NOVEL MODALITIES FOR PREECLAMPSIA PREVENTION: A ROLE FOR EXERCISE TRAINING AND 5-AMINOIMIDAZOLE-4-CARBOXAMIDE-1-β-D-RIBOFURANOSIDE (AICAR) ADMINISTRATION

by

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DISSERTATION ABSTRACT

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Title: Novel Modalities for Preeclampsia Prevention: A Role for Exercise Training and 5-Aminoimidazole-4-Carboxamide-1-β-D-Ribofuranoside (AICAR) Administration

Preeclampsia (PE) remains one of the most enigmatic and pervasive conditions developed during pregnancy and is a leading cause of maternal and fetal morbidity and mortality throughout the world. Afflicting nearly 5-8% of pregnancies in the Unites States, PE is most commonly characterized by an increase in blood pressure and high protein excretion near or after the 20th week of gestation. Unfortunately, few effective treatments are available, and the only "cure" is delivery. While the molecular pathogenesis of PE remains undefined, an interruption in placental blood flow, or placental ischemia, is widely observed as a primary contributor to the syndrome progression. Furthermore, to investigate the role of both pharmacological and non-pharmacological modalities to prevent placental ischemia induced hypertension, we employed a robust model of reduced utero-placental perfusion pressure (RUPP) in the pregnant rat.

First, in Chapter IV, exercise initiated during gestation was not effective in the prevention of RUPP-induced hypertension, whereas exercise training prior to and continued through gestation prevented the increase in blood pressure. Though the molecular contributions to this effect are undefined, the effects appear to be independent of angiogenic balance restoration.

Finally, in Chapter V, administration of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was explored as a novel pharmacological modality to prevent the onset of hypertension and endothelial dysfunction in the RUPP model. As hypothesized, AICAR ameliorated the RUPP-induced hypertension, and the anti-hypertensive effect in the RUPP appears to be dependent on the restoration of angiogenic balance in the maternal plasma.

This dissertation includes previously unpublished and published co-authored material.

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Bauer, A.J., Banek, C.T., Needham, K.M., Regal, J. & Gilbert, J.S. (2013). Pravastatin attenuates hypertension, oxidative stress and angiogenic imbalance in the reduced utero-placental perfusion pressure hypertensive rat. *Hypertension*, 61(5):1103-10.

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Gilbert, J.S, Banek, C.T., Bauer, A.J., Gingery, A. & Needham, K.M. (2012). Exercise training attenuates placental ischemia induced hypertension and angiogenic imbalance in the rat. *Hypertension*, 60(6):1545-51.

Gilbert, J.S., Banek, C.T., Katz, V., Babcock, S.A. & Regal, J.F. (2012). Complement activation in pregnancy: Too much of a good thing? *Hypertension*, 60(5):1114-6.

Banek, C.T., Bauer, A.J., Gingery, A. & Gilbert, J.S. (2012). Timing of ischemic insult alters fetal growth trajectory, maternal angiogenic balance and markers of renal oxidative stress in the pregnant rat. *American Journal of Physiology: Regulatory, Integrative, and Comparative Physiology*, 303(6):R658-64.

Gilbert, J.S, Banek, C.T., Bauer, A.J., Gingery, A. & Dreyer, H.C. (2012). Placental and vascular adaptations to exercise training before and during pregnancy in the rat. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* 303(5):R520-6.

Gilbert, J.S., Bauer, A.J., Gingery, A., Banek, C.T. & Chasson, S. (2012). Circulating and utero-placental adaptations to chronic placental ischemia in the rat. *Placenta*, 33(2): 100-5.

Gilbert, J.S., Bauer, A.J., Gilbert, S.A.B. & Banek, C.T. (2012). The opposing roles of anti-angiogenic factors in cancer and preeclampsia. *Frontiers in Bioscience*, 4: 2752-69.

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Gilbert, J.S. & Banek, C.T. (2011). Sex differences in the developmental programming of adult disease. Sex Hormones ISBN 978-953-307-856-4. Editor: R. K. Dubey. InTech. Print and Online.

Yoshimura, A., Banek, C.T., Yusubov, M.S., Nemykin, V.N. & Zhdankin, V.V. (2011) Preparation, X-ray structure, and reactivity of 2-Iodylpyridines: recyclable hypervalent iodine(V) reagents. *Journal of Organic Chemistry*, 20;76(10):3812-9.

Zagulyaeva, A.A., Banek, C.T., Yusubov, M.S. & Zhdankin, V.V. (2010). Hofmann Rearrangement of Carboxamides Mediated by Hypervalent Iodine Species Generated in situ from Iodobenzene and Oxone: Reaction Scope and Limitations. *Organic Letters,* 12(20): 4644-7.

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For my mother and father.

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CHAPTER I

INTRODUCTION

"A headache accompanied by heaviness and convulsions during pregnancy is considered bad" - Hippocrates, 400 B.C. [1]

HISTORICAL PERSPECTIVE

Near the beginning of my graduate career, I recall a striking point shared in conversation with a prominent obstetrician, Dr. James Martin, in which he emphasized that preeclampsia (PE) has been observed and documented for several millennia; yet, we have only really begun to understand cause of the symptoms within the last 30-40 years. This remark, cemented in my mind, was equally as exciting as it was discouraging for a young, bright-eyed scientist. To elaborate on Dr. Martin's point, preeclampsia (PE) remains one of the most enigmatic and feared conditions in maternal and fetal medicine to this day, despite over 4000 years of documented research and observation [2]. With the accepted nomenclature, etiology, and diagnostic criteria changing several times over the course of several centuries, treatment and prevention of PE has been unfortunately limited.

Much of the remaining mystery of PE has stemmed from its predecessor, eclampsia, in which violent convulsions and pitting edema have been observed throughout world medical history, particularly by the Indian, Greek, Egyptian,

and Chinese historians, as early as 2000 B.C. [2-4]. First described by François Boissier de Sauvages de Lacroix in the 18th century, the term "eclampsia" is derived from the Greek word *eklampsis*, which roughly translates "to shine forth" [1,2]. It is unclear why this term was used to describe eclamptic women, but the late Dr. Leon Chesley has noted in his elaborate historical reviews [2,3] the term was also used to describe non-pregnant epileptic subjects, which points to its focus on suddenness of tonic-clonic seizures shared between the two maladies. Interestingly, Chesley also reported that the Greeks were the first to recognize the harbingers of eclamptic presentation, in which the women would report of visual or cerebral disturbances such as headaches, blurred vision, and general malaise prior to the presentation of the eclamptic convulsions later in their pregnancies [2]. Not until the mid-late 19th century, was eclampsia recognized as "high and strong pulse" disorder [5] and often accompanied by high protein content in the urine [6,7]. Following the invention and clinical integration of the sphygmomanometer [8], Vaquez and Nobelcourt are credited with the first report of high blood pressure in eclampsia (and the undefined preeclampsia) [3]. This may be the most-defining moment in preeclampsia-eclampsia research, as it gave physicians a measurement to predict fits, headaches, and convulsions later in pregnancy – birthing the now well-recognized term, pre-eclampsia [2,3].

Since the use of hypertension and proteinuria to define the PE development, the next substantial challenge in the research was to understand the etiology to develop possible modalities for treatment or prevention. Unfortunately, we as researchers have yet to define the exact etiology of PE, which remains a heavily researched topic even to this day and has even earned the moniker of "the disease of many theories" [9,10].

BACKGROUND AND SIGNIFICANCE

While the exact molecular and physiological mechanism of PE development remains unclear, placental hypoperfusion is widely regarded to be the initiating event in the increase in blood pressure and renal dysfunction in preeclampsia [11-13]. This placental-origin hypothesis was based primarily on the clinical observation of symptom remittance following the delivery of the placenta, as fetal delivery alone is not sufficient [9,10,14]. Further, the hypoperfusion, or ischemia, is understood to be caused by a failure of the placental-derived trophoblast cells to invade the spiral arteries of the uterus, and tapping into the maternal arterial blood supply during placentation [15-18]. The concomitant cascade of placental-derived signaling in response to ischemia was previously recognized and championed by Jim Roberts and colleagues [11], and studies performed by our laboratory [19] and others [20] since then have elegantly shown under ischemic conditions, the placenta secretes anti-angiogenic factors, most notably soluble

VEGF receptor-1 (sFlt-1) [21-23]. Karumanchi and colleagues were the first to elucidate the role of sFlt-1 in the ontology of preeclampsia in both an experimental and clinical setting [20], and has since then been one of the most widely studied signaling factors in PE research. Maynard first proposed an increase in sFlt-1 results in a neutralization of circulating vascular endothelial growth factor (VEGF) in the murine model or placental growth factor (PlGF) in the human, which results in a decreased pro-angiogenic factor bioavailability. This reduction in pro-angiogenic factor bioavailability is often referred to as angiogenic imbalance [21,24]. In turn, this imbalance leads to decreased vascular endothelial function and health, systemic oxidative stress, renal dysfunction, and ultimately, increased blood pressure [21-23].

To model the development of PE through placental ischemia, many laboratories use the reduced uterine perfusion pressure (RUPP) model (**Chapter III**, **Figure 3.1**), a well-characterized and robust model of the placental ischemia induced hypertension and model of PE [25]. Moreover, recent studies [20,26-29] from our laboratory and others have suggested restoring the angiogenic balance by increasing pro-angiogenic bioavailability and/or decreasing anti-angiogenic factor levels as possible target to mitigate the development of the hypertension in PE through. Despite the strong evidence marking the angiogenic imbalance as a target for preeclamptic symptom treatment or prevention, there remains a gap in

the experimental knowledge and clinical practice which further justifies the specific aims proposed.

Furthermore, recent clinical and experimental studies have reported intriguing effects of administering a pharmacological "mimetic of exercise" [30] in a pregnant or non-pregnant diabetic state complicated by a primary/secondary hypertension [31-36], but these studies have primarily focused on the improved glucose handling and not the favorable cardiovascular or renal effects in the hypertensive subjects. As the observed effects of these compounds, such as AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) and other adenosine monophosphate activated protein kinase (AMPK)-activators, are poorly understood [37]. Furthermore, there is a clear need to determine the mechanism and effectiveness of AICAR administration in experimental models of PE.

STATEMENT OF PROBLEM

Currently, there is a dearth of knowledge in the mechanisms by which exercise training prior to and during gestation mitigates the development of preeclampsia. Additionally, there is also a substantial gap in the scientific literature regarding the mechanisms by which pharmaceutical modalities which mimic components of exercise may be beneficial to women during pregnancies complicated by placental ischemia and hypertension. Therefore, the proposed

studies will allow the following: 1) increase the understanding of the molecular and physiological basis of exercise training during normal and hypertensive pregnancies in an effort to develop safe and efficacious treatments and preventative modalities; 2) elucidate AMPK as a potential molecular pathway by which exercise training prevents the onset of preeclamptic hypertension 3) improve the understanding of the clinical application of AICAR, an AMPK-activating compound, for treatment of hypertensive disorders during pregnancy by employing a unique and robust animal model which closely mimics the human syndrome; 4) extend the observations of exercise and AICAR administration in pregnancy to a translational application.

PURPOSE AND HYPOTHESES

The purpose of this dissertation work is to elucidate the potential for exercise training and "exercise mimicking" pharmaceuticals in the treatment and/or prevention of preeclampsia using a robust experimental animal model of the syndrome.

Specific Aim 1

The role of exercise training as a preventative modality for preeclampsia, the molecular contributions and the role in vascular and placental physiology is left unclear. It has previously been established that the bioavailability of vascular endothelial growth factor (VEGF) is crucial for the maintenance of maternal

blood pressure and vascular function in pregnancy, and in preeclampsia, circulating free VEGF is decreased. Moreover, an intriguing characteristic of exercise training is the increase of VEGF production, and these levels are even further increased in circulation in pregnant women and rats under training protocols. Therefore, Aim 1 (see **Chapter IV**) is designed to test the hypothesis that exercises training: (1) prevents the decrease in VEGF bioavailability the onset of placental ischemia induced hypertension and endothelial dysfunction in the rat RUPP model; and (2) the effects are mediated through the restoration of angiogenic balance and bioavailability of VEGF.

Parts 1-2 of Chapter IV are in preparation for publication with Haley E. Gillham, Karen W. Needham, and Jeffrey S. Gilbert as co-authors. I performed the experimental work with the technical assistance of co-authors H.E. Gillham and K.W. Needham. J.S. Gilbert provided editorial assistance and research funding.

Specific Aim 2

As exercise is recognized to combat and prevent hypertension in gestation, the molecular contributions to explain this effect remain undefined. One potential pathway that governs much of cellular energy production and usage is AMP-activated protein kinase (AMPK). Many studies using AMPK-agonists such as Metformin and AICAR in a model of primary or secondary

hypertension suggest an anti-hypertensive effect and improvement of vascular function. Moreover, AMPK and its downstream signaling targets have been reported by others to potentially mediate the production of VEGF following exercise training, but the role of AMPK in pregnancy-induced VEGF production and tissue specific stress signaling remains uncertain. Therefore, Aim 2 (see **Chapter V**) is designed to test the hypothesis that the administration of the potent AMPK-activating compound AICAR: (1) will improve VEGF bioavailability and prevent an increase in arterial pressure and vascular dysfunction in the RUPP model; (2) the anti-hypertensive effects will be dependent on the restoration of angiogenic balance and VEGF bioavailability; and (3) directly improve placental and vascular endothelial health and function through prevention of cellular stress signaling.

In Chapter V, a portion of the data in Part 1 was published in 2012 in the *American Journal of Physiology - Heart and Circulation*, with Ashley J. Bauer, Karen W. Needham, Hans C. Dreyer, and Jeffrey S. Gilbert as co-authors. I performed the experimental work along with the technical assistance of A.J. Bauer and K.W. Needham. H.C. Dreyer and J.S. Gilbert provided editorial assistance. J.S. Gilbert provided the research funding.

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CHAPTER II

LITERATURE REVIEW

The following review of literature is focused on areas germane to the research topic of preeclampsia. After addressing the normal cardiovascular physiology, the introduction to preeclampsia pathophysiology will be presented, along with a leading hypothesis of its ontology. Further, current treatments and preventative measures will be presented to lay the foundation for the remainder of the dissertation data chapters (**Chapters IV** and **V**), in which pharmacological and non-pharmacological modalities will be addressed. Finally, the dearth of knowledge in regard to treatments and prevention of preeclampsia will be discussed, establishing the need for the current research presented, as well as future investigation.

HYPERTENSION IN PREGNANCY

To begin this review, it is essential to establish the definition of hypertension, or high (arterial) blood pressure. First and foremost, hypertension is clinically defined by the repeated measurement of 140mmHg systolic and/or 90mmHg diastolic [38]. In general, hypertension is recognized to be a major contributor and predictor of cardiovascular disease related morbidity and mortality, which include chronic heart failure, acute myocardial infarction, stroke, and renal failure [38]. Additionally, hypertension is often asymptomatic and

difficult to diagnose without regular measurement, which is the reason it has earned the moniker of the "silent killer" [39].

Unfortunately, hypertension during pregnancy is one of the leading complications of pregnancy, which affects nearly 250,000 women, or up to 10% of pregnancies in the USA, annually [40]. Moreover, the timing of the onset of hypertension in pregnancy is important to the clinical diagnoses and further treatment. Four major forms of hypertension presentation during pregnancy are currently recognized by the National Institute of Health (NIH) Heart, Lung, and Blood Institute (NHLBI) [40]: Chronic hypertension (Preexisting blood pressure of >140/90mmHg); Gestational Hypertension (*De novo* hypertension in pregnancy, no proteinuria); Preeclampsia (New-onset hypertension ≥20 weeks, high protein excretion: ≥300mg/24hr); Preeclampsia superimposed on preexisting hypertension (arterial pressure before gestation >140/90mmHg, *de novo* proteinuria).

Of these four, preeclampsia is often credited as the major clinical concern, as this is often described as the most costly in terms of both health and finance. Strikingly, it is estimated that the collective financial burden produced by preeclampsia and related hypertensive disorders in pregnancy accounts for nearly $10 billion annually in the United States alone, according to the Preeclampsia Foundation (USA). Unfortunately, the diagnostic criteria,

pathogenesis, and treatment recommendations for preeclampsia remain unknown or controversial [41], which warrants further investigation into this debilitating and costly syndrome.

PATHOPHYSIOLOGY OF PREECLAMPSIA

As described briefly above, preeclampsia (PE) is a leading contributor to maternal and fetal morbidity and mortality in both the United States and worldwide [40,42]. PE has long been diagnosed at presentation of hypertension (≥140/90mmHg) and proteinuria (≥300mg/24hrs) at ≥20th week of gestation, but diagnosis criteria has recently changed to include markers of thrombocytopenia, impaired renal and/or liver function, pulmonary edema, and cerebral/visual disturbances as secondary confirmation measurements in the absence of proteinuria [41]. While the past several decades have seen a rise of nearly 40% in the incidence of preeclampsia, this is largely thought to be due to increases in the number of higher order pregnancies (multiple births), and the age at onset of pregnancy and rate of obesity [40,41]. Treatment is currently limited to supportive care (e.g. bed rest), and induction of delivery if symptoms progress to life-threatening conditions, after which symptoms typically resolve within 48–72 hours [41,43,44].

Despite the tremendous recent efforts to understand the pathophysiology of preeclampsia, the current molecular mechanisms behind PE etiology remain

unsettled. Though the molecular pathophysiology still requires further refinement, PE is widely acknowledged to initiate from poor placental perfusion (i.e. ischemia) and insufficiency, which is derived primarily from an interruption in the normal remodeling of the spiral arteries of the uterine endometrium [45-48]. Further, the resulting placental ischemia leads to a myriad of placental-derived signaling cascades which include circulating angiogenic factor imbalance, innate and adaptive immune activation, and increased sympathetic vasomotor activity, which jointly result in an increase in mean arterial pressure [49-51]. Furthermore, while it is important to acknowledge and emphasize the significance of all contributing pathways in PE pathogenesis, this literature review and the remainder of this dissertation will focus on the maintenance and restoration of angiogenic balance through both pharmacological and non-pharmacological means in PE symptom prevention.

PLACENTAL ISCHEMIA, ANGIOGENIC IMBALANCE, AND PREECLAMPSIA

Although the molecular pathophysiology of preeclampsia remains undefined, poor placental perfusion, or placental ischemia, is widely regarded as a key initiator of the syndrome [11,18,52]. The finite mechanisms by which placental ischemia is developed remain undefined [53], but inadequate cytotrophoblast invasion of the uterine spiral arteries is considered to be a primary cause of

placental hypoperfusion throughout gestation [52].

Specifically, in normal pregnancy, the spiral arteries of the maternal myometrium lining of the uterus remain tortuous and have a high resistance to blood flow in non-pregnant and early pregnancy stages. As the placenta progressively develops and nutrient supply is no longer sufficient, placental-derived cytotrophoblast cells migrate and begin to invade the spiral arteries, replacing the endothelium and smooth muscle cells. This results in an increased capacitance for blood flow and a loss of constrictive properties to circulating vasoconstrictors for the remainder of gestation [15-17]. Moreover, an interruption in this process, possibly mediated through innate and adaptive immune activation [54-57], allows the spiral arteries to maintain high resistance to arterial flow and thus resulting in a decrease in placental perfusion. For more elaborate discussion of spiral artery remodeling in normal pregnancy and PE, the reader is directed to the thorough review and discussion by Zhou and colleagues [53]. Furthermore, though the mechanism of spiral artery reformation is vital to the study of preeclampsia, our laboratory is focused on the signaling following the onset of placental ischemia and the production of placental-derived factors in response to this stress.

Since James Roberts and colleagues [12] first suggested a role of "soluble agents" produced by an ischemic placenta, there have been an extraordinary

number of studies to elucidate potential molecular contributions to the endothelial dysfunction and hypertension in PE. Following, a myriad of "soluble agents" have since been isolated and implicated in the development of PE hypertension, which include angiogenic factors, various cytokines and adipokines such as TNFα and interleukins, adaptive and innate immune system activation, and production of agonistic auto-antibodies such as the angiotensin II type 1 receptor-auto antibody (AT1-AA) [12,20,52,58,59].

Of the panoply of placental-derived signaling factors studied, few have received as much attention as the anti-angiogenic soluble splice variant of Flt-1 (i.e. VEGF receptor-1). This soluble variant of Flt-1, or sFlt-1, contains the extracellular ligand-binding domain of full length Flt-1 and acts to antagonize the normal signaling of the pro-angiogenic ligands vascular endothelial growth factor (VEGF) and placental growth factor (PlGF). Under ischemic conditions, the placenta increases its secretion of sFlt-1 into the maternal circulation [60], which in turn decreases the availability of free VEGF and PlGF. This increase in angiogenic factor antagonism, or decrease in active or available pro-angiogenic factor production, has been termed as "angiogenic imbalance" by Karumanchi and colleagues [24], and will be referred to as such through the remainder of this dissertation.

Moreover, Karumanchi and colleagues were the first to propose the critical role of sFlt-1 in the development of PE, where they observed an increased serum sFlt-1 in preeclamptic patients [20]. Additionally, adenoviral-mediated overproduction of sFlt-1 directly induced hypertension and endothelial dysfunction in a pregnancy murine model [20]. Several studies following this report [23,24,61-69] strongly support the hypothesis that the ischemic placenta contributes to endothelial cell dysfunction in the maternal vasculature by inducing an alteration in the balance of circulating levels of angiogenic/anti-angiogenic factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), and sFlt-1. Additionally, replacement of bioavailable VEGF through exogenous infusion in several rodent models of PE has also been shown to mitigate the development of endothelial dysfunction and hypertension [28,70,71]. Further, Banek and colleagues have recently reported an earlier induction of placental ischemia (day 12 vs. day 14 of gestation) results in a hypertension without the presence of angiogenic imbalance, which is suggestive of a critical role in timing and relative ischemia in the secretion of sFlt-1 and the concomitant angiogenic imbalance. Although recent data suggest circulating sFlt-1 concentrations may presage the clinical onset of preeclamptic symptoms [23,67,72,73], several studies indicate that placental hypoxia and poor placental perfusion may initiate this imbalance of angiogenic factors [60,74]. Therefore, it

remains unclear whether impaired placental perfusion initiates preeclamptic symptoms such as hypertension, endothelial dysfunction and/or increased sFlt-1, or if inadequate placental development occurs initially and is followed by a pathological rise in sFlt-1 expression and secretion [68].

Collectively, these reports have promoted further research in the treatment, predication and prevention of PE through the maintenance and restoration of angiogenic balance. Of the several published exogenous methods to treat angiogenic imbalance in PE, the current focus this dissertation is specifically on the use of voluntary exercise [27,75], a widely-recognized stimulant of VEGF production [76-78].

NITRIC OXIDE AND OXIDATIVE STRESS IN NORMAL PREGNANCY AND PREECLAMPSIA

Recent reports have indicated NO production is elevated in pregnancy as compared to non-pregnant controls, and this increase is required for normal arterial and renal vasodilation to compensate for normal blood volume expansion [79]. Studies from several groups indicate NOS inhibition in pregnant rats results in an increase in arterial pressure as well as an increase in vascular resistance, proteinuria, intrauterine growth restriction, and increased fetal morbidity [80-82]. Further, as vascular function and maintenance through VEGF signaling is crucial to the acute and chronic production NO [83-86], disruption of

normal VEGF signaling results in a decrease in endothelial function and increase in arterial pressure [87,88].

Recent data from experimental and clinical investigations suggest there is an intimate relationship between NO availability and oxidative stress in maintenance of vascular function and blood pressure [89,90]. Briefly, oxidative stress is a process in which the defense mechanisms of a tissue are overcome by an increasing production of reactive oxygen species (ROS). In regard to vascular function, during oxidative stress, an imbalance of pro- and anti-oxidant factors results in damage of the endothelial tissue cells [91]. Oxidative stress may mediate endothelial cell dysfunction and contribute to the pathophysiology of preeclampsia based on evidence of increased pro-oxidant activity along with decreased anti-oxidant protection.

Regular exercise training in healthy and hypertensive individuals has been shown to improve endothelial function via improvement of NOS expression and NO production [92-94]. Interestingly, Ramirez-Velez and colleagues have recently reported an improvement of eNOS expression and NO production in placentas from pregnant women who exercised throughout pregnancy [95]. Further, this suggests a potential mechanism by which exercise training could ameliorate systemic and placental-specific vascular dysfunction.

While NO production is often referred to as a beneficial effect, it is important to acknowledge the duality of the secondary effects of accumulated ROS, specifically superoxide anion O_2^-, can combine with NO to form the strong oxidative and nitrosylating product, peroxynitrite ($ONOO^-$) [96]. This process decreases the bioavailability of NO, and causes a further, and cascading, oxidative burst. Unsurprisingly, there is increased oxidative stress and peroxynitrate formation in the hypertensive pregnant RUPP rat [97-99] and preeclamptic pregnancies [96], which can be resolved by the administration of a superoxide anion scavenger, Tempol [97,99]. However, it should be noted the administration of an antioxidant cocktail or Vitamin C/E supplementation during pregnancy is not only ineffective in preventing PE in the clinical setting, but may increase the risk of fetal growth restriction or small for gestational age [100,101].

Additionally, a single bout of exercise in an exercise-naïve subject results in an increase in oxidative stress [102,103]. However, in repeated exercise training, an overall anti-oxidative shift is observed, likely through the accretion of a robust buffer of antioxidants and oxidizing agents [102]. Further, this anti-oxidative training effect from exercise training is also observed in pregnancy, which has been suggested to contribute the decreased incidence of preeclampsia in exercise-trained women [104-106]. Moreover, we have recently reported exercise training before and during gestation ameliorates markers of oxidative stress in the

placenta and kidney in both normal pregnant and RUPP rats [27]. Interestingly, examination of cytosolic and mitochondrial isolations in placentas of women who exercised during pregnancy show a reduction in oxidative stress, measured by O_2^- and H_2O_2 production [95]. This suggests moderate-mild exercise in pregnancy directly effects placental (anti)oxidant production and regulation. While regular exercise training prior to and during pregnancy has been shown to improve the anti-oxidative potential in experimental and clinical studies, it remains unclear if it is a primary or secondary mediator of increased blood pressure in preeclamptic subjects.

EXERCISE FOR PREECLAMPSIA PREVENTION

Mild to moderate exercise training in the non-pregnant state is widely recognized to combat long-term high blood pressure, and also decrease the incidence of hypertension [107-109]. As discussed in Chapters I and IV, similar observations are noted in pregnancy, where regular mild/moderate physical activity during normal pregnancy has beneficial effects for both fetal and maternal health throughout and following pregnancy [110-113]. Further, epidemiological studies of prenatal exercise and/or continued/initiated during pregnancy report a lowered incidence of morbidities such as gestational diabetes, gestational hypertension, fetal growth restriction, and preeclampsia [105,114-116]. Despite the past evidence of exercise as potential prevention of PE, few

hypothesis-driven mechanistic and molecular experiments have been reported.

We and others have recently observed exercise before and during gestation increases VEGF in rodents [27,75], and placental growth factor (PlGF) in women [117]. Further, exercise before and during gestation mitigates hypertension in several experimental models [27,118,119] of PE. Interestingly, the beneficial effects of exercise training before and during gestation remain similar across these two experimental models of preeclampsia, where an improvement of endothelial function and blood pressure was also associated with an increase in circulating concentration of VEGF and/or decreased sFlt-1. In addition to this story, our preliminary data suggests the effects of exercise initiated at the beginning gestation in the RUPP model are slightly different. Though we observe a similar improvement of angiogenic balance, we do not observe expected mitigation of hypertension and endothelial dysfunction. This is further discussed in Chapter IV.

In regard to fetal or placental effects we have recently reported that exercise before and during gestation improved placental efficiency (fetal weight: placental weight) [27,75] in both normal pregnancy and in RUPP dams, which suggests an improvement of placental diffusion capacity. Others have reported various effects on placental growth, and this may be due to the variations in timing, intensity, and duration of exercise between these studies [95,111,113,117-123].

Moreover, consideration of exercise naiveté and timing of exercise training may be crucial. As Larry Wolfe and colleagues have reported, maternal acid-base regulation and metabolism are impaired toward late stages of gestation, and normal carbohydrate metabolism and lactic acid use during exercise is hindered [124]. Further, placental tissue samples from women who exercised during pregnancy show an increase in NO production and decreased reactive oxygen species when compared to the sedentary/low-activity controls [95].

EXERCISE AND ANGIOGENIC BALANCE: GOVERNED BY AMPK?

Activation of skeletal muscle during exercise has been widely reported to stimulate the heterotrimeric serine/threonine protein kinase AMPK (AMP-activated protein kinase) and downstream pathways, in an intensity-dependent manner [30,125]. Additionally, the role of AMPK in acute and chronic training adaptations following exercise has been well-studied and discussed [30,126,127]. Importantly, activation of AMPK in exercising skeletal muscle reportedly mediates the production of pro-angiogenic factors, such as VEGF, through mRNA stabilization [127,128]. Specifically, Zwetsloot and colleagues, have recently reported a central role of AMPK in the stimulation of VEGF production post-exercise in transgenic model of muscle-specific AMPK knockdown, which suggests other tissues may utilize this AMPK-regulatory pathway as well. Interestingly, tissues of high metabolic activity (skeletal muscle, heart, brain, and

placenta) express a specialized γ2-isoform of AMPK-γ subunit [129], which has been suggested to tightly regulate AMPK activation because if its increased sensitivity to AMP levels [129,130]. Taken together, administration of AMPK activators or AMP analogues during pregnancy may enhance the production of VEGF/PlGF through the stimulation of placental AMPK. Indeed, the crucial roles of AMPK in normal and pathological placental development, and the potential regulation of VEGF/PlGF production are currently unclear. Therefore, we sought to elucidate the role of AMPK activation in angiogenic imbalance in the RUPP model through administration of the potent AMPK-activator AICAR (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside).

Epidemiological evidence of Metformin (1,1-dimethylbiguanide) [31,33,131] and low-dose aspirin (salicylic acid) [132-134] use during pregnancy report a reduced risk of PE and other pregnancy-related forms of hypertension. Moreover, biguanides, salicylates, nucleosides, and others drug classes alike are known stimulators of AMPK with anti-oxidative, anti-inflammatory, and anti-hypertensive effects [30,135,136]. AMPK can directly regulate eNOS activation through Ser1177 phosphorylation [137], which is suggestive of a potential vasodilatory effect in the short term. Moreover, a recent study in healthy human males confirmed this dilatory effect, in which intravenous treatment with AICAR caused an increase in heart rate and forearm blood flow paired with a decrease in arterial

pressure [36]. Similar observations have also been reported in hypertensive rats as well [34]. Additionally, there is an intriguing emergence of a positive feedback system between eNOS and AMPK, in which NO can activate the AMPK through the sGC (soluble guanylate cyclase)/cGMP signaling to increase Ca2+/CaMKK (calmodulin-dependent protein kinase kinase) activation of AMPK and further activation of eNOS and NO production [138]. Furthermore, administration of AMPK activator AICAR has been shown to mitigate hypertension in several experimental animal models complicated by high blood pressure [34-36,139], but few have directly focused on the potential anti-hypertensive effects of AMPK stimulation.

As discussed in Chapter V of this dissertation, we have recently reported AICAR treatment (50mg/kg b.i.d) ameliorated RUPP-induced hypertension, angiogenic imbalance, and endothelial dysfunction [26]. Similar to our exercise study in the RUPP model [27], AICAR improved circulating free VEGF and decreased sFlt-1 in the RUPP. Notably, placental and renal markers of oxidative stress and anti-oxidative capacity were also mitigated with AICAR treatment in the RUPP [26]. Interestingly, no detrimental effects were observed in the fetal or placental weights when normal pregnant or RUPP dams received the AICAR treatment [26]. In fact, AICAR administration mitigated RUPP resorption rates [26], suggesting an overall beneficial effect on the RUPP conceptus development.

Verily, the interpretations from these studies are limited as our previous study does not elucidate the exact role of AMPK signaling in the effects of AICAR administration, and future studies are required.

SUMMARY AND PERSPECTIVES

Preeclampsia has long been recognized as a leading cause of maternal and fetal morbidity and mortality worldwide. While great strides have been made in recent years to understand the pathological progression and ontogeny of PE, the cause of this syndrome remains enigmatic. Further, though the number of prospective studies of PE treatment and prevention has been limited, retrospective epidemiological reports have aided and focused research efforts. Of the numerous reports of potential modalities for PE prevention, exercise training has been widely recognized for its potential to decrease PE development; however, the molecular contributions are still unclear. Recently, several experimental and clinical reports have concluded exercise training before and during gestation can elicit a myriad of protective effects that collectively mitigate the development of PE.

The effects of exercise have recently been focused on factors mediating angiogenic balance, as exercise training in pregnancy has been shown to alter the expression of VEGF and PlGF. Our initial hypothesis focused on the role of VEGF production during exercise to combat the effects of sFlt-1 secretion from

the placenta, but the putative role of VEGF or PlGF in exercise training effects of PE are still unclear. Our most recent observation suggest exercise training also decreases plasma sFlt-1 in the RUPP rat (unpublished observation), which lend further explanation to the improvement in VEGF or PlGF bioavailability in previous studies from our laboratory and others. Further studies to isolate the specific roles of these angiogenic factors are underway, and should provide an interesting avenue for further clinical research.

Additionally, the role of AICAR administration has led to several exciting new hypotheses to pursue. Firstly, AICAR, or known by its moniker "the exercise mimetic", was initially used to elucidate a potential mechanism by which exercise could also be mediating the development of hypertension following placental ischemia. As hypothesized, AICAR mitigated the angiogenic imbalance, endothelial dysfunction, and hypertension in the RUPP model. These exciting results suggest AMPK could be a novel target for PE treatment and prevention. It is important to note the role of AMPK remains unknown in the effects of AICAR and exercise training in a model of PE. Nevertheless, the protective effects of AICAR in the RUPP model suggest of a novel pharmacological therapy to prevent PE development. Further, with no detrimental effects observed in the normal pregnant dams, further investigation in AICAR other potent AMPK activators should be assessed for potential PE

prophylaxis. Additional to the observations of AICAR and exercise in the RUPP model, there is a need to examine the roles of other pathological contributors to progression, including the innate and adaptive immune activation, as well as the additional role of central control of vasomotor activity.

In concert, we believe there are promising and specific roles for both exercise training and AICAR to treat or prevent the onset of preeclamptic hypertension. Furthermore, as the mechanisms of each modality are still unclear, the data chapters of this dissertation use innovative and hypothesis-driven approaches to elucidate these contributing mechanisms.

CHAPTER III

GROSS METHODOLOGY

PART 1: IN VIVO METHODOLOGY

Animal Husbandry

Studies were performed in age-matched female Sprague Dawley rats purchased from Charles River (Portage, MI) or Harlan Laboratories (Indianapolis, IN). Animals were housed in a temperature-controlled room (23°C) with a 12:12 light:dark cycle. All food and water was provided *ad libitum*. All animal experimental procedures executed were in accordance with National Institutes of Health guidelines for care and use of animals. Additionally All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Oregon.

Reduced Uteroplacental Perfusion Pressure Surgery

The RUPP procedure is a well-established and robust model for studying the link between placental ischemia induced hypertension in the pregnant rat [25,140]. In brief, hand-made silver clips were placed on the lower abdominal aorta (0.203-mm inner diameter (ID)) above the iliac bifurcation and also on branches (0.100-mm ID) of both the right and left ovarian arteries supplying the uterus on day 14 of pregnancy (term = 21). Together, these act to reduce both the main uterine artery blood flow, and any compensatory blood flow of the ovarian

arteries. **Figure 3.1** depicts the anatomy of the surgical procedure. Normal pregnant rats all underwent a sham surgery, which included the midline incision and suture.

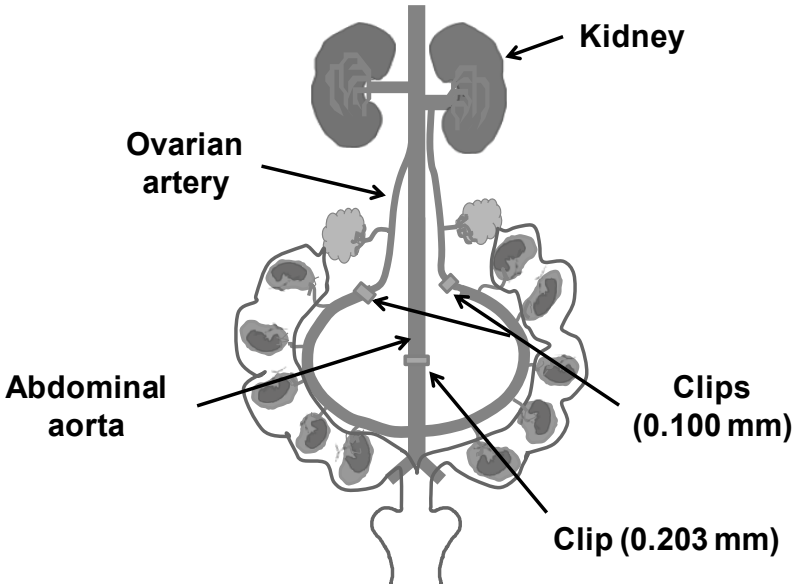

Figure 3.1. ***Reduced Utero-Placental Perfusion Pressure (RUPP) Procedure.*** On day 14 of gestation, one clip (0.203 mm ID) is placed on the abdominal aorta superior to the iliac bifurcation, and two additional clips are placed on the ovarian feeder arteries to prevent compensatory blood flow. Figure courteously provided by Jeffrey Gilbert, redepicts the previously published model details by Dr. Granger and colleagues [73].

Voluntary Exercise Training

Rats assigned to the exercise cohort were allowed 24-hour voluntary access to stainless steel rodent wire activity wheels (Lafayette Instruments, USA), and weekly running distance and time were measured by the activity wheel software (Activity Wheel Systems version 11.0, Lafayette Instruments, USA).

Voluntary wheel running was chosen over forced exercise for this study to minimize potentially deleterious effects due to stress or overtraining that have been reported previously with treadmill running and swimming in Sprague-Dawley rats.

Breeding

Animals were bred by pairing a female and male rat of similar ages in a cage with a 1″ raised wire rack. Marker of coitus was confirmed by daily monitoring for the presence of a seminal plug. Day of gestational age (dGA) was assigned as dGA 0 with the presence of at least two seminal plugs. Animals that failed to breed were excluded from the studies.

Arterial Blood Pressure Measurement

Animals were instrumented on day 18 of gestation with an indwelling catheter, and arterial pressure was determined in conscious rats on day 19 of gestation. On day 18 of gestation, a catheter of heat-stretched PE-50 polyvinyl tubing was introduced to the left common carotid artery, and advanced approximately 3cm while the animal was under isoflurane anesthesia. Catheters were exteriorized through the back of the neck with subcutaneous tunneling. On day 19 of gestation, animals were placed in Plexiglas® restraining cages, and direct pressures were monitored using a blood pressure transducer (ADInstruments) for 60 minutes following a fixed 30 minute stabilization period.

Mean arterial pressure was averaged over the recorded time. Additionally, heart rate was analyzed over the hour period from the arterial pressure measurements using LabChart 8.0 software (ADInstruments).

Necropsy and Tissue Collection

For all *in vivo* experiment termination, rats were placed under isoflurane anesthesia, and a midline ventral incision was made to isolate the abdominal aorta for arterial blood collection. Arterial blood from the abdominal aorta was collected for subsequent assays into unlined and EDTA-lined Corvac® sterile tubes for serum and plasma processing, respectively. Secondary confirmation of death was done by pneumothorax and removal of heart. Heart, kidney, fetal, and placental weights were recorded, as were resorptions and implantations. All collected tissues for subsequent analysis were flash frozen in liquid nitrogen and stored at −80°C.

Mulvany Microvessel Wire Myography

Following necropsies on day 19 of gestation, secondary and tertiary mesenteric arterioles were isolated and cleaned of all surrounding adipose and connective tissues under a dissection microscope. 1-2mm vessel segments were then mounted on two 40μm stainless steel wires and attached to a wire myograph (DMT, Denmark) to allow for isometric force recordings. Vessels were normalized to tensions that proportionally modeled 100mmHg using the

normalization module in LabChart 8.0 (ADInstruments, USA).

After a 10 minute period for equilibration in a Kreb's buffer (130mM NaCl, 4.7mM, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 1.6mM $CaCl_2$, 14.9mM $NaHCO_3$, 5.5mM glucose , pre-constriction was obtained by exposing the vessels to an isosmotic, high-potassium physiological saline solution (K-PSS) (74.7mM NaCl, 60mM KCl, 1.8 KH_2PO_4, 1.2mM $MgSO_4$, 1.6mM $CaCl_2$, 14.9mM $NaHCO_3$, 5.5mM glucose). Following a triple washout period with Kreb's buffer, a stabilized vascular constriction was achieved by the addition of the thromboxane A_2 analogue U46619 (5 μM). Endothelial-dependent vasorelaxation was evaluated with a cumulative dose response curve to acetylcholine (Ach; $1x10^{-9}$–$1x10^{-5}$ M). Further, endothelial-independent vasodilation and smooth muscle function were assessed with a cumulative dose response curve to sodium nitroprusside (SNP, $1x10^{-9}$–$1x10^{-5}$ M). Following each relaxation curve, a single dose of 0.1mM SNP was given to confirm smooth muscle integrity. At experiment termination, exposure to K-PSS was used to assess vessel viability and functional decay. Data is presented as mean±SD of the percent vasorelaxation from U46619 contraction force.

PART 2: IN VITRO METHODOLOGY

Cell Culture Technique and Conditions

Immortalized human villous trophoblast cells (BeWo) and primary human umbilical vascular endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC, USA). Prior to experimental protocols, cells were grown to manufacturer's recommendations. Briefly, cells were grown to 70-80% confluence in a $75cm^2$ flask (Corning Inc., USA) under 5% CO_2/20% O_2, and re-plated at $1x10^5$ cells/well in a 24-well plate for further treatment. BeWo cells were cultured with F12K basal media (ATCC), and HUVECs were cultured with vascular cell basal media (ATCC). Growth media were supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin (ATCC).

Vascular Endothelial Tube Formation Assay

To assess angiogenic potential in isolated sera [20,26,27,141], an *in vitro* measurement of endothelial tubule formation was used. In a 96-well plate, 30μl of growth factor-reduced Matrigel® (BD Biosciences) was added to each well, and incubated for 30 minutes at 37˚C to allow for adequate polymerization. Primary human vascular endothelial cells (HUVECs) of passages 1-3 were plated at $1x10^5$ cells/mL. Cells were suspended and cultured in a serum-free vascular cell basal medium (ATCC). Further, after overnight thaw at 4˚C, serum samples from animal treatment groups were vortexed, and added to a well to reach 5%

(v/v). Tubule formation was assessed at 100X optical zoom with a digital inverted compound microscope (Axio Observer, Zeiss) and Zen imaging and analysis software (Zeiss). Total tube count was assessed by at least two individual investigators that were blinded to the identity of the experimental groups. Values from each observer were averaged to obtain final counts.

Placental Explant Isolation and Culture

Normal pregnant dams were euthanized and whole-placentas were isolated on day 19 of gestation. Tissues were collected in ice-cold sterile PBS. Under a dissection microscope, the decidua and trophospongium were carefully dissected away to isolate the anchoring villi bundles. Approximately 10-15mg of villous tissue were excised and weighed prior to culture. The explants were cultured in a 24-well plate on 150μl of Growth Factor Reduced Matrigel® (BD Biosciences) and 1mL of 50/50 Dulbecco's Modified Eagle's Medium: Ham's F-12 with 10% Fetal Bovine Serum, 100 μg/ml streptomycin, 100 U/ml penicillin, and 25 μg/ml ascorbic acid [142-144]. Tissues were conditioned in a CO_2/O_2 gas incubator at either physiological normoxia (8% O_2) or at hypoxia (1% O_2). Experimental procedure and timeline are depicted in **Figure 3.2**.

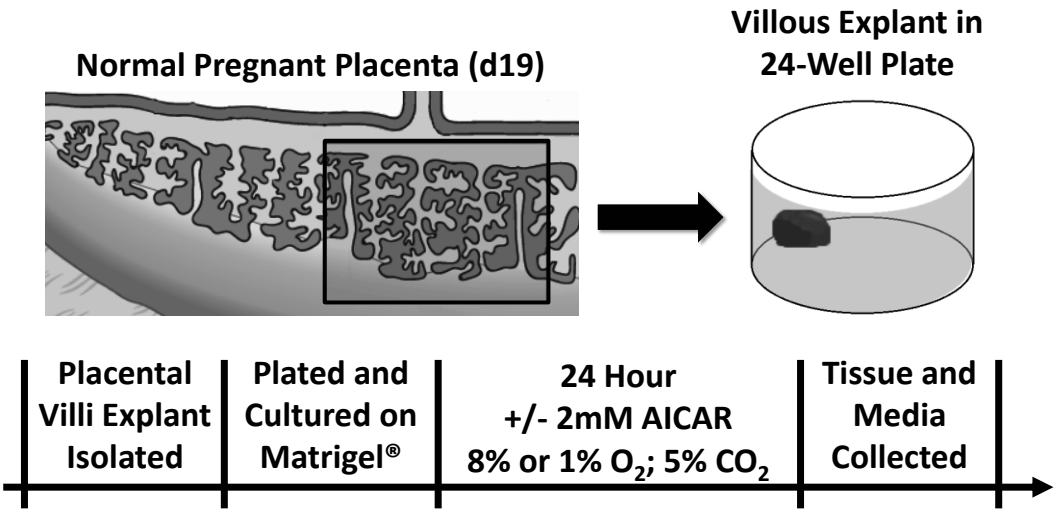

Figure 3.2. ***Placental Villi Explant Isolation and Culture Protocol.***

PART 3: TISSUE AND SAMPLE PROCESSING

Protein Extraction and Quantification

Total soluble protein was extracted from tissues and cultured cells in a radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with phenylmethanesulphonylfluoride in dimethyl sulfoxide, sodium orthovanadate and a protease inhibitor cocktail (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The concentration of soluble protein in each homogenate was determined using the bicinchoninic acid method (Pierce Biotechnology, USA). Renal tissue position (right or left) was chosen at random, and placental samples were carefully selected for middle position on either side of the uterine horns.

SDS-PAGE and Western Blotting

Protein (50 μg) was separated by electrophoresis on 4-20% sodium dodecyl sulfate (SDS) polyacrylamide separating gels (Life Technologies, USA) then transferred to polyvinylidene fluoride (PVDF) membranes (BioRad, USA) and stained with 0.5% ponceau to confirm the transfer across each gel. The images of the ponceau stained membranes were digitized with a flatbed scanner.

The membranes were then washed and incubated one hour at room temperature in commercially available non-mammalian protein blocking buffer (LI-COR Biosciences, USA). Membranes were incubated in blocking solution containing commercially available antibodies (0.1μg/mL) overnight at 4˚C. Membranes were washed and incubated for one hour with the appropriate fluorescent protein-conjugated secondary antibodies (7.5ng/ml, LI-COR Biosciences, USA). The immune-reactive bands were imaged and quantified using the Odyssey dual-channel fluorescent imaging system and Image Studio 2.0 software (LI-COR Biosciences, USA). Specificity of primary antibodies (negative controls) was evaluated by imaging membranes with the primary antibody omitted.

CHAPTER IV

EXERCISE TREATMENT TO MITIGATE PLACENTAL ISCHEMIA INDUCED HYPERTENSION AND ANGIOGENIC IMBALANCE IN AN EXPERIMENTAL MODEL OF PREECLAMPSIA

Part 1 of Chapter IV is in preparation for publication with Haley E. Gillham, Karen W. Needham, and Jeffrey S. Gilbert as co-authors. I performed the experimental work along with the technical assistance of H.E. Gillham and K.W. Needham. J.S. Gilbert provided editorial assistance. J.S. Gilbert provided editorial assistance and project funding.

Additionally, Parts 2-3 of this chapter are in preparation for publication with Haley E. Gillham, Karen W. Needham, and Jeffrey S. Gilbert as co-authors. I performed the experimental work along with the technical assistance of H.E. Gillham and K.W. Needham. J.S. Gilbert provided project funding.

PART 1: EXERCISE DURING GESTATION TO IMPROVE VEGF BIOAVAILABILITY AND MITIGATE HYPERTENSION INDUCED BY PLACENTAL ISCHEMIA

Introduction

Preeclampsia (PE) is newly defined as a new-onset hypertension accompanied by markers of one of the following: renal and liver dysfunction, thrombocytopenia, pulmonary edema, or cerebral disturbances, near or after the

20th week of gestation. PE is a leading cause of fetal and maternal morbidity and mortality in the U.S.[41] and worldwide [42]. Following the presentation of PE, treatment options are currently limited to supportive care and induction of delivery if symptoms progress [41,44]. Further, as many clinical reports suggest PE increases both the mother's and child's lifetime risk of cardiovascular and metabolic disease development [145-148], development and further investigation of possible treatments for and prevention of preeclamptic pregnancies is highly favorable to improve maternal and fetal health.

Although the etiology of preeclampsia remains unclear, poor placental perfusion is regarded by many as the initiating event in the development of hypertension and angiogenic imbalance in preeclampsia [48,140,149]. This ischemic environment stimulates several pro-hypertensive factors, including sFlt-1 (soluble Fms-like tyrosine kinase-1) and VEGF (vascular endothelial growth factor). An imbalance of pro- and anti-angiogenic factors (e.g. decreased VEGF/PlGF, increased sFlt-1) is observed to contribute to the development of hypertension in both human and animal models of PE, and improvement of the angiogenic potential through VEGF administration has promising therapeutic potential [20,28,29,150]. Similarly, non-pharmacological modalities such as exercise have been sought out to improve plasma angiogenic balance through endogenous stimulation of VEGF [27,75,117].

Exercise is well-known to mitigate hypertension in the non-pregnant state and recent studies have explored the possibility that vigorous physical activity may serve as a therapeutic or preventative modality [107-109]. We and others have observed exercise before and during gestation increases VEGF in rodents [27,75], and placental growth factor (PlGF) in women [117]. Further, we and others have previously observed exercise before and during gestation mitigates hypertension in several models [27,118,119] of preeclampsia. Nevertheless, whether these same benefits are shared with exercise training initiated at the onset of pregnancy remains unclear. Therefore, we hypothesized (**Figure 4.1**) voluntary exercise

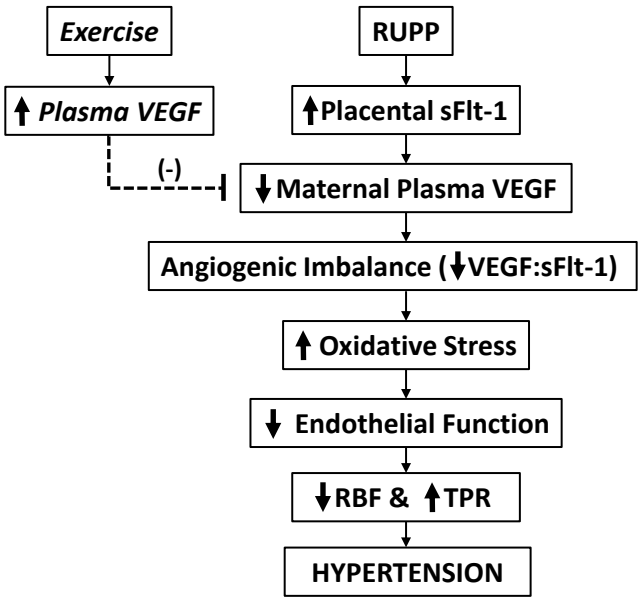

Figure 4.1. ***Overarching Exercise Hypothesis***. RUPP-induced hypertension and endothelial dysfunction is expected to be prevented by exercise training prior to and during gestation by improving plasma VEGF concentrations. RBF: Renal Blood Flow; TPR: Total Peripheral Resistance.

initiated at the beginning of gestation will improve angiogenic potential and attenuate placental ischemia induced hypertension in the rat.

Methods

Animals

Studies were performed between the University of Oregon and the University of Minnesota Medical School-Duluth with female Sprague-Dawley rats purchased from Harlan Laboratories (Indianapolis, IN) and Charles River Laboratories (Wilmington, MA). Animals were housed in a temperature-controlled room (23°C) with a 12:12 light:dark cycle. All experimental procedures executed were completed in accordance with National Institutes of Health guidelines for use and care of animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Oregon and University of Minnesota. Six-week-old rats were given one week of wheel access with three other animals to learn to use the exercise wheel [27]. The animals were then bred, and assigned day of gestation (dGA) 0 when ≥2 copulation plugs are observed [27]. Animals to exercise during pregnancy (ED) were housed separately with voluntary access to an activity wheel (Lafayette Instruments, Model 80859; Lafayette, IN), and sedentary treated animals had no wheel access. Dams were assigned to sedentary normal pregnant (NP) (n=8) and RUPP (n=8) groups or exercise during pregnancy (NP+ED (n=8); RUPP+ED (n=8)). The timeline of the

experimental protocol is depicted in **Figure 4.2**.

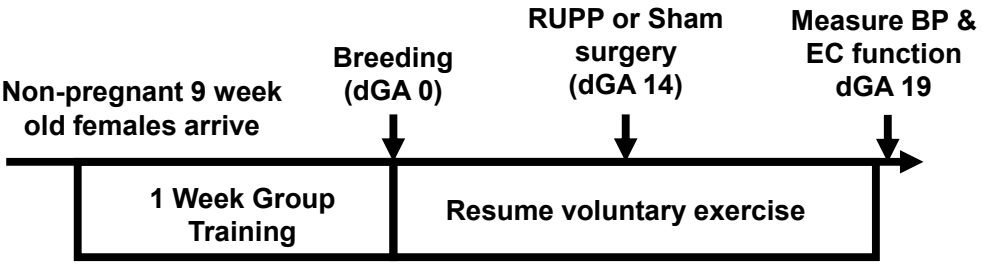

Figure 4.2. ***Voluntary Exercise Experimental Timeline.*** 9-week-old female Sprague Dawley rats train for 1 week prior to pregnancy to learn how to use the exercise wheels. Dams were then bred, and allowed access to exercise wheels upon confirmation of coitus. RUPP/sham was performed on day 14. Blood pressures and tissues collected on day 19.

Reduced Uterine Perfusion Pressure (RUPP) Procedure

The RUPP procedure is a well-characterized and widely accepted model [140] for studying the link between placental ischemia and hypertension in the pregnant rat and has been described in detail previously[27]. Briefly, a silver clip (0.203-mm inner diameter (ID)) was placed on the lower abdominal aorta, superior to the common iliac bifurcation. Two additional clips (0.100-mm ID) were placed on the branches of both the right and left ovarian arteries on day 14 of gestation (term=21 days). Half of the NP dams underwent a sham surgery, which included an abdominal midline incision and suture. After observing no differences in the angiogenic factors and blood pressures these animals were grouped with the NP rats.

Measurement of MAP in Chronically Instrumented Conscious Rats

Animals were instrumented with an indwelling arterial catheter of the common carotid artery on day 17 of gestation. On day 19, unanesthetized mean arterial pressure (MAP) was measured using a fluid-filled pressure transducer (ADInsruments, Colorado Springs, CO) [26,27,151].

Conceptus Measurements and Serum Collection

After blood pressure measurement, the dams were placed under 3% isoflurane anesthesia, and a midline ventral incision was made to isolate the abdominal aorta for arterial plasma and serum collection as reported previously [27,151]. After exsanguination, a pneumothorax and cardiac excision were used to confirm death. Blood was collected for subsequent assays into blank and EDTA-lined Corvac® tubes for serum and plasma processing, respectively (Sherwood Davis, St. Louis, MO). Fetal weight, placental weight, and number of resorptions were recorded in the manner described previously [27,151]. All tissues collected for subsequent analysis were flash frozen in liquid nitrogen and stored at -80˚C.

Enzyme-Linked Immunosorbant Assays

Plasma concentration of free VEGF was measured using commercially available enzyme linked immunosorbant assay (ELISA) kits (R&D Systems, Quantikine®; Minneapolis, MN, Part: MMV00) according to the manufacturer's directions as described previously [26,27,151].

Endothelial Tube Formation Assay

Angiogenic balance was further assessed in the serum of pregnant rats *in vitro* as previously reported [26,27] in two separate experiments and each was performed in duplicate. HUVECs (human umbilical vascular endothelial cells) were plated at $5x10^5$ cells/mL (100µl/well) onto 96-well plate lined with growth-factor-reduced Matrigel® (BD Biosciences, Bedford, MA). Five microliters of serum from each group (NP, RUPP, NP+ED, RUPP+ED) was introduced to the 100µl cell media. Cells were incubated at 37°C, 20% O_2, 5% CO_2. Total number of tubule formations per frame was assessed at 100X optical zoom with a digital inverted compound microscope and ImageJ analysis software (National Institutes of Health, Bethesda, MD). Total tube count was assessed by at least two investigators that were blinded to the identity of the experimental groups. Values from each observer were averaged to obtain final counts.

Antioxidant Capacity and Oxidative Stress

Placental tissue, amniotic fluid, and plasma oxidative stress was assessed by measuring total antioxidant capacity and lipid peroxidation, carried out as previously reported [26,152]. Total antioxidant capacity was assessed in placental tissue, amniotic fluid, and maternal plasma by measured by using a Trolox-equivalent antioxidant capacity (TEAC) assay kit (Cayman Chemical Company, Ann Arbor, MI). Additionally, lipid peroxidation was measured in maternal

serum using a TBARS (thiobarbituric acid reactive substances) commercial assay kit (Cayman Chemical Company, Ann Arbor, MI). All assays were performed in duplicate and according to the manufacturers' directions.

Statistical Analysis and Calculations

All data are presented as mean ± SD, and statistical significance was accepted when $p<.05$. Comparisons were made with a one-way analysis of variance test combined with a Bonferroni post-hoc test were employed. Statistical calculations were made with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA USA).

Results

Voluntary Running Wheel Activity

Similar to our previous observations [27], voluntary running distance on the activity wheels did not differ between the NP+ED and RUPP+ED groups throughout gestation. As reported previously [27], (*$p<.05$) the distance run in the third week of gestation was decreased in both groups. Data is presented in **Figure 4.3**.

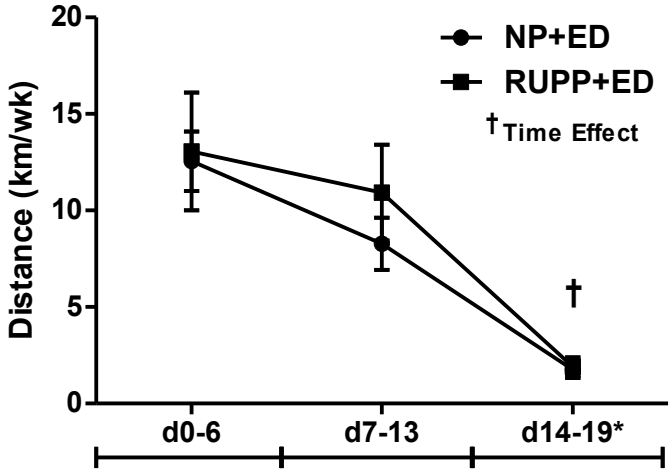

Figure 4.3. ***Exercise Measured by Weekly Distance.*** Distance per gestational week no different between NP and RUPP. Across both groups, time-dependent decrease (*p<.05 vs. Week 1 and 2) in distance run was observed in week 3. NP, normal pregnant control; RUPP, reduced uterine perfusion treatment; ED, exercise during gestation. *p<.05, time effect. Data presented as mean±SD; n=6-8 per group.

Mean Arterial Pressure

At day 19 of gestation, arterial blood pressure was increased (p<.05) (**Figure 4.4**) in the RUPP compared to NP rats. No effect of exercise was observed in either the RUPP+ED or NP+ED blood pressure measurements. Together, exercise initiated at the onset of gestation did not attenuate RUPP hypertension.

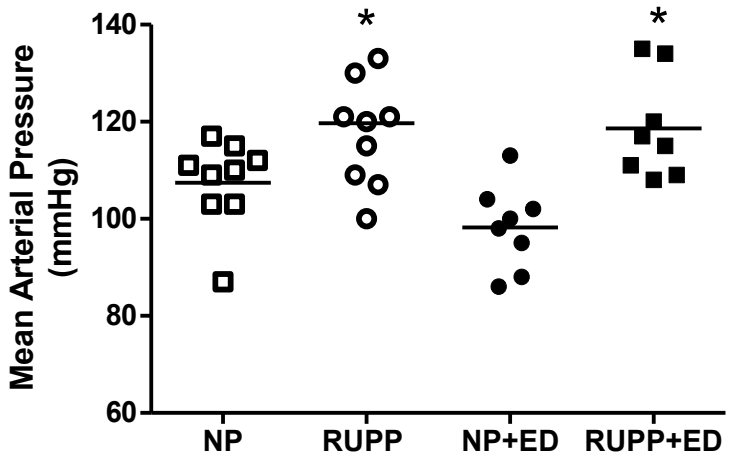

Figure 4.4. ***Mean Arterial Pressure on Day 19.*** The RUPP-induced increase (*p<.05 vs. NP) in blood pressure was slightly attenuated (p=.06) by exercise during (ED) gestation. NP 107±9; *RUPP 120±13; NP+ED 98±9; RUPP+ED 119±11 mmHg. *p<.05 vs. NP; †p<.05 vs. RUPP. Data presented as mean±SD.

Circulating VEGF and Angiogenic Potential

As displayed in **Figure 4.5**, circulating free VEGF was decreased (p<.05) in the RUPP compared to the NP group and exercise increased (p<.05) plasma VEGF in RUPP+ED but not NP+ED rats. Angiogenic potential measured by HUVEC total tubule formation was decreased (p<.05) in the RUPP compared to the NP controls, and increased (p<.05) in the RUPP+ED compared to the RUPP. Together, these data (depicted in **Figure 4.5**, bottom panel) indicate RUPP-induced angiogenic imbalance is improved with exercise during pregnancy.

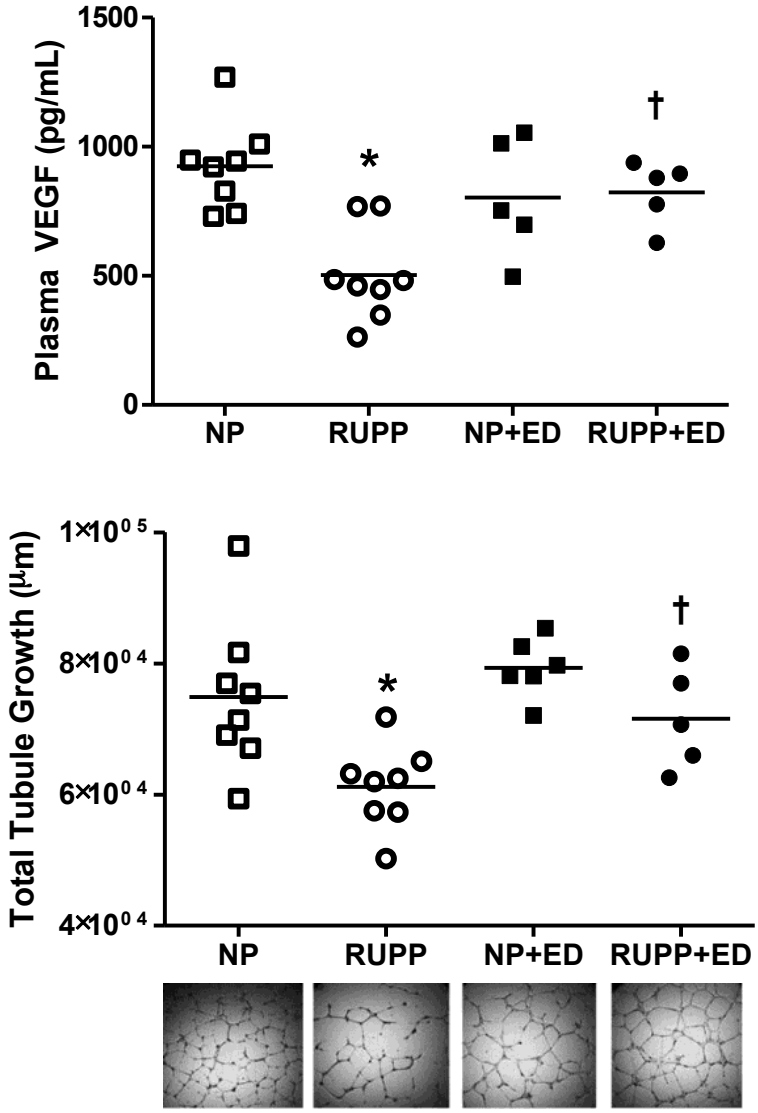

Figure 4.5. ***Plasma VEGF and Serum Angiogenic Potential.*** (Top Panel) Free VEGF levels in maternal plasma were decreased ($p<.05$) in the RUPP compared to NP, and exercise improved (†$p<.05$) the VEGF bioavailability in the RUPP+ED. NP 924±61; *RUPP 503±64; NP+ED 803±104; †RUPP+ED 824±56pg/mL. (Panel B) Primary HUVEC microtubule formation was improved (†$p<.05$) when exposed to serum from RUPP+ED dams compared to the sedentary RUPP. NP 7.5±1.5; *RUPP 6.1±0.6; NP+ED 7.9±0.5; RUPP+ED 7.2±0.7x10^4 μm. *$p<.05$ vs. NP; †$p<.05$ vs. RUPP. Data presented as mean±SD.

Tissue and Circulating Oxidative Stress and Antioxidant Capacity

RUPP decreased ($p<.05$) TEAC in amniotic fluid compared to the NP, but had no effect on placental tissue or plasma TEAC (**Figures 4.6a-c**). Exercise during RUPP pregnancy had no effect on TEAC in placenta, amniotic fluid, or maternal plasma. Amniotic fluid TEAC in NP+ED was decreased ($p<.05$) compared to the NP control, but no effect was observed in the maternal plasma or placental extract. Lipid peroxidation measured by malondialdehyde (MDA) levels in maternal serum was increased in the RUPP compared to the NP, and was not attenuated by exercise training during pregnancy in the RUPP+ED treatment group (**Figure 4.6d**).

Maternal and Fetal Morphometric Data at Necropsy

Maternal and fetal weights were decreased ($p<.05$) in the RUPP compared to NP, and exercise during gestation had no significant effect in either NP+ED or RUPP+ED groups (**Table 4.1**). Additionally, placental weights were decreased ($p<.05$) with RUPP compared to NP, as well as a decreased weight observed in the NP+ED vs. NP ($p<.05$). Placental efficiency, measured by a ratio of fetal and placental weight, was also decreased ($p<.05$) in the RUPP vs. NP, but also decreased ($p<.05$) in NP+ED. Finally, resorption percentage was increased in the RUPP compared to the NP control group, and RUPP+ED was observed to have a decreased rate of resorption compared to RUPP.

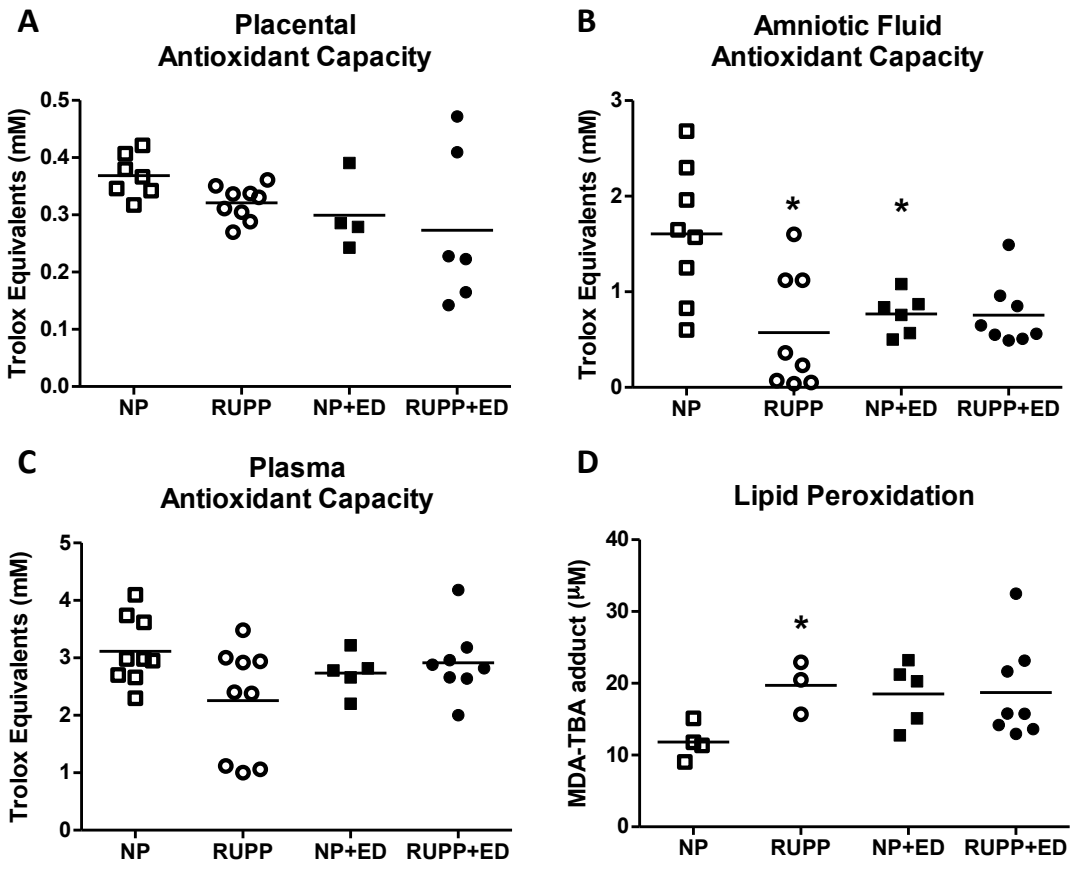

Figure 4.6. ***Tissue and Systemic Oxidative Stress and Anti-Oxidant Potential.*** (Panel A) Trolox-equivalent antioxidant capacity (TEAC) levels were not affected by RUPP placental tissue compared to the NP, and no exercise effect was observed in RUPP. NP 0.37±0.04; *RUPP 0.32±0.03; NP+ED 0.30±0.06; RUPP+ED 0.27±0.14 Trolox equivalent (μM). (Panel B) Amniotic fluid TEAC was decreased (*p<.05) in the RUPP and NP+ED compared to the NP. NP 1.61±0.71; *RUPP 0.57±0.61; NP+ED 0.77±0.21; RUPP+ED 0.76±0.34 Trolox equivalent (μM). (Panel C) Plasma TEAC was not effect by RUPP or exercise treatment. NP 3.11±0.58; *RUPP 2.26±0.95; NP+ED 2.74±0.37; RUPP+ED 2.92±0.62 Trolox equivalent (μM). (Panel D) Serum lipid peroxidation (measured by malonyldialdehyde formation) was increased (*p<.05) in RUPP with respect to NP, and no effect from exercise observed. NP 11.8±2.5; *RUPP 19.7±3.7; NP+ED 18.5±4.4; RUPP+ED 18.7±6.7. *p<.05 vs. NP; †p<.05 vs. RUPP. Data presented as mean±SD.

Table 4.1. ***Maternal and Conceptus Morphometric Data at Necropsy.***

Treatments	*Maternal Weight (g)*	*Fetal Weight (g)*	*Placental Weight (g)*	*Placental Efficiency (au)*	*% Resorption*
NP (n=9)	305.7±10.7	2.58±0.18	0.50±0.05	6.3±1.1	1.1±3.1
RUPP (n=9)	*268.2±10.1	*2.18±0.21	*0.44±0.05	*4.9±0.6	*73.1±7.2
NP+ED (n=8)	308.2±12.7	*2.15±0.16	*0.42±0.06	*4.9±1.1	2.6±1.3
RUPP+ED (n=8)	286.4±7.6	1.97±0.32	0.41±0.07	4.9±0.7	†38.2±8.6

Note. *p<.05 indicates different from NP. †p<.05 indicates different from RUPP. Data presented as mean±SD.

Discussion

The current study presents intriguing and novel findings in regard to the effects of exercise initiated at the beginning of gestation (ED) in the RUPP model of preeclampsia. In contrast to our initial hypothesis, exercise training during gestation did not prevent placental ischemia induced hypertension, despite restoration of plasma VEGF and angiogenic potential following exercise training during gestation in the RUPP dam. Additionally, ED treatment did not mitigate the RUPP-induced oxidative stress, and had no observed effect on fetal-placental development. These data collectively suggest an exercise regimen started at the beginning of gestation does not mitigate the RUPP-induced hypertension in the

rat, but promotes a restored angiogenic balance.

Though the mechanisms underlying the pathogenesis of PE are not completely defined, placental ischemia is commonly regarded as the initiating event resulting in angiogenic imbalance, renal and vascular dysfunction and increased blood pressure [45,150,153,154]. Previous clinical [20,29] and experimental [28,151] observations support the important role of impaired angiogenic balance in hypertension during preeclampsia, and mitigating this angiogenic imbalance attenuates or completely abolishes the hypertension in several rodent models of preeclampsia [28,70,71]. Indeed the current study shows that exercise during pregnancy only improves angiogenic balance in RUPP rats but does not attenuate RUPP-induced hypertension.

Exercise During Pregnancy and Angiogenic Balance

Firstly, RUPP hypertension was not affected by exercise during pregnancy only, despite the observed improvement of maternal circulating free VEGF and serum angiogenic potential. This is an interesting observation, as these data contrast with our previously reported benefits of both exercise before and during (EBD) gestation [27] and exogenous VEGF infusion [28]. To note, our previous exercise study reported an increase in plasma VEGF of approximately 80% with exercise before and during gestation in the RUPP, and the current study only reports a 60% increase. However, this is unlikely the single difference between

the EBD and ED treatments, as approximately 25% improvement of VEGF bioavailability have been reportedly effective in the mitigation of RUPP-induced hypertension [28]. Moreover, the data from the current study suggest a moderate increase in VEGF and angiogenic potential following exercise may not be the major contributor to the blood pressure lowering effect from exercise previously observed by our group [75] and others [105,115,117-119]. In concert with these previous reports, the current study suggests the previously reported benefits of EBD may be largely due to the training prior to the pregnancy as opposed to the ED treatment in the current study.

Exercise and Oxidative Stress

In our previous studies of exercise before and during (EBD) pregnancy [27,75], the EBD treatment improved antioxidant capacity and reduced oxidative stress systemically. However, the current study reports unaltered or, in some cases, exacerbated oxidative stress in the RUPP+ED and NP+ED compared to their respective controls. Together, these observations suggest exercise training prior to pregnancy may be necessary to stimulate an (anti)oxidative buffer and prevent vascular endothelial and placental dysfunction following placental ischemia [106].

Fetal and Placental Effects of Exercise Initiated During Gestation

No effects on fetal-placental morphometrics were observed in the RUPP+ED dams, indicating neither an amelioration nor exacerbation of the RUPP-induced fetal-placental growth restriction. Similar effects have been reported in women who recorded a mild-moderate exercise regimen through 20 weeks of gestation [116]. Interestingly, Clapp and colleagues reported in normal pregnant women, exercise initiated during pregnancy improved both fetal and placental weight at mid-gestation [121]; however, this stimulation of fetal-placental development was not observed in the current study's normal pregnant or RUPP controls. This difference may be attributed to the small timing variation, as these women began exercising halfway through gestation and the current study's rats began exercising immediately following breeding. Future studies should be pursued to elucidate these potential timing differences in exercise adaptations during gestation, as this could provide crucial information in which exercise could be a potential treatment following diagnosis rather than just prevention.

Exercise and Timing

One potentially important difference between the two exercise treatments, EBD and ED, could rely on the hemodynamic and vascular adaptations to exercise in the pre-trained and sedentary groups at the start or initial stages of pregnancy. Exercise is certainly well-known to stimulate both peripheral- and

central-mediated hemodynamic shifts as well as tissue neo-angiogenesis [78,155,156]. Further, uterine blood flow during exercise is decreased in both normal and complicated pregnancies during strenuous exercise [157,158], as splanchnic blood flow is decreased and skeletal muscle blood flow is quickly increased. However, Hart and colleagues concluded exercise training during normal and preeclamptic pregnancies increases uterine blood flow following a moderate exercise bout [157], but few studies have followed to support these findings since this study and ACOG contraindication [110]. It is possible the exercise training prior to pregnancy allows for an increased ischemic tolerance, perfusion capacitance, or peripheral auto-regulation of blood flow that is not developed if exercise is initiated at the beginning of gestation, as previously postulated in humans [105,122,159]. Indeed, further investigations using a molecular and hypothesis-driven approach are required to elucidate these adaptation differences observed in the current study.

Conclusion

The present data support our initial hypothesis of an improvement in VEGF availability and angiogenic potential with exercise-during (ED) treatment in RUPP. However, our hypothesis of mitigated RUPP-induced hypertension was not supported as blood pressure remained increased compared to NP. Taken together, these findings suggest exercise or training status may be an important factor when prescribing exercise during pregnancies; especially those at high risk

for hypertensive disorders.

In the next section of this chapter, we will evaluate the effectiveness of exercise before and during gestation while preventing a restoration of bioavailable VEGF. This approach will allow us to assess the role of angiogenic balance in the cardiovascular effects of exercise training prior to and during gestation in a model of placental ischemia induced hypertension.

PART 2: ISOLATING THE ROLE OF ANGIOGENIC BALANCE AND VEGF BIOAVAILABILITY IN THE ANTI-HYPERTENSIVE EFFECTS OF EXERCISE BEFORE AND DURING GESTATION IN THE RUPP MODEL OF PREECLAMPSIA

Introduction

Preeclampsia (PE) is a pervasive, pregnancy-specific syndrome defined by new onset hypertension and proteinuria. Additionally, PE is often associated with increased soluble VEGF Receptor-1 (sFlt-1), decreasing the bioavailability of vascular endothelial growth factor (VEGF) creating angiogenic imbalance leading to endothelial dysfunction and hypertension. We recently reported exercise training attenuates placental ischemia-induced hypertension and restores angiogenic balance but the mechanism remains unclear [27]. The previous section in this chapter reports exercise initiated at the start of gestation does improve VEGF bioavailability, but was not effective in lowering RUPP

hypertension, raising the question of the central role of improved plasma VEGF in the anti-hypertensive effect of exercise [27]. Specifically, we [27,75] and others [117] have recently reported exercise training in normal pregnancy improved circulating VEGF and PlGF bioavailability in rodents and humans, respectively. However, it remains unclear whether the improvement in circulating angiogenic balance is necessary for in the anti-hypertensive effects of exercise.

Moreover, we aimed to elucidate if this mechanism is contributing to the blood pressure lowering effects we have previously observed in the RUPP model. Thus, we hypothesized a sustained angiogenic imbalance through recombinant sFlt-1 infusion would antagonize VEGF bioavailability and prevent the anti-hypertensive effects of exercise in a model of placental ischemia induced hypertension.

Methods

Animals

Studies were performed at the University of Oregon with female Sprague-Dawley rats purchased from Harlan Laboratories (USA). Animals were housed in a temperature-controlled room (23°C) with a 12:12 light:dark cycle. All experimental procedures executed were completed in accordance with National Institutes of Health guidelines for use and care of animals and were approved by the University of Oregon's Institutional Animal Care and Use Committee.

Voluntary Exercise Training Protocol

Three-week-old rats were given one week of wheel access as a group of 4-5 to learn to use the activity wheel (Lafayette Instruments, USA; Model 80859) [27]. Further, the animals were then separated, and given individual access for six weeks. Following the six weeks of voluntary training, the animals were then bred, and assigned day of gestation age (dGA) 0 when ≥2 copulation plugs are observed [27]. Sedentary cohorts were housed without activity wheel access. Dams were assigned to the following treatments: normal pregnant (NP), NP+sFlt1, NP+Ex/sFlt1; RUPP, RUPP+Ex, RUPP+Ex/sFlt1. Experimental protocol timeline is depicted below (**Figure 4.7)**.

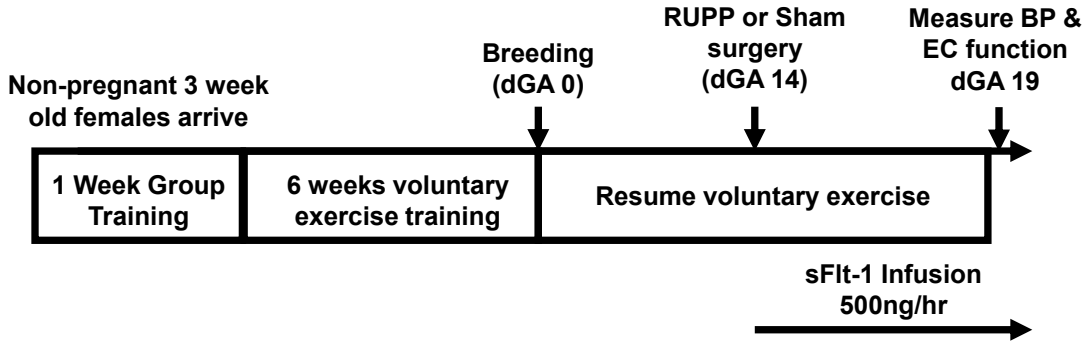

Figure 4.7. ***Voluntary Exercise Experimental Timeline****.* 3-week-old female Sprague Dawley rats train for 6 weeks prior to pregnancy. RUPP/sham is performed on day 14. Saline- or sFlt-1-filled osmotic pumps were also implanted on day 14. Blood pressures and tissues collected on day 19.

Reduced Utero-Placental Pressure (RUPP) Procedure and Osmotic Pump Implant

The RUPP procedure is a well-characterized and widely accepted model [140] for studying the link between placental ischemia and hypertension in the pregnant rat and has been described in detail previously [27]. Briefly, a silver clip (0.203-mm inner diameter (ID)) was placed on the lower abdominal aorta, superior to the common iliac bifurcation. Two additional clips (0.100-mm ID) were placed on the branches of both the right and left ovarian arteries on day 14 of gestation (term=21 days).

During the same procedure, Osmotic pumps (Alzet, USA; Model 2001) were implanted intra-peritoneal on dGA14 to chronically deliver recombinant mouse (rm)-sFlt1 (RD Systems, USA) at 500ng/ul/hr or saline vehicle containing a mouse Fc-IgG control from gestational dGA14-d19, when blood pressures and tissues were collected. This dose of sFlt-1 has been previously shown [160] to cause an increase in mean arterial pressure in pregnant rats, and this blood pressure effect was further confirmed in our pilot experiments. NP, NP+sFlt1 and RUPP data are also used in Part 3 of Chapter V as these studies were run in parallel.

Measurement of MAP in Chronically Instrumented Conscious Rats

Animals were instrumented with an indwelling arterial catheter of the left common carotid artery on day 18 of gestation. On day 19, unanesthetized mean arterial pressure (MAP) was measured directly using a fluid-filled pressure

transducer (ADInsruments, Colorado Springs, CO) [26,27,151]. Animals were allowed 30 minutes to adjust to the restraining cages, and blood pressures were recorded for the 60 minutes following.

Conceptus Measurements and Serum Collection

After blood pressure measurement, the dams were placed under 3% isoflurane anesthesia, and a midline ventral incision was made to isolate the abdominal aorta for arterial plasma and serum collection as reported previously [27,151]. After exsanguination, a pneumothorax and cardiac excision were used to confirm death. Blood was collected for subsequent assays into blank and EDTA-lined Corvac® tubes for serum and plasma processing, respectively (Sherwood Davis, St. Louis, MO). Fetal weight, placental weight, and number of resorptions were recorded in the manner described previously [27,151]. All tissues collected for subsequent analysis were flash frozen in liquid nitrogen and stored at -80˚C.

Mulvany Microvessel Wire Myography

Following necropsies on day 19 of gestation, secondary and tertiary mesenteric arterioles were isolated and cleaned of all surrounding adipose and connective tissues under a dissection microscope. Vessel segments 1-2mm in length were mounted and suspended with two 40μm stainless steel wires and attached to a wire myograph (DMT, Denmark) to allow for isometric force recordings. Vessels were normalized to tensions that proportionally modeled

100mmHg using the normalization module in LabChart 8.0 (ADInstruments, USA).

After a 10 minute period for equilibration in a Kreb's buffer (130mM NaCl, 4.7mM, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 1.6mM $CaCl_2$, 14.9mM $NaHCO_3$, 5.5mM glucose , pre-constriction was obtained by exposing the vessels to an isosmotic, high-potassium physiological saline solution (K-PSS: 74.7mM NaCl, 60mM KCl, 1.8 KH_2PO_4, 1.2mM $MgSO_4$, 1.6mM $CaCl_2$, 14.9mM $NaHCO_3$, 5.5mM glucose). Following a triple washout period with Kreb's buffer, a stabilized vascular constriction was achieved by the addition of the thromboxane A_2 analogue U46619 (5 μM). Endothelial-dependent vasorelaxation was evaluated with a cumulative dose response curve to acetylcholine (Ach; $1x10^{-9}$–$1x10^{-5}$ M). Further, endothelial-independent smooth muscle function was assessed with a cumulative dose response curve to sodium nitroprusside (SNP, $1x10^{-9}$–$1x10^{-5}$ M). Following each relaxation curve, a single dose of 0.1mM SNP was given to confirm smooth muscle integrity. At experiment termination, exposure to K-PSS was used to assess vessel viability and functional decay. Data is presented as mean±SD of the per cent vasorelaxation from U46619 contraction force.

Enzyme-Linked Immunosorbant Assays

Plasma concentration of free VEGF was measured using commercially available enzyme linked immunosorbant assay (ELISA) kits (R&D Systems,

Quantikine®; Minneapolis, MN, Part: MMV00) according to the manufacturer's directions as described previously [26,27,151].

Endothelial Tube Formation Assay

Angiogenic balance was further assessed in the serum of pregnant rats *in vitro* as previously reported [26,27] in two separate experiments and each was performed in duplicate. HUVECs (human umbilical vascular endothelial cells) were plated at $5x10^5$ cells/mL (100ul/well) onto 96-well plate lined with growth-factor-reduced Matrigel® (BD Biosciences, USA). Five microliters of serum from each group was introduced to the 100µl cell media. Cells were incubated at 37°C, 20% O_2, 5% CO_2. Average tubule length per frame was assessed at 100X optical zoom with a digital inverted compound microscope and ImageJ analysis software (National Institutes of Health, Bethesda, MD). Tubule lengths were assessed by at least two investigators that were blinded to the identity of the experimental groups. Values from each observer were averaged to obtain final counts.

Statistical Analysis and Calculations

All data are presented as mean ± SD, and statistical significance was accepted when $p<.05$. Comparisons within NP and RUPP groups were evaluated with a one-way analysis of variance test combined with a Bonferroni post-hoc test were employed. Comparison between NP and RUPP controls was analyzed

by a t-test. Statistical calculations were made with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA USA).

Results

Voluntary Running Wheel Activity

Prior to gestation, the animals averaged approximately 52.6±4.7 km/week (95% CI: 43.1-62.0). After breed, average weekly exercise distance in the first and second weeks of gestation was decreased (*$p<.05$) in comparison to average distance prior to gestation. This is likely a response to pregnancy, as virgin controls that failed to breed (n=4) resumed (39.3±3.5 km/week) their training regimen upon reintroduction to the activity cages. Average exercise distances prior to and during gestation are presented in **Figure 4.8**.

Further, a decrease (†$p<.05$) was observed in week 3 in all groups compared to average distance run in weeks 1 and 2, suggesting a gestational age dependent relationship. Moreover, no significant difference ($p>.05$) in gestational week 3 distances were observed between normal pregnant and RUPP-grouped cohorts.

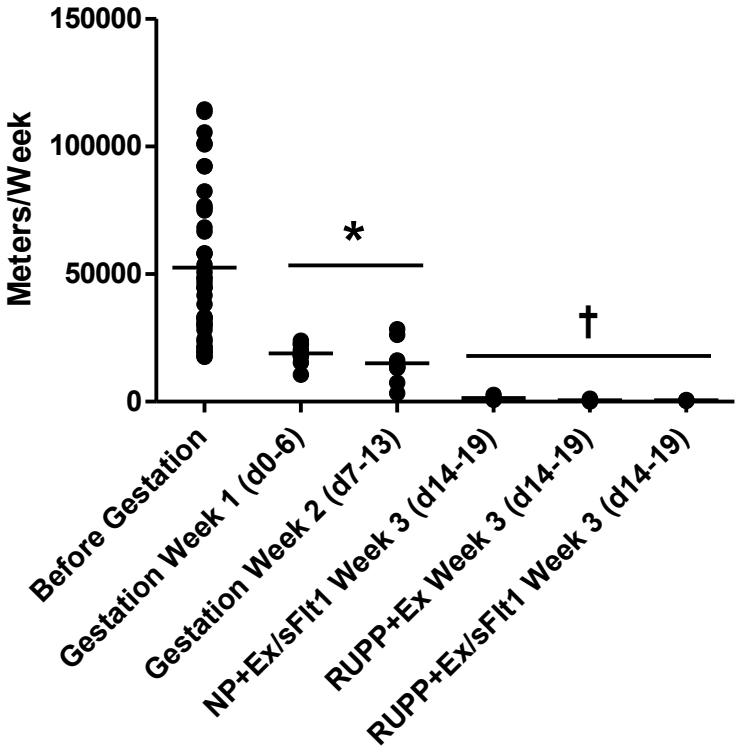

Figure 4.8. ***Weekly Exercise Distance.*** Across all animals, exercise distance per week was decreased (*p<.05) with pregnancy (Weeks 1 and 2). A further decrease (†p<.05) was observed in week 3 in all groups compared to weeks 1 and 2. *P<.05 vs. NP.

Mean Arterial Pressure

Infusion of rm-sFlt1 in NP animals resulted in an increased (p<.05) mean arterial pressure (MAP) when compared to the vehicle-treated NP controls (depicted in **Figure 4.9**). Additionally, exercise (EX) training attenuated this effect, as MAP was no longer elevated compared to NP control values. Similarly, RUPP caused an increase (p<.05) in MAP vs. NP, and Ex treatment obviated (p<.05) this effect despite infusion of rm-sFlt1.

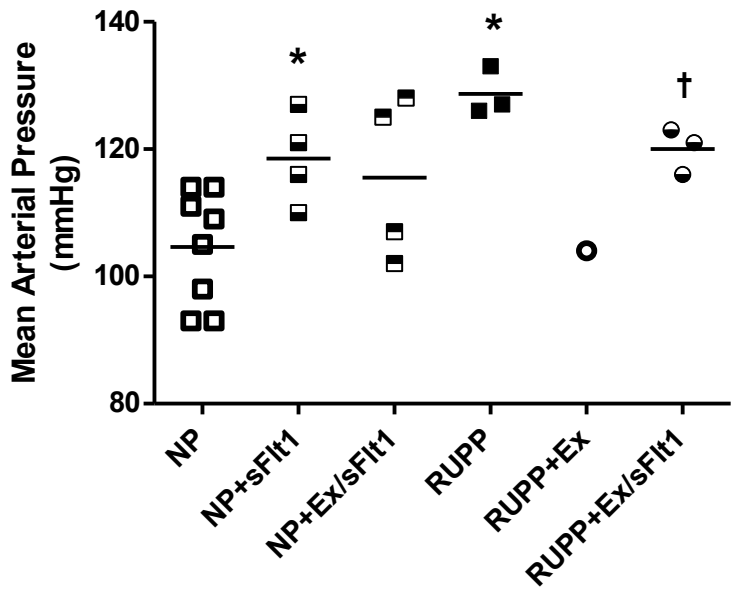

Figure 4.9. ***Mean Arterial Pressure on Day 19.*** Infusion of sFlt1 in normal pregnant dams increased (*p<.05) mean arterial pressure (MAP), and exercise (EX). RUPP had increased (*p<.05) MAP vs. NP, and Ex mitigated (†p<.05) RUPP hypertension despite a chronic infusion of sFlt-1. NP 105±9; *NP+sFlt 119±7; NP+Ex/sFlt 116±13mmHg; *RUPP 129±4; RUPP+Ex 104±N/A; †RUPP+Ex/sFlt 120±4mmHg. *p<.05 vs. NP; †p<.05 vs. RUPP. Data presented as mean±SD. RUPP+Ex sample size limited to one due to insufficient blood pressure recordings. NP, NP+sFlt1 and RUPP data is also reported in Chapter V, Part 3.

Vascular Endothelial Function

Endothelial function of mesenteric arterioles (200-400μm diameter) was assessed by a logarithmic dose-response curve to acetylcholine (Ach) following pre-constriction to thromboxane A_2 mimetic, U46619 (5μM). Normal pregnant (NP) dams receiving sFlt-1 infusion (NP+sFlt1) demonstrated a decreased (p<.05) relaxation potential compared to NP controls. Moreover, exercise training attenuated this effect, where no difference between NP+Ex/sFlt1 and NP was

observed. Additionally, RUPP caused a decrease in vasorelaxation to Ach compared to NP, which was also restored ($p<.05$) with Ex treatment. Moreover, Ex treatment improved ($p<.05$) vasorelaxation potential in the RUPP+Ex/sFlt1 cohort with respect to RUPP. Data is depicted in **Figure 4.10a-b**.

Plasma Free VEGF and sFlt-1Balance

As we have previously observed, free VEGF concentration in maternal plasma was decreased ($p<.05$) RUPP vs. NP controls. No effect of sFlt-1 infusion or exercise was observed in either NP or RUPP groups' VEGF levels. Data is depicted below in top panel of **Figure 4.11**. Likewise, sFlt-1 concentration in maternal plasma was increased ($p<.05$ vs. NP) with sFlt-1 infusion in the NP+sFlt1 dams, which confirmed successful peptide delivery (**Figure 4.11**, bottom left panel). No additional effect of exercise was observed in the NP animals. Similarly, the infusion of sFlt-1 in the RUPP+Ex/sFlt1 treatment group resulted in an increase ($p<.05$) in plasma sFlt-1 vs. RUPP controls.

Used as an indicator of angiogenic balance, the ratio of VEGF:sFlt-1 (**Figure 4.11**, bottom right panel) was decreased ($p<.05$) in NP+sFlt-1 and NP+Ex/sFlt-1 compared to NP controls. Additionally, the VEGF:sFlt-1 ratio in the RUPP+Ex/sFlt1 treatment was decreased ($p<.05$) vs. RUPP. Together, these data suggest the infusion of rm-sFlt1 maintained an elevated plasma sFlt-1, and suppression of VEGF:sFlt-1 balance through exercise treatment.

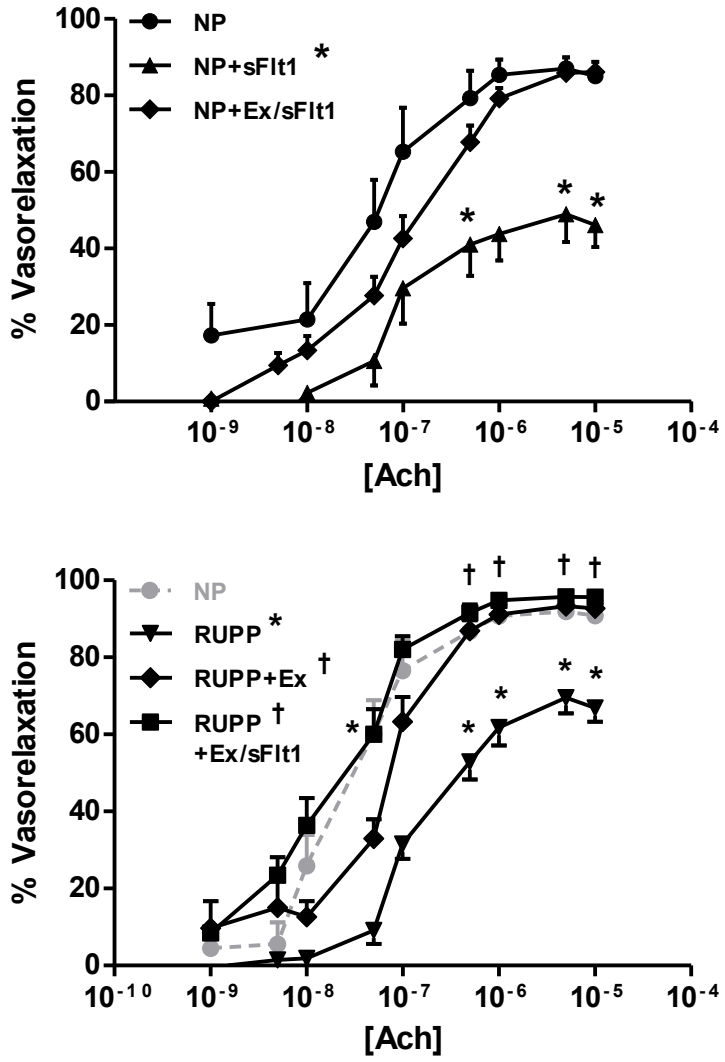

Figure 4.10. ***Mesenteric Vascular Endothelial Function.*** (Top Panel) Endothelial function measured by vasodilation of isolated mesentery arterioles to acetylcholine was improved by Ex in both NP (*p<.05) and (Bottom Panel) RUPP (†p<.05) groups, despite sFlt-1 infusion. No effects on smooth muscle function were observed in SNP curve (endothelial-independent vasodilation) (Data not shown). *p<.05 vs. NP; †p<.05 vs. RUPP. Data presented as mean±SD; n=4-8 per group. Data analyzed between groups across each dose. NP, NP+sFlt1 and RUPP data is also reported in Chapter V, Part 3.

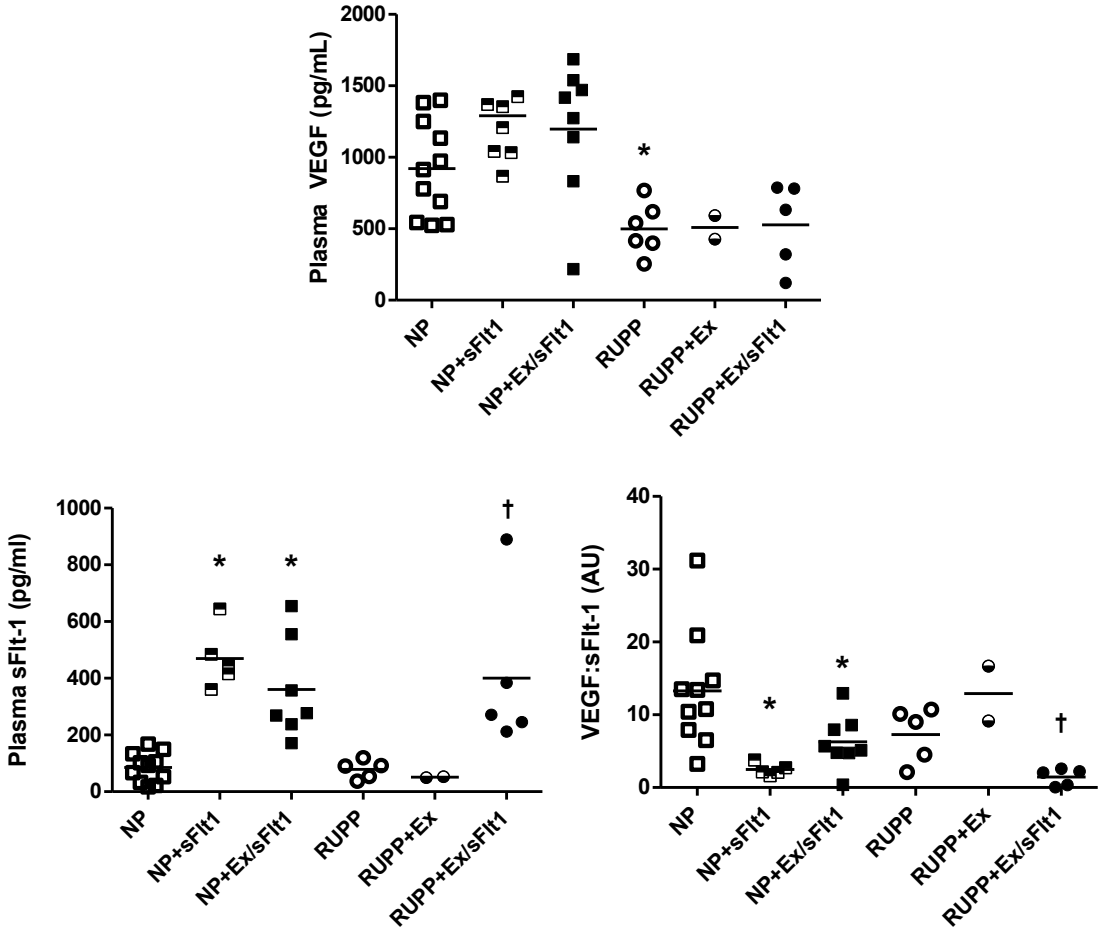

Figure 4.11. ***Plasma VEGF, sFlt-1, and VEGF:sFlt-1 Balance.*** (Top Panel) RUPP had decreased (*p<.05) bioavailable VEGF compared to NP. No effect of sFlt-1 infusion in either NP or RUPP groups were observed. NP 920±335; *NP+sFlt 1291±358; *NP/Ex+sFlt 1197±475; *RUPP 500±182; RUPP/Ex 510±117; RUPP/Ex+sFlt 528±297 pg/mL. (Bottom Left Panel) sFlt-1 was increased (*p<.05) in NP+sFlt1, and NP+Ex/sFlt1 vs. NP. Additionally, RUPP+Ex/sFlt1 treatment increased (†p<.05) plasma sFlt-1 vs. RUPP controls. NP 86±52; *NP+sFlt 470±108; *NP+Ex/sFlt 360±178; *RUPP 78±33; †RUPP+Ex 52±2; RUPP+Ex/sFlt 400±281pg/mL. (Bottom Right Panel) The ratio of bioavailable VEGF:sFlt-1 was decreased by sFlt-1 infusion in both NP+sFlt1 and NP+Ex/sFlt1 vs. NP controls. sFlt-1 infusion in RUPP+Ex/sFlt1 was further decreased compared to RUPP VEGF:sFlt-1 values. NP 13.3±8.0; *NP+sFlt 2.5±0.8; *NP+Ex/sFlt 6.3±3.6; *RUPP 7.3±3.8; †RUPP+Ex 12.9±N/A; RUPP+Ex/sFlt 1.4±1.2 AU. *p<.05 vs. NP; †p<.05 vs. RUPP. RUPP+Ex data are limited to n=2 due to insufficient sample availability. NP, NP+sFlt1 and RUPP data is also reported in Chapter V, Part 3.

Serum Angiogenic Potential

Serum angiogenic potential was assessed by culture of human vascular endothelial cells (HUVECs) in the presence of 5% (v/v) animal sera, followed by measurement of microtubule formation. Firstly, NP+sFlt1 exhibited a decreased ($p<.05$ vs. NP) average tubule size, and remained decreased ($p<.05$ vs. NP) in the NP+Ex/sFlt1 treatment group (see **Figure 4.12**). Further, serum from RUPP dams had a decreased tubule formation compared to NP controls. No additional effects of Ex or sFlt-1 infusion were observed in the RUPP group.

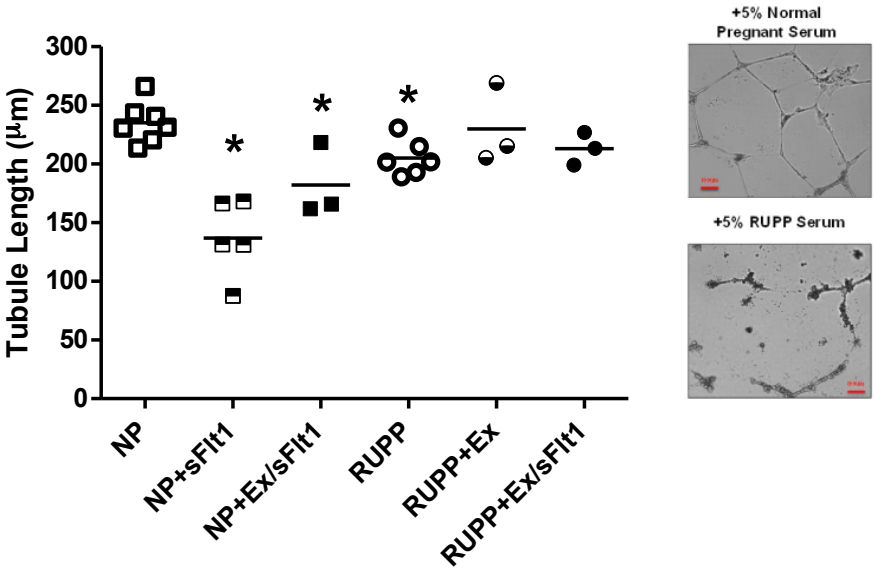

Figure 4.12. ***Serum Angiogenic Potential.*** Serum angiogenic potential was decreased in HUVEC cells exposed to serum from NP+sFlt, NP+Ex/sFlt1 and RUPP vs. NP (*$p<.05$). No additional effects were observed in the RUPP groups. NP 235±17; *NP+sFlt 137±33; NP+Ex/sFlt 182±31; *RUPP 205±15; †RUPP+Ex 230±34; RUPP+Ex/sFlt 213±14µm. A sample picture of NP vs. RUPP tubule formation is provided (scale bar = 100µm). *$p<.05$ vs. NP; †$p<.05$ vs. RUPP. Data presented as mean+SD. NP, NP+sFlt1 and RUPP data is also reported in Chapter V, Part.

Maternal and Fetal Morphometrics

No effect on maternal or conceptus morphometrics was observed between NP and NP+sFlt1. Interestingly, there was a decrease in maternal and fetal weight, as well as an increase in placental weight and fetal demise in NP+Ex/sFlt1 vs. NP. Also, as we have previously reported, RUPP caused a decrease ($p<.05$) in maternal, fetal, and placental weight compared to NP, and increased ($p<.05$ vs. NP) fetal demise rate. Ex treatment obviated ($p<.05$) resorption rate in RUPP+Ex compared to RUPP, and sFlt-1 infusion prevented this effect. Data is summarized below in **Table 4.2**.

Table 4.2. ***Maternal and Conceptus Morphometric Data at Necropsy***.

Treatments	*Maternal Weight (g)*	*Fetal Weight (g)*	*Placental Weight (g)*	*% Resorption*
NP (n=10)	311±30	2.25±0.12	0.46±0.03	2.6±5.8
NP+sFlt1 (n=8)	288±16	2.27±0.24	0.49±0.08	0.9±2.4
NP+Ex/sFlt1 (n=9)	*277±34	*2.06±0.32	*0.60±0.19	*26.8±35.4
RUPP (n=10)	*259±19	*1.79±0.26	*0.36±0.07	*68.1±23.7
RUPP+Ex (n=3)	260±18	1.98±0.06	0.39±0.04	†34.4±22.1
RUPP+Ex/sFlt1 (n=7)	245±33	1.90±0.35	0.40±0.07	46.9±1.2

Note. *$p<.05$ indicates different from NP. †$p<.05$ indicates different from RUPP. Data presented as mean±SD. NP, NP+sFlt1 and RUPP data is also reported in Chapter V, Part 3.

Discussion

To verify the role of the VEGF:sFlt-1 axis and angiogenic balance in the anti-hypertensive effects of exercise training before and during pregnancy, we employed an infusion of recombinant sFlt-1 on NP and RUPP to induce and maintain suppression of bioavailable VEGF throughout exercise treatment. It was hypothesized that the effects of exercise training in the RUPP model is dependent on the stimulation of bioavailable VEGF. To this end, we sought to antagonize VEGF signaling with an infusion of recombinant sFlt-1. As intended, sFlt-1 infusion sustained a suppression of bioavailable sFlt-1 and VEGF:sFlt-1 balance in both NP and RUPP cohorts through exercise treatment, and permitted us to isolate the role of the VEGF:sFlt-1 axis in both NP and RUPP sub-groups. Interestingly, these data collectively do not support our initial hypothesis, where exercise treatment mitigated RUPP-induced hypertension and endothelial dysfunction despite the infusion of sFlt-1 and decreased free VEGF:sFlt-1.

Blood Pressure and Exercise in Pregnancy

As previously reported by Gilbert and colleagues [27], exercise is observed to attenuate RUPP induced hypertension. Though in the current study, RUPP+Ex treatment had insufficient evidence to confirm this previous report, we observed exercise training prior to and during gestation to attenuate the RUPP hypertension despite the infusion of recombinant sFlt-1. This suggests the anti-

hypertensive effects may be independent of VEGF:sFlt-1 imbalance. Interestingly, this effect was not observed in the NP cohorts receiving sFlt-1 infusion, which suggests the effects are largely influenced by the presence of placental hypoperfusion due to RUPP. Further it is currently unclear whether these disparate effects from exercise training indicate a role of compromised placental blood flow in the anti-hypertensive mechanism, and further investigation is required.

In regard to voluntary exercise distances reported in this study, we observed a significant decrease in weekly exercise distance per week during weeks 1-2 compared to before gestation, and even further in week 3. This decrease in activity has previously observed in gestation week 3 in normal pregnant, sham controls, and RUPP dams [27,75], so the decrease in voluntary activity levels is likely not due to the sham or RUPP surgical procedures. Similarly observed in women, a decrease in voluntary activity has also been reported in exercise-trained pregnant women, who have a decrease in activity in early pregnancy, and a further decrease or indolence in the third trimester [161]. Moreover, the effects of exercise training on blood pressure are present despite a decrease in activity in week 3, which indicates the cardiovascular benefits and adaptations are likely mediated by the previous weeks' training levels.

Exercise, Angiogenic Balance, and Endothelial Function

The role of angiogenic balance, most notably through the VEGF/PlGF:sFlt-1 axis, has been a large focus of the preeclampsia research since Karumanchi's seminal report on the role of sFlt-1 over a decade ago [20]. Though the direct role of VEGF/PlGF replacement in experimental models of preeclampsia is quite clear [28,70,71], it is unclear whether this is the central mechanism for other modalities, such as exercise.

In regard to angiogenic balance, we observed a decrease in bioavailable VEGF, increase in sFlt-1, and/or decreased VEGF:sFlt-1 in the RUPP and sFlt-1 infused NP cohorts. Firstly, exercise did not improve VEGF bioavailability in the NP+Ex/sFlt1 or RUPP+Ex/sFlt1 cohorts. Similarly, endogenous plasma sFlt-1 and VEGF:sFlt-1 in plasma was also unaffected by exercise training or sFlt-1 infusion in the NP and RUPP, which has also been observed in experimental models of PE [118]. Moreover, in concert with the blood pressure data, these results suggest improved angiogenic balance in the VEGF-sFlt1 axis is not required for exercise to prevent the RUPP hypertension. Further, these data contrast with our previous hypothesis and reports [27], and suggest the anti-hypertensive effect of exercise in the RUPP may not be primarily mediated through the plasma VEGF bioavailability or sFlt-1 suppression.

Additionally, a potential convoluting point in this study is the voluntary access to the activity wheels, in which weekly exercise varied (though modestly) between animals. This in itself raises the question of an exercise dose response, and for the potential to control endogenous VEGF production. Moreover, there was no observed association between VEGF and sFlt1 expression and exercise distance (a) prior to gestation, and (b) exercise distance in week 3 of gestation (**Figure 4.13**). In summary, we did not observe any effect of either distance on the expression of VEGF or sFlt-1, as the effects appear to be dichotomous. Furthermore, the minimum training threshold to gain the cardiovascular benefits is still unknown, and would be an intriguing and elucidating investigation for the translation of the current and previous reports.

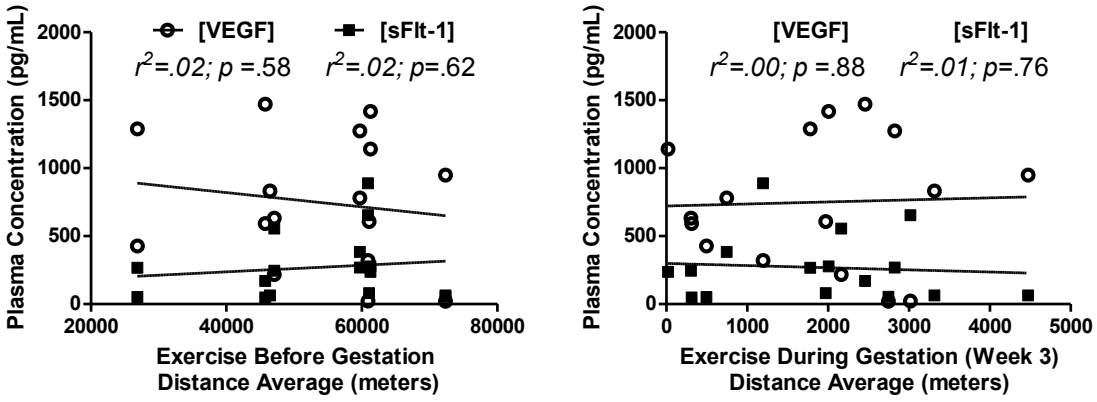

Figure 4.13. ***Relationship Between Weekly Exercise Distance and Plasma Angiogenic Factor Balance.*** Across both NP+Ex and RUPP+Ex cohorts, no relationship is observed between average weekly exercise distance prior to gestation (top panel) and during week 3 of gestation (bottom panel) and plasma concentrations of sFlt-1 and VEGF at day 19 of gestation. Data analyzed by linear regression.

Regarding the effect on endothelial function, exercise training improved vascular endothelial function in both RUPP and sFlt-infused NP dams. Further, in the RUPP cohort, endothelial function was improved regardless of sFlt-1 infusion, which further suggests these effects are not dependent on the restoration of normal VEGF:sFlt-1 plasma levels and may rely on other controlling mechanisms. Similarly, serum angiogenic potential was observed to be improved by exercise in both NP+sFlt1 and RUPP groups, regardless of sFlt-1 infusion, suggesting endothelial cell function is improved. These data also suggest an angiogenic balance may actually be restored in the sera of the treated animals, as tubule formation was improved with exercise. Together, although merely speculative at this point, these measurements of endothelial function support the role of a pathway alternative to VEGF signaling that is mediating the observed cardiovascular effects.

Study Limitations

It should be noted that the validity and "methodological consistency" of the ELISA-measured angiogenic factors, particularly VEGF, have been recently called into question by Weissengerber and colleagues. [162]. While we did not observe the wide variability and difficulty in obtaining comparable values in free VEGF, we would like to recognize that this currently is an area of concern in the preeclampsia research field. We also failed to replicate our previous published

observations in the RUPP in regard to the plasma sFlt-1 levels. Without these effects in RUPP, the interpretation and comparative quality of the angiogenic effects in RUPP should be taken into consideration in future work. Moreover, to further supplement the measurement of angiogenic balance, additional measurements of circulating sFlt-1 and serum angiogenic potential (tubule formation assay) were performed. However, we feel this is sufficient experimental evidence to appropriately interpret the current data at this time.

Additionally, the measurement of sFlt-1 was limited to one measurement at day 19, and the timing of delivery remains unknown without repeated measurements after the introduction of sFlt-1. To this end, due to observation to of increased sFlt-1 levels at day 19 in the sFlt-infused animals, we are confident in the method of delivery and the presence of circulating sFlt-1 in these animals.

Conclusion

Collectively, these data support that the anti-hypertensive effects of exercise in the RUPP model were not dependent on the restoration of VEGF:sFlt-1 balance in maternal plasma. More importantly, in a syndrome with very limited options in both prevention and treatment modalities, the previously reported beneficial effects of exercise in preventing preeclamptic-like hypertension are further supported by this study. Though the potential clinical implications are exciting, this study confirms the molecular contributions to

these effects should be further evaluated before exercise training is indicated for preeclampsia prevention. Likewise, if these the pathways activated during exercise are revealed, potential pharmacological modalities could be further explored as well. Furthermore, a potential pathway in which exercise could be functioning through will be examined in the next chapter, in which a parallel study of AMP-activated protein kinase (AMPK) mediated effects will be evaluated in the RUPP model.

CHAPTER V

5-AMINOIMIDAZOLE-4-CARBOXAMIDE-1-β-D-RIBOFURANOSIDE TREATMENT IN A RAT MODEL OF PLACENTAL ISCHEMIA INDUCED HYPERTENSION

In Chapter V, a portion of the data in Part 1 was published in 2012 in the *American Journal of Physiology - Heart and Circulation*, and Ashley J. Bauer, Karen W. Needham, Hans C. Dreyer, and Jeffrey S. Gilbert are co-authors. I performed the experimental work along with the technical assistance of A.J. Bauer and K.W. Needham. H.C. Dreyer and J.S. Gilbert provided editorial assistance. J.S. Gilbert provided project funding.

Parts 2-3 of Chapter V are in preparation for publication with Haley E. Gillham, Sarah M. Johnson, Karen W. Needham, and Jeffrey S. Gilbert as co-authors. I performed the experimental work with the technical assistance of co-authors H.E. Gillham, S.M. Johnson, and K.W. Needham. J.S. Gilbert provided project funding.

PART 1: 5-AMINOIMIDAZOLE-4-CARBOXAMIDE-1-β-D-RIBOFURANOSIDE (AICAR) ADMINISTRATION TO IMPROVE VEGF BIOAVAILABILITY AND PREVENT HYPERTENSION INDUCED BY PLACENTAL ISCHEMIA

Introduction

Preeclampsia, a pregnancy-specific syndrome, is the leading cause of fetal and maternal morbidity and mortality across the world, and unfortunately the incidence has been increasing in recent decades [10,40]. Currently, there are limited treatments available, and early delivery is often indicated to prevent further progression of the syndrome. Consequently, preeclampsia is the leading cause of pre-mature delivery, which is well-known to have numerous deleterious effects on perinatal and life-long health [10,40].

Recent clinical and experimental studies suggest dysregulation of circulating angiogenic factors results in an angiogenic imbalance that is most notably characterized by an altered ratio of pro-angiogenic (e.g. vascular endothelial growth factor; VEGF) and anti-angiogenic factors (e.g. soluble VEGF receptor-1; sFlt-1). An imbalance favoring increased anti-angiogenic factors has been strongly linked to the etiology of hypertension during preeclampsia [20,28,29,151]. Recent studies also indicate restoration of angiogenic balance can mitigate an increase in blood pressure observed in several rodent models of

preeclampsia [20,22,27,28,163]. Moreover, recent experimental and clinical observations of hypertension in pregnancy and preeclampsia have shown exercise can mitigate hypertension, restore angiogenic balance, and endothelial function [27,119]. Despite these recent findings, the exact mechanisms by which physical activity improves angiogenic balance and decreases blood pressure in pregnancy remain unclear [27,75].

Recent studies have revealed some of the metabolic changes observed due to exercise training can be stimulated pharmacologically [139,164]. Of the several pharmacological models of exercise in recent literature, AMP-activated protein kinase (AMPK) has been reported as a major regulator of or contributor to several essential metabolic adaptations observed in exercise [35,36,165-168]. Moreover, the pharmacological activation of AMPK through 5-aminoimidazole-4-carboxamide-3-ribonucleoside (i.e. AICAR) [139,164], an adenosine mimetic [37,169], and has been shown to decrease mean arterial pressure in hypertensive rats [34]. Further, activation of the AMPK pathway has been shown to regulate increases in VEGF expression [127], but whether or not this occurs with AICAR administration remains unknown. Therefore, we hypothesized (**Figure 5.1**) AICAR, a potent AMPK stimulator, would stimulate the bioavailability of VEGF in circulation, improved endothelial function, and, most importantly, abrogate the placental ischemia-induced hypertension.

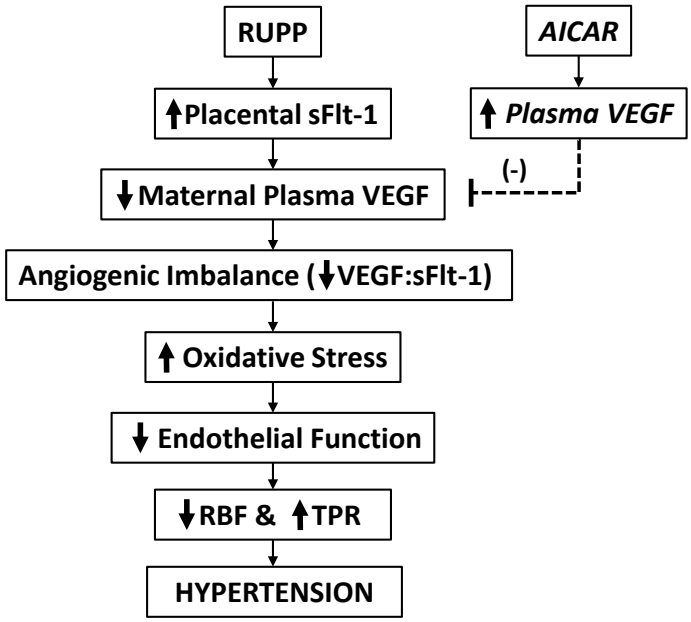

Figure 5.1. ***Overarching AICAR Hypothesis***. RUPP-induced hypertension and endothelial dysfunction is expected to be ameliorated by AICAR treatment and the concomitant VEGF stimulation. RBF: Renal blood flow; TPR: Total Peripheral Resistance

Methods

Animals

Studies were performed in timed-pregnant Sprague-Dawley rats purchased from Charles River (Wilmington, MA) or Harlan (Indianapolis, IN). Animals were housed in a temperature-controlled room (23°C) with a 12:12 light:dark cycle. All experimental procedures executed were completed in accordance with National Institutes of Health guidelines for use and care of animals and were approved by the Institutional Animal Care and Use Committee at both University of Minnesota and the University of Oregon. Dams

were assigned to normal pregnancy (NP) (n=8) and RUPP (n=8) groups with and without AICAR (Santa Cruz Biotechnology Inc., Santa Cruz, CA) treatment (NP+A (n=6); RUPP+A (n=8)). AICAR treatment was introduced by intraperitoneal (i.p.) injection at 50mg/kg twice a day [34,36,139] mixed in 0.9% sterile saline solution, and controls were treated with a 0.9% sterile saline solution vehicle. Half of the *in vivo* studies for each group were completed at each institution.

Reduced Uterine Perfusion Pressure (RUPP) Procedure

The RUPP procedure is a robust model for studying the link between placental ischemia and hypertension in the pregnant rat and has been described in detail previously [27,151]. In brief, silver clips were placed on the lower abdominal aorta (0.203-mm inner diameter (ID)) above the iliac bifurcation and also on branches (0.100 mm ID) of both the right and left ovarian arteries supplying the uterus on day 14 of gestation (term = 21 days). Four normal pregnant dams underwent a sham surgery, which included the midline incision and suture. After observing no differences in the angiogenic factors and blood pressures these animals were grouped with the normal pregnant rats.

Measurement of MAP in chronically instrumented conscious rats

Animals were instrumented on day 17 of gestation with an indwelling catheter and arterial pressure was determined in both groups of rats on day 19 of

gestation as described previously [27]. Briefly, on day 17, V-3 tubing (SCI) catheters we introduced to the left common carotid artery while the animal was under isoflurane anesthesia. Catheters were exteriorized through the back of the neck following subcutaneous tunneling. On day 19, animals were placed in restraining cages, and direct pressures were monitored using a blood pressure transducer (ADInstruments) for one hour following a 30 minute stabilization period [19]. Mean arterial pressure (MAP) was averaged over the hour time period. Additionally, heart rate was analyzed over the hour period from the arterial pressure measurements using LabChart 7.0.

Conceptus Measurements and Serum Collection

After the measurement of MAP, the dams were placed under isoflurane anesthesia and a midline ventral incision was made to isolate the abdominal aorta for plasma and serum collection as reported previously [27,151]. Blood was collected for subsequent assays into Corvac® sterile serum separator tubes (Sherwood Davis, St. Louis, MO). Blood and amniotic fluid glucose concentrations were measured as reported previously [152]. Fetal weight, placental weight, and number of resorptions were recorded in the manner described previously [27,151]. All collected tissues for subsequent analysis were flash frozen in liquid nitrogen and stored at -80˚C.

Enzyme-Linked Immunosorbant Assays

Plasma concentrations of free VEGF and sFlt-1 were measured using commercial enzyme linked immunosorbant assay kits (R&D Systems, Quantikine®; Minneapolis, MN) according to the manufacturer's directions as described previously [27,151].Oxidative stress assays

Protein Extraction and Quantitation

As described previously [27,75], total soluble protein was extracted from whole placentas in radioimmunoprecipitation assay (RIPA) lysis buffer containing phenylmethanesulphonylfluoride (PMSF) in dimethyl sulfoxide (DMSO), sodium orthovanadate and a protease inhibitor cocktail (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Total soluble cellular protein concentration was determined using the bicinchoninic acid (BCA) method (Pierce Biotechnology, Rockford, IL). Renal tissue position (right or left) was chosen at random, and placental samples were carefully selected for middle position on either side of the uterine horns [27,75].

Western Blotting

Western blotting was carried out as previously reported [75,170]. Protein (50 μg) was separated by electrophoresis on 4-20% sodium dodecyl sulfate (SDS) polyacrylamide separating gels (Life Technologies, Grand Island, NY) then transferred to nitrocellulose membranes (BioRad, Hercules, CA) and Ponceau

stained to assess the transfer across each gel. The images of the Ponceau stained membranes were digitized with a flatbed scanner.

The membranes were then incubated one hour in casein blocking solution (BioRad). Membranes were incubated in blocking solution containing commercially available antibodies (0.1μg/mL) overnight at 4˚C (phospho(Thr172) -AMPKα (40H9);AMPKα (23A3); Cell Signaling Technology). Membranes were washed and incubated for one hour with the appropriate fluorescent protein-conjugated secondary antibodies (7.5ng/ml, LI-COR Biosciences, USA). The immunoreactive bands were imaged and quantified using the Odyssey dual-channel fluorescent imaging system and Image Studio 2.0 software (LI-COR Biosciences, USA). Specificity of primary antibodies (negative controls) was evaluated by imaging membranes with the primary antibody omitted.

Endothelial Tube Formation Assay

Angiogenic balance was further assessed in the serum of pregnant rats *in vitro* as previously reported [27] in two separate experiments and each was performed in duplicate. Firstly, the sera collected from the treatment groups (NP, RUPP, NP+A, RUPP+A) were introduced to human umbilical vascular endothelial cell (HUVEC) solution (plated at $5x10^5$ cells/mL) and monitored for eight hours for tubule formation. In addition, the direct effect of AICAR was also assessed by treating cells with serum from NP and RUPP and adding 20μM

AICAR directly to the media. Total number of tubule formations per frame was assessed at 40X optical zoom with a digital inverted compound microscope and ImageJ analysis software (National Institutes of Health, Bethesda, MD). Total tube count was assessed by at least two individual investigators that were blinded to the identity of the experimental groups. Values from each observer were averaged to obtain final counts.

Statistical Analysis and Calculations

All data are presented as mean ± SD, and statistical significance was accepted when $p<.05$. Comparisons were made with a one-way analysis of variance test combined with a Bonferroni post-hoc test were employed. Statistical calculations were made with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA USA).

Results

Effect of AICAR on Blood Pressure and Heart Rate

RUPP hypertension was ameliorated with AICAR ($p<.05$) (**Figure 5.2**), and AICAR had no effect on NP blood pressure. Additionally, there were no differences in resting heart rate observed between treatment groups (Mean+SD: NP 394±18; RUPP 437±22; NP+A 451±10; RUPP+A 441±19 beats per minute).

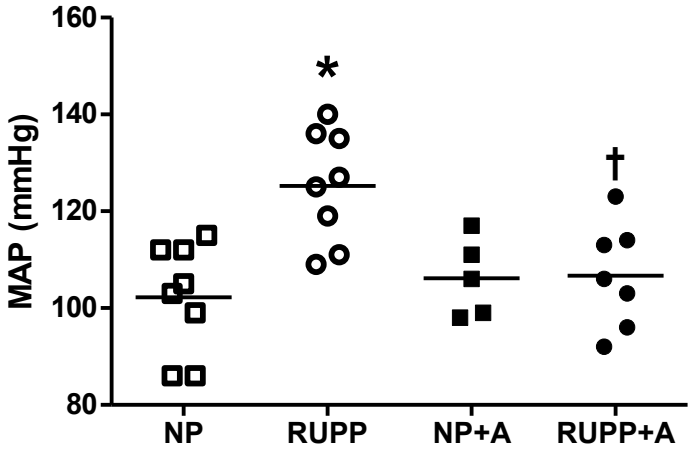

Figure 5.2. ***Mean Arterial Pressure on Day 19***. The RUPP-induced increase in blood pressure was ameliorated with AICAR (50mg/kg b.i.d.) treatment. Mean+SD: NP 102±11; *RUPP 125±12; NP+A 106±8; †RUPP+A 107±11mmHg. *p<.05 vs. NP; †p<.05 vs. RUPP.

Effects of AICAR on Plasma VEGF, sFlt-1, and Serum Angiogenic Potential

Free VEGF levels in maternal plasma were decreased (p<.05) in RUPP vs. NP, and AICAR increased (p<.05) VEGF in the RUPP when compared to RUPP untreated. Plasma sFlt-1 levels were increased (p<.05) in RUPP vs. NP, and RUPP+AICAR circulating sFlt-1 was decreased (p<.05) compared to RUPP. Data is depicted in **Figure 5.3**. Moreover, angiogenic potential was increased (p<.05) with AICAR treatment in the RUPP compared to the untreated RUPP (Figure 3A). Also, there was no difference observed between the NP and NP+A. Moreover, AICAR did not have a direct effect on HUVEC tubule formation when added to NP or RUPP sera-treated cells (**Figure 5.4**).

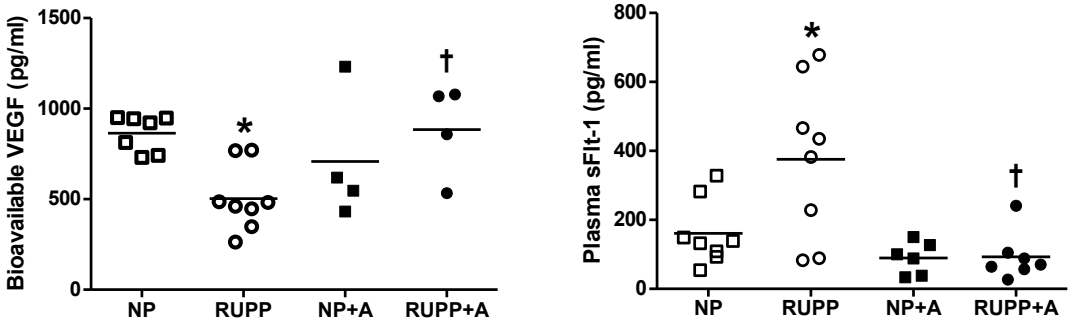

Figure 5.3 ***Plasma Concentration of Free VEGF and sFlt-1.*** (Left Panel) Free VEGF in maternal plasma was decreased (*p<.05) in the RUPP, and improved (†p<.05) in the RUPP+AICAR. Mean+SD: NP 864±100; *RUPP 503±181; NP+A 708±358; †RUPP+A 884±255 pg/mL. (Right Panel) Plasma sFlt-1 was restored back to NP levels in the RUPP treated with AICAR, and significantly decreased (P<.05) compared to RUPP controls. Mean+SD: NP 161±95; *RUPP 376±228; NP+A 93±70; †RUPP+A 89±47 pg/mL. *p<.05 vs. NP; †p<.05 vs. RUPP.

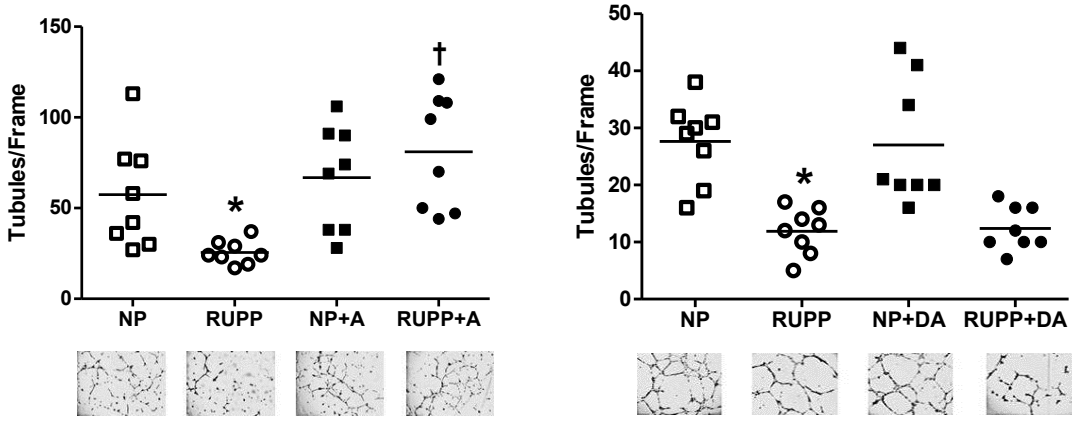

Figure 5.4. ***Serum Angiogenic Potential and Direct Angiogenic Effect of AICAR.*** (Left Panel) Angiogenic potential measured by HUVEC microtubule formation was decreased in RUPP serum vs. NP, and AICAR improved (†p<.05) tubule number in RUPP+A serum. Mean+SD: NP 57±30; *RUPP 26±7; NP+A 67±29; †RUPP+A 81±32 tubules/frame. (Right Panel) Angiogenic potential of NP or RUPP serum was not affected by the addition of AICAR directly (DA) into the culture media. Mean+SD: NP 28±7; *RUPP 12±4; NP+DA 12±4; RUPP+DA 27±11 tubules/frame. *p<.05 vs. NP; †p<.05 vs. RUPP.

Measurement of Anti-Oxidant Capacity and Oxidative Stress

Placental Trolox-equivalent antioxidant capacity (TEAC) levels were decreased in the RUPP compared to the NP ($p<.05$), and AICAR treatment obviated the decrease in the RUPP+A (**Figure 5.5a**). No effect on TEAC in the kidney was observed (**Figure 5.5b**). Additionally, placental (**Figure 5.5c**) and renal (**Figure 5.5d**) catalase activity (measured by formaldehyde production) was increased by RUPP, and decreased ($p<.05$) in RUPP+A with respect to the RUPP group.

Tissue AMPK Activation

The ratio of phosphorylated AMPKα (pAMPKα) to total AMPKα, was increased with AICAR treatment in RUPP placenta ($p<.05$) compared to untreated RUPP, NP, and NP+AICAR animals (**Figure 5.6**).

Maternal and Fetal Morphometric and Metabolic Data at Necropsy

Maternal weights and fetal weights were decreased ($p<.05$) in the RUPP compared to NP, and AICAR had no significant effect. Placental weight was decreased ($p<.05$) in RUPP vs. NP, and AICAR mitigated this decrease. Total litter viability per cent was decreased ($p<.05$) in RUPP vs. NP, and AICAR remediated the RUPP resorption. Lastly, blood glucose and amniotic fluid glucose were not changed with AICAR treatment in either normal pregnant or RUPP. The previous data is summarized in **Table 5.1**.

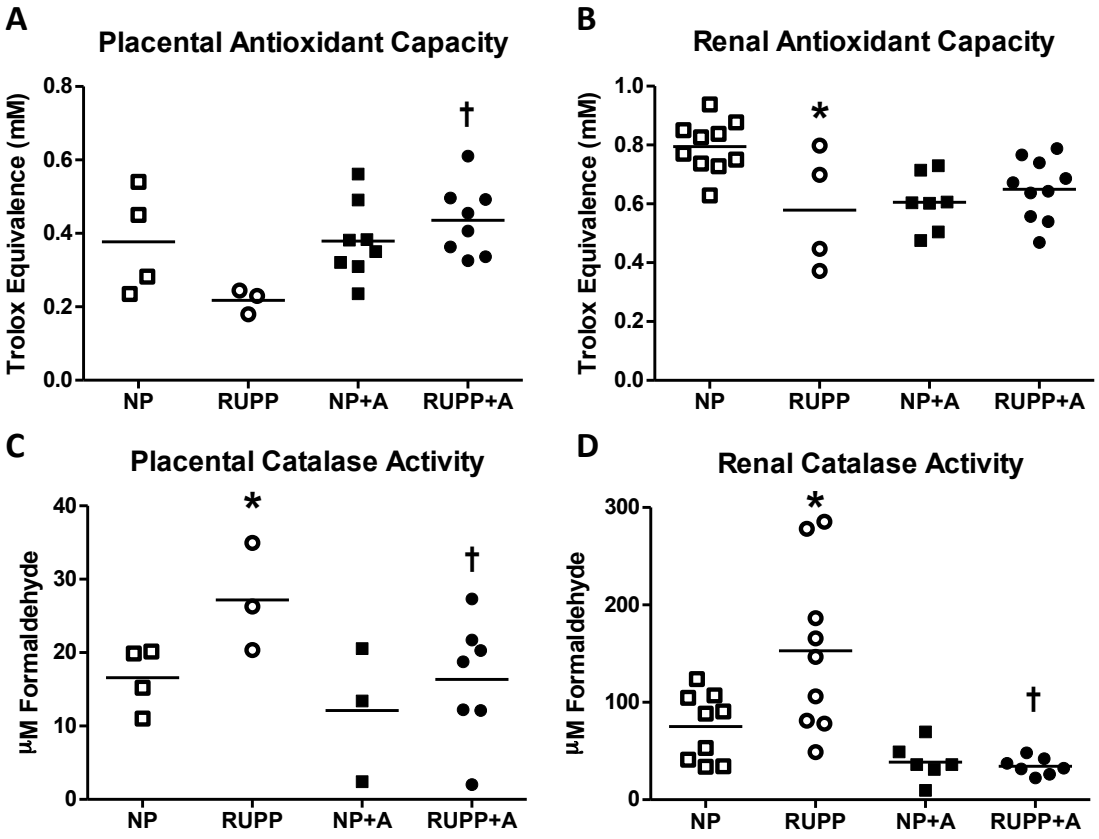

Figure 5.5. ***Placental and Renal Anti-Oxidant Capacity and Oxidative Stress.*** (Panel A) Trolox-equivalent antioxidant capacity (TEAC) levels were decreased in the RUPP placenta compared to the NP (*p<.05), and AICAR obviated (†p<.05) the decrease in the RUPP+A. Mean+SD: NP 0.38±0.14; *RUPP 0.20±0.05; NP+A 0.38±0.09; †RUPP+A 0.48±0.13 mM. (Panel B) Renal TEAC levels were decreased (*p<.05) in RUPP vs. NP, and unaffected by AICAR treatment in RUPP. NP 0.79±0.09; *RUPP 0.61±0.10; NP+A 0.58±0.20; †RUPP+A 0.65±0.10 mM.. (Panel C) Placental catalase activity (measured by formaldehyde production) was increased (*p<.05) in the RUPP vs. NP, and decreased (†p<.05) in RUPP+A with respect to RUPP. Mean+SD: NP 16.6±4.3; *RUPP 27.2±6.0; NP+A 17.1±5.1; †RUPP+A 18.2±4.2 µM. (Panel D) Renal catalase activity was increased (*p<.05) in the RUPP vs. NP, and remediated (†p<.05) in RUPP+A with respect to RUPP. Mean+SD: NP 75.2±34.8; *RUPP 152.9±85.4; NP+A 38.6±19.9; †RUPP+A 34.3±9.0 µM. *p<.05 vs. NP; †p<.05 vs. RUPP.

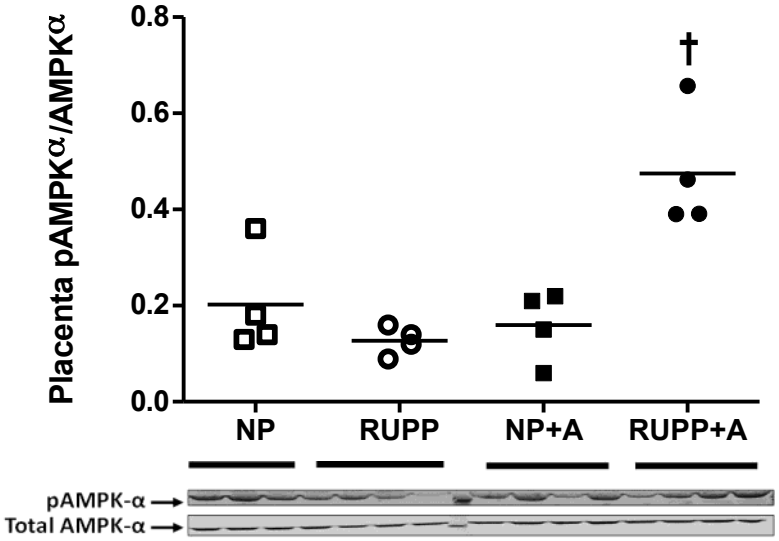

Figure 5.6. ***Placental AMPK Phosphorylation.*** The ratio of phosphorylated AMPKα (pAMPKα) to total AMPKα was increased (†p<.05) with AICAR in RUPP+A placenta compared to RUPP. Mean+SD: NP 0.19±0.08; *RUPP 0.18±0.09; NP+A 0.16±0.07; †RUPP+A 0.33±0.18 μM.. *p<.05 vs. NP; †p<.05 vs. RUPP.

Discussion

The present study is the first to investigate the treatment of placental ischemia induced hypertension with 5-aminoimidazole-4-carboxamide-3-ribonucleoside (AICAR), which has revealed novel evidence of restoration of angiogenic balance and abrogation of hypertension in an experimental model of hypertension in preeclampsia. Foremost, the hypertension observed in our reduced uterine perfusion pressure (RUPP) model was abrogated with intraperitoneal 50mg/kg b.i.d. AICAR treatment. Secondly, maternal angiogenic balance was restored in the RUPP following AICAR treatment, by both

increasing the circulating free VEGF and decreasing circulating sFlt-1. These observations were further supported by the increased microtubule formation in HUVECs treated with serum from the RUPP+AICAR vs. RUPP, which indicates an increased angiogenic potential and suggests an increased endothelial function with AICAR in the RUPP. Next, the RUPP-associated increase in oxidative stress markers were attenuated in both placenta and kidney when treated with AICAR. When viewed in concert, these findings suggest AICAR treatment may have several beneficial effects on the hypertensive phenotype of a model of placental ischemia-induced hypertension.

Table 5.1. ***Maternal and Conceptus Morphometric and Metabolic Data.***

Treatments	*Maternal Weight (g)*	*Fetal Weight (g)*	*Placental Weight (g)*	*% Resorption*	*Maternal Blood Glucose (mg/dL)*	*Amniotic Fluid Glucose (mg/dL)*
NP (n=8)	337.8±4.5	2.27±0.04	0.48±0.01	95.6±1.9	82.6±7.4	137.4±15.1
RUPP (n=8)	*294.5±10.8	*2.05±0.06	*0.42±0.02	*47.9±8.5	101.7±7.7	129.8±13.6
NP+A (n=5)	342.3±7.7	2.34±0.05	0.50±0.03	98.8±1.2	81.0±13.8	166.7±33.5
RUPP+A (n=7)	*305.0±4.7	*2.08±0.06	†0.46±0.02	†69.8±11.1	79.0±9.2	99.7±31.7

Note. *p<.05 indicates different from NP. †p<.05 indicates different from RUPP. Data presented as mean±SD.

Effects of AICAR on Blood Pressure and Endothelial Function

Administration of an adenosine mimetics similar to AICAR, such as Metformin, has long been recognized to improve cardiovascular function and lower blood pressure in non-pregnant hypertensives [33,131], but the mechanisms remain uncharacterized [31-33,171]. Further, recent studies have shown AICAR can directly improve endothelial function and lower blood pressure in rodent models of hypertension and improved conduit vessel function [34,172]. Therefore, our observation that AICAR treatment lowers blood pressure is in agreement with previous reports in other models of hypertension [34-36], and we are the first to report these effects in any model of hypertension during pregnancy.

It has been noted that the blood pressure effects observed in this model could potentially be directly from AICAR interaction on the vascular endothelial cells, as endothelial-cell-mediated vasorelaxation to AICAR has been shown to be dependent on both NO and EDHF production [172]. However, the vasodilatory properties observed by Ford and colleagues *ex vivo* are transient [172], and the mechanisms mediating chronic effects of AICAR remain unclear [35]. In the present study blood pressure was recorded more than 12 hours after the final intraperitoneal injection of AICAR, suggesting the maternal cardiovascular effects of AICAR in the present study were due to chronic signaling changes such as restoration of angiogenic balance rather than previously reported acute

mechanisms [172]. In particular, our present data suggests the effects of AICAR administration may be due to chronic changes in factors such as circulating free VEGF and sFlt-1 concentrations that in turn can influence cardiovascular and renal function, rather than direct, acute vasodilatory effects of AICAR administration. These observations are further supported by our tube formation data which shows chronic *in vivo* treatment alters circulating factors and increases tubule formation while acute treatment with AICAR in the media has no effect. This also suggests that cells other than ECs may be responsible for the changes that promote tube formation. Further studies are underway to identify which cells respond to AICAR in a manner consistent with pro-angiogenic function.

It has been well established AICAR can mimic many of the metabolic effects observed in moderate physical activity, such as mitochondrial biogenesis [166-168], decreased lipogenesis [139], increased glucose tolerance [35,36,139,165,168,173], and AMPK stimulation [139,165,172,174]. Moreover, our lab has recently reported exercise training prior to and during gestation has several beneficial effects on maternal blood pressure and angiogenic balance [27]. While the present study reports that AICAR administration appears to have similar cardiovascular and angiogenic balance effects as exercise in RUPP animals [27,75], an interesting difference is AICAR did not directly stimulate the production of VEGF in the normal

pregnant rats [27]. In comparison to a recent study of adenosine administration and hypoxia-dependent mechanisms in placental explants [143], the current study also suggests these effects on the angiogenic factors may be ischemia- or hypoxia-dependent. In addition, this study also suggests gestational AICAR treatment may hold promise as a therapeutic independent of its use as a mimetic of exercise. While these findings are intriguing, further studies are required to further elucidate the mechanisms by which AICAR lowers blood pressure and restores angiogenic balance.

Angiogenic Imbalance and AICAR

The effects of AICAR on angiogenic balance present an interesting story as AICAR administration appears to regulate both VEGF and sFlt-1. Indeed, our initial hypothesis only considered effects on VEGF via AMPK activation, as the regulation of sFlt-1 via AMPK is unclear at this point. Also, it is important to note the changes following AICAR administration on the angiogenic balance and blood pressure were only observed in the RUPP, and not the normal pregnant (NP). This further suggests a possible mechanism that is mediated by the placental ischemia and/or hypoxia. Interestingly, AICAR has been used to improve ischemia-reperfusion injury in cardiac tissue during coronary bypass surgery [175], which, unsurprisingly, may have similar effects in other tissues such as the placenta. Moreover, these specific placental ischemia-dependent

mechanisms have been proposed before in *in vitro* models [143], but, we are the first to report this effect of adenosine-mimetic administration in a model of placental ischemia-induced hypertension. These observations are further supported by our placental tissue analysis of AMPK phosphorylation, in which AICAR-induced increases in phosphorylation of AMPK was dependent on the placental ischemia induced by the RUPP procedure and not observed in the NP placenta. Taken together with the MAP and angiogenic balance data, AICAR has no measured effect on the normal pregnant dams in this study. We also observed that chronic AICAR did not stimulate AMPK phosphorylation in skeletal muscle in either NP or RUPP groups. Although these findings are interesting, further studies are required to determine if this is a tissue or pregnancy specific observation or if the molecular changes are dependent on hypoxia or chronic ischemia.

Systemic and Organ-Specific Oxidative Stress and Anti-Oxidant Capacity

In addition to the effects on the angiogenic balance, RUPP-associated renal and placental markers of oxidative stress were reduced with AICAR treatment. Previous work has shown that oxidative stress plays an important role in preeclampsia and RUPP hypertension [100,140] and our present observations suggest that the effects of AICAR may be mediated by a combination of the inhibition of the formation of reactive oxygen species and the stimulation of antioxidant molecules. Similar to our observations with angiogenic balance this effect was

only observed in the RUPP groups following AICAR treatment, suggesting a dependence on the presence of placental-ischemia. While the attenuation of these oxidative stress markers in the RUPP+AICAR coincide with the stimulation of bioavailable VEGF and a concomitant production or maintenance of downstream anti-oxidative molecules, whether the observed effects are dependent on the VEGF remains unclear. Further studies of the direct and indirect antioxidant properties of AICAR are required to identify the exact pathways underlying the present observations.

Study Limitations

While we feel the present results are exciting, it is important to recognize they are not without limitations. While our current hypothesis focused on the previously reported links between purinergic signaling and VEGF, other angiogenic factors important in preeclampsia such as sEng [163,176] may also be affected by AICAR and further studies are planned to evaluate these possibilities. Moreover, this study does not specifically address the exact role of AMPK signaling and additional studies are planned to evaluate the exact role of that pathway in angiogenic balance. Lastly, the source of the changes in VEGF and sFlt-1 in the present study remain unknown, so further studies are required to isolate the source. Nevertheless, studies are planned to further investigate the role of AMPK on AICAR's effects *in vivo*. Further studies are currently aimed at

the effects of AICAR specific to pregnancy, the cell-type-specific (e.g. placental, vascular endothelial cell, skeletal muscle) effects, and the source of and contribution to VEGF and sFlt-1 control.

Conclusion

Taken together, the effects of AICAR in the RUPP model reveal an interesting potential role of AMPK activation on angiogenic balance and normotension restoration. Also presented are encouraging therapeutic potential for placental ischemia-induced hypertension, by restoring angiogenic balance, abrogating RUPP-induced hypertension, and having no reportable deleterious effects on the fetal-placental unit. While these results are promising, further pharmacological studies are required to elucidate the exact mechanisms by which AICAR lowers blood pressure and restores angiogenic balance.

In the next section, Part 2, the effects of AICAR on the placenta and vascular endothelium will be explored to further elucidate the mechanism by which AICAR may be acting to prevent the RUPP-induced hypertension and endothelial dysfunction.

PART 2: PLACENTAL AND VASCULAR-SPECIFIC EFFECTS OF AICAR ON ANGIOGENIC FACTOR SECRETION AND CELL STRESS SIGNALING

Introduction

In Part 2 of this data chapter, tissue-specific effects of AICAR administration are explored in vascular endothelium, placental cytotrophoblasts, as well as placental villous syncytium. Our laboratory has recently reported the potent AMP-activated kinase (AMPK) agonist 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) prevents an increase in arterial blood pressure and restores angiogenic balance (e.g. increased ratio of VEGF to sFlt-1) in rats with placental ischemia induced hypertension [26]. However, the central molecular mechanisms linking AMPK and VEGF/sFlt-1 secretion under ischemic stress remain unknown. Moreover, stimulation of the AMPK pathway has recently been reported to increase VEGF expression [127,128], which has also been suggested to be the mediating mechanism in the prevention of placental ischemia induced hypertension in preeclampsia. Interestingly, the administration of adenosine, which is a known agonist for AMPK activation, reportedly decreased sFlt-1 production in placental villi under hypoxic conditions [143]. Also, AMPK activation is observed to mitigate apoptotic and ER stress signaling cascades in various tissues, including vascular endothelium [130,135,174,177], which is further suggestive of its protective properties.

Though AICAR reportedly improves the VEGF and sFlt-1 balance in maternal circulation in the RUPP model [26], the tissue specificity also remains in question. Several reports have concluded storage pools of releasable sFlt-1 are primarily sequestered and sourced from the placenta and peripheral vascular endothelium during preeclampsia and normal pregnancy [65,68,74,178-180], which may be tissues affected under AICAR treatment. Therefore, it is important to investigate each tissue *in vitro* to further understand the physiological applications of AICAR treatment in preeclampsia.

Thus, the aim of this study was to model placental ischemia with human placental cytotrophoblast (BeWo), primary umbilical vascular endothelial (HUVEC) cells, and rat placental villous explants cultured under physiological hypoxic conditions to determine if AICAR modifies angiogenic factor secretion. Further, we hypothesize AICAR treatment will prevent the imbalance of VEGF and sFlt-1 expression by placental tissue explants culture under hypoxic conditions.

Methods

Animals

All experimental procedures executed were completed in accordance with National Institutes of Health guidelines for use and care of animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at the

University of Oregon. Placentas for villous explant culture were isolated on day 19 of gestation from six timed-pregnant Sprague-Dawley rats purchased from Harlan Laboratories (Indianapolis, IN). Animals were housed in a temperature-controlled room (23°C) with a 12:12 light:dark cycle.

Placental Explant Isolation and Culture

Normal pregnant dams were euthanized and whole-placentas were isolated on day 19 of gestation. Tissues were collected in ice-cold sterile PBS. Under a dissection microscope, the decidua and trophospongium were carefully dissected away to isolate the anchoring villi bundles. Approximately 10-15mg of villous tissue were excised and weighed prior to culture. The explants were cultured in a 24-well plate on 150µl of Growth Factor Reduced Matrigel® (BD Biosciences) and 1mL of 50/50 Dulbecco's Modified Eagle's Medium: Ham's F-12 with 100 µg/ml streptomycin, 100 U/ml penicillin, and 25 µg/ml ascorbic acid. Tissues were conditioned in a CO_2/O_2 gas incubator at either physiological normoxia (8% O_2) or at hypoxia (1% O_2) in the presence of 2mM AICAR for 24 hours.

HUVEC and BeWo Cell Culture Technique and Conditions

Immortalized human villous trophoblast cells (BeWo) and primary human umbilical vascular endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC, USA). Prior to experimental protocols, cells

were grown to manufacturer's recommendations. Briefly, cells were grown to 70-80% confluence in a 75cm^2 flask (Corning Inc., USA) under 5% CO_2/20% O_2, and re-plated at 1x10^5 cells/well in a 24-well plate for further treatment. BeWo cells (Passages 20-30) were cultured with F12K basal media (ATCC), and HUVECs (passages 2-4) were cultured with vascular cell basal media (ATCC). Growth medias were supplement with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin (ATCC).

Following, cells were sub-cultured to study the effects of AICAR under physiological normoxic (8% O_2) and hypoxic (1% O_2) conditions. Cells were transferred to a 6-well culture plate at 1x10^5 cells/well (1 mL), and grown to ~70% confluence. After a serum starvation (~1-2 hours), cells were washed twice with sterile PBS. Sterile-filtered, serum-less media supplemented with or without AICAR (2mM) was introduced to the cells and cultured at the specified conditions for 24 hours. Conditioned media and cells were collected for further analysis. All treatments were run in sextuplet.

Enzyme-Linked Immunosorbant Assays

Plasma concentrations of free VEGF and sFlt-1 were measured using species-matched commercial ELISA (enzyme linked immunosorbant assay) kits according to the manufacturer's directions (R&D Systems, Quantikine®; Minneapolis, MN) as described previously [27,151].

Statistical Analysis and Calculations

All data are presented as mean ± SD, and statistical significance was accepted when $p<.05$. Comparisons were made with a one-way analysis of variance test combined with a Bonferroni post-hoc test were employed. Statistical calculations were made with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA USA).

Results

Angiogenic Factor Production in Rat Placental Explant Culture

In cultured placental villous explants, isolated at day 19 of normal gestation, the production of VEGF was increased ($p<.05$) with 24 hours of 1% O_2 treatment compared to normoxic controls. Additionally, no effect of AICAR on VEGF production was observed under either 8% or 1% O_2 conditions (**Figure 5.7**, top panel). Similarly, an increased ($p<.05$) production of sFlt-1 was observed in explants cultured in 1% O_2 conditions, however, this effect was abrogated by the administration of AICAR, suggesting AICAR's angiogenic effects may be hypoxic and sFlt-1-specific (**Figure 5.7**, bottom panel).

Cytotrophoblast and Vascular Endothelium Response to AICAR Under Hypoxic Stress

In regard to angiogenic factor regulation in BeWo cells (human cytotrophoblast) under hypoxic stress, VEGF production was moderately increased ($p<.05$ vs. 8% O_2 control) with 24 hour exposure to hypoxic condition,

and no effect of AICAR was observed (see **Figure 5.8**). Moreover, no effect of oxygen or AICAR administration on sFlt-1 production was observed.

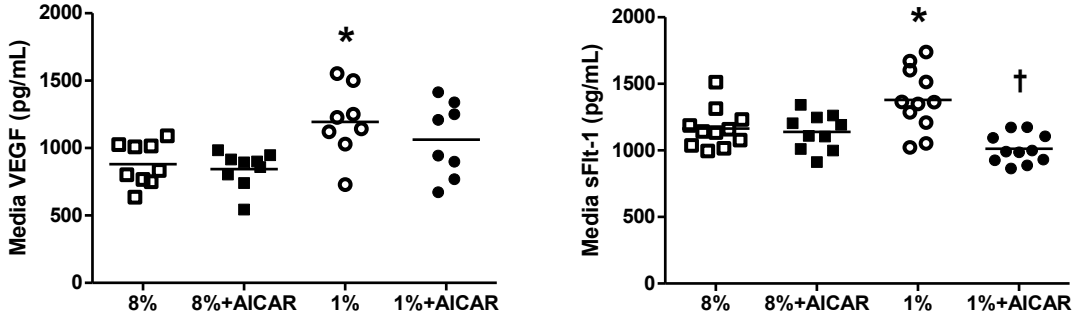

Figure 5.7. ***Placental Explant Secretion of VEGF and sFlt-1.*** (Top Panel) VEGF levels in the conditioned media were increased when exposed to 1% O_2 conditions (*p<.05), and AICAR had no effect under either oxygen conditions. Mean+SD: 8% 881 ±157; 8%+AICAR 844 ±134; *1% 1194 ±262; 1%+A 1062±277 pg/mL. (Bottom Panel) Secretion of sFlt-1 was stimulated (*p<.05) in 1% O_2 conditions, and the effect was reversed (†p<.05) in 1%+AICAR. Mean+SD: 8% 1165 ±150; 8%+AICAR 1139 ±135; *1% 1379 ±236; 1%+A 1012±110 pg/mL. *p<.05 vs. 8% control; †p<.05 vs. 1% control.

Moreover, production of angiogenic factors VEGF and sFlt-1 were markedly effected by AICAR, where AICAR increased VEGF secretion well as obviated the expression of sFlt-1. Overall, this data suggests AICAR may directed towards the vascular endothelial tissue to improve angiogenic factor balance in regard to the VEGF:sFlt-1 axis. Data is depicted below in **Figure 5.9**.

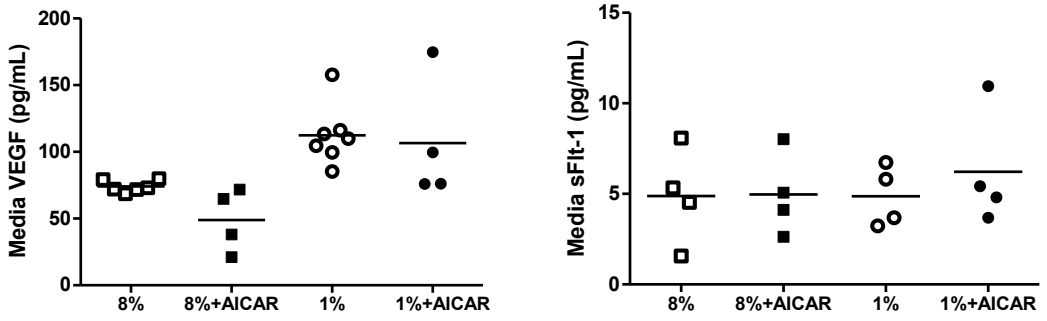

Figure 5.8. ***BeWo Angiogenic Factor Production.*** (Top Panel) VEGF secretion from BeWo cytotrophoblasts was not affected by culture under 1% O_2 conditions, and AICAR had no effect under either oxygen conditions. Mean+SD: 8% 74 ±5; 8%+AICAR 49 ±24; *1% 112 ±23; 1%+A 107±47 pg/mL. (Bottom Panel) Secretion of sFlt-1 from BeWo cells were not affected by oxygen nor AICAR treatment. Mean+SD: 8% 5 ±3; 8%+AICAR 5 ±2; *1% 5 ±2; 1%+A 6±3 pg/mL. *p<.05 vs. 8% control; †p<.05 vs. 1% control.

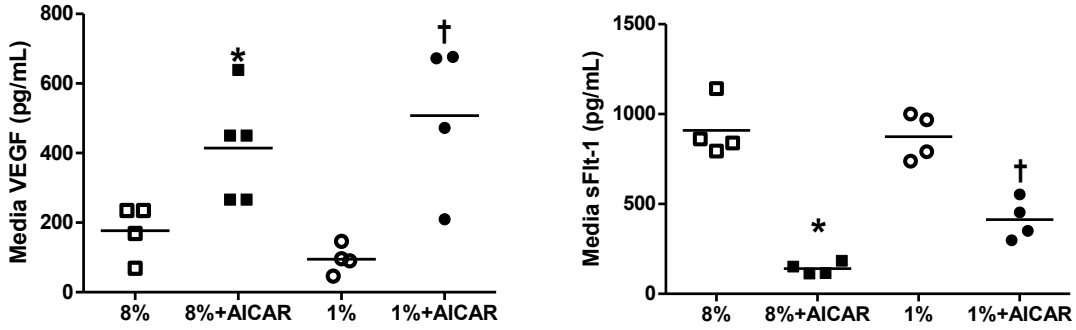

Figure 5.9. ***HUVEC VEGF and sFlt-1 Secretion.*** (Top Panel) VEGF concentration in conditioned media was increased (*†p<.05) with AICAR treatment under both 8% and 1% O_2 conditions compared to their respective controls. Mean+SD: 8% 177±78; 8%+AICAR 414±156; *1% 95±41; 1%+A 508±221 pg/mL. (Bottom Panel) Secretion of sFlt-1 was reduced with AICAR treatment in both 8% and 1% O_2 conditions compared to their respective controls. Mean+SD: 8% 910±158; 8%+AICAR 141±34; *1% 874±129; 1%+A 414±113 pg/mL. *p<.05 vs. 8% control; †p<.05 vs. 1% control.

Discussion

Sufficient placental health and function is essential to the development of a fetus throughout gestation. In preeclamptic pregnancies, placental function is often reduced, due to a failure to adapt the ischemic environment which results in the concomitant apoptotic activation [181-183]. Regarding the current study, we hypothesize AICAR treatment will prevent the imbalance of VEGF and sFlt-1 expression by placental tissue explants culture under hypoxic conditions. Further, several novel and exciting findings are presented, which begin to elucidate the tissue-specific effects of AICAR and understand its role in preeclampsia treatment and prevention.

AICAR and Angiogenic Factor Production

To measure the effect of AICAR in the hypoxic placental tissue, placental villi bundles were isolated from rat placentas and cultured under 8% or 1% O_2 conditions to model placental ischemic conditions. Similar to previous reports [143,184], secretion of VEGF and sFlt-1 were increased under 24 hour hypoxic culture. Moreover, as hypothesized, angiogenic factor secretion was normalized by AICAR treatment in placental villous explants. Supporting our initial hypothesis, sFlt-1 production under hypoxia was attenuated with AICAR treatment, whereas VEGF production remained unaffected. Together, these observations further confirm our previous *in vivo* findings in the RUPP model [26]. These data are

substantiated by previous reports from George and colleagues [143], where adenosine treatment suppressed the production of sFlt-1 under hypoxia in placental villous explant culture. As AICAR is considered as an adenosine mimetic [37], these mechanisms are likely very closely related or shared. Taken together, the current study confirms the potential of AICAR and other similar molecules in the control of angiogenic factor secretion, specifically sFlt-1, from the placenta under ischemic or hypoxic stress. It is important to recognize that it is unclear whether the stimulation of VEGF is a direct result from suppression of the antagonistic sFlt-1, and further studies are required.

Additionally, the role of AMPK remains unclear at this time as this was not measured, and further study is required its contributions to angiogenic factor release. A recent study [185] in cardiac remodeling in hypertension reported a direct effect of AMPK activation on the inhibition of NFAT (nuclear factor of activated T cells) family activity, which is a known positive regulator of sFlt-1 and Flt-1 production [186-188]. To this end, this pathway should be further investigated in placental tissue to elucidate this fascinating signaling process for application in preeclampsia.

In regard to cell-specificity, the effects of AICAR appear to be particular to vascular endothelial cells, as observed in the HUVEC culture data. Firstly, cultured cytotrophoblasts (BeWo) were not responsive to AICAR treatment,

which suggests the effects on angiogenic factor production observed in the villous explant culture are not mediated directly through the cytotrophoblasts. Interestingly, AICAR suppressed sFlt-1 release in vascular endothelial cells under hypoxic conditions, and also improved VEGF secretion. These observations provide an intriguing avenue for further investigation in the hypoxic-specificity in the effects of AICAR, and the potential role for AMPK activation as a central mediator.

Study Limitations

Though these experiments begin to elucidate the cell-specific effects of AICAR that may explain the previous *in vivo* observations in Part 1 of this data chapter, there are several questions remaining. Firstly, the role of AMPK in the angiogenic factor and cell stress signaling remains untested, and further experiments are required. A chemical or genetic antagonism of AMPK activity could be combined with the current experimental protocol to elucidate this intriguing question. Further, examination of angiogenic factor mRNA expression would be beneficial to reveal the effects of AICAR treatment on sFlt-1 and VEGF expression, and if the observed sFlt-1 suppression is indeed the cause for an increase in VEGF in the conditioned media.

Additionally, the timeline of the protein expression changes was not analyzed in any of the experiments. For instance, for how long and how quickly

after hypoxic exposure does the expression of sFlt-1 increase? Though this is critical information in the minutia of the pharmacology and clinical application, we feel this was outside of the scope of the current study.

Conclusion

Collectively, the present study provides an interesting role for AICAR in the angiogenic factor secretion profile and cell stress under hypoxic conditions, and supports the concept of AICAR administration in models of placental ischemia. Moreover, this project suggests the improvement of free VEGF in our previous *in vivo* report appears to result from the suppression of sFlt-1 secretion from placental explants under hypoxic stress, which provides an intriguing and novel target for preeclamptic angiogenic imbalance. Though further studies are required to fully elucidate the molecular mechanisms, the potential clinical applications of AICAR are exciting and provide an avenue for pharmacological research and development for preeclampsia treatment and prevention.

In Part 3 of this data chapter, we will exam the role of the angiogenic imbalance, in regard to the sFlt-1:VEGF axis, in the effects of AICAR in placental ischemia induced hypertension and angiogenic imbalance.

PART 3: ELUCIDATING THE ROLE OF ANGIOGENIC BALANCE AND VEGF BIOAVAILABILITY IN THE ANTI-HYPERTENSIVE EFFECTS OF AICAR ADMINISTRATION IN THE RUPP MODEL

Introduction

Though the etiology of preeclampsia (PE) is unclear, recent studies have implicated the imbalance of angiogenic factors, most notably through the VEGF/PlGF:sFlt-1 axis. This imbalance has been a large focus of the preeclampsia research since Karumanchi's seminal report on the role of sFlt-1 over a decade ago [20]. Since then, several experimental modalities studied have been focused on the improvement of angiogenic balance to mitigate placental ischemia induced hypertension [26,27,71,118,141], primarily through the restoration of circulating VEGF to counteract the rise in sFlt-1.

Furthermore, as reported in Parts I-II of this data chapter, AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) remediated the hypertension and angiogenic imbalance in the RUPP model, potentially through the stimulation of VEGF production and/or the suppression of sFlt-1. Indeed, the overall anti-hypertensive effects are promising, but the role of angiogenic imbalance remains in question in regard to the observed cardiovascular effects of AICAR in a model of placental ischemia induced hypertension. Therefore, we hypothesized the anti-hypertensive effects of AICAR would be prevented by

maintaining an angiogenic imbalance by chronic infusion of recombinant mouse (rm)-sFlt1 in an animal model of PE.

Methods

Animals

All experimental procedures executed were completed in accordance with National Institutes of Health guidelines for use and care of animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Oregon. Studies were performed between the University of Oregon and the with timed-pregnant Sprague-Dawley rats purchased from Harlan Laboratories (Indianapolis, IN). Animals were housed in a temperature-controlled room (23°C) with a 12:12 light:dark cycle.

Reduced Uterine Perfusion Pressure (RUPP) Procedure

The RUPP procedure is a well-characterized and widely accepted model [140] for studying the link between placental ischemia and hypertension in the pregnant rat and has been described in detail previously[27]. Briefly, a silver clip (0.203-mm inner diameter (ID)) was placed on the lower abdominal aorta, superior to the common iliac bifurcation. Two additional clips (0.100-mm ID) were placed on the branches of both the right and left ovarian arteries on day 14 of gestation (term=21 days). NP dams underwent a sham surgery, which included an abdominal midline incision and suture. After observing no

differences in the angiogenic factors and blood pressures these animals were grouped with the NP rats.

AICAR and Recombinant sFlt-1 Administration

On gestational day 14, dams were assigned to normal pregnancy (NP) (n=8) and RUPP (n=8) groups with and without AICAR (Santa Cruz Biotechnology Inc., Santa Cruz, CA) treatment (NP+A (n=6); RUPP+A (n=8)). Animals received with AICAR from days 14-19 with intraperitoneal (i.p.) injection of 50mg/kg b.i.d. by a 0.5mL 0.9% saline vehicle. All other animals received a 0.5mL 0.9% saline vehicle injection as control.

Further, recombinant mouse VEGF-R1/Flt-1-Fc chimera (R&D Systems, Minneapolis, MN, #471-F1-100; 500ng/ul/h) or vehicle (0.9% sterile saline) was chronically administered i.p. from days 14-19 of gestation by mini-osmotic pump (Alzet, Cupertino, CA, Model 2001). Control groups received a vehicle (0.9% saline). This dose has previously been observed to increase arterial pressure and plasma sFlt-1 levels in pregnant rats [176], and was further confirmed in our pilot studies. NP, NP+sFlt1 and RUPP data are also reported in Part 2 of Chapter IV as these studies were run in parallel.

Measurement of MAP in Chronically Instrumented Conscious Rats

Animals were instrumented with an indwelling arterial catheter of the left common carotid artery on day 18 of gestation. On day 19, unanesthetized mean

arterial pressure (MAP) was measured using a fluid-filled pressure transducer (ADInsruments, Colorado Springs, CO) [26,27,151].

Conceptus Measurements and Serum Collection

After blood pressure measurement, the dams were placed under 3% isoflurane anesthesia, and a midline ventral incision was made to isolate the abdominal aorta for arterial plasma and serum collection as reported previously [27,151]. After exsanguination, a pneumothorax and cardiac excision were used to confirm death. Blood was collected for subsequent assays into blank and EDTA-lined Corvac® tubes for serum and plasma processing, respectively (Sherwood Davis, St. Louis, MO). Fetal weight, placental weight, and number of resorptions were recorded. All tissues collected for subsequent analysis were flash frozen in liquid nitrogen and stored at -80˚C.

Mulvany Microvessel Wire Myography

Following necropsies on day 19 of gestation, secondary and tertiary mesenteric arterioles were isolated and cleaned of all surrounding adipose and connective tissues under a dissection microscope. 1-2mm vessel segments were then mounted on two 40μm stainless steel wires and attached to a wire myograph (DMT, Denmark) to allow for isometric force recordings. Vessels were normalized to tensions that proportionally modeled 100mmHg using the normalization module in LabChart 8.0 (ADInstruments, USA).

After a 10 minute period for equilibration in a Kreb's buffer (130mM NaCl, 4.7mM, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 1.6mM $CaCl_2$, 14.9mM $NaHCO_3$, 5.5mM glucose , pre-constriction was obtained by exposing the vessels to an isosmotic, high-potassium physiological saline solution (K-PSS) (74.7mM NaCl, 60mM KCl, 1.8 KH_2PO_4, 1.2mM $MgSO_4$, 1.6mM $CaCl_2$, 14.9mM $NaHCO_3$, 5.5mM glucose). Following a triple washout period with Kreb's buffer, a stabilized vascular constriction was achieved by the addition of the thromboxane A_2 analogue U46619 (5 μM). Endothelial-dependent vasorelaxation was evaluated with a cumulative dose response curve to acetylcholine (Ach; $1x10^{-9}$–$1x10^{-5}$ M) . Further, endothelial-independent smooth muscle function was assessed with a cumulative dose response curve to sodium nitroprusside (SNP, $1x10^{-9}$–$1x10^{-5}$ M). Following each relaxation curve, a single dose of 0.1mM SNP was given to confirm smooth muscle integrity. At experiment termination, exposure to K-PSS was used to assess vessel viability and functional decay. Data is presented as mean±SD of the per cent vasorelaxation from U46619 contraction force.

Enzyme-Linked Immunosorbant Assays

Plasma concentration of free VEGF was measured using commercially available enzyme linked immunosorbant assay (ELISA) kits (R&D Systems, Quantikine®; Minneapolis, MN, Part: MMV00) according to the manufacturer's directions as described previously [26,27,151].

Endothelial Tube Formation Assay

Angiogenic balance was further assessed in the serum of pregnant rats *in vitro* as previously reported [26,27] in two separate experiments and each was performed in duplicate. HUVECs (human umbilical vascular endothelial cells) were plated at $5x10^5$ cells/mL (100µl/well) onto 96-well plate lined with growth-factor-reduced Matrigel® (BD Biosciences, Bedford, MA). Five microliters of maternal serum was introduced to the 100µl cell media. Cells were incubated at 37°C, 20% O_2, 5% CO_2. Average tubule length was assessed at 100X optical zoom with a digital inverted compound microscope and Zen 2012 software (Carl Zeiss, Inc., Thornwood NY).

Statistical Analysis and Calculations

All data are presented as mean ± SD, and statistical significance was accepted when $p<.05$. Comparisons within NP and RUPP groups were evaluated with a one-way analysis of variance test combined with a Bonferroni post-hoc test were employed. Comparison between NP and RUPP controls was analyzed by an unpaired t-test. Statistical calculations were made with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA USA).

Results

Mean Arterial Pressure

At day 19 of gestation, arterial pressure in the rm-sFlt1 infused normal pregnant cohort (NP+sFlt1) was increased ($p<.05$) in comparison to NP controls, and remained elevated ($p<.05$ vs. NP) despite AICAR treatment (50mg/kg, b.i.d.). Moreover, RUPP-induced hypertension was remediated ($p<.05$) with AICAR treatment, and this effect was reversed ($p<.05$) with rm-sFlt1 infusion. Data is depicted in **Figure 5.10**.

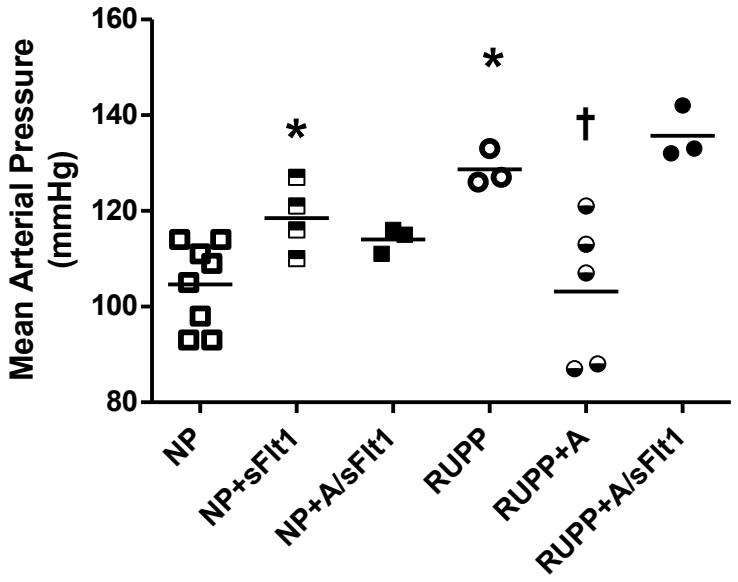

Figure 5.10. ***Mean Arterial Pressure on Day 19.*** Infusion of sFlt1 in normal pregnant dams increased (*$p<.05$) mean arterial pressure (MAP). RUPP increased (*$p<.05$) MAP vs. NP, and AICAR mitigated (†$p<.05$) mitigated this effect in RUPP+A, but not RUPP+A/sFlt1. Mean+SD: NP 105±9; *NP+sFlt 119±7; NP+A/sFlt1 114±3; *RUPP 129±4; †RUPP+A 103±15; RUPP+A/sFlt 136±6 mmHg. *$p<.05$ vs. NP; †$p<.05$ vs. RUPP. n=3-8 per group. NP, NP+sFlt1 and RUPP data is replicated from Chapter IV, Part 2.

Plasma Angiogenic Balance: VEGF and sFlt-1

The concentration of plasma free VEGF was not affected by sFlt-1 infusion in the NP+sFlt1 or NP+A/sFlt1 treatment groups in comparison to the NP control. As we have previously reported [26,27,151], RUPP decreased bioavailable VEGF when compared to NP controls, and AICAR reversed this effect. Moreover, the parallel treatment sFlt-1 infusion with AICAR blocked this effect on VEGF bioavailability in the RUPP (**Figure 5.11**, top panel).

Additionally, plasma sFlt-1 levels were increased ($p<.05$) with sFlt-1 infusion in the NP and RUPP cohorts compared to their respective controls, which confirmed successful peptide delivery. Further, AICAR treatment did not have an effect NP+A/sFlt1 or RUPP+A/sFlt1 compared to the NP and RUPP groups, respectively (**Figure 5.11**, bottom left panel). Moreover, the ratio of plasma VEGF:sFlt-1 remained decreased in the sFlt-1 infused NP and RUPP controls despite AICAR treatment, which suggests the VEGF:sFlt-1 angiogenic imbalance was maintained by sFlt-1 infusion (**Figure 5.11**, bottom right panel).

Vascular Endothelial Function

Endothelium-dependent vasodilation potential was assessed in isolated mesenteric arterioles (200-400μm diameter) by a logarithmic dose response to acetylcholine (Ach) (**Figure 5.12**). With respect to NP controls, rm-sFlt1 infusion decreased ($p<.05$) relaxation potential in the NP+sFlt1 group, and this effect was

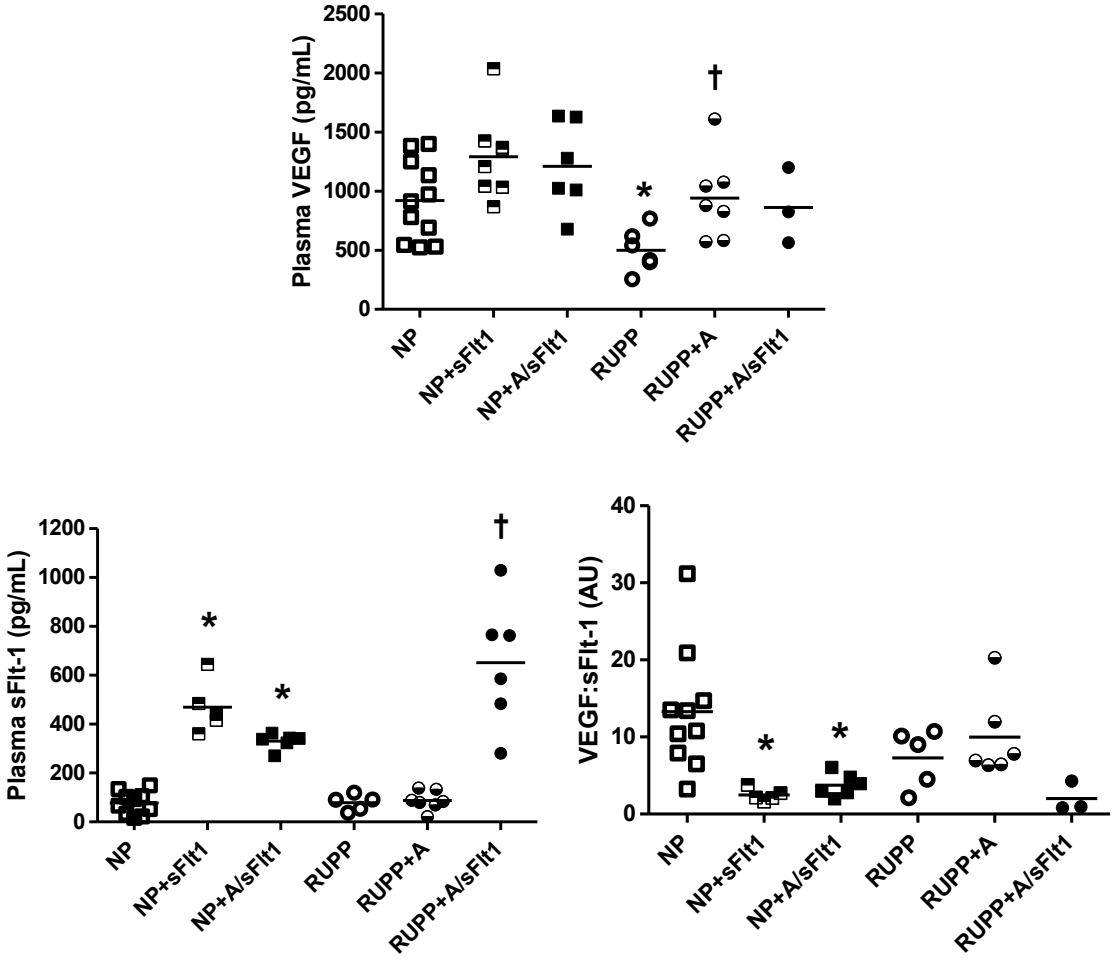

Figure 5.11. ***Plasma VEGF, sFlt-1, and VEGF:sFlt-1 Balance.*** (Top Panel) RUPP had decreased (*p<.05) bioavailable VEGF compared to NP. No effect of sFlt-1 infusion in either NP or RUPP groups were observed. NP 920±335; *NP+sFlt 1291±358; *NP/A+sFlt 1209±378; *RUPP 500±182; RUPP/A 941±355; RUPP/A+sFlt 862±319 pg/mL. (Bottom Left Panel) sFlt-1 was increased (*p<.05) in NP+sFlt1, and NP+A/sFlt1 vs. NP. Additionally, RUPP+A/sFlt1 treatment increased (†p<.05) plasma sFlt-1 vs. RUPP controls. NP 76±47; *NP+sFlt 470±108; *NP+A/sFlt 330±32; *RUPP 78±33; †RUPP+A 88±40; RUPP+A/sFlt 651±260 pg/mL. (Bottom Right Panel) The ratio of bioavailable VEGF:sFlt-1 was decreased by sFlt-1 infusion in both NP+sFlt1 and NP+A/sFlt1 vs. NP controls. sFlt-1 infusion in RUPP+A/sFlt1 was further decreased compared to RUPP VEGF:sFlt-1 values. NP 13.3±8.0; *NP+sFlt 2.5±0.8; *NP+A/sFlt 3.8±1.5; *RUPP 7.3±3.8; †RUPP+A 10.0±5.5; RUPP+A/sFlt 2.3±1.7 AU. *p<.05 vs. NP; †p<.05 vs. RUPP. n=3-10 per group. NP, NP+sFlt1 and RUPP data is replicated from Chapter IV, Part 2.

ameliorated with the addition of AICAR treatment (**Figure 5.12**, top panel).

In regard to RUPP, vasorelaxation to Ach was blunted ($p<.05$ vs. NP), and AICAR treatment reversed ($p<.05$ vs. RUPP) this effect (**Figure 5.12**, bottom panel), even with the addition of sFlt-1 infusion in the RUPP+A and RUPP+A/sFlt1 cohorts. Additionally, no treatment effects were observed in smooth muscle function, assessed by vasorelaxation to sodium nitroprusside, in either NP- or RUPP-treatment sub-groups.

Serum Angiogenic Potential

Serum angiogenic potential was assessed by culture of human vascular endothelial cells (HUVECs) in the presence of 5% (v/v) animal sera, followed by measurement of microtubule formation. As reported in the previous chapter, sFlt1-infused NP animals (NP+sFlt1) had a decreased ($p<.05$ vs. NP) angiogenic potential, and AICAR treatment in NP+A/sFlt1 dams restored normal tubule formation potential (**Figure 5.13**). Moreover, RUPP sera caused a decrease ($p<.05$) in tubule formation compared to NP controls. Further, AICAR treatment in both RUPP+A and RUPP+A/sFlt1 treatment groups had no effect on tubule formation with respect to RUPP controls.

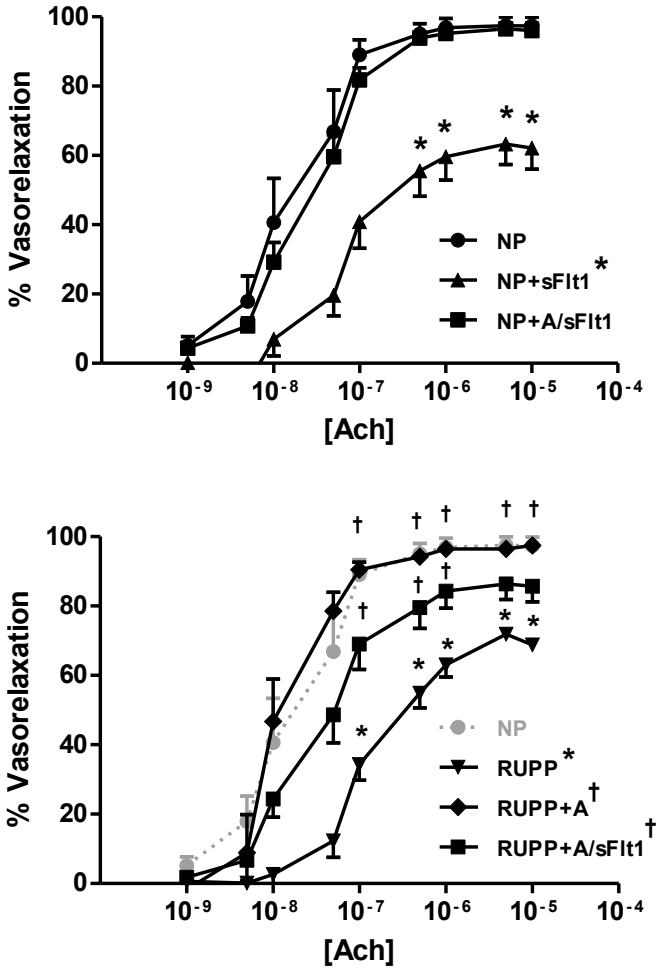

Figure 5.12. ***Mesenteric Arteriole Vascular Endothelial Function.*** (Top Panel) After constriction to thromboxane mimetic U46619, NP+sFlt1 dams exhibited a decreased (*p<.05) vasodilation potential to matched doses of acetylcholine (Ach) compared to NP, AICAR reversed this effect despite infusion of sFlt-1 in NP+A/sFlt1. (Bottom Panel) RUPP caused a decrease relaxation to Ach compared to NP (gray), and AICAR reversed (†p<.05) this effect in both RUPP+A and RUPP+A/sFlt1. Data presented as mean+SD; n=4-6 per group. Data analyzed between groups across each dose. NP, NP+sFlt1 and RUPP data is replicated from Chapter IV, Part 2.

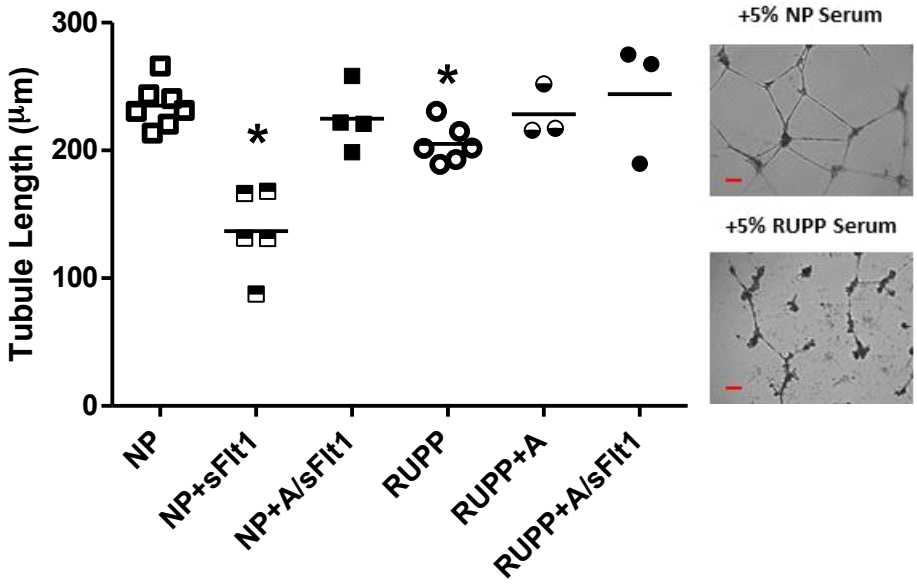

Figure 5.13. ***Serum Angiogenic Potential.*** Serum angiogenic potential was decreased in NP+sFlt and RUPP vs. NP (*p<.05). Ex treatment in RUPP improved (†p<.05) tubule formation vs. RUPP. Mean+SD: NP 247±27; *NP+sFlt 177±48; NP+A/sFlt 225±25; *RUPP 176±39; †RUPP+A 236±23; RUPP+A/sFlt 248±39um. A sample picture of NP vs. RUPP tubule formation is provided (scale bar = 100μm). *p<.05 vs. NP; †p<.05 vs. RUPP. NP, NP+sFlt1 and RUPP data is replicated from Chapter IV, Part 2.

Maternal and Conceptus Morphometrics

As hypothesized and previously reported, RUPP caused a decrease (p<.05 vs. NP) in maternal, fetal, and placental weight, as well as an increased (p<.05 vs. NP) rate of fetal demise. Infusion of rm-sFlt1 in NP caused a decrease in maternal weight, but had no effects on fetal or placental morphometrics. As previously reported [26], AICAR treatment ameliorated the rate of fetal demise, however, we did not observe an effect maternal, fetal, or placental weights. With the addition

of rm-sFlt1 infusion, rates of fetal demise were increased, and a decrease in placental weight was also observed. Data is summarized in **Table 5.2**.

Table 5.2. ***Maternal and Conceptus Morphometric Data at Necropsy.***

Treatments	*Maternal Weight (g)*	*Fetal Weight (g)*	*Placental Weight (g)*	*% Resorption*
NP (n=10)	311±8	2.28±0.07	0.50±0.02	1.5±1.5
NP+sFlt1 (n=8)	*288±6	2.27±0.08	0.49±0.03	0.8±0.9
NP+A/sFlt1 (n=6)	*267±12	2.41±0.21	0.51±0.01	8.5±4.3
RUPP (n=10)	*259±6	*1.82±0.14	*0.37±0.03	*73.0±10.3
RUPP+A (n=7)	267±8	1.97±0.04	0.45±0.02	†58.3±4.2
RUPP+A/sFlt1 (n=6)	250.2±9.2	1.79±0.20	†0.43±0.02	70.0±14.3

Note. *P<.05 indicates different from NP. †P<.05 indicates different from RUPP. Data presented as mean±SD. NP, NP+sFlt1 and RUPP data is replicated from Chapter IV, Part 2.

Discussion

The current study presents several intriguing and novel findings regarding AICAR treatment in a model of placental ischemia induced hypertension. Indeed, the data from this project support our initial hypothesis and suggest AICAR's anti-hypertensive effects are dependent on a restored angiogenic imbalance. An unexpected finding was the restoration of endothelial

function in the RUPP appears to be partially independent of an improvement of plasma VEGF:sFlt-1 angiogenic balance.

AICAR, Angiogenic Balance, and Mean Arterial Pressure

Similar to our previous observation [26], AICAR restored the angiogenic balance and endothelial function in the RUPP dams. Moreover, we are the first to report sFlt1-induced hypertension in the normal pregnant dams was not affected with AICAR treatment, which further suggests the mechanism of AICAR is specific to the RUPP and potentially dependent on placental ischemia. This explanation for the specificity to RUPP remains unclear, as AICAR has been shown to effectively lower blood pressure in several non-pregnant animal models of hypertension [34,35,172].

One potential explanation, in concert with our previous observations [26,189], is AICAR's pharmacological mechanism is mediated through the suppression of sFlt-1 production. Further, this would account for the absence of effect on NP+sFlt1 blood pressure, as well as the RUPP infused with sFlt-1. It should be noted that AICAR and AMPK activation may also improve VEGF production as Ouchi and others have previously reported [128]; however, we have not observed this effect in the NP [26]. Much work remains needed in this topic, to elucidate the molecular mechanisms of this intriguing effect.

A potential mechanism that may account for this difference is a potential

difference in extracellular fluid volume. Briefly, plasma volume has been observed to be decreased in PE women [190-192] as well as the RUPP rat [193], and should be accounted for in drug administration in pregnancy [194]. Though plasma and extracellular fluid volume was not directly measured in the current study, one could speculate a difference in blood volume may impact the effective dose received. As a difference in plasma volume is not necessarily accounted for by total bodyweight normalization, further investigation is required.

AICAR and Endothelial Function

Indeed, AICAR improved endothelial-dependent vasorelaxation in mesenteric arterioles in both NP and RUPP cohorts despite a concomitant infusion of sFlt-1. Together, there appears to be an alternative mechanism by with AICAR improved endothelial function, as angiogenic imbalance of the VEGF:sFlt-1 axis was maintained throughout AICAR treatment. Assumingly, if the effects of AICAR were specifically targeted on restoring endothelial function, the sFlt1-induced hypertension in the NP cohort would be expected to be ameliorated; yet, this was not observed. Indeed, AICAR treatment improved the endothelial function in both NP+sFlt-1 and RUPP cohorts, and these effects appear to be independent of angiogenic balance restoration through the VEGF:sFlt-1 axis. This may be due an increase in eNOS activation or coupling in vascular endothelium and smooth muscle cells, which are directly activated by

AMPK [137,174,195]; nevertheless, these effects were not measured in the present study and should be pursued in the future. Moreover, Chen and colleagues have demonstrated the Ser1177 residue is phosphorylated with chronic AMPK activation in endothelial cells [137], which suggests a long term effect on NO synthesis rather than the acute outcome previously presumed by Ford and colleagues [34]. Though the specific contributions of VEGF and sFlt1 remain unexamined, the role of VEGF-sFlt1 axis balance in this model of PE appears to play an essential role. Further studies are underway to isolate the roles of VEGF and sFlt1 in the promising therapeutic potential of AICAR treatment.

Furthermore, endothelial function was improved in both hypertensive treatment groups, RUPP and NP+sFlt1, while the effect on blood pressure appears to be limited to the RUPP treatment. The reason remains unknown, but may include mechanisms that were not addressed in this study, which may include the immune, central, or other humoral signaling that has been recently implicated in preeclampsia etiology [51,196-202]. For instance, administration of AICAR in endothelial culture has been previously shown to decrease monocyte adhesion and inflammatory cytokine release [203], which could contribute to the cardiovascular effects observed in the current study.

Study Limitations

Though the findings in this study are intriguing, these experiments are not without limitation. Indeed, the infusion of sFlt-1 was shown to effectively sustain angiogenic imbalance through the VEGF:sFlt-1 axis, this approach does not dissect the individual roles of VEGF and sFlt-1 control in the effects of AICAR. However, the ELISA data clearly demonstrates the VEGF:sFlt-1 angiogenic imbalance in maintained with rm-sFlt1 infusion, which suggests the anti-hypertensive effect of AICAR treatment is largely dependent on the restoration of angiogenic balance. To this end, additional studies using pharmacological or genetic dissection methods are required to isolate the individual roles of sFlt-1 and VEGF regulation under AICAR treatment.

It should also be noted that the validity of the circulating VEGF measurement by ELISA (purchased from R&D Systems, Minneapolis, MN) have recently been challenged by Weissgerber and colleagues [162]. While we did not observe the wide variability and difficulty in obtaining comparable values in free VEGF as Weissgerber has previously reported, we would like to recognize that this currently is an area of concern in preeclampsia research. We also failed to replicate our previous published observations in the RUPP in regard to the plasma sFlt-1 levels. Without these effects in RUPP, the interpretation and comparative quality of the angiogenic effects in RUPP should be taken into

consideration in future studies. In response to this concern, additional measurements of circulating sFlt-1 and serum angiogenic potential (tubule formation assay) to further support this data. To this end, we feel this is sufficient experimental evidence to appropriately interpret the current data and the role of VEGF bioavailability in the effects of AICAR in the RUPP model.

Conclusion

In concert with our previous studies, the current study confirms AICAR treatment following RUPP can abrogate he development of hypertension and endothelial dysfunction. Moreover, the effects of AICAR appear to be dependent on the restoration of angiogenic balance in the VEGF:sFlt-1 axis. Furthermore, while the present study begins to dissect the pharmacological mechanism of AICAR treatment in the RUPP model of preeclampsia, further research is required to reveal the mechanisms of AICAR's promising anti-hypertensive treatment in preeclampsia.

CHAPTER VI

CONCLUSION

MAIN FINDINGS

In Chapter IV, the exercise training studies revealed several interesting findings in the RUPP model of placental ischemia induced hypertension. First and foremost, the hypertension in the RUPP model was attenuated, and even obviated in the treatment of exercise prior to and during gestation. The role of exercise treatment that is initiated at gestation ostensibly is not effective in remediating the hypertensive response to placental ischemia. Additionally, as hypothesized, exercise training improved angiogenic balance through increasing bioavailable VEGF, and decreasing sFlt-1, the role of VEGF:sFlt-1. However, when the VEGF:sFlt-1 balance was sustained throughout treatment, the role of angiogenic balance restoration was not essential for the blood pressure lowering effect in the RUPP dams. Together, these findings provide an intriguing and novel role for exercise to prevent preeclamptic-like development in the RUPP rat, which also provide a strong base for continued research in the molecular mechanism of the observed anti-hypertensive effect.

Regarding the results following AICAR treatment, there was a remarkable anti-hypertensive effect in the RUPP model, while having no observed deleterious effects on the fetal and placental growth in either normal pregnant or

RUPP cohorts. Moreover, the drug appears to have a direct effect on sFlt-1 secretion *in vivo*, as well as *in vitro* models of hypoxic conditions in isolated placental explants and cultured vascular endothelial cells. This effect alone is quite notable, as increased plasma sFlt-1 remains extremely difficult to prevent or control in clinical practice [204]. To note, AICAR's anti-hypertensive effects were not observed in the NP+sFlt1 PE model, and were reversed by sFlt-1 infusion in the RUPP, which together suggests the effects of AICAR are dependent on placental ischemia as well as the potential suppression of sFlt-1.

In concert, the treatments investigated in this dissertation offer unique and novel treatment modalities in the prevention of preeclamptic-like hypertension in the pregnant rat (outlined in **Figure 6.1**), and potentially the preeclampsia in humans. It is with great hope that these effects are translated to human pregnancies, and mediate the onset of this costly and arduous syndrome.

Limitations

Though the findings in this dissertation are exciting, it is recognized these studies were not performed without limitation. Firstly, in regard to exercise treatment, the exercise distance run by each rat was not controlled for across or within individual studies. To control for these distances, the mode of voluntary exercise by activity wheel would likely require a substitution forced activity and daily monitoring, which may add an additional convoluting variable of stress.

Indeed, these components would be beneficial to isolate and dissect the ideal exercise training time and intensity; however, these fall outside of the current scope of the projects and should be pursued in future studies.

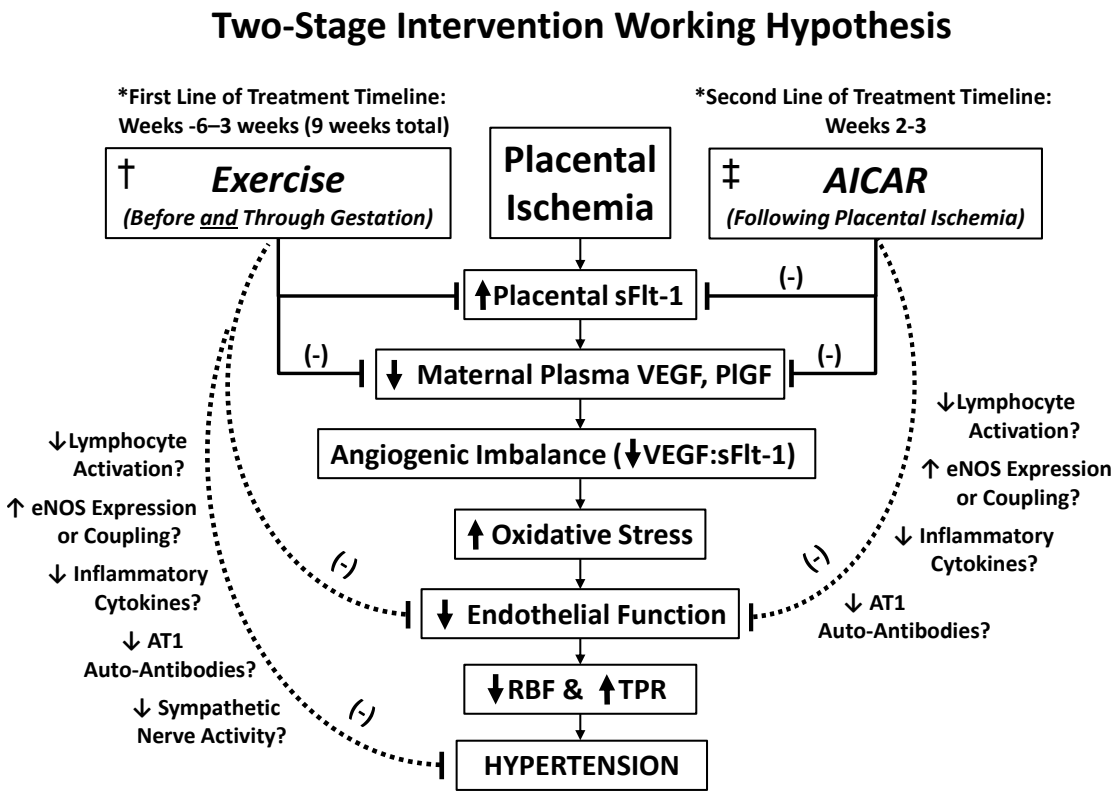

Figure 6.1. ***New Working Hypothesis for Exercise and AICAR Treatments.*** We report placental-ischemia-induced hypertension is mitigated by exercise training prior to and throughout gestation in the RUPP rat (†Specific Aim 1). Further, endothelial function and the hypertension were improved with exercise, and were not dependent on the restoration of VEGF:sFlt-1 angiogenic balance. Additionally, AICAR treatment (‡Specific Aim 2) is reported to ameliorated placental ischemia induced hypertension, and is indeed dependent on restored VEF:sFlt-1 balance. Moreover, AICAR did improve endothelial function independent of restored angiogenic balance, which suggests an alternative, contributing pathway to the mechanism. Together, these effects are hypothesized to be offer two stages of intervention to prevent the preeclamptic pathology following increased placental ischemia. RBF: Renal blood flow; TPR: Total peripheral resistance.

An additional limitation to be recognized in the data interpretation is the effect of AICAR or EBD (Exercise Before and During gestation) on angiogenic balance within the VEGF:sFlt-1 axis. Firstly, the VEGF concentrations reported across each *in vivo* study vary widely, and this is likely due to the methodological inconsistencies raised recently by Dr. Weissgerber and colleagues [162]. Further, the previously reported effects of RUPP on plasma sFlt-1 were not replicated in the later studies of each chapter. Though these rats presented with increased mean arterial pressure in comparison to sham/vehicle controls, the expected increase in plasma sFlt-1 was not observed. This may be due to methodological inconsistency within the assay, but may also indicate the current cohort of rats did not respond to RUPP in the predicted manner. Further, Banek and colleagues have recently reported the timing of the RUPP procedure in the gestational timeline is essential to provoke the imbalance of VEGF and sFlt-1 [9]. Specifically, animals that underwent the RUPP surgery on day 12 vs. day 14 of gestation presented with an increased mean arterial pressure in the five days following surgery, yet no effect on plasma sFlt-1. Furthermore, it is possible the animals in the current studies that did not respond to RUPP as hypothesized were mistimed in pregnancy, as only the presence of the seminal plug was used to time the pregnancy (day 0 at plug day). To avoid this potential convoluting factor in future work, Doppler confirmation, as well as fetal size/weight should be utilized

in parallel to seminal plug observation to control for gestational timing.

FUTURE DIRECTIONS

While the effects of exercise before and during gestation are quite exciting, several key questions remain to be answered in order to understand the mechanisms of the cardiovascular effects. Firstly, the role of VEGF:sFlt-1 balance in the maternal circulation appears to be vital to the cardiovascular effects of AICAR treatment, whereas exercise remediated the placental ischemia induced hypertension despite a sustained VEGF:sFlt-1 imbalance. This difference may be suggestive of a novel pathway which was not accounted for in the initial hypothesis, which may include the other humoral, immune, or neural contributions to PE pathology discussed in Chapter II. Furthermore, the role of VEGF and sFlt-1 in the effects of exercise appears to be secondary in the overall effect in the RUPP model. This observation is quite intriguing, as it suggests treatment of preeclampsia may be pursued through mechanisms outside of angiogenic factors.

An additional question remaining in this story is the direct effects of both exercise and AICAR treatments on the systemic and organ-specific hemodynamics. Exercise is well known to further increase stroke volume and cardiac output that is already elevated with pregnancy, as well as further decrease total peripheral resistance [123,156,205,206]. In regard to AICAR, only one

study has been completed in healthy men to suggest acute effects of AICAR administration may improve cardiac output and decrease peripheral vascular resistance. Unfortunately, the cardiovascular effects of chronic AICAR administration remain poorly understood [36], and require further investigation. Further, as placental blood flow is assumed to be reduced by the RUPP's mechanical restriction of the primary source of arterial blood flow, AICAR and exercise are likely to cause tissue-specific adaptations at the tissue level to thrive under such ischemic conditions. These effects are yet to be elucidated, and should be pursued in future studies.

INFERENCE AND APPLICATION OF EXERCISE AND AICAR TREATMENT TO PREVENT PREECLAMPSIA

While both exercise prior to and during pregnancy and AICAR treatment mitigated the model placental ischemia induced hypertension. Taken together, the clinical application from these studies is intriguing, where it allows the potential interventional moments at two moments during the preconception and late-gestation phases.

As discussed in Chapter IV, the role of exercise in prevention of the hypertension and vascular dysfunction following placental ischemia is dependent on the time of training initiation in the pregnant rat. Specifically, if exercise was initiated 6 weeks previous to, and sustained through gestation, the

RUPP hypertension was ameliorated, whereas training initiated following conception was not effective. Combined with the very supportive epidemiological reports, these studies demonstrate a potential prophylactic modality for women planning on becoming pregnant. Though exciting, the mechanisms and long-term effects of the exercise training treatment still require thorough investigation.

Indeed, while exercise initiated at gestation in the RUPP model was not effective in mitigating the hypertension at the end of gestation, AICAR treatment following RUPP appears to fill that void of treatment. As the effects described in Chapter V suggest an intriguing role for AICAR treatment, the preventative effects were also paralleled by no observed deleterious effects in either the normal pregnant or RUPP. Further, these findings also open a door for the further exploration of AICAR to extend the length or gestation or even use as a prophylactic pharmaceutical for preeclampsia. Certainly, the offspring of these AICAR-treated pregnancies were not thoroughly studied, and much further investigation is required.

Though admittedly speculative at this point, this collection of work outlines an intriguing, and relatively inexpensive, approach to prevent the onset of preeclampsia if impairment of placental perfusion is to occur. In the ideal clinical situation, the physician could advise a woman who is planning on

becoming pregnant to start a mild-moderate exercise training regimen (e.g. jogging, swimming, bicycling, etc.). Once pregnancy is confirmed, the woman could be advised to continue a mild-moderate exercise training program throughout the remainder of gestation. From our findings in the RUPP rat, and in the recent epidemiological reports, exercise training could prevent the development of hypertension that may develop if there is an impaired placental perfusion due to spiral arterial malformation. In addition to this theoretical application, if a woman is not regularly exercising prior to gestation, could AICAR be used following the detection of interrupted placental perfusion? Since we have observed no deleterious effects in either the normal pregnant or RUPP dams, the potential for prophylactic treatment with AICAR remains possible. However, long-term studies in cardiovascular and neural development of the offspring are required to make this conclusion and begin clinical trial.

Together, these modalities may offer two stages of defense against the development of hypertension and endothelial dysfunction associated with preeclampsia. These findings in this dissertation are clearly an exciting and promising venture to be further pursued through both basic science and clinical research to tackle one of the oldest and detrimental threats to maternal and fetal health worldwide.

REFERENCES CITED

1. Chesley, L. C. A short history of eclampsia. *Obstet. Gynecol.* **1974,** *43* (4), 559-602.

2. Chesley, L. C. History and epidemiology of preeclampsia-eclampsia. *Clinical obstetrics and gynecology* **1984**.

3. Chesley, L. C. *Hypertensive Disorders in Pregnancy;* 1st ed.; Appleton-Century-Crofts: New York NY, 1978.

4. Bell, M. J. A historical overview of preeclampsia-eclampsia. *J Obstet. Gynecol. Neonatal Nurs.* **2010,** *39* (5), 510-518.

5. Ballantyne, J. W. Sphygmographic tracings in puerperal eclampsia. *Edinburgh Medicine Journal* **1885,** (30), 1007-1020.

6. Lever, J. C. Cases of puerperal convulsions with remarks. *Guy's Hospital Reports* **1843,** (2), 495-517.

7. Rayer, P. F. *Traite des maladies des reins et des alterations de la secretion urinaire;* Bailliere: Paris, 1837.

8. Booth, J. A short history of blood pressure measurement. *Proc. R. Soc. Med.* **1977,** *70* (11), 793-799.

9. Roberts, J. M.; Cooper, D. W. Pathogenesis and genetics of pre-eclampsia. *Lancet* **2001,** *357* (9249), 53-56.

10. Sibai, B.; Dekker, G.; Kupferminc, M. Pre-eclampsia. *Lancet* **2005,** *365* (9461), 785-799.

11. Roberts, J. M.; Taylor, R. N.; Musci, T. J.; Rodgers, G. M.; Hubel, C. A.; McLaughlin, M. K. Preeclampsia: an endothelial cell disorder. *Am J Obstet Gynecol* **1989,** *161* (5), 1200-1204.

12. Roberts, J. M.; Taylor, R. N.; Goldfien, A. Clinical and biochemical evidence of endothelial cell dysfunction in the pregnancy syndrome preeclampsia. *Am J Hypertens* **1991,** *4* (8), 700-708.

13. Hanretty, K. P.; Whittle, M. J.; Rubin, P. C. Doppler uteroplacental waveforms in pregnancy-induced hypertension: a re-appraisal. *Lancet* **1988,** *1* (8590), 850-852.

14. Redman, C. W. Current topic: pre-eclampsia and the placenta. *Placenta* **1991,** *12* (4), 301-308.

15. Brosens, I. A.; Robertson, W. B.; Dixon, H. G. The role of the spiral arteries in the pathogenesis of preeclampsia. *Obstet Gynecol Annu.* **1972,** *1*, 177-191.

16. Gerretsen, G.; Huisjes, H. J.; Elema, J. D. Morphological changes of the spiral arteries in the placental bed in relation to pre-eclampsia and fetal growth retardation. *Br. J Obstet Gynaecol.* **1981,** *88* (9), 876-881.

17. Piinenborg R; Anthony J; Davey DA; Rees A; Tiltman A; Vercruysse L; van Assche A Placental bed spiral arteries in the hypertenive disorders of pregnancy. *Br J Obstet Gynaecol* **1991,** *98* (7), 648-655.

18. Fisher, S. J.; Roberts JM Defects in Placentation and Placental Perfusion. In *Chelsey's Hypertensive Disorders in Pregnancy*, 2nd ed.; Lindheimer, M. D., Roberts JM, Cunningham F.G., Eds.; Appleton & Lange: Stanford, CT, 1999; pp 377-394.

19. Gilbert, J. S.; Babcock, S. A.; Granger, J. P. Hypertension Produced by Reduced Uterine Perfusion in Pregnant Rats Is Associated With Increased Soluble Fms-Like Tyrosine Kinase-1 Expression. *Hypertension* **2007,** *50*, 1142-1147.

20. Maynard, S. E.; Min, J. Y.; Merchan, J.; Lim, K. H.; Li, J.; Mondal, S.; Libermann, T. A.; Morgan, J. P.; Sellke, F. W.; Stillman, I. E.; Epstein, F. H.; Sukhatme, V. P.; Karumanchi, S. A. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J. Clin. Invest* **2003,** *111* (5), 649-658.

21. Maynard, S.; Epstein, F. H.; Karumanchi, S. A. Preeclampsia and Angiogenic Imbalance. *Annual Review of Medicine* **2008,** *59* (1), 61-78.

22. Maynard, S. E.; Venkatesha, S.; Thadhani, R.; Karumanchi, S. A. Soluble Fms-like tyrosine kinase 1 and endothelial dysfunction in the pathogenesis of preeclampsia. *Pediatr Res* **2005,** *57* (5 Pt 2), 1R-7R.

23. Levine, R. J.; Maynard, S. E.; Qian, C.; Lim, K. H.; England, L. J.; Yu, K. F.; Schisterman, E. F.; Thadhani, R.; Sachs, B. P.; Epstein, F. H.; Sibai, B. M.; Sukhatme, V. P.; Karumanchi, S. A. Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med* **2004,** *350* (7), 672-683.

24. Bdolah, Y.; Sukhatme, V. P.; Karumanchi, S. A. Angiogenic imbalance in the pathophysiology of preeclampsia: newer insights. *Semin. Nephrol* **2004,** *24* (6), 548-556.

25. Granger, J. P.; LaMarca, B. B.; Cockrell, K.; Sedeek, M.; Balzi, C.; Chandler, D.; Bennett, W. Reduced uterine perfusion pressure (RUPP) model for studying cardiovascular-renal dysfunction in response to placental ischemia. *Methods Mol Med* **2006,** *122*, 383-392.

26. Banek, C. T.; Bauer, A. J.; Needham, K. M.; Dreyer, H. C.; Gilbert, J. S. AICAR administration ameliorates hypertension and angiogenic imbalance in a model of preeclampsia in the rat. *Am. J Physiol Heart Circ. Physiol* **2013,** *304* (8), H1159-H1165.

27. Gilbert, J. S.; Banek, C. T.; Bauer, A. J.; Gingery, A.; Needham, K. M. Exercise training attenuates placental ischemia induced hypertension and angiogenic imbalance in the rat. *Hypertension* **2012,** *60* (6), 1545-1551.

28. Gilbert, J. S.; Verzwyvelt, J.; Colson, D.; Arany, M.; Karumanchi, S. A.; Granger, J. P. Recombinant Vascular Endothelial Growth Factor 121 Infusion Lowers Blood Pressure and Improves Renal Function in Rats With Placental Ischemia-Induced Hypertension. *Hypertension* **2009,** *55* (2), 380-385.

29. Ohkuchi, A.; Hirashima, C.; Matsubara, S.; Takahashi, K.; Matsuda, Y.; Suzuki, M. Threshold of soluble fms-like tyrosine kinase 1/placental growth factor ratio for the imminent onset of preeclampsia. *Hypertension* **2011,** *58* (5), 859-866.

30. Narkar, V. A.; Downes, M.; Yu, R. T.; Embler, E.; Wang, Y. X.; Banayo, E.; Mihaylova, M. M.; Nelson, M. C.; Zou, Y.; Juguilon, H.; Kang, H.; Shaw, R. J.; Evans, R. M. AMPK and PPARd Agonists Are Exercise Mimetics. *Cell* **2008,** *134* (3), 405-415.

31. Giugliano, D.; De, R. N.; Di, M. G.; Marfella, R.; Acampora, R.; Buoninconti, R.; D'Onofrio, F. Metformin improves glucose, lipid metabolism, and reduces blood pressure in hypertensive, obese women. *Diabetes Care* **1993,** *16* (10), 1387-1390.

32. Agarwal, N.; Rice, S. P. L.; Bolusani, H.; Luzio, S. D.; Dunseath, G.; Ludgate, M.; Rees, D. A. Metformin Reduces Arterial Stiffness and Improves Endothelial Function in Young Women with Polycystic Ovary Syndrome: A Randomized, Placebo-Controlled, Crossover Trial. *Journal of Clinical Endocrinology Metabolism* **2010,** *95* (2), 722-730.

33. Velazquez, E. M.; Mendoza, S.; Hamer, T.; Sosa, F.; Glueck, C. J. Metformin therapy in polycystic ovary syndrome reduces hyperinsulinemia, insulin resistance, hyperandrogenemia, and systolic blood pressure, while facilitating normal menses and pregnancy. *Metabolism* **1994,** *43* (5), 647-654.

34. Ford, R. J.; Teschke, S. R.; Reid, E. B.; Durham, K. K.; Kroetsch, J. T.; Rush, J. W. AMP-activated protein kinase activator AICAR acutely lowers blood pressure and relaxes isolated resistance arteries of hypertensive rats. *J Hypertens.* **2012,** *30* (4), 725-733.

35. Buhl, E. S.; Jessen, N.; Pold, R.; Ledet, T.; Flyvbjerg, A.; Pedersen, S. B.; Pedersen, O.; Schmitz, O.; Lund, S. Long-Term AICAR Administration Reduces Metabolic Disturbances and Lowers Blood Pressure in Rats Displaying Features of the Insulin Resistance Syndrome. *Diabetes* **2002,** *51* (7), 2199-2206.

36. Bosselaar, M.; Smits, P.; van Loon, L. J. C.; Tack, C. J. Intravenous AICAR During Hyperinsulinemia Induces Systemic Hemodynamic Changes but Has No Local Metabolic Effect. *The Journal of Clinical Pharmacology* **2012,** *51* (10), 1449-1458.

37. Dixon, R.; Gourzis, J.; McDermott, D.; Fujitaki, J.; Dewland, P.; Gruber, H. AICA-riboside: safety, tolerance, and pharmacokinetics of a novel adenosine-regulating agent. *J Clin. Pharmacol.* **1991,** *31* (4), 342-347.

38. James, P. A.; Oparil, S.; Carter, B. L.; Cushman, W. C.; Dennison-Himmelfarb, C.; Handler, J.; Lackland, D. T.; LeFevre, M. L.; MacKenzie, T. D.; Ogedegbe, O.; Smith, S. C., Jr.; Svetkey, L. P.; Taler, S. J.; Townsend, R. R.; Wright, J. T., Jr.; Narva, A. S.; Ortiz, E. 2014 evidence-based guideline for the management of high blood pressure in adults: report from the panel members appointed to the Eighth Joint National Committee (JNC 8). *JAMA* **2014,** *311* (5), 507-520.

39. Calhoun, D. A.; Jones, D.; Textor, S.; Goff, D. C.; Murphy, T. P.; Toto, R. D.; White, A.; Cushman, W. C.; White, W.; Sica, D.; Ferdinand, K.; Giles, T. D.; Falkner, B.; Carey, R. M. Resistant hypertension: diagnosis, evaluation, and treatment: a scientific statement from the American Heart Association Professional Education Committee of the Council for High Blood Pressure Research. *Circulation* **2008,** *117* (25), e510-e526.

40. Roberts, J. M.; Pearson, G.; Cutler, J.; Lindheimer, M. Summary of the NHLBI Working Group on Research on Hypertension During Pregnancy. *Hypertension* **2003,** *41* (3), 437-445.

41. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. *Obstet. Gynecol.* **2013,** *122* (5), 1122-1131.

42. Conde-Agudelo, A.; Villar, J.; Lindheimer, M. World Health Organization systematic review of screening tests for preeclampsia. *Obstet. Gynecol.* **2004,** *104* (6), 1367-1391.

43. Schroeder, B. M. Practice Guidelines ACOG Practice Bulletin on Diagnosing and Managing Preeclampsia and Eclampsia. *American family physician* **2002**.

44. WHO Recommendations for Prevention and Treatment of Pre-Eclampsia and Eclampsia. *Geneva: World Health Organization* **2011**.

45. McMaster, M. T.; Zhou, Y.; Fisher, S. J. Abnormal placentation and the syndrome of preeclampsia. *Semin. Nephrol.* **2004,** *24* (6), 540-547.

46. Red-Horse, K.; Drake, P. M.; Fisher, S. J. Human pregnancy: the role of chemokine networks at the fetal-maternal interface. *Expert. Rev. Mol. Med.* **2004,** *6* (11), 1-14.

47. Gilbert, J. S.; Ryan, M. J.; LaMarca, B. B.; Sedeek, M.; Murphy, S. R.; Granger, J. P. Pathophysiology of hypertension during preeclampsia: linking placental ischemia with endothelial dysfunction. *Am J Physiol Heart Circ Physiol* **2008,** *294* (2), H541-H550.

48. Maynard, S. E.; Karumanchi, S. A. Angiogenic factors and preeclampsia. *Semin. Nephrol.* **2011,** *31* (1), 33-46.

49. Mutter, W. P.; Karumanchi, S. A. Molecular mechanisms of preeclampsia. *Microvascular Research* **2008,** *75* (1), 1-8.

50. Saito, S.; Shiozaki, A.; Nakashima, A.; Sakai, M.; Sasaki, Y. The role of the immune system in preeclampsia. *Mol. Aspects Med.* **2007,** *28* (2), 192-209.

51. Schobel, H. P.; Fischer, T.; Heuszer, K.; Geiger, H.; Schmieder, R. E. Preeclampsia -- A State of Sympathetic Overactivity. *The New England Journal of Medicine* **1996,** *335* (20), 1480-1485.

52. Conrad, K. P.; Benyo, D. F. Placental cytokines and the pathogenesis of preeclampsia. *Am J Reprod Immunol.* **1997,** *37* (3), 240-249.

53. Zhou, Y.; Gormley, M. J.; Hunkapiller, N. M.; Kapidzic, M.; Stolyarov, Y.; Feng, V.; Nishida, M.; Drake, P. M.; Bianco, K.; Wang, F.; McMaster, M. T.; Fisher, S. J. Reversal of gene dysregulation in cultured cytotrophoblasts reveals possible causes of preeclampsia. *J Clin. Invest* **2013,** *123* (7), 2862-2872.

54. Hunkapiller, N. M.; Gasperowicz, M.; Kapidzic, M.; Plaks, V.; Maltepe, E.; Kitajewski, J.; Cross, J. C.; Fisher, S. J. A role for Notch signaling in trophoblast endovascular invasion and in the pathogenesis of pre-eclampsia. *Development* **2011,** *138* (14), 2987-2998.

55. Chatterjee, P.; Weaver, L. E.; Doersch, K. M.; Kopriva, S. E.; Chiasson, V. L.; Allen, S. J.; Narayanan, A. M.; Young, K. J.; Jones, K. A.; Kuehl, T. J.; Mitchell, B. M. Placental Toll-like receptor 3 and Toll-like receptor 7/8 activation contributes to preeclampsia in humans and mice. *PLoS. ONE.* **2012,** *7* (7), e41884.

56. Goulopoulou, S.; Matsumoto, T.; Bomfim, G. F.; Webb, R. C. Toll-like receptor 9 activation: a novel mechanism linking placenta-derived mitochondrial DNA and vascular dysfunction in pre-eclampsia. *Clin. Sci. (Lond)* **2012,** *123* (7), 429-435.

57. Xie, F.; Turvey, S. E.; Williams, M. A.; Mor, G.; von, D. P. Toll-like receptor signaling and pre-eclampsia. *Am. J Reprod. Immunol.* **2010,** *63* (1), 7-16.

58. Rinehart, B. K.; Terrone, D. A.; Lagoo-Deenadayalan, S.; Barber, W. H.; Hale, E. A.; Martin, J. N., Jr.; Bennett, W. A. Expression of the placental cytokines tumor necrosis factor alpha, interleukin 1beta, and interleukin 10 is increased in preeclampsia. *Am J Obstet Gynecol* **1999,** *181* (4), 915-920.

59. Wallukat, G.; Homuth, V.; Fischer, T.; Lindschau, C.; Horstkamp, B.; Jupner, A.; Baur, E.; Nissen, E.; Vetter, K.; Neichel, D.; Dudenhausen, J. W.; Haller, H.; Luft, F. C. Patients with preeclampsia develop agonistic autoantibodies against the angiotensin AT1 receptor. *J Clin Invest* **1999,** *103* (7), 945-952.

60. Makris, A.; Thornton, C.; Thompson, J.; Thomson, S.; Martin, R.; Ogle, R.; Waugh, R.; McKenzie, P.; Kirwan, P.; Hennessy, A. Uteroplacental ischemia results in proteinuric hypertension and elevated sFLT-1. *Kidney Int* **2007,** *71* (10), 977-984.

61. Wolf, M.; Shah, A.; Lam, C.; Martinez, A.; Smirnakis, K. V.; Epstein, F. H.; Taylor, R. N.; Ecker, J. L.; Karumanchi, S. A.; Thadhani, R. Circulating levels of the antiangiogenic marker sFLT-1 are increased in first versus second pregnancies. *Am J Obstet Gynecol* **2005,** *193* (1), 16-22.

62. Thadhani, R. I.; Johnson, R. J.; Karumanchi, S. A. Hypertension during pregnancy: a disorder begging for pathophysiological support. *Hypertension* **2005,** *46* (6), 1250-1251.

63. Thadhani, R.; Mutter, W. P.; Wolf, M.; Levine, R. J.; Taylor, R. N.; Sukhatme, V. P.; Ecker, J.; Karumanchi, S. A. First trimester placental growth factor and soluble fms-like tyrosine kinase 1 and risk for preeclampsia. *J Clin Endocrinol Metab* **2004,** *89* (2), 770-775.

64. Shibata, E.; Rajakumar, A.; Powers, R. W.; Larkin, R. W.; Gilmour, C.; Bodnar, L. M.; Crombleholme, W. R.; Ness, R. B.; Roberts, J. M.; Hubel, C. A. Soluble fms-like tyrosine kinase 1 is increased in preeclampsia but not in normotensive pregnancies with small-for-gestational-age neonates: relationship to circulating placental growth factor. *J Clin Endocrinol Metab* **2005,** *90* (8), 4895-4903.

65. Rajakumar, A.; Michael, H. M.; Rajakumar, P. A.; Shibata, E.; Hubel, C. A.; Karumanchi, S. A.; Thadhani, R.; Wolf, M.; Harger, G.; Markovic, N. Extra-placental expression of vascular endothelial growth factor receptor-1, (Flt-1) and soluble Flt-1 (sFlt-1), by peripheral blood mononuclear cells (PBMCs) in normotensive and preeclamptic pregnant women. *Placenta* **2005,** *26* (7), 563-573.

66. Levine, R. J.; Lam, C.; Qian, C.; Yu, K. F.; Maynard, S. E.; Sachs, B. P.; Sibai, B. M.; Epstein, F. H.; Romero, R.; Thadhani, R.; Karumanchi, S. A. Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *N Engl J Med* **2006,** *355* (10), 992-1005.

67. Lam, C.; Lim, K. H.; Karumanchi, S. A. Circulating angiogenic factors in the pathogenesis and prediction of preeclampsia. *Hypertension* **2005,** *46* (5), 1077-1085.

68. Karumanchi, S. A.; Bdolah, Y. Hypoxia and sFlt-1 in preeclampsia: the "chicken-and-egg" question. *Endocrinology* **2004,** *145* (11), 4835-4837.

69. Karumanchi, S. A.; Maynard, S. E.; Stillman, I. E.; Epstein, F. H.; Sukhatme, V. P. Preeclampsia: a renal perspective. *Kidney Int* **2005,** *67* (6), 2101-2113.

70. Woods, A. K.; Hoffmann, D. S.; Weydert, C. J.; Butler, S. D.; Zhou, Y.; Sharma, R. V.; Davisson, R. L. Adenoviral Delivery of VEGF121 Early in Pregnancy Prevents Spontaneous Development of Preeclampsia in BPH/5 Mice. *Hypertension* **2011,** *57* (1), 94-102.

71. Li, Z.; Zhang, Y.; Ying Ma, J.; Kapoun, A. M.; Shao, Q.; Kerr, I.; Lam, A.; O'Young, G.; Sannajust, F.; Stathis, P.; Schreiner, G.; Karumanchi, S. A.; Protter, A. A.; Pollitt, N. S. Recombinant Vascular Endothelial Growth Factor 121 Attenuates Hypertension and Improves Kidney Damage in a Rat Model of Preeclampsia. *Hypertension* **2007,** *50* (4), 686-692.

72. Tranquilli, A. L.; Bezzeccheri, V.; Giannubilo, S. R.; Scagnoli, C.; Mazzanti, L.; Garzetti, G. G. Amniotic vascular endothelial growth factor (VEGF) and nitric oxide (NO) in women with subsequent preeclampsia. *European Journal of Obstetrics & Gynecology and Reproductive Biology* **2004,** *113* (1), 17-20.

73. Rana, S.; Karumanchi, S. A.; Levine, R. J.; Venkatesha, S.; Rauh-Hain, J. A.; Tamez, H.; Thadhani, R. Sequential Changes in Antiangiogenic Factors in Early Pregnancy and Risk of Developing Preeclampsia. *Hypertension* **2007,** *50* (1), 137-142.

74. Nevo, O.; Soleymanlou, N.; Wu, Y.; Xu, J.; Kingdom, J.; Many, A.; Zamudio, S.; Caniggia, I. Increased expression of sFlt-1 in in vivo and in vitro models of human placental hypoxia is mediated by HIF-1. *Am J Physiol Regul Integr Comp Physiol* **2006,** *291* (4), R1085-R1093.

75. Gilbert, J. S.; Banek, C. T.; Bauer, A. J.; Gingery, A.; Dreyer, H. C. Placental and vascular adaptations to exercise training before and during pregnancy in the rat. *Am. J Physiol Regul. Integr. Comp Physiol* **2012,** *303* (5), R520-R526.

76. Breen, E. C.; Johnson, E. C.; Wagner, H.; Tseng, H. M.; Sung, L. A.; Wagner, P. D. Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. *J Appl. Physiol (1985.)* **1996,** *81* (1), 355-361.

77. Kraus, R. M.; Stallings, H. W., III; Yeager, R. C.; Gavin, T. P. Circulating plasma VEGF response to exercise in sedentary and endurance-trained men. *Journal of Applied Physiology* **2004,** *96* (4), 1445-1450.

78. Hansen, A. H.; Nielsen, J. J.; Saltin, B.; Hellsten, Y. Exercise training normalizes skeletal muscle vascular endothelial growth factor levels in patients with essential hypertension. [Article]. *Journal of Hypertension* **2010,** *28* (6), 1176-1185.

79. Sladek, S. M.; Magness, R. R.; Conrad, K. P. Nitric oxide and pregnancy. *Am J Physiol* **1997,** *272* (2 Pt 2), R441-R463.

80. Conrad, K. P. Animal models of pre-eclampsia: do they exist? *Fetal Medicine Review* **1990,** *2*, 67-88.

81. Kassab, S.; Miller, M. T.; Hester, R.; Novak, J.; Granger, J. P. Systemic Hemodynamics and Regional Blood Flow During Chronic Nitric Oxide Synthesis Inhibition in Pregnant Rats. *Hypertension* **1998,** *31* (1), 315-320.

82. Hefler, L. A.; Tempfer, C. B.; Moreno, R. M.; O'Brien, W. E.; Gregg, A. R. Endothelial-derived nitric oxide and angiotensinogen: blood pressure and metabolism during mouse pregnancy. *Am. J Physiol Regul. Integr. Comp Physiol* **2001,** *280* (1), R174-R182.

83. Hood, J. D.; Meininger, C. J.; Ziche, M.; Granger, H. J. VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. *The American Journal Of Physiology* **1998,** *274* (3, Part 2), H1054-H1058.

84. Kroll, J.; Waltenberger, J. A novel function of VEGF receptor-2 (KDR): rapid release of nitric oxide in response to VEGF-A stimulation in endothelial cells. *Biochem. Biophys. Res. Commun.* **1999,** *265* (3), 636-639.

85. Kroll, J.; Waltenberger, J. VEGF-A induces expression of eNOS and iNOS in endothelial cells via VEGF receptor-2 (KDR). *Biochem. Biophys. Res. Commun.* **1998,** *252* (3), 743-746.

86. He, H.; Venema, V. J.; Gu, X.; Venema, R. C.; Marrero, M. B.; Caldwell, R. B. Vascular endothelial growth factor signals endothelial cell production of nitric oxide and prostacyclin through flk-1/KDR activation of c-Src. *J Biol. Chem.* **1999,** *274* (35), 25130-25135.

87. Izzedine, H.; Ederhy, S.; Goldwasser, F.; Soria, J. C.; Milano, G.; Cohen, A.; Khayat, D.; Spano, J. P. Management of hypertension in angiogenesis inhibitor-treated patients. *Annals of Oncology* **2009,** *20* (5), 807-815.

88. Izzedine, H.; Rixe, O.; Billemont, B.; Baumelou, A.; Deray, G. Angiogenesis inhibitor therapies: focus on kidney toxicity and hypertension. *Am. J Kidney Dis.* **2007,** *50* (2), 203-218.

89. Cheng, H. F.; Harris, R. C. Cyclooxygenases, the kidney, and hypertension. *Hypertension* **2004,** *43* (3), 525-530.

90. Schnackenberg, C. G.; Welch, W. J.; Wilcox, C. S. Normalization of Blood Pressure and Renal Vascular Resistance in SHR With a Membrane-Permeable Superoxide Dismutase Mimetic : Role of Nitric Oxide. *Hypertension* **1998,** *32* (1), 59-64.

91. Walsh, S. W. Maternal-placental interactions of oxidative stress and antioxidants in preeclampsia. *Semin. Reprod Endocrinol* **1998,** *16* (1), 93-104.

92. Higashi, Y.; Sasaki, S.; Kurisu, S.; Yoshimizu, A.; Sasaki, N.; Matsuura, H.; Kajiyama, G.; Oshima, T. Regular aerobic exercise augments endothelium-dependent vascular relaxation in normotensive as well as hypertensive subjects: role of endothelium-derived nitric oxide. *Circulation* **1999,** *100* (11), 1194-1202.

93. Hambrecht, R.; Wolf, A.; Gielen, S.; Linke, A.; Hofer, J.; Erbs, S.; Schoene, N.; Schuler, G. Effect of exercise on coronary endothelial function in patients with coronary artery disease. *N. Engl. J Med.* **2000,** *342* (7), 454-460.

94. Clarkson, P.; Montgomery, H. E.; Mullen, M. J.; Donald, A. E.; Powe, A. J.; Bull, T.; Jubb, M.; World, M.; Deanfield, J. E. Exercise training enhances endothelial function in young men. *J Am. Coll. Cardiol.* **1999,** *33* (5), 1379-1385.

95. Ramirez-Velez, R.; Bustamante, J.; Czerniczyniec, A.; Aguilar de Plata, A. C.; Lores-Arnaiz, S. Effect of exercise training on Enos expression, NO production and oxygen metabolism in human placenta. *PLoS. ONE.* **2013,** *8* (11), e80225.

96. Roggensack, A. M.; Zhang, Y.; Davidge, S. T. Evidence for peroxynitrite formation in the vasculature of women with preeclampsia. *Hypertension* **1999,** *33* (1), 83-89.

97. Sedeek, M. H.; Sholook, M. M.; Blazli, C.; Abram, S. R.; Chandler, D. L.; Granger, J. P. Tempol, but not Vitamin E & C, decreases the blood pressure response to a chronic reduction in uterine perfusion pressure in pregnant rats. *Faseb Journal* **2004,** *18* (4), A740.

98. Sedeek, M. H.; Alexander, B. T.; Sholook, M. M.; Chandler, D. L.; Abram, S. R.; Granger, J. P. Renal cortical NADPH oxidase and superoxide dismutase activity in response to reduced uterine perfusion pressure in pregnant rats. *Faseb Journal* **2004,** *18* (4), A740.

99. Sedeek, M. H.; Wang, Y. P.; Granger, J. P. Increased oxidative stress in a rat model of preeclampsia. *American Journal of Hypertension* **2004,** *17* (5), 142A.

100. Poston, L.; Igosheva, N.; Mistry, H. D.; Seed, P. T.; Shennan, A. H.; Rana, S.; Karumanchi, S. A.; Chappell, L. C. Role of oxidative stress and antioxidant supplementation in pregnancy disorders. *Am. J Clin. Nutr.* **2011,** *94* (6 Suppl), 1980S-1985S.

101. Poston, L.; Briley, A. L.; Seed, P. T.; Kelly, F. J.; Shennan, A. H. Vitamin C and vitamin E in pregnant women at risk for pre-eclampsia (VIP trial): randomised placebo-controlled trial. *Lancet* **2006,** *367* (9517), 1145-1154.

102. Ji, L. L. Antioxidants and oxidative stress in exercise. *Proc. Soc. Exp. Biol. Med.* **1999,** *222* (3), 283-292.

103. Leaf, D. A.; Kleinman, M. T.; Hamilton, M.; Barstow, T. J. The effect of exercise intensity on lipid peroxidation. *Med. Sci. Sports Exerc.* **1997,** *29* (8), 1036-1039.

104. Sorensen, T. K.; Williams, M. A.; Lee, I. M.; Dashow, E. E.; Thompson, M. L.; Luthy, D. A. Recreational physical activity during pregnancy and risk of preeclampsia. *Hypertension* **2003,** *41* (6), 1273-1280.

105. Weissgerber, T. L.; Wolfe, L. A.; Davies, G. A. The role of regular physical activity in preeclampsia prevention. *Med Sci Sports Exerc.* **2004,** *36* (12), 2024-2031.

106. Yeo, S.; Davidge, S. T. Possible beneficial effect of exercise, by reducing oxidative stress, on the incidence of preeclampsia. *J Womens Health Gend. Based. Med.* **2001,** *10* (10), 983-989.

107. Hagberg, J. M.; Brown, M. D. Does exercise training play a role in the treatment of essential hypertension? *J Cardiovasc. Risk* **1995,** *2* (4), 296-302.

108. Pescatello, L. S.; Franklin, B. A.; Fagard, R.; Farquhar, W. B.; Kelley, G. A.; Ray, C. A.; This pronouncement was written for the American College of Sports Medicine by Exercise and Hypertension. *Medicine & Science in Sports & Exercise* **2004,** *36* (3).

109. Hagberg, J. M.; Park, J. J.; Brown, M. D. The role of exercise training in the treatment of hypertension: an update. *Sports Med.* **2000,** *30* (3), 193-206.

110. ACOG Committee on Obstetric Practice Committee opinion #267: exercise during pregnancy and the postpartum period. *Obstetrics & Gynecology* **2002,** *99* (1), 171-173.

111. Clapp, J. F., III; Capeless, E. L. Neonatal morphometrics after endurance exercise during pregnancy. *Am. J. Obstet. Gynecol.* **1990,** *163* (6 Pt 1), 1805-1811.

112. Clapp, J. F., III Morphometric and neurodevelopmental outcome at age five years of the offspring of women who continued to exercise regularly throughout pregnancy. *J. Pediatr.* **1996,** *129* (6), 856-863.

113. Clapp, J. F., III; Kim, H.; Burciu, B.; Lopez, B. Beginning regular exercise in early pregnancy: effect on fetoplacental growth. *Am J Obstet. Gynecol.* **2000,** *183* (6), 1484-1488.

114. Campbell, M. K.; Mottola, M. F. Recreational exercise and occupational activity during pregnancy and birth weight: a case-control study. *Am. J. Obstet. Gynecol.* **2001,** *184* (3), 403-408.

115. Dempsey, J. C.; Butler, C. L.; Williams, M. A. No need for a pregnant pause: physical activity may reduce the occurrence of gestational diabetes mellitus and preeclampsia. *Exerc. Sport Sci. Rev.* **2005,** *33* (3), 141-149.

116. Marcoux, S.; Brisson, J.; Fabia, J. The effect of leisure time physical activity on the risk of pre-eclampsia and gestational hypertension. *J Epidemiol. Community Health* **1989,** *43* (2), 147-152.

117. Weissgerber, T. L.; Davies, G. A. L.; Roberts, J. M. Modification of angiogenic factors by regular and acute exercise during pregnancy. *Journal of Applied Physiology* **2010,** *108* (5), 1217-1223.

118. Genest, D. S.; Falcao, S.; Michel, C.; Kajla, S.; Germano, M. F.; Lacasse, A. A.; Vaillancourt, C.; Gutkowska, J.; Lavoie, J. L. Novel Role of the Renin-Angiotensin System in Preeclampsia Superimposed on Chronic Hypertension and the Effects of Exercise in a Mouse Model. *Hypertension* **2013**.

119. Falcao, S.; Bisotto, S.; Michel, C.; Lacasse, A. A.; Vaillancourt, C.; Gutkowska, J.; Lavoie, J. L. Exercise training can attenuate preeclampsia-like features in an animal model. *J Hypertens.* **2010,** *28* (12), 1057-1062.

120. Clapp, J. F., III; Kim, H.; Burciu, B.; Schmidt, S.; Petry, K.; Lopez, B. Continuing regular exercise during pregnancy: effect of exercise volume on fetoplacental growth. *Am J Obstet. Gynecol.* **2002,** *186* (1), 142-147.

121. Clapp, J. F., III; Rizk, K. H. Effect of recreational exercise on midtrimester placental growth. *Am. J Obstet. Gynecol.* **1992,** *167* (6), 1518-1521.

122. Clapp, J. F., III The effects of maternal exercise on fetal oxygenation and feto-placental growth. *Eur. J Obstet. Gynecol. Reprod. Biol.* **2003,** *110 Suppl 1*, S80-S85.

123. Lotgering, F. K.; Gilbert, R. D.; Longo, L. D. Maternal and fetal responses to exercise during pregnancy. *Physiol Rev.* **1985,** *65* (1), 1-36.

124. Kemp, J. G.; Greer, F. A.; Wolfe, L. A. Acid-base regulation after maximal exercise testing in late gestation. *J Appl. Physiol (1985.)* **1997,** *83* (2), 644-651.

125. Richter, E. A.; Ruderman, N. B. AMPK and the biochemistry of exercise: implications for human health and disease. *Biochem. J* **2009,** *418* (2), 261-275.

126. Jorgensen, S. B.; Richter, E. A.; Wojtaszewski, J. F. Role of AMPK in skeletal muscle metabolic regulation and adaptation in relation to exercise. *J Physiol* **2006,** *574* (Pt 1), 17-31.

127. Zwetsloot, K. A.; Westerkamp, L. M.; Holmes, B. F.; Gavin, T. P. AMPK regulates basal skeletal muscle capillarization and VEGF expression, but is not necessary for the angiogenic response to exercise. *The Journal of Physiology* **2008,** *586* (24), 6021-6035.

128. Ouchi, N.; Shibata, R.; Walsh, K. AMP-activated protein kinase signaling stimulates VEGF expression and angiogenesis in skeletal muscle. *Circ. Res.* **2005,** *96* (8), 838-846.

129. Cheung, P. C.; Salt, I. P.; Davies, S. P.; Hardie, D. G.; Carling, D. Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding. *Biochem. J* **2000,** *346 Pt 3*, 659-669.

130. Moffat, C.; Ellen, H. M. Metabolic functions of AMPK: aspects of structure and of natural mutations in the regulatory gamma subunits. *IUBMB. Life* **2010,** *62* (10), 739-745.

131. Glueck, C. J.; Goldenberg, N.; Pranikoff, J.; Loftspring, M.; Sieve, L.; Wang, P. Height, weight, and motor-social development during the first 18 months of life in 126 infants born to 109 mothers with polycystic ovary syndrome who conceived on and continued metformin through pregnancy. *Hum. Reprod.* **2004,** *19* (6), 1323-1330.

132. Sibai, B. M.; Gordon, T.; Thom, E.; Caritis, S. N.; Klebanoff, M.; McNellis, D.; Paul, R. H. Risk factors for preeclampsia in healthy nulliparous women: a prospective multicenter study. The National Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Units. *Am. J Obstet. Gynecol.* **1995,** *172* (2 Pt 1), 642-648.

133. Imperiale, T. F.; Petrulis, A. S. A meta-analysis of low-dose aspirin for the prevention of pregnancy-induced hypertensive disease. *JAMA* **1991,** *266* (2), 260-264.

134. Wallenburg, H. C.; Dekker, G. A.; Makovitz, J. W.; Rotmans, P. Low-dose aspirin prevents pregnancy-induced hypertension and pre-eclampsia in angiotensin-sensitive primigravidae. *Lancet* **1986,** *1* (8471), 1-3.

135. Hou, X.; Song, J.; Li, X. N.; Zhang, L.; Wang, X.; Chen, L.; Shen, Y. H. Metformin reduces intracellular reactive oxygen species levels by upregulating expression of the antioxidant thioredoxin via the AMPK-FOXO3 pathway. *Biochem. Biophys. Res. Commun.* **2010,** *396* (2), 199-205.

136. Steinberg, G. R.; Kemp, B. E. AMPK in Health and Disease. *Physiol Rev.* **2009,** *89* (3), 1025-1078.

137. Chen, Z. P.; Mitchelhill, K. I.; Michell, B. J.; Stapleton, D.; Rodriguez-Crespo, I.; Witters, L. A.; Power, D. A.; Ortiz de Montellano, P. R.; Kemp, B. E. AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett.* **1999,** *443* (3), 285-289.

138. Zhang, J.; Xie, Z.; Dong, Y.; Wang, S.; Liu, C.; Zou, M. H. Identification of nitric oxide as an endogenous activator of the AMP-activated protein kinase in vascular endothelial cells. *J Biol. Chem.* **2008,** *283* (41), 27452-27461.

139. Pold, R.; Jensen, L. S.; Jessen, N.; Buhl, E. S.; Schmitz, O.; Flyvbjerg, A.; Fujii, N.; Goodyear, L. J.; Gotfredsen, C. F.; Brand, C. L.; Lund, S. Long-Term AICAR Administration and Exercise Prevents Diabetes in ZDF Rats. *Diabetes* **2005,** *54* (4), 928-934.

140. Li, J.; LaMarca, B.; Reckelhoff, J. F. A model of preeclampsia in rats: the reduced uterine perfusion pressure (RUPP) model. *Am. J Physiol Heart Circ. Physiol* **2012,** *303* (1), H1-H8.

141. Bauer, A. J.; Banek, C. T.; Needham, K.; Gillham, H.; Capoccia, S.; Regal, J. F.; Gilbert, J. S. Pravastatin attenuates hypertension, oxidative stress, and angiogenic imbalance in rat model of placental ischemia-induced hypertension. *Hypertension* **2013,** *61* (5), 1103-1110.

142. Genbacev, O.; Zhou, Y.; Ludlow, J. W.; Fisher, S. J. Regulation of human placental development by oxygen tension. *Science* **1997,** *277* (5332), 1669-1672.

143. George, E. M.; Cockrell, K.; Adair, T. H.; Granger, J. P. Regulation of sFlt-1 and VEGF secretion by adenosine under hypoxic conditions in rat placental villous explants. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **2010,** *299* (6), R1629-R1633.

144. Benyo, D. F.; Miles, T. M.; Conrad, K. P. Hypoxia stimulates cytokine production by villous explants from the human placenta. *J Clin Endocrinol Metab* **1997,** *82* (5), 1582-1588.

145. Evans, C. S.; Gooch, L.; Flotta, D.; Lykins, D.; Powers, R. W.; Landsittel, D.; Roberts, J. M.; Shroff, S. G. Cardiovascular System During the Postpartum State in Women With a History of Preeclampsia. *Hypertension* **2011,** *58* (1), 57-62.

146. Melchiorre, K.; Sutherland, G. R.; Liberati, M.; Thilaganathan, B. Preeclampsia is associated with persistent postpartum cardiovascular impairment. *Hypertension* **2011,** *58* (4), 709-715.

147. Bellamy, L.; Casas, J. P.; Hingorani, A. D.; Williams, D. J. Pre-eclampsia and risk of cardiovascular disease and cancer in later life: systematic review and meta-analysis. *BMJ* **2007,** *335* (7627), 974.

148. Irgens, H. U.; Reisater, L.; Irgens, L. M.; Lie, R. T.; Roberts, J. M. Long term mortality of mothers and fathers after pre-eclampsia: population based cohort study Pre-eclampsia and cardiovascular disease later in life: who is at risk? *BMJ* **2001,** *323* (7323), 1213-1217.

149. Karumanchi, S. A.; Epstein, F. H. Placental ischemia and soluble fms-like tyrosine kinase 1: cause or consequence of preeclampsia? *Kidney Int.* **2007,** *71* (10), 959-961.

150. Banek, C.; Gilbert, J. Approaching the threshold for predicting preeclampsia: monitoring angiogenic balance during pregnancy. *Hypertension* **2011,** *58* (5), 774-775.

151. Banek, C. T.; Bauer, A. J.; Gingery, A.; Gilbert, J. S. Timing of ischemic insult alters fetal growth trajectory, maternal angiogenic balance and markers of renal oxidative stress in the pregnant rat. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* **2012,** *303* (6), R658-R664.

152. Heltemes, A.; Gingery, A.; Soldner, E. L.; Bozadjieva, N.; Jahr, K. N.; Johnson, B. K.; Gilbert, J. S. Chronic placental ischemia alters amniotic fluid milieu and results in impaired glucose tolerance, insulin resistance and hyperleptinemia in young rats. *Exp. Biol. Med. (Maywood.)* **2010,** *235* (7), 892-899.

153. Alexander, B. T.; Bennett, W. A.; Khalil, R. A.; Granger, J. P. Preeclampsia: linking placental ischemia with cardiovascular-renal dysfunction. *News Physiol Sci.* **2001,** *16*, 282-286.

154. Tjoa, M. L.; Levine, R. J.; Karumanchi, S. A. Angiogenic factors and preeclampsia. *Front Biosci.* **2007,** *12*, 2395-2402.

155. Rowell, L. B.; O'Leary, D. S. Reflex control of the circulation during exercise: chemoreflexes and mechanoreflexes. *J Appl. Physiol (1985.)* **1990,** *69* (2), 407-418.

156. Rauramo, I.; Forss, M. Effect of exercise on maternal hemodynamics and placental blood flow in healthy women. *Acta Obstet. Gynecol. Scand.* **1988,** *67* (1), 21-25.

157. Hart, A.; Morris, N.; Osborn, S.; Wright, H. Effective uterine bloodflow during exercise in normal and pre-eclamptic pregnancies. *Lancet* **1956,** *271* (6941), 481-484.

158. Hackett, G. A.; Cohen-Overbeek, T.; Campbell, S. The effect of exercise on uteroplacental Doppler waveforms in normal and complicated pregnancies. *Obstet. Gynecol.* **1992,** *79* (6), 919-923.

159. Weissgerber, T. L.; Wolfe, L. A.; Davies, G. A.; Mottola, M. F. Exercise in the prevention and treatment of maternal-fetal disease: a review of the literature. *Appl. Physiol Nutr Metab* **2006,** *31* (6), 661-674.

160. Bridges, J. P.; Gilbert, J. S.; Colson, D.; Dukes, M.; Babcock, S. A.; Ryan, M. J.; Granger, J. P. Soluble Flt-1 induces hypertension and vascular dysfunction in pregnant rats. *The FASEB Journal* **2008,** *22* (1_MeetingAbstracts), 969.

161. Collings, C. A.; Curet, L. B.; Mullin, J. P. Maternal and fetal responses to a maternal aerobic exercise program. *Am. J. Obstet. Gynecol.* **1983,** *145* (6), 702-707.

162. Weissgerber, T. L.; McConico, A.; Knudsen, B. E.; Butters, K. A.; Hayman, S. R.; White, W. M.; Milic, N.; Miller, V. M.; Garovic, V. D. Methodological Differences Account for Inconsistencies in Reported Free VEGF Concentrations in Pregnant Rats. *Am. J. Physiol Regul. Integr. Comp Physiol* **2014**.

163. Venkatesha, S.; Toporsian, M.; Lam, C.; Hanai, J.; Mammoto, T.; Kim, Y. M.; Bdolah, Y.; Lim, K. H.; Yuan, H. T.; Libermann, T. A.; Stillman, I. E.; Roberts, D.; D'Amore, P. A.; Epstein, F. H.; Sellke, F. W.; Romero, R.; Sukhatme, V. P.; Letarte, M.; Karumanchi, S. A. Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat Med* **2006,** *12* (6), 642-649.

164. Jessen, N.; Pold, R.; Buhl, E. S.; Jensen, L. S.; Schmitz, O.; Lund, S. Effects of AICAR and exercise on insulin-stimulated glucose uptake, signaling, and GLUT-4 content in rat muscles. *J Appl. Physiol* **2003,** *94* (4), 1373-1379.

165. Russell, R. R., III; Bergeron, R.; Shulman, G. I.; Young, L. H. Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. *Am. J Physiol* **1999,** *277* (2 Pt 2), H643-H649.

166. Frier, B. C.; Wan, Z.; Williams, D. B.; Stefanson, A. L.; Wright, D. C. Epinephrine and AICAR-induced PGC-1alpha mRNA expression is intact in skeletal muscle from rats fed a high-fat diet. *Am. J Physiol Cell Physiol* **2012,** *302* (12), C1772-C1779.

167. Frier, B. C.; Hancock, C. R.; Little, J. P.; Fillmore, N.; Bliss, T. A.; Thomson, D. M.; Wan, Z.; Wright, D. C. Reductions in RIP140 are not required for exercise- and AICAR-mediated increases in skeletal muscle mitochondrial content. *J Appl. Physiol* **2011,** *111* (3), 688-695.

168. Lemieux, K.; Konrad, D.; Klip, A.; Marette, A. The AMP-activated protein kinase activator AICAR does not induce GLUT4 translocation to transverse tubules but stimulates glucose uptake and p38 mitogen-activated protein kinases alpha and beta in skeletal muscle. *FASEB J* **2003,** *17* (12), 1658-1665.

169. Karagounis, L. G.; Hawley, J. A. The 5' adenosine monophosphate-activated protein kinase: regulating the ebb and flow of cellular energetics. *Int. J Biochem. Cell Biol.* **2009,** *41* (12), 2360-2363.

170. Bailey, A. N.; Hocker, A. D.; Vermillion, B. R.; Smolkowski, K.; Shah, S. N.; Jewett, B. A.; Dreyer, H. C. MAFbx, MuRF1, and the stress-activated protein kinases are upregulated in muscle cells during total knee arthroplasty. *Am. J Physiol Regul. Integr. Comp Physiol* **2012,** *303* (4), R376-R386.

171. Wang, X. F.; Zhang, J. Y.; Li, L.; Zhao, X. Y.; Tao, H. L.; Zhang, L. Metformin improves cardiac function in rats via activation of AMP-activated protein kinase. *Clin. Exp. Pharmacol. Physiol* **2011,** *38* (2), 94-101.

172. Ford, R. J.; Rush, J. W. E. Endothelium-dependent vasorelaxation to the AMPK activator AICAR is enhanced in aorta from hypertensive rats and is NO and EDCF dependent. *American Journal of Physiology - Heart and Circulatory Physiology* **2011,** *300* (1), H64-H75.

173. Salt, I. P.; Connell, J. M.; Gould, G. W. 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) inhibits insulin-stimulated glucose transport in 3T3-L1 adipocytes. *Diabetes* **2000,** *49* (10), 1649-1656.

174. Martinez-Martin, N.; Blas-Garcia, A.; Morales, J. M.; Marti-Cabrera, M.; Monleon, D.; Apostolova, N. Metabolomics of the effect of AMPK activation by AICAR on human umbilical vein endothelial cells. *Int. J Mol. Med.* **2012,** *29* (1), 88-94.

175. Corton, J. M.; Gillespie, J. G.; Hawley, S. A.; Hardie, D. G. 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? *Eur. J Biochem.* **1995,** *229* (2), 558-565.

176. Bridges, J. P.; Gilbert, J. S.; Colson, D.; Gilbert, S. A.; Dukes, M. P.; Ryan, M. J.; Granger, J. P. Oxidative Stress Contributes to Soluble Fms-Like Tyrosine Kinase-1 Induced Vascular Dysfunction in Pregnant Rats. *Am J Hypertens* **2009,** *22* (5), 564-568.

177. Terai, K.; Hiramoto, Y.; Masaki, M.; Sugiyama, S.; Kuroda, T.; Hori, M.; Kawase, I.; Hirota, H. AMP-activated protein kinase protects cardiomyocytes against hypoxic injury through attenuation of endoplasmic reticulum stress. *Mol. Cell Biol.* **2005,** *25* (21), 9554-9575.

178. Nevo, O.; Many, A.; Xu, J.; Kingdom, J.; Piccoli, E.; Zamudio, S.; Post, M.; Bocking, A.; Todros, T.; Caniggia, I. Placental Expression of Soluble fms-Like Tyrosine Kinase 1 is Increased in Singletons and Twin Pregnancies with Intrauterine Growth Restriction. *Journal of Clinical Endocrinology Metabolism* **2008,** *93* (1), 285-292.

179. Zhao, S.; Gu, Y.; Fan, R.; Groome, L. J.; Cooper, D.; Wang, Y. Proteases and sFlt-1 Release in the Human Placenta. *Placenta* **2010,** *31* (6), 512-518.

180. Weissgerber, T. L.; Rajakumar, A.; Myerski, A. C.; Edmunds, L. R.; Powers, R. W.; Roberts, J. M.; Gandley, R. E.; Hubel, C. A. Vascular Pool of Releasable Soluble VEGF Receptor-1 (sFLT1) in Women With Previous Preeclampsia and Uncomplicated Pregnancy. *J Clin. Endocrinol. Metab* **2014,** *99* (3), 978-987.

181. Burton, G. J.; Yung, H. W.; Cindrova-Davies, T.; Charnock-Jones, D. S. Placental endoplasmic reticulum stress and oxidative stress in the pathophysiology of unexplained intrauterine growth restriction and early onset preeclampsia. *Placenta* **2009,** *30 Suppl A*, S43-S48.

182. Straszewski-Chavez, S. L.; Abrahams, V. M.; Mor, G. The Role of Apoptosis in the Regulation of Trophoblast Survival and Differentiation during Pregnancy. *Endocr Rev* **2005,** *26* (7), 877-897.

183. Al-Gubory KH; Fowler PA; Garrel C The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. *Int J Biochem Cell Biol* **2010,** *42* (10), 1634-1650.

184. Genbacev, O.; Schubach, S. A.; Miller, R. K. Villous culture of first trimester human placenta--model to study extravillous trophoblast (EVT) differentiation. *Placenta* **1992,** *13* (5), 439-461.

185. Chan, A. Y.; Dolinsky, V. W.; Soltys, C. L.; Viollet, B.; Baksh, S.; Light, P. E.; Dyck, J. R. Resveratrol inhibits cardiac hypertrophy via AMP-activated protein kinase and Akt. *J Biol. Chem.* **2008,** *283* (35), 24194-24201.

186. Jinnin, M.; Medici, D.; Park, L.; Limaye, N.; Liu, Y.; Boscolo, E.; Bischoff, J.; Vikkula, M.; Boye, E.; Olsen, B. R. Suppressed NFAT-dependent VEGFR1 expression and constitutive VEGFR2 signaling in infantile hemangioma. *Nat. Med.* **2008,** *14* (11), 1236-1246.

187. Hogan, P. G.; Chen, L.; Nardone, J.; Rao, A. Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev.* **2003,** *17* (18), 2205-2232.

188. Zhou, C. C.; Ahmad, S.; Mi, T.; Xia, L.; Abbasi, S.; Hewett, P. W.; Sun, C.; Ahmed, A.; Kellems, R. E.; Xia, Y. Angiotensin II induces soluble fms-Like tyrosine kinase-1 release via calcineurin signaling pathway in pregnancy. *Circ. Res.* **2007,** *100* (1), 88-95.

189. Banek, C. T.; Gillham, H. E.; Johnson, S. M.; Dreyer, H. C.; Gilbert, J. S. 5-aminoimidazole-4-carboxamide-3-ribonucleoside (AICAR) Decreases Soluble Fms-like Tyrosine Kinase-1 (sFlt-1) and Attenuates Endoplasmic Reticulum Stress in Rat Placental Villi Explants. *Hypertension* **2013,** *62*, A269.

190. Salas, S. P.; Marshall, G.; Gutierrez, B. L.; Rosso, P. Time Course of Maternal Plasma Volume and Hormonal Changes in Women With Preeclampsia or Fetal Growth Restriction. *Hypertension* **2006,** *47* (2), 203-208.

191. Silver, H. M.; Seebeck, M.; Carlson, R. Comparison of total blood volume in normal, preeclamptic, and nonproteinuric gestational hypertensive pregnancy by simultaneous measurement of red blood cell and plasma volumes. *Am. J. Obstet. Gynecol.* **1998,** *179* (1), 87-93.

192. Hays, P. M.; Cruikshank, D. P.; Dunn, L. J. Plasma volume determination in normal and preeclamptic pregnancies. *Am. J. Obstet. Gynecol.* **1985,** *151* (7), 958-966.

193. Hines, T.; Connell, K.; Sims, R.; Rice, C. Baroreflex regulation and plasma volume in hypertensive pregnant rats with reduced uterine perfusion. *FASEB J* **2006,** *20*, A774.

194. Costantine, M. M. Physiologic and pharmacokinetic changes in pregnancy. *Front Pharmacol.* **2014,** *5*, 65.

195. Pang, T.; Rajapurohitam, V.; Cook, M. A.; Karmazyn, M. Differential AMPK phosphorylation sites associated with phenylephrine vs. antihypertrophic effects of adenosine agonists in neonatal rat ventricular myocytes. *AJP: Heart and Circulatory Physiology* **2010,** *298* (5), H1382-H1390.

196. Xia, Y.; Kellems, R. E. Angiotensin receptor agonistic autoantibodies and hypertension: preeclampsia and beyond. *Circ. Res.* **2013,** *113* (1), 78-87.

197. LaMarca, B.; Wallace, K.; Herse, F.; Wallukat, G.; Martin, J. N., Jr.; Weimer, A.; Dechend, R. Hypertension in response to placental ischemia during pregnancy: role of B lymphocytes. *Hypertension* **2011,** *57* (4), 865-871.

198. Dechend, R.; Homuth, V.; Wallukat, G.; Muller, D. N.; Krause, M.; Dudenhausen, J.; Haller, H.; Luft, F. C. Agonistic antibodies directed at the angiotensin II, AT1 receptor in preeclampsia. *J Soc Gynecol Investig.* **2006,** *13* (2), 79-86.

199. Hines, T.; Beauchamp, D.; Rice, C. Baroreflex Control of Sympathetic Nerve Activity in Hypertensive Pregnant Rats with Reduced Uterine Perfusion. *Hypertension in Pregnancy* **2007,** *26* (3), 303-314.

200. Greenwood, J. P.; Scott, E. M.; Stoker, J. B.; Walker, J. J.; Mary, D. A. S. G. Sympathetic Neural Mechanisms in Normal and Hypertensive Pregnancy in Humans. *Circulation* **2001,** *104* (18), 2200-2204.

201. LaMarca, B. D.; Ryan, M. J.; Gilbert, J. S.; Murphy, S. R.; Granger, J. P. Inflammatory cytokines in the pathophysiology of hypertension during preeclampsia. *Curr. Hypertens. Rep.* **2007,** *9* (6), 480-485.

202. LaMarca, B.; Parrish, M.; Ray, L. F.; Murphy, S. R.; Roberts, L.; Glover, P.; Wallukat, G.; Wenzel, K.; Cockrell, K.; Martin, J. N., Jr.; Ryan, M. J.; Dechend, R. Hypertension in response to autoantibodies to the angiotensin II type I receptor (AT1-AA) in pregnant rats: role of endothelin-1. *Hypertension* **2009,** *54* (4), 905-909.

203. Salminen, A.; Hyttinen, J. M.; Kaarniranta, K. AMP-activated protein kinase inhibits NF-kappaB signaling and inflammation: impact on healthspan and lifespan. *J Mol. Med. (Berl)* **2011,** *89* (7), 667-676.

204. Thadhani, R.; Kisner, T.; Hagmann, H.; Bossung, V.; Noack, S.; Schaarschmidt, W.; Jank, A.; Kribs, A.; Cornely, O. A.; Kreyssig, C.; Hemphill, L.; Rigby, A. C.; Khedkar, S.; Lindner, T. H.; Mallmann, P.; Stepan, H.; Karumanchi, S. A.; Benzing, T. Pilot study of extracorporeal removal of soluble fms-like tyrosine kinase 1 in preeclampsia. *Circulation* **2011,** *124* (8), 940-950.

205. Clapp, J. F., III Exercise during pregnancy. A clinical update. *Clin. Sports Med.* **2000,** *19* (2), 273-286.

206. Rauramo, I.; Forss, M. Effect of exercise on placental blood flow in pregnancies complicated by hypertension, diabetes or intrahepatic cholestasis. *Acta Obstet. Gynecol. Scand.* **1988,** *67* (1), 15-20.