N-linked sequins, one on the second extracellular loop and one on the N-terminus itself, and glycosylation at these sites can account for up to 60 kDa of PAR-2's molecular weight, which can ranges from 45 to 100 kDa [49]. Given the higher band in all groups at about 90 kDa, it is believed that both native and glycosylated PAR-2 were detected at similar levels in NP, LP, and ePE PAs. PAR-1 contains the most N-linked

glycosylation sites of all 4 PAR types, three on the N-terminus and two on the extracellular loop [50, 52]. This can result in changes in its molecular weight, which has been shown to range from 34 - 100 kDa [50]. In the PAR-1 blot, a prominent band at 70 kDa appeared in the LP group accompanied by lesser bands in the NP and ePE group at that molecular weight. It is believed that expression of native PAR-1 was similar in PAs from all three groups, but that protein levels of glycosylated PAR-1 were increased in LP PAs. PAR-1 glycosylation is thought to be important for cell surface receptor expression [50]. These results suggest that PARs -1 and -2 are expressed in PAs in rats during pregnancy and PE, and furthermore that levels of glycosylated PAR-1 are greater during healthy pregnancy, but those levels are dampened to near non-pregnant levels in ePE.

The current study showed that thrombin was a modest vasoconstrictor in PAs in female rats. In human and porcine coronary arteries, it caused vasodilation, yet vasoconstriction in porcine pulmonary and canine cerebral arteries [10 - 12]. However, little is known about thrombin's vasoactive properties in cerebral vessels in rats, and its impacts during pregnancy and experimental PE. PARs 1 and 2 can mediate COX activation and NOS production [24, 44]. Thrombin-induced vasoconstriction was similar with and without COX and NOS inhibition, indicating that there are additional cellular pathways besides COX or NOS by which thrombin could activate PARs and cause vasoconstriction. Using real-time confocal microscopy, thrombin was shown to induce increases in intracellular calcium and contraction in cerebral arteries less than 50 μm in diameter [46]. Considering the PAs utilized in the current study were of similar size, thrombin's modest

vasoconstrictive effects may occur through the activation of the PARs of the smooth muscle in cerebrovasculature.

3.6 Conclusion

The hypercoagulable state induced by pregnancy, and super-hypercoagulable state of PE, drives both protection against bleeding and the risk of cardiovascular events that can be life-threatening for pregnant women. Rising maternal morbidity and mortality, in large part by maternal stroke, presents a clear need to focus on the reactivity of cerebrovasculature to conditions induced during pregnancy and PE. It is well established that PE causes widespread maternal vascular dysfunction, and it has been previously shown that ePE adversely affects PA structure and function [13, 14, 16]. The current study found that thrombin and prothrombin were elevated in plasma in ePE rats compared to NP and LP rats. Thrombin was found in the CSF of rats of all groups and was significantly greater in LP CSF. Additionally, COX and NOS inhibition caused greater constriction in PAs from healthy pregnant rats than in NP, but that increase in constriction was not seen in ePE. Finally, the findings in the current study indicate that thrombin is modestly vasoconstrictive in the cerebrovasculature of rats during pregnancy and ePE.

In combination with ePE-driven vascular dysfunction, thrombin-induced vasoconstriction in PAs could increase vascular resistance, which may lead to decreased cerebral blood flow to the frontoparietal WM tracts of the sensorimotor cortex and basal ganglia. Increased vascular resistance can lead to greater shear stress on vascular walls and endothelial damage, further contributing to the endothelial dysfunction in PE [5]. In the event of maternal stroke, hypoperfusion to structures so critical for cognitive function could exacerbate ischemia and hypoxia; and ultimately magnify neuronal injury, worsening stroke outcome. However, the extent to which thrombin-induced vasoconstriction

contributes to worsened stroke outcome during pregnancy compared to other driving factors remains to be investigated. The way in which thrombin contributes to stroke risk is likely related to its procoagulant potential in addition to its vasoactive influences, and the relative contribution of these factors is likely difficult to isolate. Understanding the effects of thrombin on the cerebrovascular during pregnancy and PE may allow for development of more precise stroke risk assessments and inform recommendations to help protect pregnant women in the short- and long-term.

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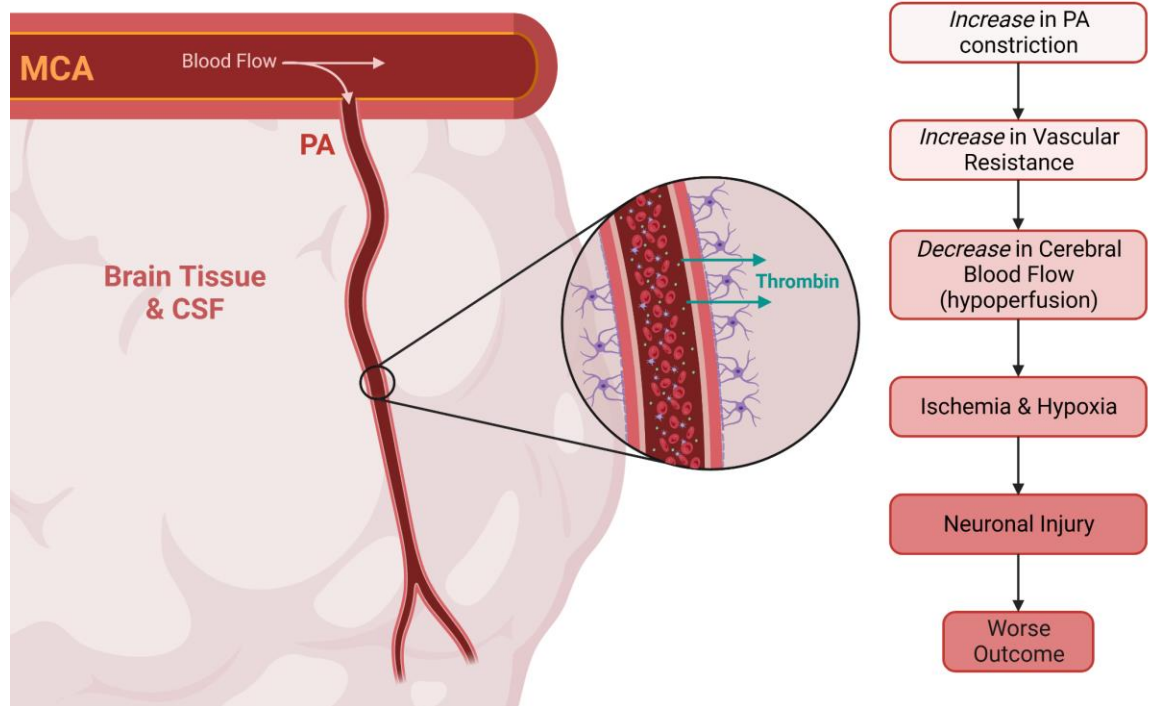
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**CHAPTER 4: DISCUSSION, FUTURE WORK, AND
CONCLUSION**

4.1 Discussion, Future Work, and Conclusions

Stroke in pregnancy is a leading cause of maternal morbidity and mortality throughout the world [1, 2]. The upregulation of coagulation factors, such as thrombin, and overall hypercoagulable state associated with pregnancy are key contributors to the 3-fold increase in stroke risk for pregnant women and subsequent 6-fold increased stroke risk associated with preeclamptic pregnancies [2]. The results of the current study suggest that thrombin, a procoagulation factor known to increase during pregnancy and PE, is a modest vasoconstrictor in intraparenchymal arterioles in the brains of rats, likely by activation of PARs in vessel wall smooth muscle cells, and that the vasoconstrictive effects persist in pregnancy and PE [3]. Thrombin's vasoconstrictive effects may lead to decreased lumen diameter of the penetrating arterioles, which could increase vascular resistance. Importantly, increased vascular resistance could lead to decreased cerebral blood flow, or hypoperfusion, in the sensorimotor cortex and frontoparietal white matter tracts that the PAs perfuse. Not only is proper perfusion crucial in physiological conditions, but in the event of stroke in the MCA region, hypoperfusion through the PAs that may be providing collateral flow around the stroke could increase ischemia and hypoxia in that peri-infarct region. Increased ischemia and hypoxia would then contribute to greater neuronal injury and ultimately worsen stroke outcome (Figure 4.1). It is important to note that the way in which thrombin contributes to stroke risk is likely related to its vasoactive influences in addition to its prothrombotic potential and that the relative contributions of these factors is likely difficult to ascertain.

Figure 4.1: Graphical illustration of proposed mechanism by which thrombin may contribute to maternal stroke outcome.



Furthermore, thrombin's modest vasoconstrictive effects likely occur through the activation of the PARs of the smooth muscle in cerebrovasculature. WB analysis showed that protein levels of native PAR-1 were similar in NP, LP, and ePE PAs yet protein levels of what is thought to be glycosylated PAR-1 is increased in LP PAs as compared to NP. Additionally, ePE PAs did not express that same increase in glycosylated PAR-1. These results suggest that PAR-1 levels or PAR-1 glycosylation may increase during pregnancy and could have a physiological, and potentially protective, effect that is disrupted by ePE. PAR-1 glycosylation is thought to be important for cell surface expression of the receptor

[3], which could indicate that increased PAR-1 cell surface expression in cerebrovasculature presents a physiologically protective mechanism in pregnancy, likely by way of the intracellular cascades that activated PAR-1 interacts with. PAR signaling is known to activate a variety of intracellular signaling pathways by interacting with heterotrimeric G-proteins, namely G_q and G_i [4, 5]. Further investigation is necessary to determine what signaling pathways are specifically associated with PAR activation in cerebrovasculature during pregnancy. Where PAR expression has been reported in various regions of human and rat brains such as the hippocampus and striatum, PARs may also be expressed in the vasculature of those regions [6, 7]. Comparative IHC imaging and WB analysis should be performed to investigate PAR expression in other cerebrovascular regions like hippocampal arterioles [6, 7]. Specifically, coronal brain sections could be stained to investigate colocalization of PARs within cerebrovasculature beds, as a limitation of the current study was not having proper sample size to quantitatively colocalize PAR-1 and -2 in the smooth muscle cells of PAs.

In the current study, indomethacin and L-NAME were administered to the isolated and pressurized PAs to inhibit COX and NOS. Inhibiting those pathways isolated thrombin's vasoactive effects to the smooth muscle cells of the vessel walls. However, it cannot be definitively concluded based on the results of the current study that thrombin-induced vasoconstriction occurs strictly through activation of PARs. Takahashi et al. studied endothelium-denuded retinal arterioles to investigate thrombin reactive in smooth muscle cells and found that intraluminal administration of thrombin elicited similar vasoconstriction to extraluminal administered thrombin [8]. Additionally, that study

investigated thrombin reactivity for the retinal arterioles in the presence of pharmacological inhibitors of PARs and protein kinase C (PKC) and found that blocking PARs and PKC reduced thrombin-induced vasoconstriction [8]. These techniques could be applied to an isolated vessel study of cerebral arterioles to further elucidate the mechanisms by which thrombin is vasoactive in the brain and if thrombin's vasoactive are to PAR-activated in PAs. If thrombin-induced constriction persists with PAR-inhibition, then thrombin may interact with other cellular mechanisms in cerebrovasculature. Furthermore, thrombin-induced constriction could be compared to the percent constriction caused by a pharmacological PAR-1 activating peptide and intracellular calcium levels could be immunohistochemically monitored in real-time using confocal imaging.

The current study showed that thrombin and prothrombin were significantly elevated in the plasma from ePE rats compared to NP and LP plasma. This study also found thrombin to be present in the CSF of NP, LP, and ePE rats. In contrast the plasma thrombin levels, thrombin levels in CSF were significantly elevated in LP CSF rather than in ePE. While increased plasma levels aligned with our hypotheses, the presence of in CSF at the recorded posed additional questions. CSF thrombin levels were notably greater in all groups compared to the corresponding thrombin plasma levels. As shown in Table 4.1 and Figure 4.2, CSF thrombin were orders of magnitude greater than plasma thrombin levels. Additional investigation and techniques may be necessary to better compare plasma and CSF thrombin concentrations. When investigating serum and plasma thrombin concentrations as biomarkers of Alzheimer's disease development, Malecka and Ferapontova 2021 utilized a direct electrocatalysis based on oxygen reduction reactions

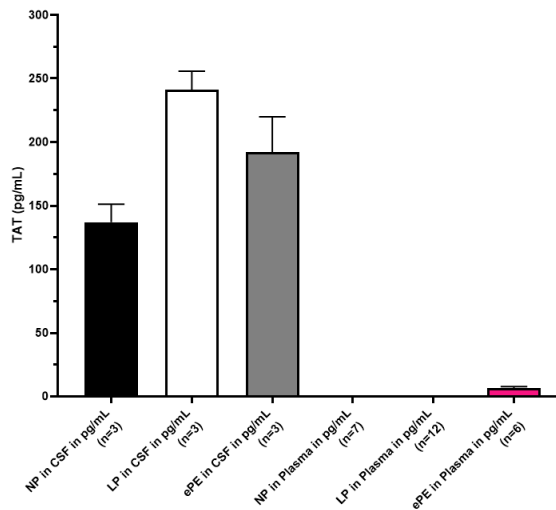
and DNAzyme labeling to detect femtomolar levels of thrombin [9]. Techniques such as this may provide more sensitive and selective methods to determine thrombin in rat CSF with lower sample volumes thus avoiding the need to pool samples to fulfil the required volumes of ELISA kits, which was a limitation of the current study. Where CSF thrombin levels were increased in LP rats, there may be a physiological function for thrombin' vasoactive in intraparenchymal vessels during pregnancy; potentially in relation to the increased levels of glycosylated PAR-1 seen in the LP group. Thrombin can serve a neuro-physiological function, such as supporting neuronal development, cellular proliferation and differentiation or other thrombin-induced cellular functions that may be an advantageous, and potentially protective, change in the brain during pregnancy or may be a result of the unique cerebrovascular adaptations to pregnancy [10]. These results not only suggest that thrombin is expressed or produced by cerebral cells in rats, but that the expression is up-regulated in healthy pregnancy but not in a pathological state of pregnancy. Ultimately, the results of this study highlight that thrombin plays not only a coagulatory but a vasoactive role in cerebrovasculature during pregnancy and PE, and may contribute to worsened maternal stroke outcome.

Table 4.1:

Thrombin-antithrombin (TAT) levels (average \pm SEM) in plasma and CSF from NP, LP, and ePE rats in pg/mL obtained by ELISAs.

	TAT in Plasma (pg/mL)	TAT in CSF (pg/mL)
NP average	0.486 \pm 0.011	137 \pm 14.224
LP average	0.557 \pm 0.011	241 \pm 14.844
ePE average	6.598 \pm 1.248	192 \pm 28.000

Figure 4.2: *Thrombin-antithrombin (TAT) levels (average \pm SEM) in plasma and CSF from NP, LP, and ePE rats in pg/mL obtained by ELISAs.*



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APPENDIX A: SUPPLEMENTAL MATERIAL

Figure 5.1: Myogenic reactivity of PAs. (A) Pressure-diameter curves of PAs from nonpregnant (NP), late pregnant (LP), and experimental preeclamptic (ePE) rats and (B) percent tone across the pressure range 20 – 120 mmHg.

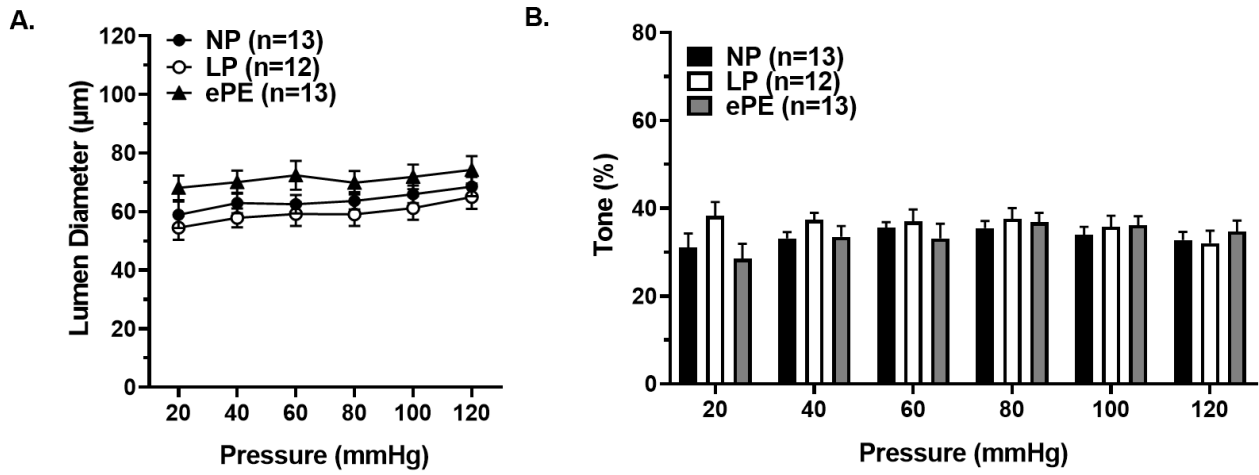


Figure 5.2: Myogenic reactivity of PAs before and after thrombin. (A) Pressure-diameter curves of PAs from nonpregnant (NP), late pregnant (LP), and experimental preeclamptic (ePE) rats; and (B) pressure-diameter curves of PAs from NP, LP, and ePE rats when bathed in 50 mU/mL thrombin. (C) Percent tone across the pressure range 20 – 120 mmHg; (D) percent tone across the pressure range 20 – 120 mmHg of vessels bathed in 50 mU/mL thrombin.

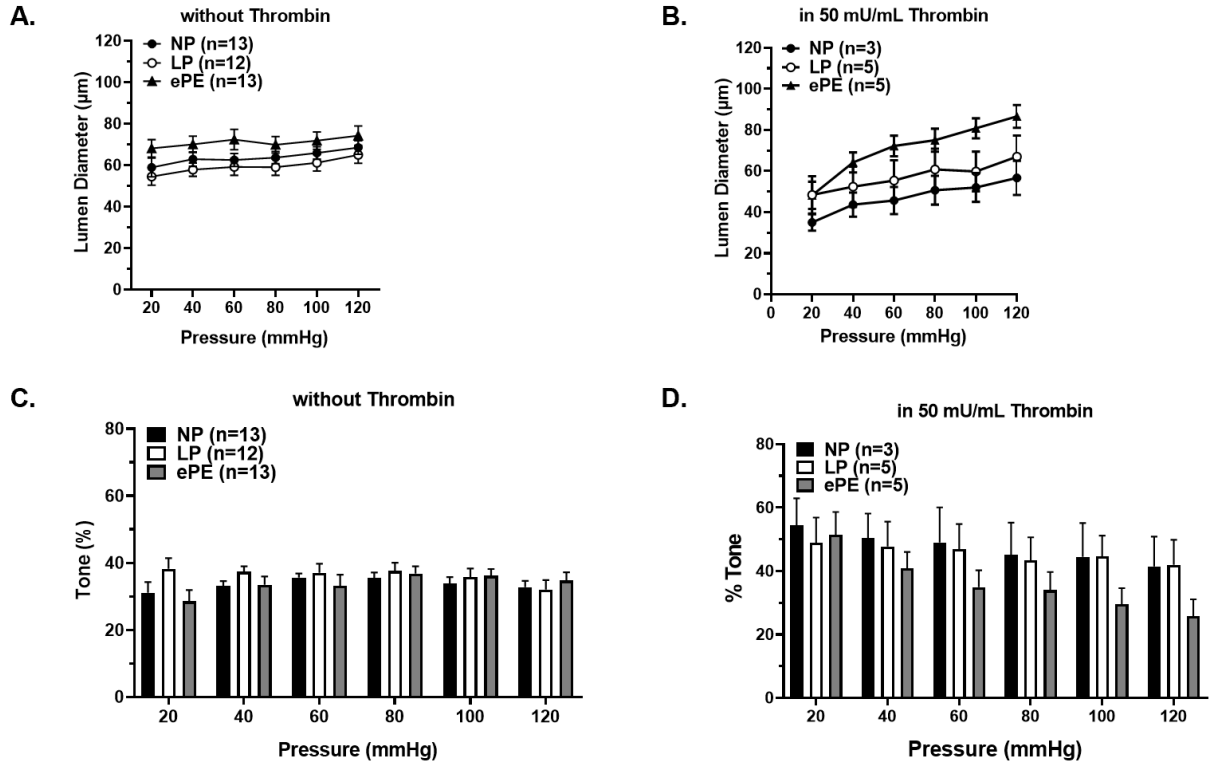


Figure 5.3: The role of small- and intermediate-conductance calcium-activated potassium channels in vasodilation. (A) Lumen diameters of PAs from nonpregnant (NP), late pregnant (LP), and experimental preeclamptic (ePE) rats and (B) percent reactivity to SK/IK channel activation.

