The role of complement system activation in placental ischemia-induced hypertension

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Dedication

This work is dedicated to my family and KJN.

Abstract

Preeclampsia is a pregnancy-specific condition characterized by new-onset hypertension and proteinuria associated with placental ischemia. Because no cure exists besides parturition, preeclampsia remains a leading cause of maternal and perinatal death and morbidity. New management strategies are urgently needed to attenuate maternal symptoms and prolong gestation. Immune activity is normally heightened in pregnancy and has been shown to increase even further in preeclampsia, as evidenced by elevations in innate immune complement activation products, including C3a. Decreased circulating free vascular endothelial growth factor (VEGF) is a known contributor to preeclampsia and previous studies have demonstrated a link between VEGF and complement. We therefore hypothesized that complement activation is critical to placental ischemia-induced hypertension. To test this, we used the Reduced Utero-placental Perfusion Pressure (RUPP) model of placental ischemia in the rat to induce hypertension and explore the effects of inhibiting complement activation and antagonizing a specific complement receptor in this model. The data demonstrate that complement activation occurred following placental ischemia and administration of soluble complement receptor 1, an inhibitor of complement activation, successfully prevented complement activation and abrogated the hypertension without influencing circulating free VEGF concentrations. To determine the specific complement component responsible, we used a C3a receptor antagonist to inhibit C3a-mediated cellular responses that may be important in placental ischemia-induced hypertension. The C3a receptor antagonist attenuated the hypertension and improved placental efficiency and did not affect circulating free VEGF, suggesting complement may be important in hypertension apart from circulating free VEGF concentrations. Overall, these data suggest a potentially valuable role for specific complement inhibition in managing the symptoms of preeclampsia.

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Introduction

The following investigates the complex physiological relationships involved in preeclampsia, a multi-systemic condition specific to pregnancy. Using the integrative principle of feedback loops, we manipulated several key factors implicated as important to the pathophysiology of preeclampsia. We incorporated a broad scope of complexities, ranging from molecular to whole-organism, to study preeclampsia from a unique integrative perspective.

Chapter 1

Background

Pathophysiology of preeclampsia

Preeclampsia is a complex condition specific to human pregnancy that is manifest by new-onset maternal hypertension and proteinuria, often accompanied by intrauterine growth restriction (Steegers et al., 2010). Preeclampsia is a widespread disorder affecting approximately 2-8% of pregnancies (Gilbert et al., 2007; Maynard et al., 2008) and it involves multiple systems, making it a leading cause of maternal and perinatal morbidity and mortality. Currently, no definitive cures exist besides delivery of the conceptus, and lack of treatment may lead to progression to eclampsia with seizures and death. Extensive studies have identified various pathophysiological characteristics of the condition, yet the etiology of preeclampsia remains unclear (Sibai et al., 2005; Steegers et al., 2010). Understanding the underlying mechanisms of preeclampsia may facilitate the development of effective management options to improve both maternal and fetal health.

Clinically, preeclampsia is defined as hypertension (140/90 mmHg) and proteinuria (300 mg protein in the urine measured over 24 hours) and is typically associated with widespread endothelial damage and dysfunction. Symptoms may become evident as early as 20 weeks ("early-onset" if before 34 weeks, "late-onset" after 34 weeks). Early-onset preeclampsia has a different profile than late-onset and is associated with more pronounced angiogenic imbalance, more severe intrauterine growth restriction, and higher morbidity (Cunningham et al., 2009) than late-onset. Variants of preeclampsia exist, such as Hemolysis, Elevated Liver enzymes, and Low Platelets (HELLP) syndrome, and the development of seizures with hypertension and proteinuria indicates progression to eclampsia.

Healthy pregnancies require numerous physiological changes to occur to accommodate proper placental, embryonic and ultimately fetal development. In early human development, embryonic cells differentiate into an outer layer of trophoblastic cells and an inner mass of cells called the embryoblast around eight days post-fertilization. The trophoblastic cells normally burrow into the endometrial layer of the uterine wall to anchor the embryo and eventually establish the placenta. These cells are responsible for downregulating epithelial adhesion molecule expression and instead assume adhesion molecules characteristic of endothelial cells, a process termed pseudovasculogenesis. During this time, coiled maternal blood vessels called spiral arteries partially uncoil and dilate to reduce resistance and increase blood flow to the placenta. Inadequate trophoblastic invasion results in abnormalities in maternal arterial remodeling and ultimately leads to abnormal placentation, reduced uterine perfusion, and

placental ischemia (Caluwaerts et al., 2004; Bainbridge et al., 2009), which are implicated as critical precursors to preeclampsia (Gilbert et al., 2008).

Because the spiral arteries do not properly dilate in women destined to be preeclamptic, uterine circulation passes through higher resistance vessels and does not supply adequate blood to the feto-placental unit. The ischemic (and therefore, hypoxic) placenta stimulates the production and release of a suite of factors that are associated with endothelial dysfunction, hypertension, and oxidative stress. Alterations in these factors, including soluble fms-like tyrosine kinase 1 (sFlt-1) (a soluble form of the receptor to vascular endothelial growth factor (VEGF)), VEGF, endoglin, and placental growth factor (PlGF) have been demonstrated to contribute to the development of preeclampsia in both low- and high-risk women (Powers et al., 2010).

Other factors are thought to contribute to the pathophysiology of preeclampsia and are discussed below. These are summarized in Table 1 and include nitric oxide, angiotensin, VEGF, sFlt-1, and immune factors.

Table 1. Factors involved in the pathophysiology of preeclampsia.

Factor	Function	Levels in preeclamptic pregnancy vs normal pregnancy
Nitric oxide (NO)	Vasodilator; necessary component for angiogenesis	Decreased
Angiotensin	Regulates blood pressure through renin-angiotensin-aldosterone system	Increased
Vascular endothelial growth factor (VEGF), placental growth factor (PlGF)	Vasodilators through NO stimulation; pro-angiogenic	Decreased
Soluble fms-like tyrosine kinase-1 (sFlt-1)	Vasoconstrictor; soluble receptor for VEGF	Increased
Interleukin-6 CD4+ T cells	Markers of inflammation	Increased
Complement activation products (C3a, C5a, Bb)	Indicate innate immune response	Increased

Nitric oxide. Oxidative stress, or the presence of reactive oxygen species overwhelming the buffering effects of antioxidants, has long been considered a primary cause in the development of endothelial dysfunction leading to the manifestation of symptoms in preeclampsia (Roberts & Cooper, 2001). An increase in oxidative stress has been demonstrated following placental ischemia and may have a role in renal dysfunction and hypertension (Sedeek et al., 2008). Several factors indicative of oxidative stress have recently been identified in women with early-onset preeclampsia, such as 8-isoprostane (Wikström et al., 2009), malondialdehyde (Rani et al., 2010), and myeloperoxidase (Kurdoglu et al., 2012), and onset of placental ischemia precedes an apparent decrease in

innate antioxidant activity (Sedeek et al., 2008). In preeclampsia, endothelial nitric oxide synthase (eNOS) has decreased activity and uncouples to produce superoxide rather than nitric oxide (NO). NO is a necessary component for angiogenesis (Ahmed, 2011) and its production is increased by vascular endothelial growth factor (VEGF) receptor stimulation. Studies have shown that inhibition of VEGF receptors leads to a decrease in NO and, consequently, a lack of vasodilation (Robinson et al., 2010). Decreases in NO are also associated with hypertension in preeclampsia (Li et al., 2012).

NO and prostaglandins also mediate increases in glomerular filtration rate (GFR) and renal plasma flow (RPF) associated with decreased vascular resistance and increased blood volume. In normal pregnancy, maternal blood volume increases approximately 40-50% compared to non-pregnant volume (Conrad et al., 2009). The increase in GFR and RPF are seemingly independent of the larger blood volume (Conrad et al., 2009), and clinical analysis has shown this normal increase in blood volume to be minimized or even absent in women with eclampsia (Sibai & Mabie, 1991). Preeclampsia is associated with a decrease in glomerular filtration rate (GFR) and renal perfusion, which, in concert with the often observed hyperuricemia, result in overall decreased renal clearance and proteinuria. Proteinuria is diagnosed by the presence of 300 mg of protein in the urine over a 24-hour period and is indicative of renal endothelial damage.

Angiotensin. Hypertension is defined as a systolic blood pressure greater than or equal to 140 mmHg or a diastolic blood pressure greater than or equal to 90 mmHg. The renin-angiotensin-aldosterone system is commonly considered the root of many hypertensive conditions, and women with preeclampsia have higher placental expression

of angiotensinogen and angiotensin I receptor mRNAs, and higher levels of angiotensin II in the placenta (Anton et al., 2008). In addition, it has been long recognized that preeclamptic women are hypersensitive to angiotensin II (Gilbert et al., 2008). Angiotensin II is known to stimulate induction of matrix metalloproteinase-9, an enzyme that breaks down extracellular matrix to aid in tissue remodeling, and transforming growth factor (TGF)-β, a pro-angiogenic peptide (Dumont et al., 2007; Venkatesha et al., 2006). Placental increases in TGF-β concentrations have been observed in preeclamptic women and may be implicated as a contributor to the pathogenesis of preeclampsia because of its deterring impact on the syncitialization process (Raymond et al., 2011).

VEGF. Endothelial dysfunction leading to hypertension and proteinuria may stem from an imbalance in circulating angiogenic factors. VEGF is a pro-angiogenic factor and has a demonstrated role in decreasing blood pressure and vascular tonicity through stimulating NO and vasodilatory prostacyclin activity. Placental growth factor, part of the VEGF family, is well-known to be decreased in circulation of preeclamptic women, thus causing reduced activation of its receptors and subsequent diminished NO and prostacyclin stimulation (Yang et al., 2001; Maynard et al., 2003). VEGF inhibition has been documented to result in hypertension and proteinuria in clinical trials of cancer treatment (Yang et al., 2011).

sFlt-1. In addition to decreased circulating VEGF, excess soluble fms-like tyrosine kinase-1 (sFlt-1) in placental tissue and amniotic fluid has been measured in preeclamptic pregnancies (Maynard et al., 2003). sFlt-1 is a soluble receptor for VEGF that is increased in preeclampsia and antagonizes circulating VEGF and placental growth

factor (PlGF), another pro-angiogenic vasodilatory factor reduced in preeclampsia, to prevent VEGF interaction with membrane-bound receptors. sFlt-1 may be cleaved from Flt-1 to exclude the transmembrane domain or it may occur as a splice variant. Overexpression of sFlt-1 results in hypertension, endothelial dysfunction, and proteinuria (Maynard et al., 2003). Adjusting this imbalance in pro- and anti-angiogenic factors may be key to managing symptoms of preeclampsia.

Immune factors. The immune system is heavily implicated in the pathogenesis of preeclampsia. In normal pregnancy, immune responses are heightened to prevent fetal rejection, but excessive immune activation occurs in preeclampsia and HELLP syndrome. The complement activation products C3a, C5a, and Bb are elevated in preeclampsia (Vinatier et al., 1995; Lynch et al., 2011, Lynch et al., 2008), and increased circulating complement component 3 is associated with hypertension in humans (Engström et al., 2007). Preeclampsia is also a state of heightened systemic inflammation as evidenced by an increase of a number of inflammatory markers, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, and $CD4^+$ T cells (Dekker et al., 1999).

Certain risk factors, such as an observed increase in anti-angiogenic factors like sFlt-1, may predict the onset of preeclampsia and the presence of these risk factors may impact the severity of maternal and neonatal outcomes (Uzan et al., 2011; Ahmed, 2011). Other risk factors include obesity, ethnicity, compromised immunity, and excessive activation of C-reactive protein in response to inflammatory stimuli (Wolf et al., 2001; Cunningham et al., 2009). Uric acid has been shown to inhibit trophoblast invasion and

endothelial cell apoptosis due to its antioxidant effects (Bainbridge et al., 2009) and hyperuricemia has been evident in women that subsequently develop preeclampsia. Though a wide spectrum of predictive factors has been identified, none have demonstrated promise as a feasible target for treatment and the only effective resolution for preeclampsia remains delivery of the placenta and concurrent birth. Various pharmacological treatments (i.e. dietary antioxidants (Wang et al., 2011), calcium (Vest et al., 2012), aspirin (Powers et al., 2010), magnesium sulfate to discourage onset of seizures (Vest et al., 2012; LaMarca et al., 2011)) for preeclampsia have been postulated to encourage gestation to reach full-term, but none have proven entirely successful, and testing the utility of these treatments is ethically complicated. Thus, various animal models mimicking symptoms of preeclampsia have been characterized and may be used to assess the viability of numerous management strategies.

Animal models of preeclampsia

Several effective animal models have been developed to study the pathogenesis and clinical manifestations of preeclampsia and potential treatment options therein (Sunderland et al., 2010). Though no single model fully encompasses all aspects of the condition, certain models may be utilized to target specific clinical symptoms or examine the role(s) of factors implicated as important in the onset or progression of the condition, as in genetically modified animal models. The most relevant models include the symptoms central to preeclampsia—hypertension and proteinuria. These symptoms have been observed in models using placental ischemia (Alexander et al., 2001; Li et al.,

2012), autoantibodies (Wallukat et al., 1999), overexpression of sFlt-1 in the rat (Kumasawa et al., 2011; Maynard et al., 2003), sFlt-1 infusion (Bridges et al., 2009; Murphy et al., 2011), complement component deficiencies (C1q-deficient C1q-/- (Singh, 2011) or Crry-/- mice (Molina, 2005)), and vasoconstriction through nitric oxide inhibition (Yallampalli & Garfield, 1993; Molnár et al., 1994).

Autoantibodies to angiotensin II type 1 receptor (AT1-AA) have been demonstrated to serve as a precursor to endothelial dysfunction in rat cardiomyocytes (Wallukat et al., 1999), a central characteristic of preeclampsia. Infusion of AT1-AA in pregnant mice resulted in hypertension, proteinuria, and glomerular endotheliosis (Li et al., 2012), and AT1-AA infusion during the third trimester in pregnant rats led to hypertension, increased plasma sFlt-1, increased placental and renal endothelin-1, and oxidative stress (Li et al., 2012). AT1-AA may be involved in induction of oxidative stress through generation of reactive oxygen species (ROS) through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and are also capable of stimulating sFlt-1 production in the trophoblast (Wallukat et al., 1999; Zhou et al., 2008). These effects are evident in AT1-AA animal models (Herse & LaMarca, 2013). Additionally, AT1-AA has been demonstrated to lessen the invasive nature of trophoblastic cells and may be involved in abnormal pseudovasculogenesis (Zhou et al., 2008). Interestingly, the cardiovascular effects observed with AT1-AA in pregnancy occur to a much lesser degree in a non-pregnant animal, indicating that the state of pregnancy is more sensitive to disruptions in autoantibody concentrations. AT1-AA had no effect on sFlt-1 concentrations in non-pregnant animals.

Elevated levels of circulating sFlt-1 are observed in preeclampsia, and previous studies have shown that inducing overexpression of sFlt-1 in murine models causes hypertension, proteinuria, and endothelial dysfunction in pregnant animals (Alexander et al., 2004; Kumasawa et al., 2011). This phenotype is greatly exacerbated in the absence of eNOS in non-pregnant eNOS-/- mice (Li et al., 2012). sFlt-1 is known to be secreted by the ischemic placenta (Gilbert et al., 2007; Makris et al., 2006), and placental ischemia is also associated with decreased glomerular filtration rate, renal plasma flow, and renal nitric oxide synthase seen in preeclampsia (Gilbert et al., 2010; Alexander et al., 2001). Maynard et al (2003) demonstrated that overexpression of sFlt-1 produced hypertension and glomerular endotheliosis in both pregnant and non-pregnant rats, indicating that sFlt-1 acts on the maternal endothelium apart from the placenta, perhaps through its antagonism of VEGF (the prominent measurable vasodilator in the rat) and placental growth factor (PlGF). PlGF is a pro-angiogenic homolog of VEGF that is the prominent vasodilator in humans. Thus, excessive circulating sFlt-1, whether originating endogenously from an ischemic placenta or administered exogenously, contributes to the preeclamptic phenotype in murine models.

Various immune factors have been manipulated in murine models to produce a preeclamptic-like condition. Mice deficient in the complement component 1q (C1q) develop hypertension, proteinuria, angiogenic imbalance of VEGF and sFlt-1, and endotheliosis (Singh et al., 2011). Preeclamptic symptoms including hypertension and proteinuria, and characteristics such as endothelial damage also result with infusion of inflammatory markers into pregnant rats, such as tumor necrosis factor (TNF)-α

(Alexander et al., 2002), interleukin (IL)-6, and $CD4^+$ T cells (Li et al., 2012), implicating the relevance of the immune system in the pathophysiology and perhaps pathogenesis of preeclampsia.

Reduced utero-placental perfusion pressure model of preeclampsia

Though elevated levels of sFlt-1, AT1-AA, and inflammatory components are associated with symptoms of preeclampsia, it is not established that these factors are involved in its onset. Placental ischemia, however, is a known precursor to preeclampsia due to abnormal spiral artery remodeling and models have been developed that mechanically reduce blood supply to the placentae, and these models successfully induce symptoms of preeclampsia, including hypertension and proteinuria.

Reduced utero-placental perfusion pressure (RUPP) has been used in the dog, rat, rabbit, sheep, and non-human primate and results in a 20-80% decrease in arterial perfusion of the uterus in the pregnant animal (Li, 2012) and mimics early-onset preeclampsia (Gilbert et al., 2007). The rat is a useful instrument in studying pregnancy because its trophoblast invasion is similarly deep to that of humans (Caluwaerts et al., 2004). In the rat, silver clips are placed on the lower abdominal aorta (ID 0.203 mm) superior to the iliac bifurcation and on both uterine arteries (ID 0.100 mm) to prevent any compensatory increase in blood flow to the uterus (Figure 1). This partial obstruction of blood flow results in a suite of physiological changes that closely represent the preeclamptic phenotype. Features observed in the rat RUPP model include hypertension, proteinuria, decreased glomerular filtration rate (renal dysfunction), decreased renal

plasma flow, and increased vascular reactivity (Li et al., 2012). Additionally, the RUPP procedure results in increased vasoconstriction evidenced by increased contractility of isolated renal arterial smooth muscle cells in response to angiotensin II (Murphy et al., 2003). Alterations in vasoactive substances contribute to this response, such as decreases in vasodilatory factors (i.e. VEGF, PlGF) and increases in vasoconstrictive factors (i.e. increased AT1-AA, sEng, ET-1 (LaMarca et al., 2008)). Studies using heparin have reported increases in sFlt-1 in the RUPP model, but recent experiments in the RUPP model (Lillegard et al., 2013) and in humans (Searle et al., 2011) have demonstrated no increase in sFlt-1 in the absence of heparin, suggesting the hypertension caused by the RUPP procedure is independent of an increase in circulating sFlt-1. Certain inflammatory cytokines (i.e. TNF-α (LaMarca et al., 2008), IL-6 (LaMarca et al., 2011) and oxidative stress (measured by 8-isoprostane and malondialdehyde) are also known to increase in the rat RUPP model, thus contributing to hypertension. Pups of dams that undergo the RUPP procedure are documented to develop intrauterine growth restriction, though their placentation occurs normally.

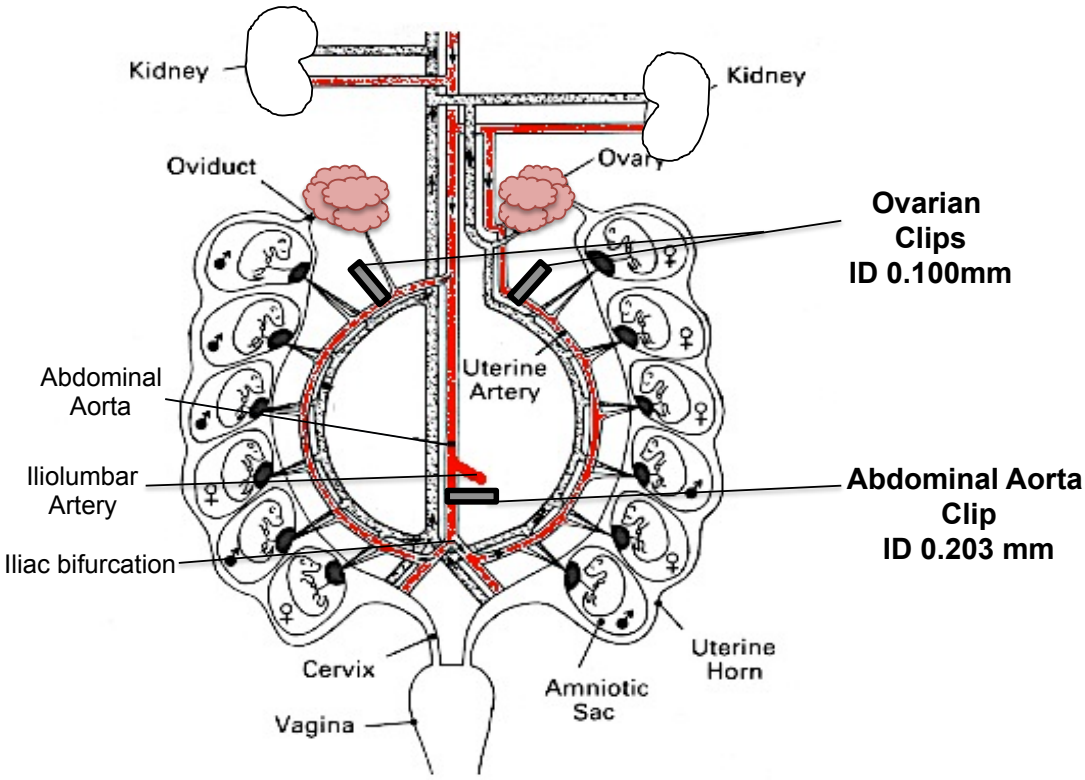

Figure 1. The Reduced Utero-placental Perfusion Pressure (RUPP) model of placental ischemia. On gestational day 14 of a 21-day term, silver clips are placed on the lower abdominal aorta (ID 0.203 mm) and on both uterine arcades (ID 0.10 mm) to induce placental ischemia, resulting in hypertension on gestational day 19. Illustration modified from Even, M et al; *J. Reprod. Fert.* (1992) 96, 709-716.

The RUPP model in the rat does not fully mimic human preeclampsia. As aforementioned, placentation in the rat occurs normally, so studies using this model must focus on the physiology of symptoms occurring after the onset of placental ischemia. RUPP rats do not exhibit glomerular endotheliosis, which is an important characteristic of preeclampsia (Conrad et al., 2009). The presence of proteinuria is inconsistent in the RUPP model (Granger et al., 2006) and this may be due to the short period of elevated blood pressure and altered angiogenic balance (JS Gilbert, Personal Communication). However, these limitations do not hinder the utility of the RUPP model in exploring

possible management or treatment options to manage hypertension, prolong gestation and promote maternal and perinatal health.

A study by Gilbert et al. (2010) demonstrated that VEGF infusion attenuated hypertension and restored impaired glomerular filtration rate and endothelial dysfunction in rats with reduced uterine perfusion in a dose-dependent manner. Furthermore, VEGF-antagonism treatments used to control tumor growth may result in the development of hypertension in non-pregnant patients (van Heeckeren et al., 2007). These data indicate that VEGF plays a critical role in the hypertension following preeclampsia (Figure 2). Other studies of exogenous VEGF administration in experimental models have shown beneficial effects on renal recovery time (Masuda et al., 2001; Kim et al., 2000) and attenuation of post-cyclosporin A-induced nephropathy, hypertension, and endothelial dysfunction (Kang et al., 2001). Furthermore, inhibition of VEGF activity has yielded several important results, such as increased apoptosis and proteinuria and decreased glomerular capillary repair in a rat model of mesangioproliferative nephritis (Ostendorf et al., 1999). VEGF activity at its receptors is also known to contribute to increased superoxide dismutase expression (an enzyme critical for NO formation) and endothelial NO synthesis (Maynard et al., 2003), so decreased VEGF may result in dampened NO synthesis leading to decreased vasodilation.

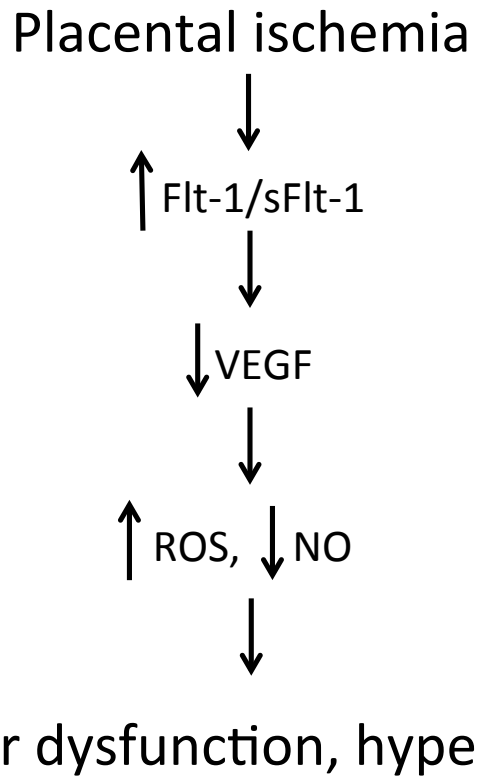

Figure 2. Role of free VEGF in placental ischemia-induced hypertension. Placental ischemia precedes an increase in Flt-1/sFlt-1, which consequently decreases bioavailable VEGF. Decreased free VEGF leads to an increase in reactive oxygen species and decrease in NO to result in vascular dysfunction and hypertension. VEGF infusion attenuates hypertension associated with placental ischemia.

Pravastatin, a commonly used inhibitor of a cholesterol-forming enzyme, has been examined in numerous animal models of preeclampsia. Singh et al (2011) utilized pravastatin in complement-deficient mice that reversed hypertension and induced placental growth factor (PlGF) expression to ameliorate endothelial dysfunction. Pravastatin has also been used to attenuate oxidative stress and hypertension in RUPP rats (Bauer et al., 2013) and preeclamptic symptoms in pregnant rats with overexpressed sFlt-1 (Saad et al., 2013; Fox et al., 2011; Kumasawa et al., 2010).

Certainly, novel therapeutic strategies are urgently needed to manage preeclamptic symptoms and promote maternal and fetal health for the full gestational term. Thus, unique avenues must be explored to cover the spectrum of pathophysiological outcomes associated with preeclampsia. VEGF is important to hypertension in preeclampsia and is associated with complement; Singh et al. (2011) demonstrated that complement-deficient mice exhibited a preeclamptic phenotype with increased blood pressure and decreased circulating VEGF. Because complement activation is increased in women with preeclampsia, we have focused on the complement system as a potential therapeutic target in managing preeclampsia.

The complement system

The immune system is a highly complex network of specialized cells and proteins critical to an organism's defense against pathogenic invasion and infection. There are two primary subsets of immunity: the adaptive immune system and the innate immune system. The adaptive immune system is a delayed but specific response that involves various effector cells (B cells and T cells) capable of targeting pathogens expressing foreign ("non-self") antigens. The innate system is an immediate response that is non-specific and recognizes foreign or invasive bodies. The innate system initiates inflammation through various leukocytes and induces the adaptive response (i.e. antibody production from B cells and immune response trafficking or direct damage from T cells). Activated leukocytes release chemotactic substances to recruit these specialized effector cells, and the innate response heavily dictates the subsequent adaptive response (Le Bon

et al., 2002). The complement system is a central component of the innate immune system that functions to eliminate infection and pathogenic invasion, opsonize antigens for phagocytic uptake, regulate the adaptive immune response, and mediate inflammation.

Activation of the complement system

Various circulating plasma proteins comprise the complement system and are synthesized mainly in hepatocytes and macrophages, though some production may occur in endothelial cells, fibroblasts, and monocytes. Activation of the complement cascade can occur through various stimuli and different stimuli induce activation of one of three pathways: classical, lectin, or alternative. Once activated, each pathway produces a series of enzymatic cleavage products to form the membrane attack complex through the terminal lytic pathway, which creates a pore in the cell membrane to ultimately lyse the invading organism (Figure 3).

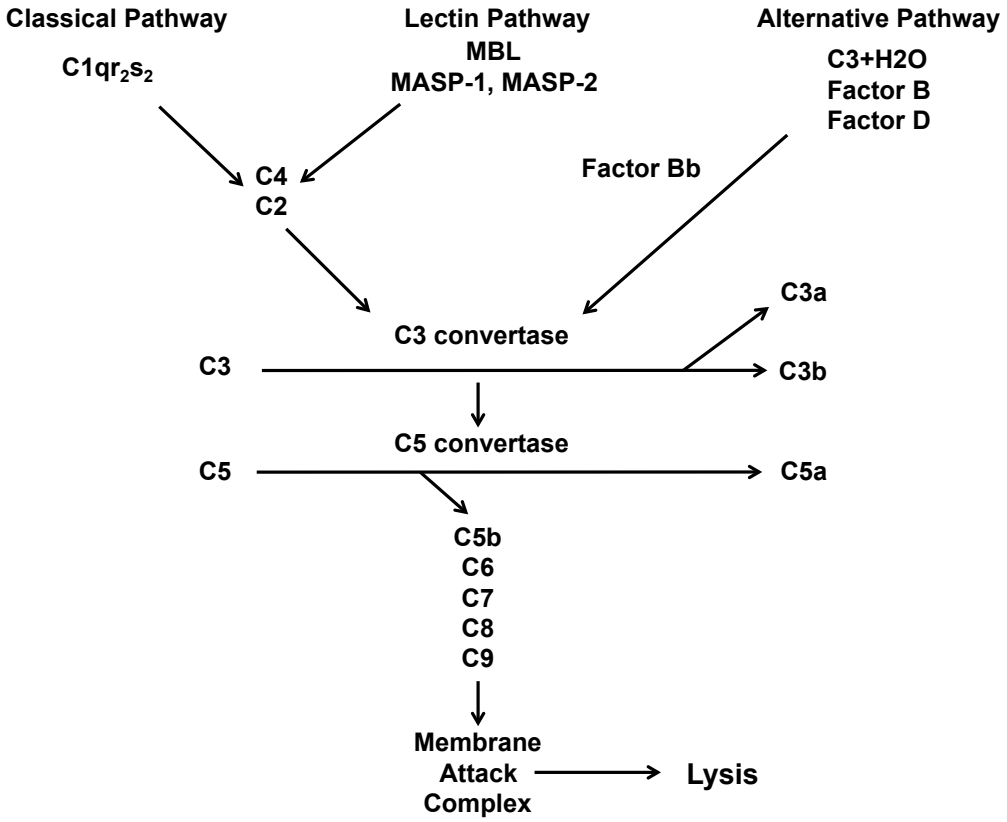

Figure 3. Complement activation cascade. Activation of the complement system initiates a cascade of enzymatic and proteolytic cleavage and synthesis events leading to the formation of the membrane attack complex, which is responsible for disrupting the cellular membrane of the invader and cell lysis.

Classical pathway activation occurs in response to antigen binding with antibody. Binding of the $C1qr_2s_2$ complex to IgG or IgM results in activation to a C1 esterase, which initiates cleavage of C4. Once C4 is cleaved, the C4b fragment can covalently bind to surfaces to act as an opsonizing agent. C4b interacts with C2, which is cleaved by C1s to form the classical pathway C3 convertase C4b2a.

The lectin pathway is activated by polysaccharides common to many bacteria that bind to mannose-binding lectin (MBL) and ficolins. MBL-associated serine proteases (MASP-1 and MASP-2) interact with these compounds to cleave C4 and C2 and thus form the classical pathway C3 convertase C4b2a apart from C1.

Alternative pathway activation is initiated by a number of foreign surfaces, including those of zymosan, endotoxin, and pure carbohydrates. This pathway involves a different set of proteins that activate after C3 molecules spontaneously change to allow hydrolysis of the C3 thioester bond. The new C3 H_2O compound binds to Factor B, which is then cleaved by Factor D to form the unstable C3 convertase, C3bBb. Properdin (P) stabilizes the convertase.

Central to all three pathways is the formation of the C3 convertase (C4b2a, classical pathway; C3bBbP, alternative pathway), which is responsible for cleaving C3. Amplification of the system occurs with the formation of the C3 convertase due to its ability to cleave multiple C3 molecules. Cleavage of C3 yields two immediate products: C3a, a small molecular weight product that remains in circulation, and C3b, which covalently binds to surfaces. C3b, like C4b, may interact with complement receptor 1 (CR1) or its soluble fragment to opsonize and clear immune complexes, or it may further the complement cascade to form the membrane attack complex. In order to further the complement cascade, C3b binds to the C3 convertase to form the C5 convertase (C4b2a3b, classical pathway; C3bBb3bP, alternative pathway) that enzymatically cleaves C5 into C5a and C5b. C5a remains in circulation and mediates inflammatory responses.

C5b is the component critical for formation of the membrane attack complex (MAC), C5b-9, in the cell membrane.

Degradation of C3b results in a number of smaller fragments (C3bi, C3dg, C3d) that interact with Complement Receptors 1-4. These receptors are located on many cell types, including primate red blood cells, neutrophils, monocytes, macrophages, eosinophils, follicular dendritic cells, B cells, and certain T cells. Interactions at complement receptors result in inflammatory and protective responses, like host defense, signaling to cells of humoral immunity, and clearance of immune complexes.

Complement activation is inherently regulated by a number of fluid-phase and membrane-bound inhibitors that act before and after assembly of the convertase and during formation of the MAC. Dysregulation or excessive activation of the complement cascade results in an overabundance of active complement cleavage products that have widespread effects, such as cell lysis and excessive activation of the complement components (resulting in a complement deficiency). Complement deficiency in these cases may be protective, as demonstrated by Ioannou et al (2012) in a lupus-prone mouse model of ischemia/reperfusion injury. Various complement inhibitors have been developed to inhibit damaging consequences of excessive complement activation.

C3a/C5a

C3a and C5a are complement activation products that bind to their specific receptors to manifest anaphylactic symptoms, such as chemotaxis, smooth muscle contraction, increased vascular permeability, and mast cell degranulation (Schraufstatter

et al., 2001; Ames et al., 1997; Hartmann et al., 1997). They are responsible for the release of inflammatory mediators from white blood cells and their receptors are widely expressed.

C5a is a well-characterized and very potent inflammatory peptide (anaphylatoxin) that is known to cause platelet aggregation and recruit polymorphonuclear leukocytes (PMN) and stimulate their release of ROS to damage the unrecognized cell membrane (Arumugam et al., 2004). In the event of high concentrations of ROS, the arachidonic acid pathway may shift to increase production of thromboxane (TX) A_2 (Greer et al., 1991). TXA_2, a product of cyclooxygenase and a mediator of vasoconstriction and platelet aggregation, is implicated as a mediator in the C5a/C5a-desArg-induced pressor response (transient increase in blood pressure) observed in guinea pig (Fraser et al., 1989) and the C5a/ C5a-desArg-induced transient hypotension in the rat (Proctor et al., 2009). Furthermore, these transient changes in blood pressure were normalized with the cyclooxygenase inhibitor indomethacin (Fraser et al., 1989; Proctor et al., 2009), indicating that TXA_2 is important in the C5a-mediated vasoactivity.

Like C5a, C3a is a potent anaphylatoxin and is important in cellular activation of mast cells, monocytes, PMN, and certain basophils (Petering et al., 2000). Following complement activation, C3/C3a is approximately 15 times more concentrated in the plasma than C5/C5a (Feinberg, 2006). C3a is involved in eosinophil (but not PMN) chemotactic functions and ROS release (DiScipio et al., 1999; Petering et al., 2000; Hartmann et al., 1997) and is known to play a role in modulating TNF-α and IL-1β synthesis (Takabayashi et al., 1996). Fischer et al (1999) demonstrated that C3a

contributes significantly to inflammation and immune regulation through IL-6 modulation. C3a has also been shown to act directly on the microvasculature to cause vasoconstriction, vascular leakage, and platelet aggregation (Björk et al., 1985). Recombinant human C3a and C3a analog peptides produce a transient pressor response when injected intravenously in the rat (Proctor et al., 2009), and C3a has been observed to increase in the rat RUPP model of placental ischemia concurrent with hypertension (Lillegard et al., 2013).

To determine the functions of C3a in various systems, synthetic analogs have been developed. It has been shown that the highly conserved essential amino acid sequence for C3a-specificity is LGLAR (Petering et al., 2000), and the synthetic peptide WWGKKYRASKLGLAR possesses the same biological functions and cationic charge as native human C3a (Bellows-Peterson et al., 2012). The peptide is an effective agonist of C3a at the C3a receptor (C3aR), and Proctor et al. (2009) demonstrated a bolus injection of this peptide induced a transient hypertensive response in the non-pregnant rat.

Complement inhibitors

A number of complement antagonists and inhibitors have been identified and characterized. An exogenous soluble form of complement receptor 1 (sCR1) inhibits formation of the C3 and C5 convertases by displacing the C3b and C4b binding domains (Weisman et al., 1990; Katyal et al., 2004), thus preventing the formation of anaphylatoxins and the membrane attack complex. sCR1 has proven beneficial in animal

models of preeclampsia (Lillegard et al., 2013) and ischemia/reperfusion injury and inflammation by reducing tissue damage (Arumugam et al., 2004).

Recently, Burwick & Feinberg (2013) presented a case study providing evidence for the utility of specific complement component inhibition in managing preeclampsia and HELLP syndrome. C5 inhibition by eculizumab in a patient with HELLP syndrome resulted in amelioration of clinical symptoms and improved abnormal laboratory values of LDH, free hemoglobin-binding haptoglobin, aspartate aminotransferase, and platelet count, thus prolonging gestation 17 days. These data suggest that preventing C5a and C5b-9 (MAC) may be beneficial in managing severe preeclampsia or HELLP syndrome.

The C3a receptor (C3aR) has been implicated as a pathophysiological factor in various inflammatory conditions. It is a G protein-coupled receptor with two distinctive features: a wide distribution throughout tissues and an exceptionally long (>170 aa) second extracellular domain (Ames et al., 2001). C3aR-/- mice demonstrated an increased susceptibility to LPS challenge, indicating the importance of the receptor in endotoxin-induced septic shock (Kildsgaard et al., 2000). Additionally, C3aR has been implicated as a contributor to allergic asthma; ovalbumin-sensitized guinea pigs lacking functional C3aR have a decreased bronchial reactivity when challenged with allergen (Regal & Klos, 2000). Thus, efforts have been put forth to develop a selective antagonist for the C3aR.

In 2001, Ames et al identified and optimized N^2-[(2,2-diphenylethoxy)acetyl]-L-arginine (SB290157) as a selective C3aR antagonist. It is a competitive non-peptide that has been shown to block C3a-mediated receptor internalization and Ca^{2+} mobilization in

human, mouse, and guinea pig C3aR-labeled rat basophilic leukemia cells without disrupting C5aR activity (Ames et al., 2001). SB290157 effectively inhibits C3a-mediated chemotaxis in a human mast cell line known to express the C3aR and naturally respond to C3a chemotaxis, but did not affect these actions mediated by C5a. SB290157 also blocks the spasmogenic effects of C3a as evidenced by a dose-dependent decrease in rat caudal artery contractile response after stimulation and inhibits the C3a-mediated release of ATP from guinea pig platelets (Ames et al., 2001). In an LPS-induced airway neutrophilia model in the guinea pig, C3aR antagonism with SB290157 resulted in a greatly reduced neutrophil recruitment. Further, SB290157 attenuated edema in a rat model of adjuvant arthritis (Ames et al., 2001) and reduced oxidative stress-mediated tissue damage in a mouse model of cerebral ischemia (Mocco et al., 2006).

Additional studies have established the utility of SB290157 in a number of inflammatory conditions. As mentioned, the C3a analog peptide produced a transient hypertension in the rat, and Proctor et al. (2009) demonstrated that antagonizing the C3aR with SB290157 prevented the C3a peptide-mediated pressor response in the rat in a dose-dependent manner. Using an acute rat model of ischemia/reperfusion, Proctor et al. (2004) demonstrated that C3aR antagonism resulted in amelioration of several key features of intestinal ischemia/reperfusion injury, including an increase in serum alanine transaminase indicative of liver damage, edema caused by increased vascular permeability, and damage to the mucosa. However, in the same study, SB290157 itself produced a transient hypertensive response and neutropenia, mimicking the action of

intravenously-infused C3a to guinea pigs (Regal & Klos, 2000) and rats (Proctor et al., 2009).

Mathieu et al (2005) further investigated the specific cellular activity of SB290157 and revealed agonist activity of the compound in certain cellular systems. In rat basophilic leukemia cells expressing human C3aR, various concentrations of SB290157 decreased the C3a-induced calcium mobilization response in a dose-dependent manner, but the antagonist itself also stimulated calcium mobilization (Mathieu et al., 2005). Importantly, Mathieu et al. postulated that the agonistic effects of SB290157 may be attributable to receptor density and distribution; in cells with low receptor expression (i.e. guinea pig platelets), SB290157 has no agonist effects, but agonist properties were evident in systems with high receptor expression (Mathieu et al., 2005; Therien, 2005). Therefore, results using this compound should be carefully interpreted. However, among the few developed C3aR antagonists, none have been identified to be more specific or potent (Ames et al., 2001). SB290157 is currently the most promising C3aR antagonist available, and antagonism of the C3aR may offer some therapeutic promise in offsetting symptoms associated with inflammatory conditions.

Complement in pregnancy/preeclampsia

Pregnancy is a state of heightened innate immune function, complement activation, and inflammation in order to prevent rejection of the fetus. In normal pregnancy, the absolute number and activation of leukocytes increase, while T cell function has been shown to be downregulated (Luppi, 2003). Numerous studies have

demonstrated a correlation between immune-compromised women and incidence of preeclampsia (Vinatier et al., 1995), and preexisting inflammation (as in obesity) has been demonstrated as a risk factor for developing preeclampsia due to increased vascular polymorphonuclear leukocyte (PMN) infiltration (Lynch et al., 2011; Walsh, 2007).

Complement components are increased in normal pregnancy and are further increased in preeclampsia (Denny et al., 2013; Haeger et al., 1992), and specifically early-onset preeclampsia (Lim et al., 2012), and the complement component 3 (C3) is increased in hypertension (Engström et al., 2007). C3a, C5a, and Bb are increased early in gestation in women with preeclampsia (Lynch et al., 2011; Derzsy et al., 2013; Lynch et al., 2008) and the presence of placental C4d is associated with preeclampsia (Buurma et al., 2012). Recently our group has demonstrated that general complement inhibition with exogenous soluble complement receptor 1 (sCR1) attenuates the hypertension seen in the rat RUPP model of preeclampsia (Lillegard et al., 2013). Long-term general complement inhibition is not a viable therapeutic option since it places the mother and fetus at risk for infection, so it is necessary to determine which particular components of the cascade are critical to pregnancy-induced hypertension.

SB290157 has been used in the angiotensin II type 1 receptor antibody mouse model of preeclampsia to decrease hypertension and proteinuria at a 30 mg/kg dose (Wang et al., 2012). Antagonism of the C3aR also effectively blocked the increase in sFlt-1 and significantly increased small placental and fetal weights. This study was the first to identify the utility of SB290157 in ameliorating symptoms of preeclampsia in a pregnant animal and indicated the importance of C3a-mediated activity in the

pathophysiology of preeclampsia in the presence of elevated antibody concentrations. Thus far, no investigations have explored C3aR antagonism or the activity of SB290157 in the context of placental ischemia-induced hypertension.

Given this information, we resolved to investigate the role of complement system activation in placental ischemia-induced hypertension, first using a general complement inhibitor, and then a specific complement receptor antagonist for the C3a receptor. Our studies primarily focused on the effect of complement inhibition or antagonism on complement activity and blood pressure, and secondarily the effects on angiogenic factors and fetal and placental data. We sought to identify whether complement activation directly influences blood pressure or mediates blood pressure through angiogenic factors.

Rationale & hypothesis: Chapter 2

Despite major advances in obstetric and perinatal care, preeclampsia remains incurable aside from delivery of the placenta. Preeclampsia is a leading cause of death and morbidity for both mother and offspring, and premature delivery to attenuate maternal symptoms places the neonate at great risk for health complications. Thus, novel strategies are urgently needed to manage the symptoms of preeclampsia and prolong gestation. Excessive complement activation has been observed in women with preeclampsia. Placental ischemia is widely accepted as a critical precursor to preeclampsia, and complement deficiency in animal models is protective against ischemia/reperfusion injury. Vascular endothelial growth factor has recently been linked to complement activation in placental dysfunction in a mouse model of spontaneous miscarriage. By using the Reduced Utero-placental Perfusion Pressure (RUPP) model of placental ischemia in the rat, we can induce the symptoms of preeclampsia following onset of placental ischemia, including an increase in complement activation and the complement activation product C3a. We hypothesize that complement activation is critical to placental ischemia-induced hypertension, and that broadly inhibiting complement activation with a soluble form of the endogenous complement receptor CR1 (sCR1) will attenuate the hypertension caused by placental ischemia in RUPP rats (Figure 4).

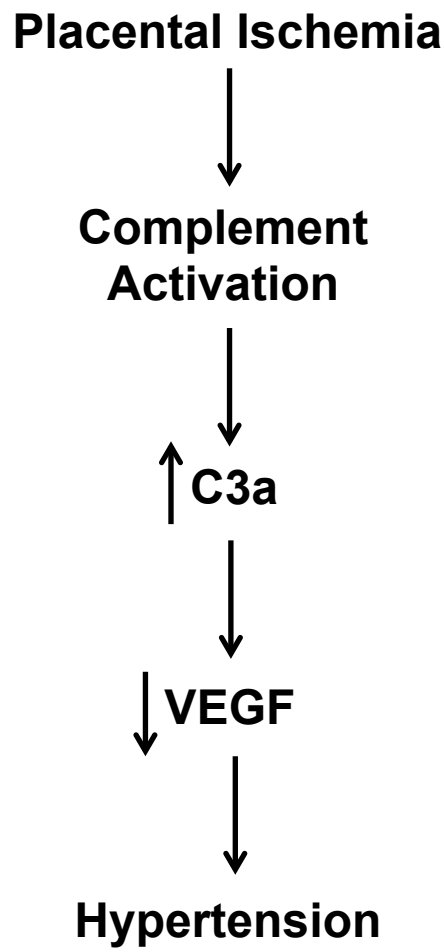

Figure 4. Hypothesis. Placental ischemia leads to complement activation resulting in increased C3a, decreased VEGF, and ultimately hypertension.

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Chapter 2

Complement activation is critical for placental ischemia-induced hypertension in the rat

Overview

Preeclampsia is a major obstetric problem defined by new-onset hypertension and proteinuria associated with compromised placental perfusion. Although activation of the complement system is increased in preeclampsia compared to normal pregnancy, it remains unclear whether excess complement activation is a cause or consequence of placental ischemia. Therefore, we hypothesized that complement activation is critical for placental ischemia-induced hypertension. We employed the reduced utero-placental perfusion pressure (RUPP) model of placental ischemia in the rat to induce hypertension in the third trimester and evaluated the effect of inhibiting complement activation with a soluble recombinant form of an endogenous complement regulator, human complement receptor 1 (sCR1; CDX-1135). On day 14 of a 21-day gestation, rats received either RUPP or Sham surgery and 15 mg/kg/day sCR1 or saline intravenously on days 14-18. Circulating complement component 3 decreased and complement activation product C3a increased in RUPP vs Sham ($p<0.05$), indicating complement activation had occurred. Mean arterial pressure (MAP) measured on day 19 increased in RUPP vs Sham rats (109.8±2.8 mmHg vs 93.6±1.6 mmHg). Treatment with sCR1 significantly reduced elevated MAP in RUPP rats (98.4±3.6 mmHg, $p<0.05$) and reduced C3a production. Vascular endothelial growth factor (VEGF) decreased in RUPP compared to Sham rats, and the decrease in VEGF was not affected by sCR1 treatment. Thus, these studies have

identified a mechanistic link between complement activation and the pregnancy complication of hypertension apart from free plasma VEGF and have identified complement inhibition as a potential treatment strategy for placental ischemia-induced hypertension in preeclampsia.

Key Words: pregnancy; hypertension; preeclampsia; complement; C3a; placental ischemia; vascular endothelial growth factor

Abbreviations:

MAP, mean arterial pressure

RUPP, reduced utero-placental perfusion pressure

sCR1, soluble complement receptor 1

sFlt-1, soluble fms-like tyrosine kinase-1

SNP, sodium nitroprusside

VEGF, vascular endothelial growth factor

Introduction

The pregnancy-specific condition preeclampsia is a leading cause of maternal and fetal morbidity and mortality (Steegers et al., 2010). Preeclampsia is hallmarked by new-onset hypertension (systolic blood pressure of >140 mmHg or a diastolic blood pressure of >90 mmHg) and proteinuria (>300 mg/L) over a period of 24 hours. The initiating event of preeclampsia is unknown. Inadequate trophoblast invasion is postulated to impair maternal spiral artery remodeling in the placenta that is necessary to accommodate increased blood flow required for pregnancy. This impaired spiral artery remodeling results in placental ischemia, accompanied by imbalances in angiogenic factors including vascular endothelial growth factor (VEGF) and soluble fms-like tyrosine kinase-1 (sFlt-1) (Baker et al., 1995; Maynard et al., 2008), oxidative stress (Hubel et al., 1999; Mistry et al., 2013), endothelial dysfunction (Gilbert et al., 2008) and inflammation with excessive complement activation (Lynch and Salmon, 2010). The impaired placental perfusion leads to hypertension, proteinuria and intrauterine growth restriction of the fetus (Steegers et al., 2010). Although many factors have been identified as contributors to this condition, delivery of the placenta remains the only definitive treatment and new treatment strategies are needed.

In pregnancy, maternal mechanisms are evoked to prevent an immune response against the fetus and involve both innate and adaptive immunity (Denny et al., 2013; Lynch and Salmon, 2010), including the complement system. The complement system is an enzymatic amplification system composed of endogenous plasma proteins that normally operates at a low steady state. The components are sequentially activated by

any of three pathways (classical, lectin, and alternative) to function in host defense and inflammation and lead to pathogen opsonization (targeting for destruction) and/or lysis. Central to all three pathways is formation of a C3 convertase that cleaves complement component 3 (C3) into C3a, a fluid phase inflammatory product, and C3b, a larger fragment that covalently binds to surfaces and is an essential part of the enzyme C5 convertase. Cleavage of C5 into C5a and C5b by C5 convertase can lead to formation of membrane attack complex, C5b-9, and lysis of the target. In uncomplicated pregnancies, a heightened inflammatory state is evident with the cleavage product C3a normally increasing in circulation as gestation progresses (Lynch and Salmon, 2010) and the acute phase protein C3 increasing (Baines et al., 1974).

Regulation of this complement activation is important for a successful pregnancy as demonstrated by the inability of offspring to survive if mice lack the normal membrane-bound complement regulator Crry (Xu et al., 2000). In addition, complement component C1q is necessary for normal placental development since C1q-deficient mice exhibit abnormal trophoblast migration and remodeling of spiral arteries and do not develop a normal placenta (Singh et al., 2011). In the rat, C3 synthesized in the uterus is necessary for normal fetal development and has been identified as an early post-implantation embryotrophic growth factor (Usami et al., 2010).

Though complement is necessary for a normal pregnancy, excessive activation of complement and generation of active fragments may be an important contributor to adverse pregnancy outcomes such as spontaneous miscarriage and preeclampsia. In mouse models of spontaneous miscarriage and antiphospholipid syndrome, complement

activation product C5a is important in fetal loss and growth restriction (Girardi et al., 2003; Girardi et al., 2006; Holers et al., 2002; Qing et al., 2011). In preeclamptic pregnancies, elevated concentrations of complement activation products Bb, C3a and C5a have been observed both early in pregnancy as well as near term (Denny et al., 2013; Derzsy et al., 2010; Lynch et al., 2011; Lynch and Salmon, 2010). It is unclear whether this excessive complement activation contributes to placental ischemia and/or placental ischemia causes complement activation and contributes to hypertension. Since complement system activation has also been implicated in pathogenesis of ischemia/reperfusion injury in many different organs (Gorsuch et al., 2012) and the complement cleavage products C3a and C5a are vasoactive (Proctor et al., 2009; Regal and Klos, 2000), we hypothesized that complement activation occurs as a result of placental ischemia and leads to pregnancy-induced hypertension (Figure 4). To test this hypothesis, we used the well-established reduced utero-placental perfusion pressure (RUPP) model of placental ischemia-induced hypertension in rat that mimics features of preeclampsia. In the RUPP model, placental ischemia is induced in the third trimester of pregnancy resulting in a reduction in blood flow to the uteroplacental units and hypertension in the mother. Our studies were designed to determine if complement system activation is increased due to placental ischemia in the RUPP model and to evaluate the effectiveness of complement inhibition using a soluble form of an endogenous complement regulator, soluble complement receptor 1 (sCR1), to attenuate hypertension seen in this model.

Materials and Methods

Reduced utero-placental perfusion pressure (RUPP) procedure and sCR1 administration

The reduced utero-placental perfusion pressure (RUPP) procedure was employed to achieve chronic placental ischemia in the pregnant rat as described previously (Alexander et al., 2001; Crews et al., 2000; Gilbert et al., 2007a; Granger et al., 2006). In brief, surgical procedures were performed with timed pregnant Sprague Dawley dams (Crl:CD IGS, Charles River Laboratories, Portage, MI) under isoflurane anesthesia. All animal experiments were submitted to and approved by the University of Minnesota Institutional Animal Care and Use Committee and conformed to National Institutes of Health guidelines. On day 14 of pregnancy, the jugular vein was cannulated and the cannula exteriorized to the back of the neck. Heparin was not used in cannulas due to its complement inhibitory properties, and a 25% dextrose lock solution was used to maintain cannula patency. For RUPP surgery under isoflurane anesthesia, a vertical midline incision was made, the lower abdominal aorta isolated and a sterile silver clip (0.203 mm ID) placed around the aorta above the iliac bifurcation. This procedure reduces uterine perfusion pressure in the gravid rat by ~40%. Since compensation of blood flow to the placenta occurs in pregnant rats through an adaptive increase in ovarian blood flow, both right and left uterine arcades are clipped at the ovarian end, right before the first segmental artery using a silver clip (0.100 mm ID). All control dams underwent a sham operation, differing only in the absence of clips. sCR1 or saline vehicle (15 mg/kg/day in a volume of 2.6 ml/kg) was administered intravenously every 24 hours beginning 15-30 min prior to RUPP or Sham surgery. Selected experiments used 30 mg/kg/day sCR1.

sCR1 (CDX-1135; Celldex Therapeutics, Needham, MA) was prepared under endotoxin-free conditions, dialyzed against nonpyrogenic normal saline, and aliquoted and stored at -80°C. Rats were randomly assigned to one of four experimental groups based on surgical procedure and drug treatment: 1) RUPP surgery receiving a normal saline vehicle (RUPP Saline); 2) RUPP surgery receiving sCR1 (RUPP sCR1); 3) sham surgery receiving a normal saline vehicle (Sham Saline); 4) sham surgery receiving sCR1 (Sham sCR1).

Measurement of mean arterial pressure and tissue collection

An intra-arterial carotid catheter was placed on gestation day 18 under isoflurane anesthesia for measurement of mean arterial pressure (MAP) in unanesthetized restrained animals on day 19 of gestation. Serum, plasma, and carotid arteries were harvested on day 19 as described previously (Alexander et al., 2001; Gilbert et al., 2007a; Gilbert et al., 2007b; Gilbert et al., 2010). The uterus was exteriorized, the total number of viable and resorbed pups counted and the pups and placentae of the right horn weighed to determine placental efficiency (fetal weight/placental weight). From the left horn, select utero-placental units from the ovarian, middle, and cervical uterine regions were fixed in 10% neutral buffered formalin for histological analysis.

Complement measurements

C3a. Serum concentrations of C3a were measured by Western immunoblot as previously described for guinea pig C3a (Regal and Klos, 2000). The primary antibody used for immunodetection was IgG fraction of rabbit polyclonal antibody to the 9 carboxy-terminal amino acids of rat C3a (Research Genetics, Inc., Huntsville, AL). The

blot was probed with a 1:2,500 dilution of primary antibody followed by 80 ng/mL of goat anti-rabbit IgG coupled to horseradish peroxidase (Pierce, Rockford, IL). Images were acquired as described previously (Gilbert et al., 2012) using incubation with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Rockford, IL) for 5 minutes, image capture with FluoroChem camera (AlphaInnotech, San Leadro, CA), and pixel density quantification with Un-Scan-It Gel Analysis Software (Silk Scientific, Orem, UT). Dilutions of a standard pool of rat serum complement-activated by yeast were used to construct a standard curve on each gel and a regression equation was used to calculate the relative amount of C3a in the unknown samples. Relative amounts of C3a in each sample were expressed as C3a units/µl based on signal intensity of 1 µl of standard pool of rat serum activated by yeast.

C3 ELISA. C3 was determined as previously described for mouse C3 (Taktak and Stenning, 1992) with modifications for rat using goat anti-rat C3 IgG fraction (MP Biomedicals, LLC. Solon, OH) as the capture antibody and a peroxidase-conjugated goat IgG fraction to rat complement C3 antibody for detection (1:8,000, MP Biomedicals, LLC. Solon, OH). The concentration of C3 in each sample was expressed relative to a rat serum standard with one C3 unit/µl representing the optical density of a 1:4,000 dilution of that standard.

Total complement hemolytic activity. The inverse dilution of serum that causes 50% hemolysis of sensitized sheep erythrocytes (CH_{50}) was determined as an indicator of total complement pathway function as previously described (Larsen et al., 2001).

Carotid artery myography

Uncannulated carotids were excised during necropsy, cleaned of adipose tissue, and a 1-2 mm segment cut from each carotid for myography. Carotid segments were placed in Krebs-Henseleit buffer (Regal et al., 1980) in DMT system baths (Model 610M, Danish Myo Technology, Aarhus, Denmark), normalized to transmural pressure of 100 mmHg, and equilibrated for 60 minutes with 3-4 washes of Krebs-Henseleit buffer as previously described (Gilbert et al., 2010). Segments were pre-contracted with thromboxane mimetic U46619 ($5.7x10^{-7}$ M), followed by addition of half-log increments of acetylcholine ($1.38x10^{-8}$ M to $4.14x10^{-4}$ M) to assess endothelial-dependent relaxation. After washing, vessels were again contracted with U46619 and relaxed in response to half-log doses of sodium nitroprusside ($1.0x10^{-8}$ M to $1.12x10^{-3}$ M) to assess endothelial-independent relaxation. Finally, after washing, a cumulative concentration response curve to U46619 verified vessel reactivity at the end of the experiment.

Plasma VEGF and sFlt-1 assays

Circulating free VEGF and sFlt-1 concentrations in EDTA plasma collected on day 19 of gestation were measured using commercially available kits for Mouse VEGF and Mouse sVEGF R1 from R&D Systems (Quantikine, Minneapolis, MN) according to manufacturer's directions and as described previously (Gilbert et al., 2007b; Gilbert et al., 2010).

Statistical analyses

Data are presented as mean or geometric mean ± SE, and differences were considered significant when $p<0.05$. Data were analyzed using two-way ANOVA with three individual contrasts considered most relevant for comparison of means: Sham

Saline vs RUPP Saline, RUPP Saline vs RUPP sCR1, and Sham Saline vs Sham sCR1. C3a, CH_{50} and VEGF values were logged to meet model assumptions.

Results

sCR1 significantly inhibits placental ischemia-induced increase in MAP

MAP increased in RUPP Saline compared to Sham Saline dams (Figure 5). 15 mg/kg/day sCR1 treatment on days 14-18 markedly attenuated the elevated MAP in the RUPP group with no difference between the two Sham groups. Selected experiments were conducted using 30 mg/kg/day sCR1 on days 14-18 with a similar significant inhibition of MAP ($p<0.05$ vs RUPP Saline; RUPP sCR1 at 30 mg/kg MAP 100.3±4.6 mm Hg).

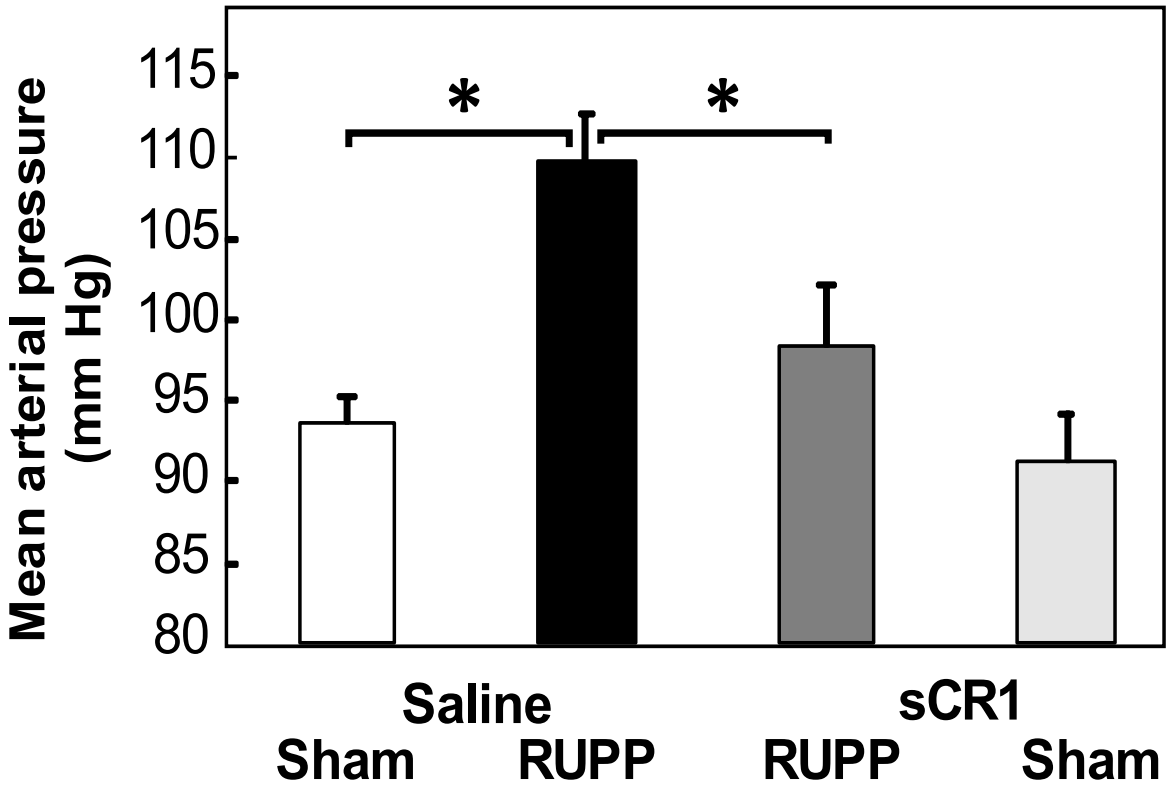

Figure 5. sCR1 significantly inhibits placental ischemia-induced increase in mean arterial pressure (MAP). The increase in MAP in RUPP Saline (n=22) compared to Sham Saline rats (n=19) was significantly inhibited by daily iv administration of 15 mg/kg sCR1 (RUPP sCR1, n=9). MAP did not differ between Sham sCR1 (n=6) and Sham Saline groups. Values represent mean ± SE of MAP measured day 19 of gestation (term=21 days). *$p<0.05$ for indicated comparisons.

sCR1 inhibits placental ischemia-induced complement activation

With excessive complement activation, the cleavage of C3 can outpace new synthesis of protein leading to reduced C3. As seen in Figure 6, the RUPP procedure results in significantly decreased C3 in the circulation, suggesting excessive complement activation occurred and C3 substrate was being consumed. To more directly assess complement activation and the effect of sCR1, C3a in circulation was determined. Placental ischemia induced a significant increase in C3a when compared to controls

(Figure 7). However, sCR1 effectively inhibited this complement activation as evidenced by decreased C3a concentrations in RUPP sCR1 animals. As previously reported (Balta et al., 2011) and confirmed in our studies, RUPP surgery with clip placement in a non-pregnant female rat did not increase blood pressure. In addition, C3a did not significantly change over a 5-day period in a non-pregnant female with RUPP surgery and clip placement (C3a change of 0.05±0.07 units/µl).

The efficacy of sCR1 in inhibiting the complement system *in vivo* was also evaluated by measuring the ability of serum collected on gestation day 19 to lyse antibody-coated sheep erythrocytes via a total hemolytic complement assay. Total hemolytic complement activity was not different in RUPP Saline and Sham Saline groups, but treatment with sCR1 significantly decreased the CH_{50} in serum from both RUPP and Sham animals (Figure 8). In fact, CH_{50} was significantly less in Sham sCR1 animals compared to RUPP sCR1 animals suggesting that it was a more effective inhibitor in control animals.

Placentae of RUPP and Sham animals were examined in all treatment groups by immunohistochemistry using a polyclonal anti-rat C3 antibody to determine if C3 deposition was evident. No difference in intensity or location of immunoreactive C3 was detected between the two groups (data not shown).

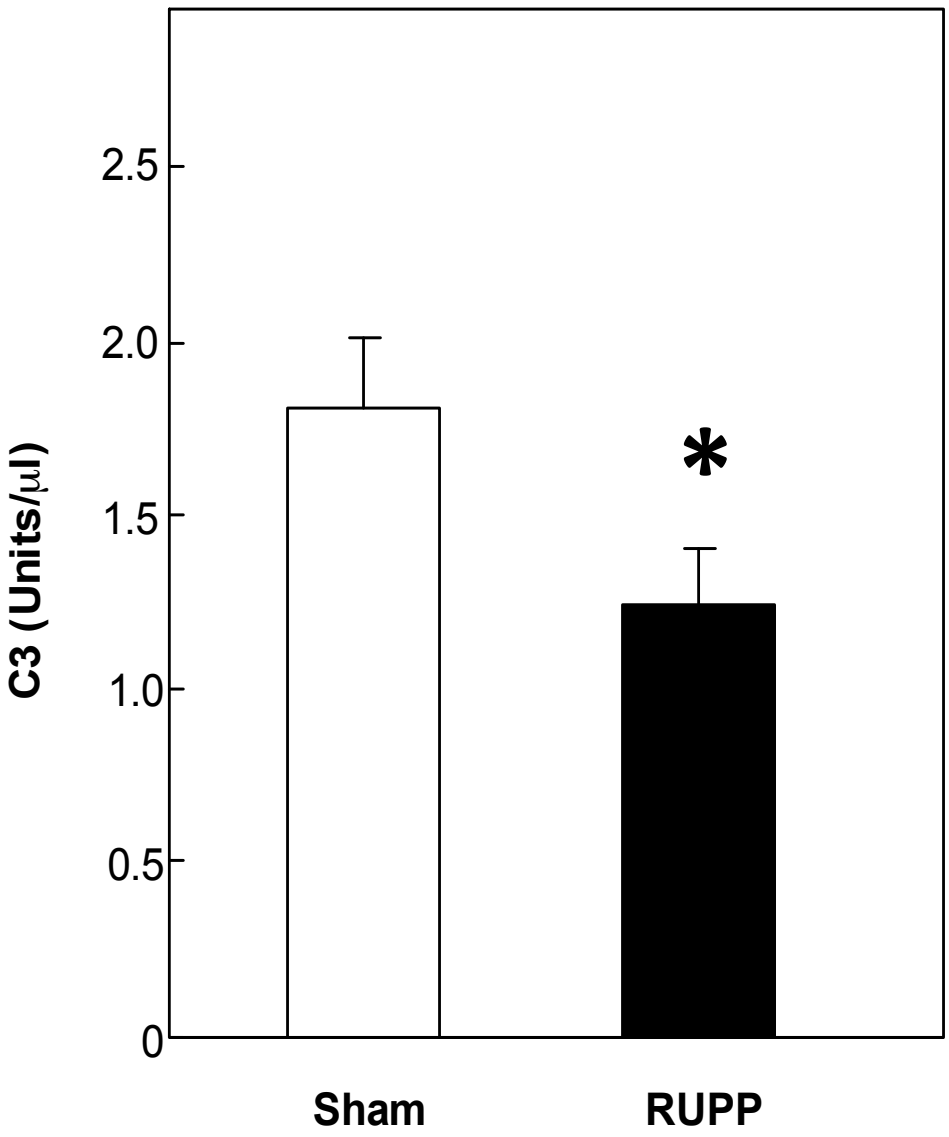

Figure 6. Complement component C3 is reduced in serum obtained day 19 of gestation in RUPP Saline (n=10) compared to Sham Saline rats (n=12). Units of C3 are relative to a rat serum standard as described in Materials and Methods. *p<0.05 vs Sham.

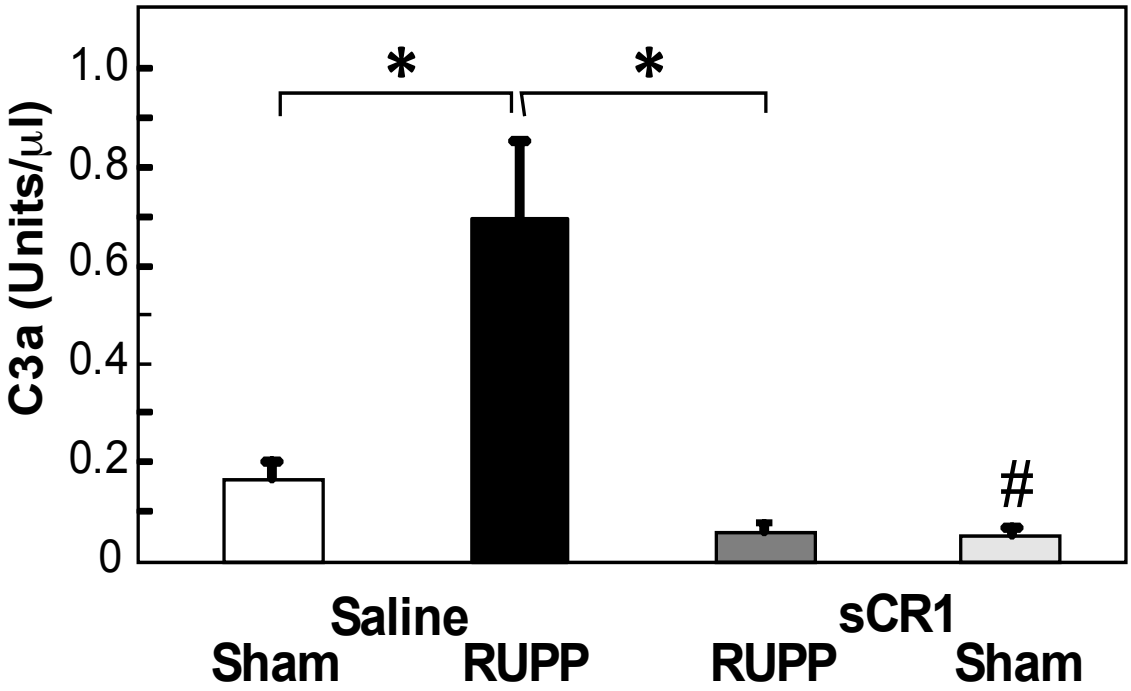

Figure 7. sCR1 significantly inhibits placental ischemia-induced increase in C3a. C3a concentrations increased in RUPP Saline (n=13) compared to Sham Saline dams (n=12). Treatment with 15 mg/kg/day sCR1 decreased C3a serum concentrations in both RUPP (n=5) and Sham (n=6) groups compared to RUPP Saline. Values represent geometric mean ± SE of C3a units/μl in serum obtained day 19 of gestation. Units of C3a are relative to a standard pool of yeast activated rat serum as described in Materials and Methods. *$p<0.05$ for indicated comparisons, #$p<0.05$ comparing Sham Saline to Sham sCR1.

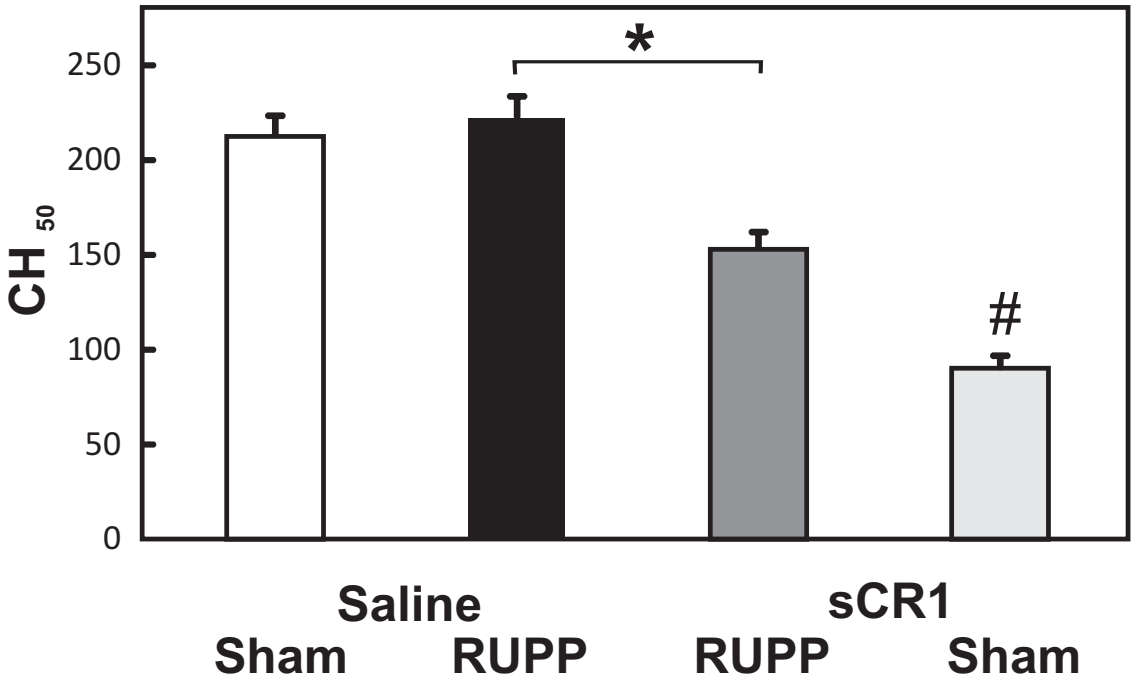

Figure 8. Treatment with 15 mg/kg/day sCR1 for 5 days significantly reduced CH_{50} in serum of RUPP and Sham animals. The CH_{50} in RUPP Saline (n=12) and Sham Saline (n=12) groups were not different. Treatment with sCR1 significantly decreased CH_{50} in serum collected on day 19 from RUPP sCR1 (n=5) and Sham sCR1 (n=6) compared to controls. Values represent geometric mean ± SE of CH_{50} in serum collected day 19 of gestation. *$p<0.05$ for indicated comparisons, #$p<0.05$ comparing Sham Saline to Sham sCR1. Sham sCR1 vs RUPP sCR1 was also tested for this outcome and was statistically different, $p<0.05$.

sCR1 does not affect the VEGF decrease observed in placental ischemia

Hypertension following placental ischemia is associated with a decreased free plasma VEGF and increased sFlt-1, and infusion of VEGF attenuates placental ischemia-induced hypertension (Gilbert et al., 2010). No increase in circulating sFlt-1 was observed in RUPP vs Sham (118.4±19.4 pg/mL vs 114.9±20.4 pg/mL) with measured values near the detection limit of the assay. VEGF significantly decreased with placental ischemia (Figure 9) and no restoration of VEGF was evident in RUPP animals with sCR1

treatment. The sFlt-1/VEGF ratio did not differ amongst treatment groups (data not shown).

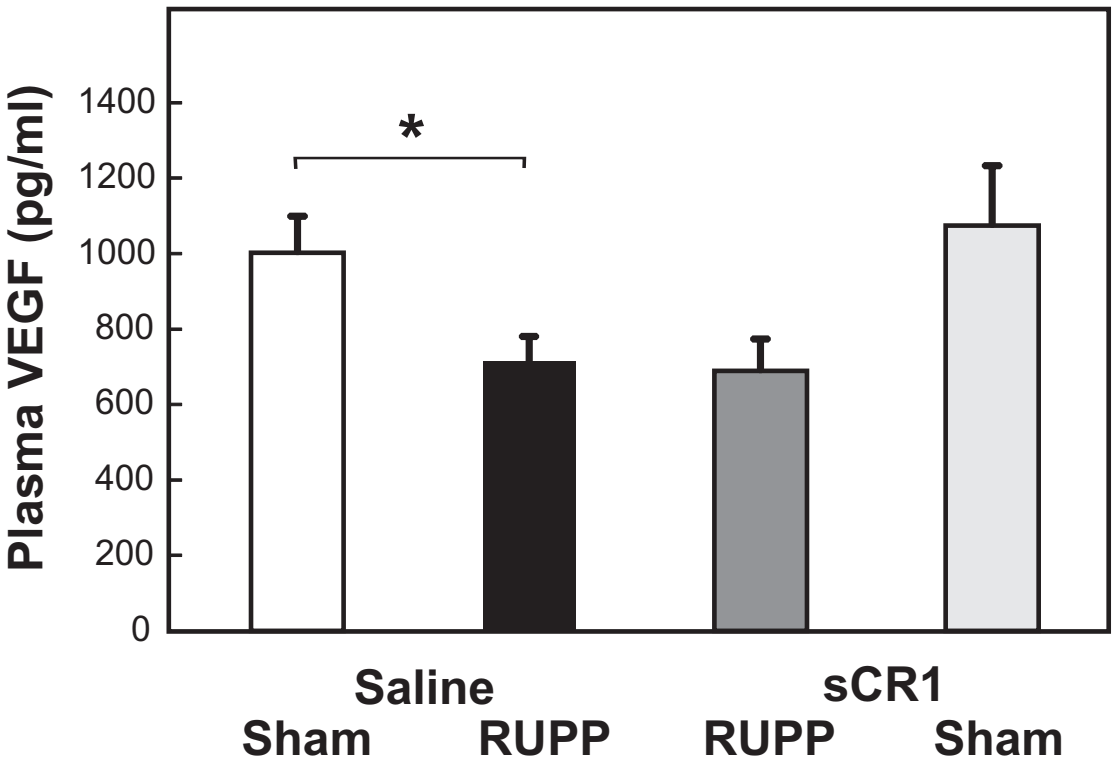

Figure 9. sCR1 treatment did not reverse RUPP-induced decrease in free VEGF concentrations in plasma. RUPP Saline animals (n= 21) had a decrease in VEGF compared to Sham Saline animals (n= 19). VEGF concentrations did not change in RUPP sCR1 animals (n=9) compared to RUPP Saline animals. Concentration of VEGF did not differ in Sham Saline and Sham sCR1 (n=6) animals. Values represent geometric mean ± SE of free VEGF measured by ELISA in plasma obtained from animals day 19 of gestation. *$p<0.05$ for indicated comparisons.

sCR1 alters endothelial-independent arterial relaxation

The contractile response to submaximal concentration of thromboxane mimetic U46619 did not differ between RUPP and Sham animals. The RUPP procedure did not significantly alter endothelial-dependent acetylcholine-induced relaxation of the carotid

artery (Figure 10A). Histological analysis of select vessels clearly showed intact endothelium. Dilation in response to the endothelial-independent vasodilator sodium nitroprusside (SNP) was also not altered in RUPP compared to Sham (data not shown). In carotid arteries from either RUPP or Sham animals treated with sCR1, the vasodilatory response to SNP was enhanced (Figure 10B) with no effect on acetylcholine-induced relaxation (data not shown). sCR1 itself did not relax an isolated carotid artery from a pregnant animal or cause relaxation of an artery pre-contracted with U46619 (data not shown). In addition, presence of sCR1 in the myography bath did not significantly alter vasodilation to either acetylcholine or SNP in carotid artery from a normal pregnant rat (data not shown).

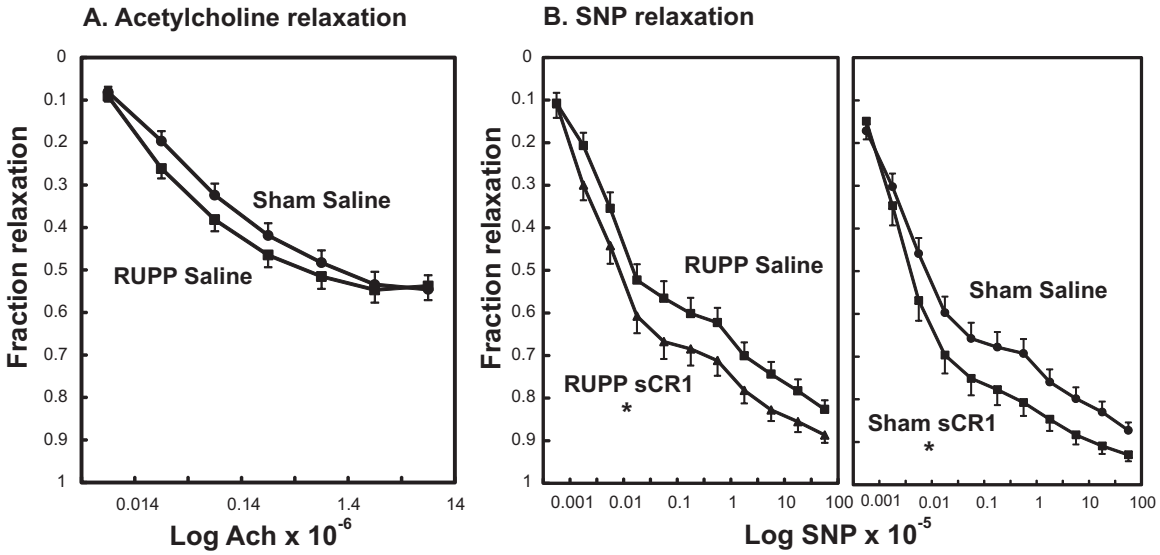

Figure 10. Effect of placental ischemia and sCR1 on relaxation responses of carotid artery *in vitro*. Values represent mean ± SE of the fraction relaxation of arteries pre-contracted with thromboxane mimetic U46619. **A**. Carotid arteries isolated from RUPP Saline (n=16) or Sham Saline (n=13) animals relaxed to increasing concentrations of acetylcholine with no significant treatment effect. **B**. sCR1 enhances endothelial-independent relaxation in carotid arteries isolated from RUPP and Sham animals. Carotid arteries isolated from animals either treated with saline or sCR1 were pre-

contracted with U46619 and the fraction relaxation to increasing concentrations of sodium nitroprusside (SNP) determined. *Repeated measures ANOVA demonstrated a significant sCR1 effect in carotid arteries from RUPP and Sham animals. Values represent geometric mean ± SE of 6-16 animals is each group.

sCR1 does not affect the RUPP-induced increase in fetal resorptions

Fetal resorptions were significantly increased in RUPP Saline and RUPP sCR1 groups compared to Sham Saline or Sham sCR1 groups with no significant sCR1 treatment effect (Figure 11). Neither average placental weights nor placental efficiencies (fetal weight/placental weight) were significantly different amongst any of the treatment groups (data not shown).

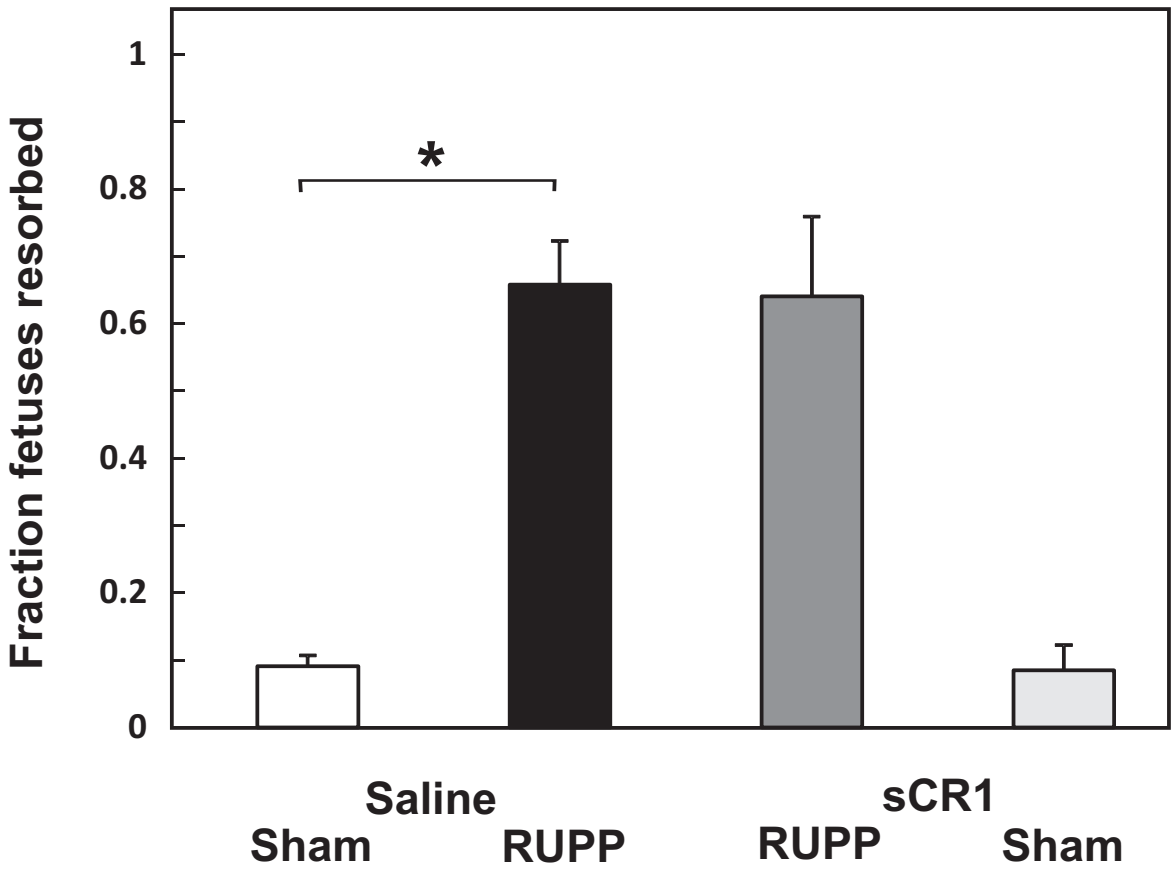

Figure 11. sCR1 treatment did not affect RUPP-induced fetal resorptions. RUPP Saline animals (n= 22) had an increase in resorptions compared to Sham Saline animals (n= 19). The fraction of fetal resorptions did not change in RUPP sCR1 animals (n=9) compared to RUPP Saline animals. Values for Sham Saline did not differ compared to Sham sCR1 (n=6). Values represent mean ± SE of the fraction of resorbed fetuses on day 19 of gestation. *$p<0.05$ for indicated comparisons.

Discussion

Our studies are the first to demonstrate that inhibition of complement activation attenuates development of high blood pressure following placental ischemia in pregnancy, indicating that complement activation is a critical event leading to placental ischemia-induced hypertension. Using the RUPP model, we established that complement activation occurs in concert with placental ischemia-induced hypertension and that sCR1

effectively inhibits complement activation to attenuate this hypertension. Previous studies had indicated an important role for angiogenic imbalance in mediating the blood pressure increase (Gilbert et al., 2010), but inhibition of complement activation attenuated placental ischemia-induced hypertension regardless of concurrent decreases in free plasma VEGF. These studies provide evidence for an important pathway leading to hypertension distinct from angiogenic imbalance and highlight the potential utility of inhibiting complement activation to manage hypertension in pregnancy.

Numerous mediators have been implicated in the pathogenesis of pregnancy-induced hypertension including TNFα, sFlt-1, endothelin, oxidative stress, CD4+ T cells and agonistic autoantibody to angiotensin II type 1 receptor (Li et al., 2012). Many animal models of preeclampsia have been described using a single mediator as a discrete initiating event leading to symptoms of preeclampsia. However, preeclampsia is a heterogeneous disorder with varying degrees of angiogenic imbalance and autoantibody production, so we chose to use a model that employs placental ischemia as the initiating event for the wide range of characteristics that mimic preeclampsia to determine the role of complement system activation. Placental ischemia is a consistent finding in preeclampsia and does not presume the mechanistic pathways leading to the hypertension. In addition, placental ischemia as the initiator of hypertension allows concurrent inflammatory and angiogenic pathways to operate in situ resulting in hypertension. The RUPP procedure is an established and effective means of inducing hypertension following placental ischemia in animal models and closely mimics many

characteristics of preeclampsia (Li et al., 2012) without inducing symptoms of severe preeclampsia (Balta et al., 2011).

During pregnancy, complement activation has been implicated in pathogenesis of hypertension in two different studies in mice; abnormal placental development (C1q deficiency) or infusion of autoantibody to angiotensin II type 1 receptor. The C1q-deficient model investigates events leading to placental ischemia as well as resulting from placental ischemia. Mice deficient in C1q exhibit abnormal placental development during pregnancy resulting in preeclamptic symptoms including high blood pressure. Thus C1q early in pregnancy is essential for normal placentation and to maintain normal blood pressure throughout pregnancy (Singh et al., 2011). In third trimester pregnant mice with the placenta already formed, adoptive transfer of human autoantibodies to the angiotensin II type I receptor (AT1-AA) results in symptoms resembling preeclampsia including increased blood pressure. A C3a antagonist prevents the AT1-AA-induced hypertension indicating that excessive complement activation during pregnancy mediates hypertension initiated by the immune complex formation of AT1-AA with its receptor (Wang et al., 2012; Zhou et al., 2008). Whether complement activation following placental ischemia involves AT1-AA is a subject of future investigations.

Inhibition via sCR1 targets complement activation products C3b and C4b to promote their degradation and accelerate decay of convertase enzymes in the complement cascade. sCR1 has been extensively used to assess the importance of complement system activation in numerous rodent models of autoimmune and inflammatory diseases (Goodfellow et al., 1997; Piddlesden et al., 1994) and was clearly effective in inhibiting

both C3a production and resultant hypertension in the RUPP model. The sCR1 reduction in CH_{50} at day 19 was significant but not as marked as some previously published studies. sCR1 effectiveness in inhibiting total hemolytic complement activity may be limited by the 5 day treatment course in our study, consistent with the observations made by Pratt et al (Pratt et al., 1997) who demonstrated rat anti-human sCR1 antibody production after several days that limits effectiveness. sCR1 is also known to bind C1q (Klickstein et al., 1997) and MBL (Ghiran et al., 2000) and thus may affect processes beyond inhibition of the complement cascade at the level of the C3 and C5 convertases. In our rat studies, sCR1 treatment does not begin until day 14 of gestation so any possible effects on C1q would be restricted to those occurring after placentae are established. Our studies address the role of complement activation downstream of placental ischemia as opposed to the previously reported role for complement upstream of placental ischemia in a C1q-deficient mouse (Singh et al., 2011).

Clearly, inhibition of complement activation has the potential to increase susceptibility to infection and limits its usefulness for long term therapy. Any human use of sCR1 or other complement inhibitors would employ appropriate immunizations and close monitoring similar to that used with the anti-C5 antibody eculizumab for treatment of paroxysmal nocturnal hemoglobinuria (Kelly et al., 2010). A recent case report using eculizumab in a woman with preeclampsia/HELLP syndrome successfully normalized lab values and prolonged pregnancy by 17 days suggesting that therapeutic manipulation of the complement system during pregnancy may be feasible (Burwick and Feinberg, 2013).

Data from previous investigations show significant changes in circulating pro- and anti-angiogenic factors following the RUPP procedure; specifically, RUPP rats exhibit decreased VEGF and increased sFlt-1 compared to Sham (Gilbert et al., 2007b; Gilbert et al., 2010). A decrease in free plasma VEGF was apparent in our studies, but was not restored with sCR1 treatment, indicating that complement activation mediates hypertension regardless of changes in free plasma VEGF. This is in contrast to studies of fetal rejection and growth restriction in the mouse where inhibition of C5 resulted in an increase in plasma free VEGF and a decrease in sFlt-1 (Girardi et al., 2006). In our study, inhibiting complement activation did not restore VEGF but attenuated hypertension introducing the possibility that low VEGF may result in increased complement activation. A recent report (Keir, 2012) suggests that decreased VEGF in the kidney might allow excessive complement activation to occur due to a decrease in complement regulators.

Unlike other studies in the RUPP model, heparin was not used in our experimental protocol due to its demonstrated ability to inhibit complement activation (Girardi et al., 2004). It is known that heparin treatment in coronary angiography results in elevated sFlt-1 and that sFlt-1 increases in the circulation of mice treated with heparin (Searle et al., 2011). In a recent abstract, heparin is also reported to displace sFlt-1 from rat placenta and increase circulating sFlt-1 (George, 2012). Thus, it is possible that the lack of increased circulating sFlt-1 in our study may be due to its continued sequestration in the placenta in absence of added heparin in the experimental protocol. Regardless,

hypertension following placental ischemia was evident in the absence of changes in circulating sFlt-1.

A difference in endothelial-dependent relaxation of aorta or carotid from RUPP rats vs Sham was not detected in our study suggesting that endothelial dysfunction of the larger blood vessels did not occur. This is in contrast to the spectrum of impaired endothelial-dependent relaxation of carotid and aorta reported in Sprague Dawley rats obtained from Harlan following RUPP surgery (Crews et al., 2000; Gilbert et al., 2010). In light of previous findings that inhibition of NO synthase increases blood pressure more in Sprague Dawley rats from Harlan compared to Charles River, our data support the possibility of differences in the relative contribution of endothelium-derived NO to carotid artery function in Charles River rats compared to Harlan (Buhimschi et al., 2001; Pollock and Rekito, 1998). In addition, more recent studies (Griffin et al., 2012) have also reported differences in nephropathy susceptibility and systemic and renal hemodynamic responses to the NO synthase inhibitor L-NAME in rats from the two suppliers. Therefore, differences in the effect of the RUPP procedure on endothelial-dependent relaxation may be due to strain differences in Sprague Dawley rats obtained from different distributors. Treatment with sCR1 *in vivo* resulted in greater carotid relaxation in both the RUPP and Sham groups in response to SNP, indicating complement may be acting independently of endothelium; however, sCR1 itself did not have direct effects on the vessel so further investigations are warranted.

Our data are first to demonstrate that inhibiting complement system activation may be a novel therapeutic strategy for managing hypertension following placental

ischemia in preeclampsia. Using a well-characterized and highly relevant model of preeclampsia in the rat, we have demonstrated a mechanistic link between complement activation and hypertension in pregnancy. Our data suggest that placental ischemia, a consistent feature of preeclampsia, leads to complement activation and hypertension in the rat by a pathway distinct from VEGF. General complement inhibition may not be an optimal therapeutic strategy for preeclampsia, however, because of the potential increased risk of maternal and fetal infection. Certainly, further research is necessary to identify how complement is activated following placental ischemia and to determine the complement activation product(s) (e.g. C3a, C5a, C5b-9) responsible.

Acknowledgements

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Disclosures

Dr. Marsh is employed by Celldex Therapeutics, Inc. and Celldex Therapeutics, Inc. provided the sCR1 used in this study. The other authors have no conflicts to report.

Rationale & hypothesis: Chapter 3

Inhibition of complement activation with sCR1 successfully attenuated placental ischemia-induced hypertension with no effect on free VEGF. sCR1 itself is not a clinically preferred treatment because it abolishes the maternal innate response, potentially leaving the mother vulnerable to infectious agents. Therefore, our goal was to identify the complement activation product that is key to placental ischemia-induced hypertension. C3a is a potent anaphylatoxin that, when complement is activated, is approximately 15 times more concentrated than C5a in plasma. Elevated C3 is associated with hypertension and increased C3a is documented in preeclampsia. C3a is known to cause vasoconstriction, and acute administration of C3a produces a pressor response in the rat. The C3a-induced pressor response is abolished with the competitive selective C3a receptor antagonist, SB290157, and a high dose of SB290157 has been shown to effectively abrogate angiotensin II type 1 receptor antibody-induced hypertension in a mouse model of preeclampsia. Thus, we hypothesize that C3a is the complement activation product responsible for placental ischemia-induced hypertension, and that inhibiting the C3a/C3aR interaction with SB290157 will attenuate the hypertension caused by placental ischemia in RUPP rats (Figure 12). Since no restoration of free VEGF occurred with sCR1-mediated complement inhibition, our working hypothesis is modified to include the possibility that complement and VEGF act independently to influence blood pressure in the rat model of placental ischemia-induced hypertension.

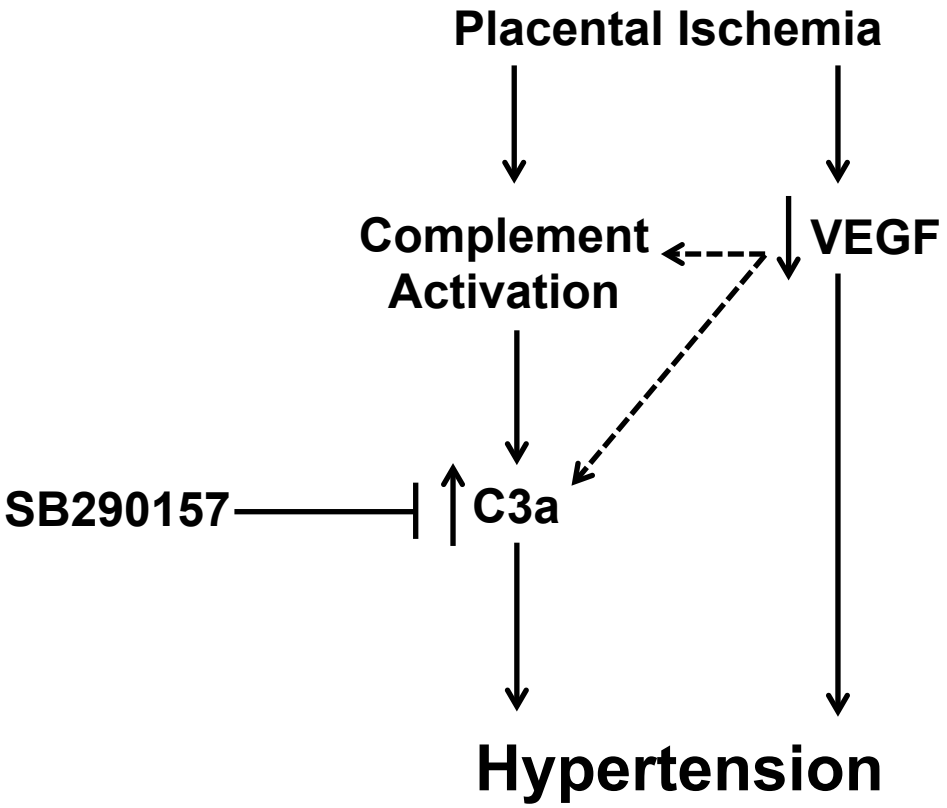

Figure 12. Hypothesis. Placental ischemia leads to complement activation resulting in increased C3a that leads to hypertension. SB290157 is a C3a receptor antagonist and we hypothesize that antagonizing the C3a receptor will attenuate hypertension mediated by C3a. Because general complement inhibition did not restore free VEGF concentrations, we now hypothesize that decreases in free VEGF may occur prior to complement activation or act in parallel to contribute to hypertension.

Chapter 3

Effect of C3a receptor antagonism on placental ischemia-induced hypertension in the rat

Overview

Preeclampsia remains one of the leading causes of maternal and perinatal morbidity and mortality in pregnancy. Previous studies have established that the immune response is elevated in pregnancy, and excessive activation of the complement system, a component of the innate immune response, occurs in preeclampsia. Our recent studies show that broadly inhibiting complement activation with soluble complement receptor 1 (sCR1) attenuated placental ischemia-induced hypertension in the rat without affecting concentrations of pro-angiogenic vascular endothelial growth factor (VEGF), thus providing evidence for the importance of complement activation itself in pregnancy-induced hypertension. We then sought to identify which specific portion of the complement cascade was critical. We hypothesized that C3a, a potent anaphylatoxin that is increased in preeclampsia, is essential to placental ischemia-induced hypertension. We used the reduced utero-placental perfusion pressure (RUPP) model of placental ischemia-induced hypertension in the rat and blocked the C3a receptor (C3aR) with the selective, competitive antagonist SB290157. 5 mg/kg SB290157 attenuated placental ischemia-induced hypertension in the RUPP dams with no effect on the Sham group as measured by arterial catheter in unanesthetized rats. Circulating free VEGF concentrations were not changed with SB290157, indicating the decrease in free VEGF occurs before or in parallel with complement activation. SB290157 did not improve reduced fetal or

placental weights in the RUPP group. Further, acute administration of a C3a analog peptide caused a pressor response in anesthetized, unrestrained pregnant and non-pregnant rats, and 100 μg/kg SB290157 abolished this pressor response in both non-pregnant and pregnant rats. Thus, antagonism of the C3aR may offer some therapeutic benefit in managing hypertension in preeclampsia.

Introduction

Preeclampsia is a serious obstetric complication characterized by new-onset maternal hypertension and proteinuria. Because preeclampsia is largely untreatable apart from parturition, it is a major cause of maternal and perinatal death. New therapeutic strategies are urgently needed to manage the symptoms of this condition and thereby promote gestational success for mother and fetus.

Though the definitive cause remains unknown, multiple factors are implicated in the pathogenesis and/or manifestation of gestational hypertension and preeclampsia. Imbalances in pro- and anti-angiogenic factors have been measured in women with preeclampsia, including a decrease in bioavailable pro-angiogenic vascular endothelial growth factor (VEGF) and an increase in circulating soluble fms-like tyrosine kinase-1 (sFlt-1), the receptor for VEGF (Maynard et al., 2003). In an animal model of placental ischemia, exogenous administration of VEGF attenuated placental ischemia-induced hypertension in a dose-dependent manner, suggesting that free VEGF is critical to blood pressure regulation during pregnancy (Gilbert et al., 2010).

In pregnancy, the immune response must be tightly regulated to prevent rejection of the fetus. The immune response involves the adaptive immune system, which is comprised of differentiated lymphocytes and is responsible for a specific but delayed response, and the innate immune system, which enables an immediate but non-specific response. The complement system is part of the innate immune system and is an enzymatic amplification system composed of plasma proteins. Normally, the complement system operates at a low steady state and responds to heal or eliminate pathogenic infection. However, excessive activation of complement can be harmful, and increases in activation products (i.e. C3a, C5a, Bb) have been observed across a wide spectrum of conditions, and previous studies have shown that women with preeclampsia have elevated levels of complement activation products (Lynch et al., 2011). In addition, there is evidence for an association between high concentrations of circulating complement component 3 (C3) and hypertension (Engström et al., 2007). Studies using various animal models have shown that administration of complement activation products can increase blood pressure (Regal et al., 1980; Proctor et al., 2009), and our group recently demonstrated that inhibiting the complement cascade with soluble complement receptor 1 (sCR1) attenuated hypertension in the reduced uterine perfusion pressure (RUPP) model of placental ischemia-induced hypertension in the rat. sCR1 did not restore the decrease in free VEGF in the RUPP model, indicating that complement activation does not influence the decrease in free VEGF, but changes in circulating VEGF levels may act in parallel to or occur before complement activation to affect blood pressure.

Activation of the complement system from various stimuli results in cleavage of the complement component 3 yielding C3a, an anaphylatoxin, and C3b, an opsonizing agent also critical to furthering the complement cascade to ultimately destroy the foreign body. C3a is a small molecular weight product that is known to mediate inflammation and vascular smooth muscle contraction (Proctor et al., 2009). Several reports have identified the direct roles of C3a in causing vessel contraction (Denny et al., 1978; Björk et al., 1985; Qu, 2009), vascular leakage, and platelet aggregation (Björk et al., 1985). It has also been shown to play a role in regulating tumor necrosis factor-α (Takabayashi et al., 1996), which affects endothelial cells through inflammatory mediators and reportedly contributes to pregnancy-induced hypertension (LaMarca et al., 2008). C3a has been shown to be involved in modulating IL-6 to produce inflammation and further regulate immune responses (Fischer et al., 1999). However, the physiological roles of C3a have not been thoroughly characterized.

Previous studies have utilized exogenous C3a and/or C3a receptor (C3aR) agonists and antagonists to explore the hypertensive response in animal models (Bao et al., 2005; Wang et al., 2012; Proctor et al., 2009). Intravenous injection of the C3a analog peptide WWGKKYRASKLGLAR, referred to hereon as C3a peptide, and recombinant human C3a (rhC3a) both result in a rapid and transient increase in blood pressure in a non-pregnant rat (Proctor et al., 2009). The C3aR antagonist SB290157 is known to effectively inhibit C3a activity *in vivo* and *in vitro* by blocking C3a-induced calcium mobilization and receptor internalization in human neutrophils, and, importantly, by blocking a C3a-induced increase in muscular contractile response (Qu, 2009). Proctor

et al. demonstrated that administration of SB290157 in the rat attenuated the increased blood pressure following complement activation by cobra venom factor (Proctor et al., 2006) or C3a peptide (Proctor et al., 2009), and the compound itself induced neutropenia. These studies suggest that C3a is a putative mediator of hypertension, but thus far, no studies have examined the therapeutic potential of using a C3aR antagonist in pregnancy-induced hypertension. Therefore, we sought to identify C3a as an important product of complement system activation responsible for placental ischemia-induced hypertension. To test this, we used the C3a receptor antagonist, SB290157, which has been shown to have high efficacy and specificity for the C3aR in the rat (Ames et al., 2001).

Studies have shown that acute administration of the C3a analog produced a transient hypertension and SB290157 blocked this response in a dose-dependent manner (Proctor et al., 2009), and we previously demonstrated that mechanically inducing ischemia in the placenta via the RUPP procedure results in increased circulating C3a and hypertension (Lillegard et al., 2013). Thus, we hypothesize that C3a is an important factor in placental ischemia-induced hypertension and intrauterine growth restriction and SB290157 will attenuate hypertension in the pregnant rat by inhibiting the C3a-mediated hypertensive responses independent of VEGF (Figure 12).

Materials & Methods

Reduced utero-placental perfusion pressure (RUPP) procedure

The RUPP procedure was performed on gestational day 14 to achieve chronic placental ischemia in the pregnant rat on gestational day 19 as described in Chapter 2. In

brief, timed pregnant Sprague Dawley rats from Charles River (approximately 250 g at time of shipment) were anesthetized with 2-3% isoflurane. The jugular vein was catheterized and due to the effect of heparin on complement activation, a 25% dextrose lock solution was used to maintain catheter patency. Silver clips were placed on the lower abdominal aorta superior to the iliac bifurcation and on the ovarian arteries between the ovaries and the first pup to induce placental ischemia. All animal experiments were submitted to and approved by the University of Minnesota Institutional Animal Care and Use Committee and conformed to National Institutes of Health guidelines.

SB290157 preparation

SB290157 (Merck KGaA, Darmstadt, Germany) was suspended in 10% ethanol/saline to make a 5 mg/ml stock solution. Animals were injected intravenously with either SB290157 (5 mg/kg) or 10% ethanol/saline vehicle daily from day 14 through day 18 and were randomly assigned to one of the following treatment groups: 1) rats that underwent the RUPP surgery receiving 5 mg/kg SB290157 (RUPP SB), n=9; 2) rats that underwent the RUPP surgery receiving 10% EtOH/Saline vehicle (RUPP Veh), n=7; 3) rats that underwent a sham surgery receiving 10% EtOH/Saline vehicle (Sham Veh), n=7; 4) rats that underwent a sham surgery receiving 5 mg/kg SB290157 (Sham SB), n=6.

Mean arterial pressure measurements and tissue collection

Mean arterial pressure (MAP) was measured as detailed in Chapter 1 via intra-arterial catheter in unanesthetized restrained animals on day 19 of gestation and serum and plasma were harvested as described previously (Gilbert et al., 2010; Gilbert et al.,

2007; Alexander et al., 2001). Fetal and placental weights were collected from non-resorbed pups. Utero-placental units were selected from the ovarian, middle, and cervical uterine regions on the left horn and were fixed in 10% neutral buffered formalin for histological analysis.

VEGF plasma assays

Circulating free VEGF concentrations in EDTA plasma collected on day 19 of gestation were measured using commercially available kits for Mouse VEGF from R&D Systems (Quantikine, Minneapolis, MN) according to manufacturer's directions and as described previously.

C3a pressor response in nonpregnant and pregnant rats

To verify that C3a produces a transient increase in blood pressure (pressor response) in anesthetized, unrestrained nonpregnant rats as previously reported, we used the potent C3a analog peptide described by Ember et al (1991) (WWGKKYRASKLGLAR, AnaSpec, Fremont, CA) to induce a transient hypertension. C3a peptide was dissolved in 0.9% sterile saline (Baxter, Deerfield, IL) to make a 0.25 mg/ml stock solution.

Nonpregnant rats were injected with 3 mg ketamine (Phoenix, St. Joseph, MO) and 0.6 mg xylazine (AnaSed, Shenandoah, IA) and received a jugular catheter for peptide administration and a carotid catheter for blood pressure measurements as described above. After the resting blood pressure stabilized for approximately 15 minutes, C3a peptide (30 ug/kg) was given and blood pressures were again allowed to stabilize. Once blood pressures returned to resting values, norepinephrine (Sigma-

Aldrich, St. Louis, MO) was given (80 μg/kg) to serve as a positive control and verify the rat vasculature was responsive.

Complement measurements

C3a Serum concentrations were measured by Western immunoblot as previously described for guinea pig C3a (Regal et al., 2000) and as outlined in Chapter 1. Images were acquired using incubation with SuperSignal West Pico Maximum Sensitivity Substrate (Thermo Fisher Scientific, Rockford, IL) for 5 minutes, image capture with FluoroChem camera (AlphaInnotech, San Leadro, CA), and pixel density quantification with Image J (National Institutes of Health, Bethesda, MD). Dilutions of a standard pool of rat serum activated by yeast were used to construct a standard curve on each gel and a regression equation was used to calculate the relative amount of C3a in the unknown samples. Relative amounts of C3a in each sample were expressed as C3a units/μl based on signal intensity of 1 μl of standard pool of rat serum activated by yeast.

Total complement hemolytic activity. The inverse dilution of serum that causes 50% hemolysis of sensitized sheep erythrocytes (CH_{50}) was determined as an indicator of total complement pathway function as previously described in Chapter 1. The ability of SB290157 to inhibit complement activation in vitro was also assessed by incubating SB290157 or 10% EtOH/saline vehicle with a pool of non-pregnant rat serum and determining whether the CH_{50} was affected.

Statistical analyses

Data were analyzed using two-way ANOVA and are presented as mean ± SE. Differences were considered significant when $p<0.05$. Three individual contrasts were

considered most relevant for comparison of means: Sham Veh vs RUPP Veh, RUPP Veh vs RUPP SB, and Sham Veh vs Sham SB. In the acute study, relevant contrasts were pregnant vs nonpregnant and C3a vs SB + C3a.

Results

C3aR antagonism by SB290157 attenuates placental ischemia-induced hypertension

On gestational day 19, rats that underwent the RUPP procedure (n=7) had an increase in mean arterial pressure (MAP) when compared to those that received a sham surgery (n=7) (109±2 vs 94±2 mmHg, respectively) as measured by arterial catheter (Figure 13). Antagonism of the C3aR with SB290157 significantly attenuated the hypertension in the RUPP animals (101±2 mmHg, n=9) with no effect on the Sham animals (95±2 mmHg, n=6) *$p<0.05$ for indicated comparisons.

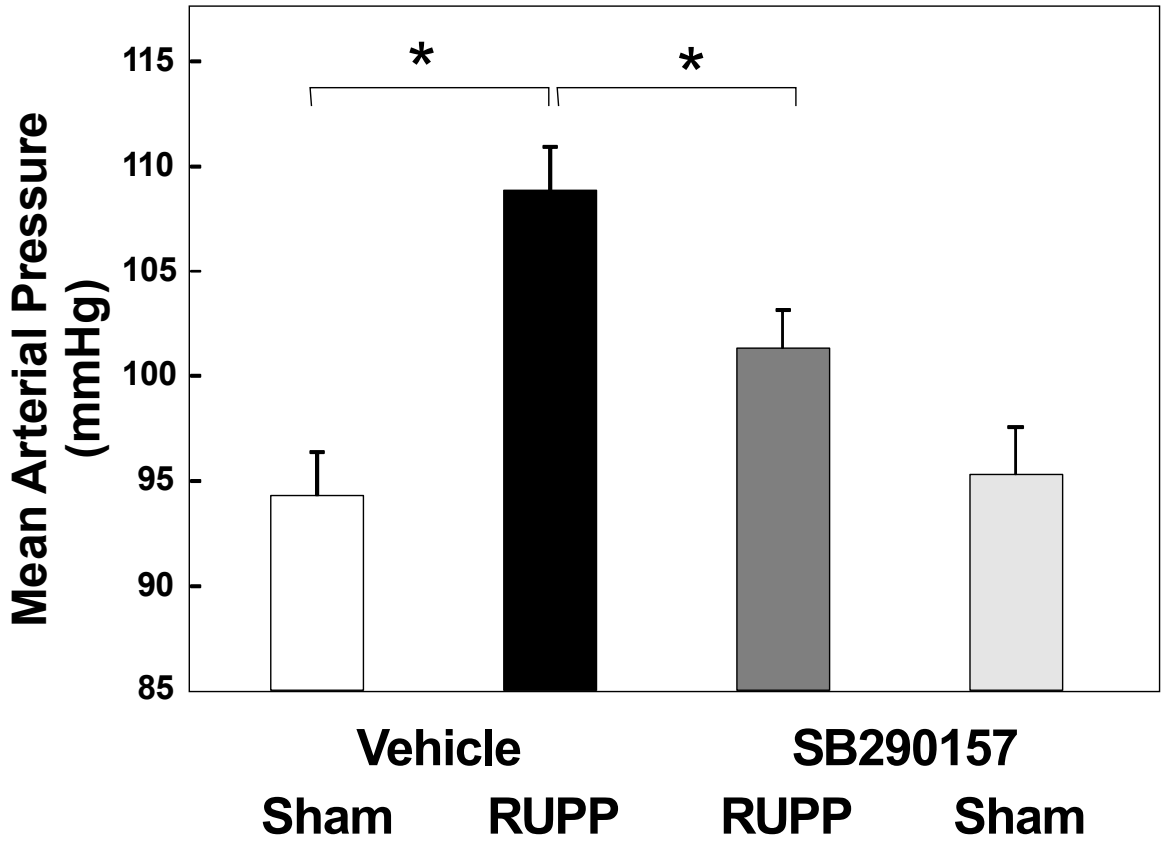

Figure 13. Mean arterial pressure was increased in the RUPP Veh group (109±2 mmHg, n=7) compared to Sham Veh (94±2 mmHg, n=7). SB290157 attenuated RUPP-induced hypertension (RUPP SB 101±2 mmHg, n=9) with no effect on the Sham group (Sham SB 95±2 mmHg, n=6). Data is presented as mean ± SE, *p<0.05 for indicated comparisons.

SB290157 does not alter VEGF in placental ischemia

Free plasma VEGF concentrations decreased slightly in vehicle-treated RUPP rats when compared to vehicle-treated Sham rats (RUPP Veh 1152.3±149.7 pg/ml, n=5, vs 1424.14±138.6 pg/ml, n=7), but the difference did not reach statistical significance (Figure 14). SB290157 did not have any effect on VEGF in RUPP or Sham rats (RUPP SB 1141±122.2 pg/ml, n=9, vs Sham SB 1430±149.7 pg/ml, n=6).

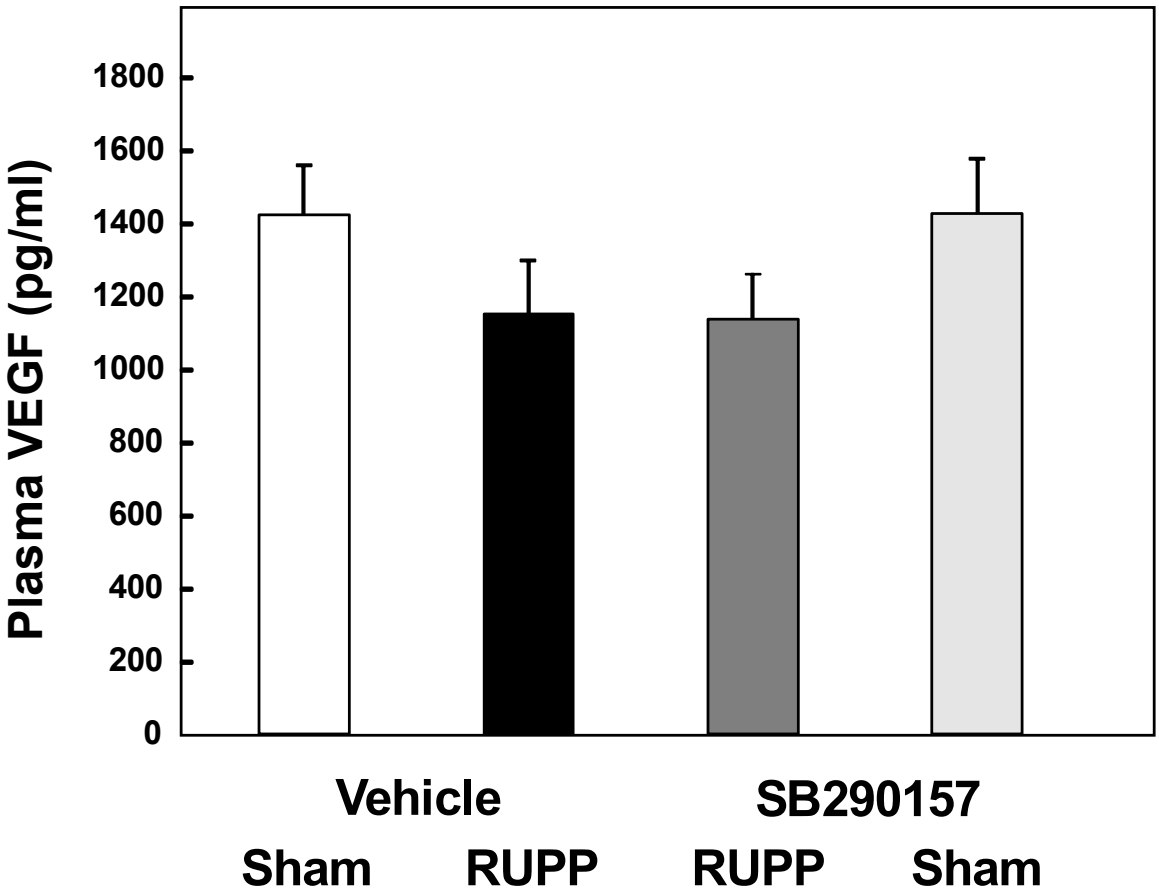

Figure 14. VEGF decreased slightly but not significantly in RUPP Veh rats (1152.3±149.7 pg/ml, n=5) as compared to Sham Veh (1424.1±138.6 pg/ml, n=7). No changes occurred with administration of SB290157 in either the RUPP (RUPP SB 1141±122.2 pg/ml, n=9) or Sham groups (Sham SB 1430±149.7 pg/ml, n=6). Data is presented as mean ± SE.

SB290157 does not affect decreased fetal weights caused by placental ischemia but improves placental efficiency in RUPP dams

The RUPP procedure predictably resulted in fetal growth restriction (Figure 15) when compared to Sham (RUPP Veh 2.24±0.07 g, n=7 vs Sham Veh 2.52±0.07 g, n=7) (*$p<0.05$). SB290157 did not improve fetal weights in the RUPP group and did not affect the Sham group (RUPP SB 2.20±0.07 g, n=8; Sham SB 2.51±0.07 g, n=6). Fetal resorptions were not changed by SB290157 (data not shown).

Placental weights were not different between RUPP Veh, Sham Veh, and Sham SB (0.45±0.02 g, n=7; 0.46±0.01 g, n=7; 0.45±0.01 g, n=6, respectively), but average total placental weight was decreased in the RUPP SB group (0.38±0.01 g, n=8) (Figure 16). Thus, placental efficiency (or mean pup weight per mean placental weight) in the RUPP SB group was significantly higher (5.77±0.13, n=8) than RUPP Veh (5.01±0.20, n=7) (*$p<0.05$) but was not different from Sham Veh (5.46±0.16, n=7) or Sham SB (5.57±0.20, n=6) (Figure 17).

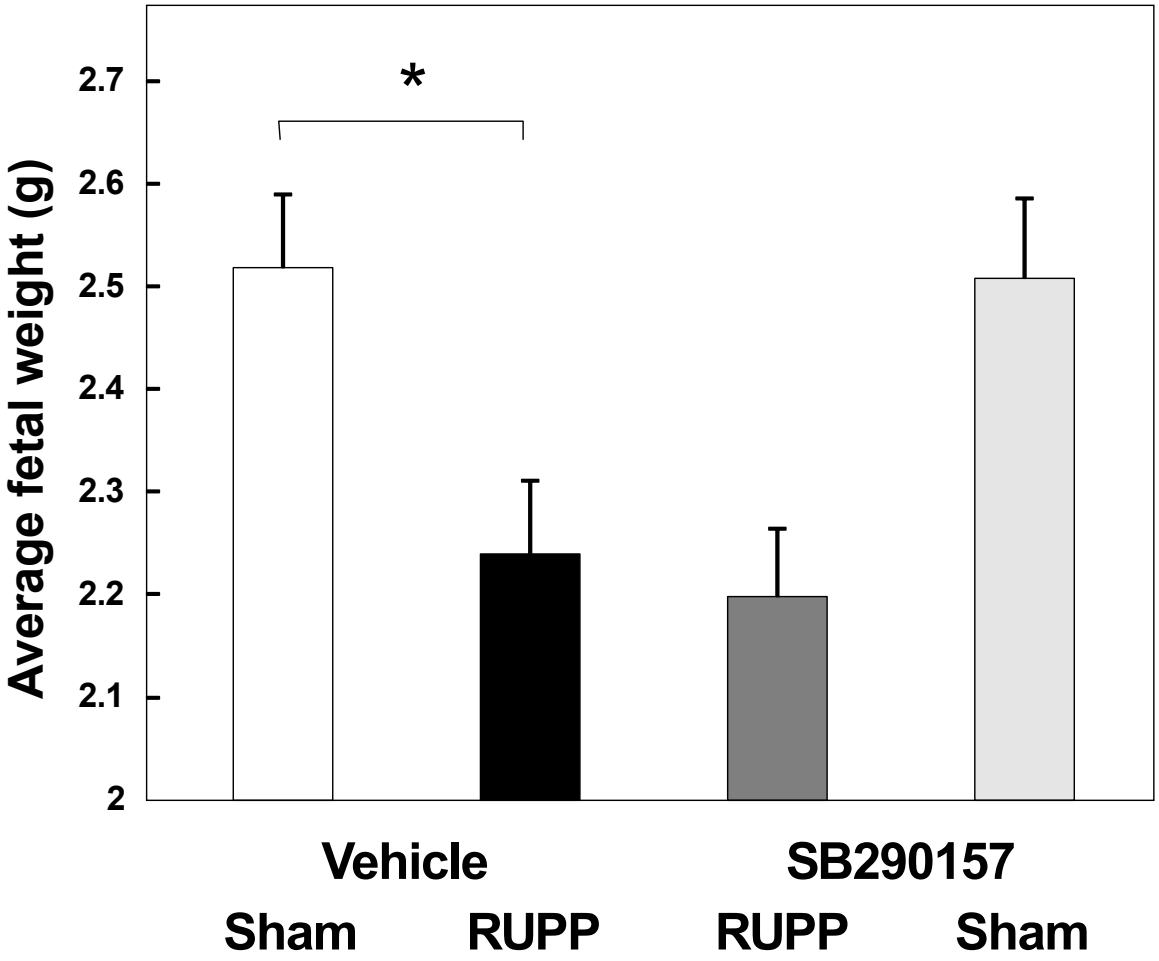

Figure 15. As expected, the average fetal weight decreased in dams that underwent the RUPP surgery (RUPP Veh 2.24±0.07 g, n=7 vs Sham Veh 2.52±0.07 g, n=7). SB290157 did not restore fetal weights in the RUPP animals (RUPP SB 2.20±0.07 g, n=8) and had

no effect on the Sham group (Sham SB 2.51±0.07 g, n=6). Data is presented as mean ± SE, *p<0.05 for indicated comparisons.

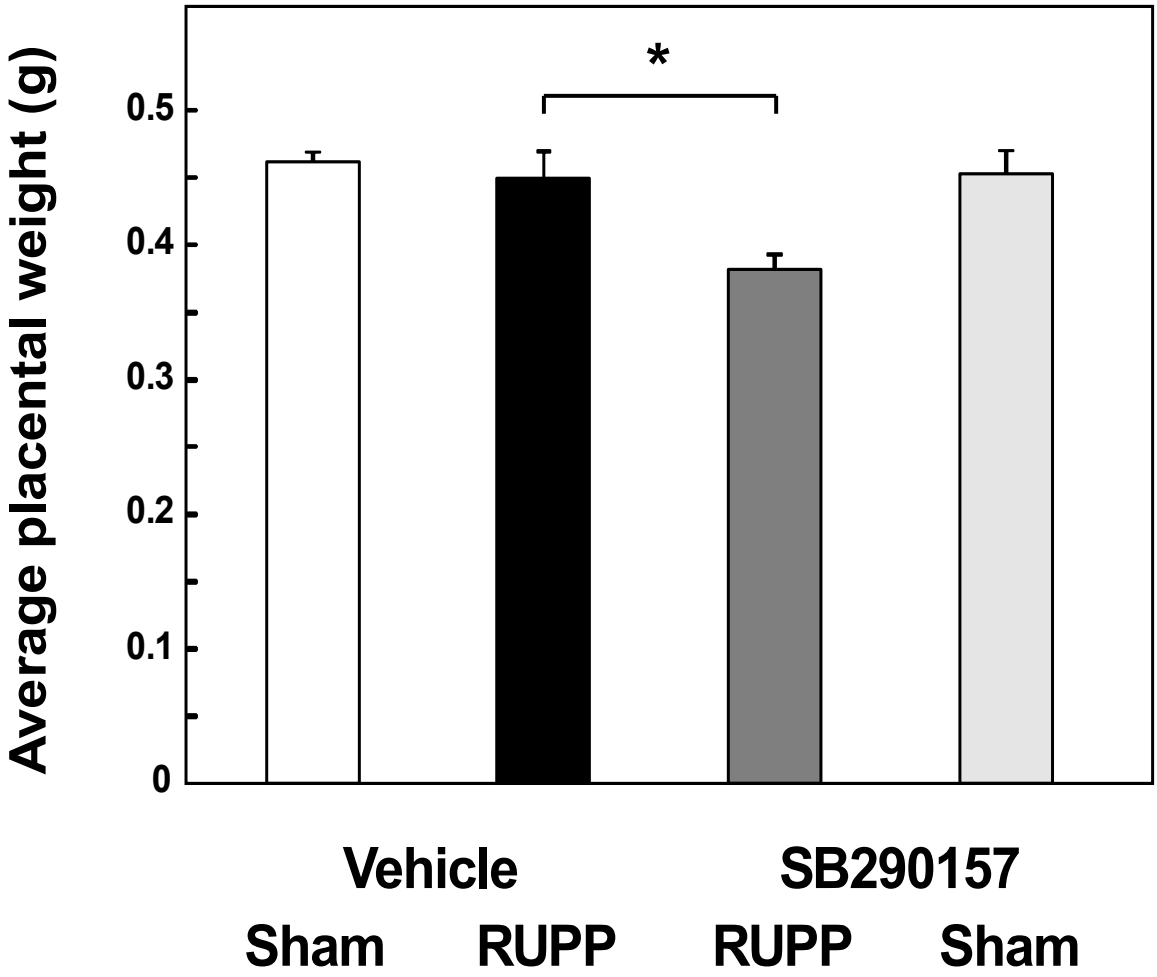

Figure 16. Average placental weight decreased in the RUPP SB group (0.38±0.01 g, n=8) vs the other three groups (Sham Veh 0.46±0.01 g, n=7; RUPP Veh 0.45±0.02 g, n=7; Sham SB 0.45±0.01 g, n=6). Data is presented as mean ± SE, *p<0.05 for indicated comparisons.

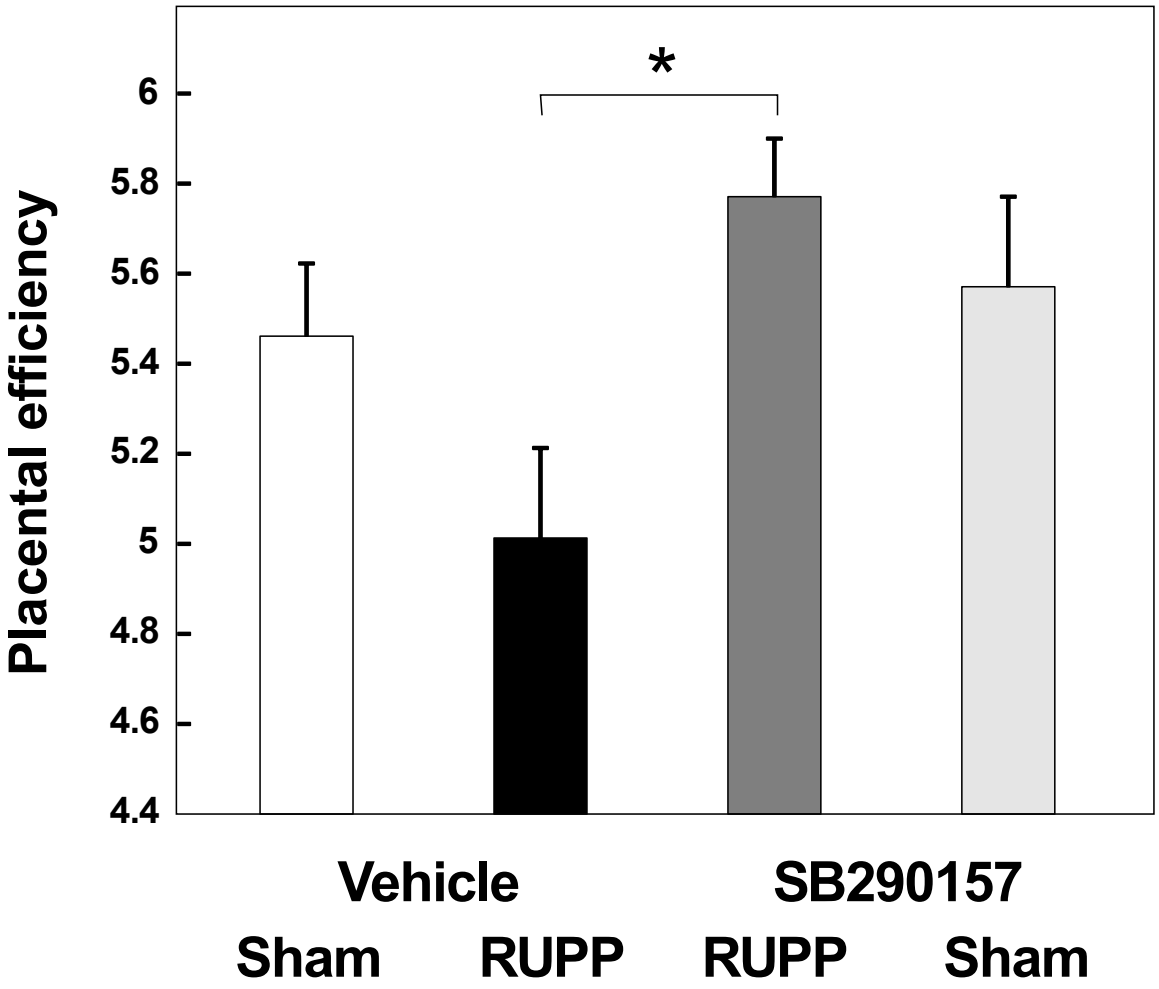

Figure 17. Placental efficiency (total mean fetal weight:total mean placental weight) trended toward a decrease in the RUPP Veh as compared to Sham Veh, though the difference was not significant (RUPP Veh 5.01±0.20, n=7 vs Sham Veh 5.46±0.16, n=7). However, RUPP SB dams exhibited a significantly greater placental efficiency compared to RUPP Veh (RUPP SB 5.77±0.13, n=8) (*$p<0.05$). No change occurred in the Sham dams with SB (Sham SB 5.57±0.20, n=6). Data is presented as mean ± SE.

SB290157 decreases circulating C3a in RUPP dams but not Sham dams

As seen in Figure 18, RUPP Veh dams had increased circulating serum C3a as compared to Sham Veh (RUPP Veh 0.39±0.07 units C3a/µL, n=7 vs Sham Veh 0.21±0.03, n=6) (*$p<0.05$). RUPP animals receiving SB290157 had decreased C3a

compared to RUPP Veh (RUPP SB 0.22±0.03, n=9) (*p<0.05), but C3a in Sham animals receiving SB290157 was not different (Sham SB 0.10±0.05, n=6).

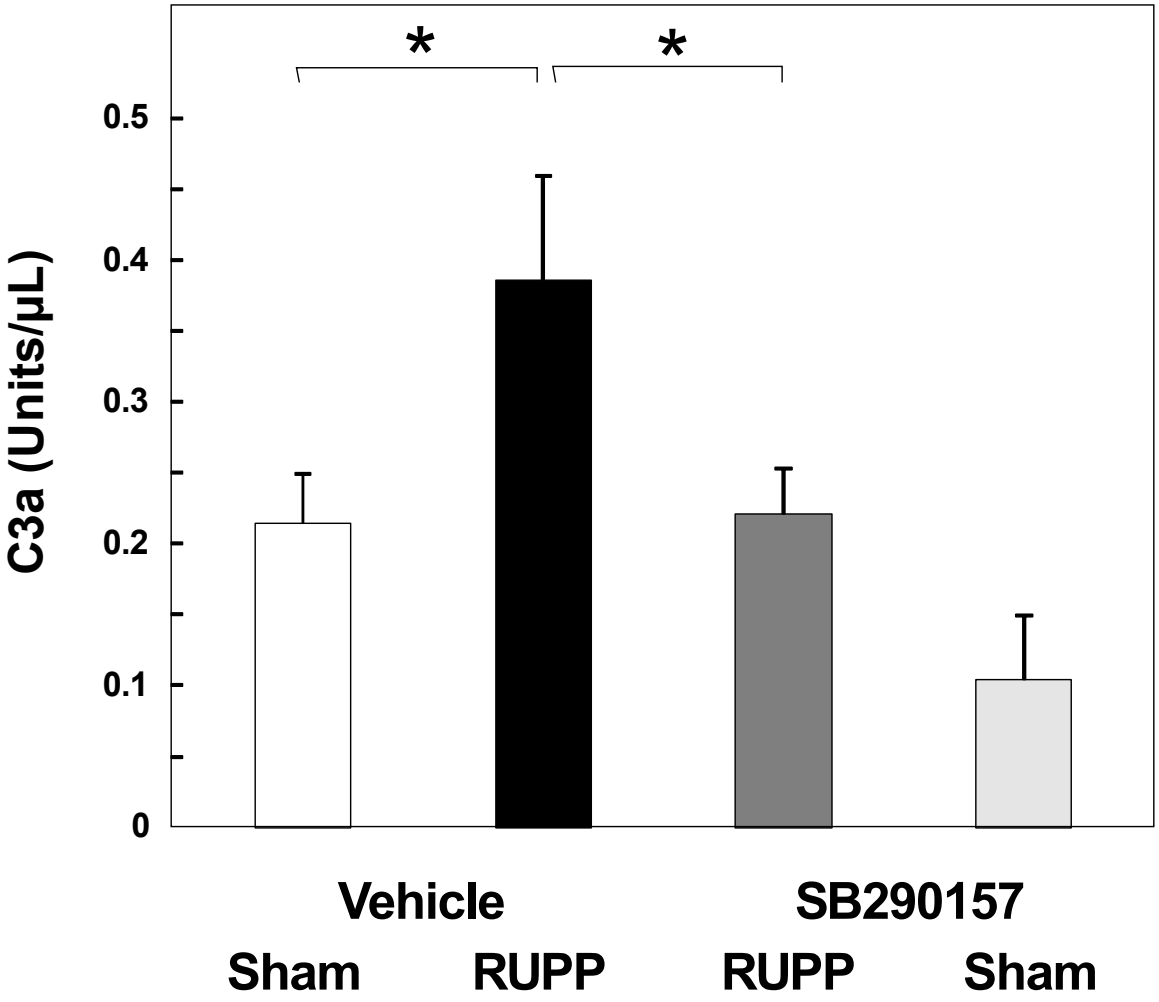

Figure 18. The complement activation product C3a increased in serum in response to the RUPP procedure (RUPP Veh 0.39±0.07 units C3a/µL, n=7 vs Sham Veh 0.21±0.03, n=6), consistent with our previous findings that RUPP increases complement activation. C3a concentrations were decreased with SB290157 in the RUPP group (RUPP SB 0.22±0.03, n=9) but not in the Sham rats (Sham SB 0.10±0.05, n=6). Data is presented as mean ± SE, *p<0.05 for indicated comparisons.

SB290157 inhibits the C3a-induced pressor response

An intravenous bolus injection of 30 μg/kg C3a peptide in anesthetized, unrestrained pregnant dams on gestational day 19 resulted in a rapid and transient pressor response (36.9±5.7% increase from resting MAP, n=6) as measured by arterial catheter (Figure 19). Resting blood pressure in pregnant anesthetized rats was 77.8±3.1 mmHg (data not shown). The same dose of the peptide yielded a 35.8±6.8% increase from resting MAP in non-pregnant rats of a comparable age and size (n=8). Resting blood pressure in non-pregnant anesthetized rats was 83.6±5.4 mmHg (data not shown).

Contrary to reports by Proctor et al (2004), MAP was not affected by the SB290157 (100 μg/kg) bolus injection itself in non-pregnant rats (9.1±7.0% change from resting, n=5). MAP was similarly unaffected in pregnant rats (2.3±2.5% change from resting, n=4).

15 minutes following SB290157 administration, 30 μg/kg C3a peptide was given. SB290157 attenuated the C3a-induced pressor response (0.74±5.4% change from resting MAP in non-pregnant rats, n=5; 0.07±1.7% change from resting MAP in pregnant rats, n=4).

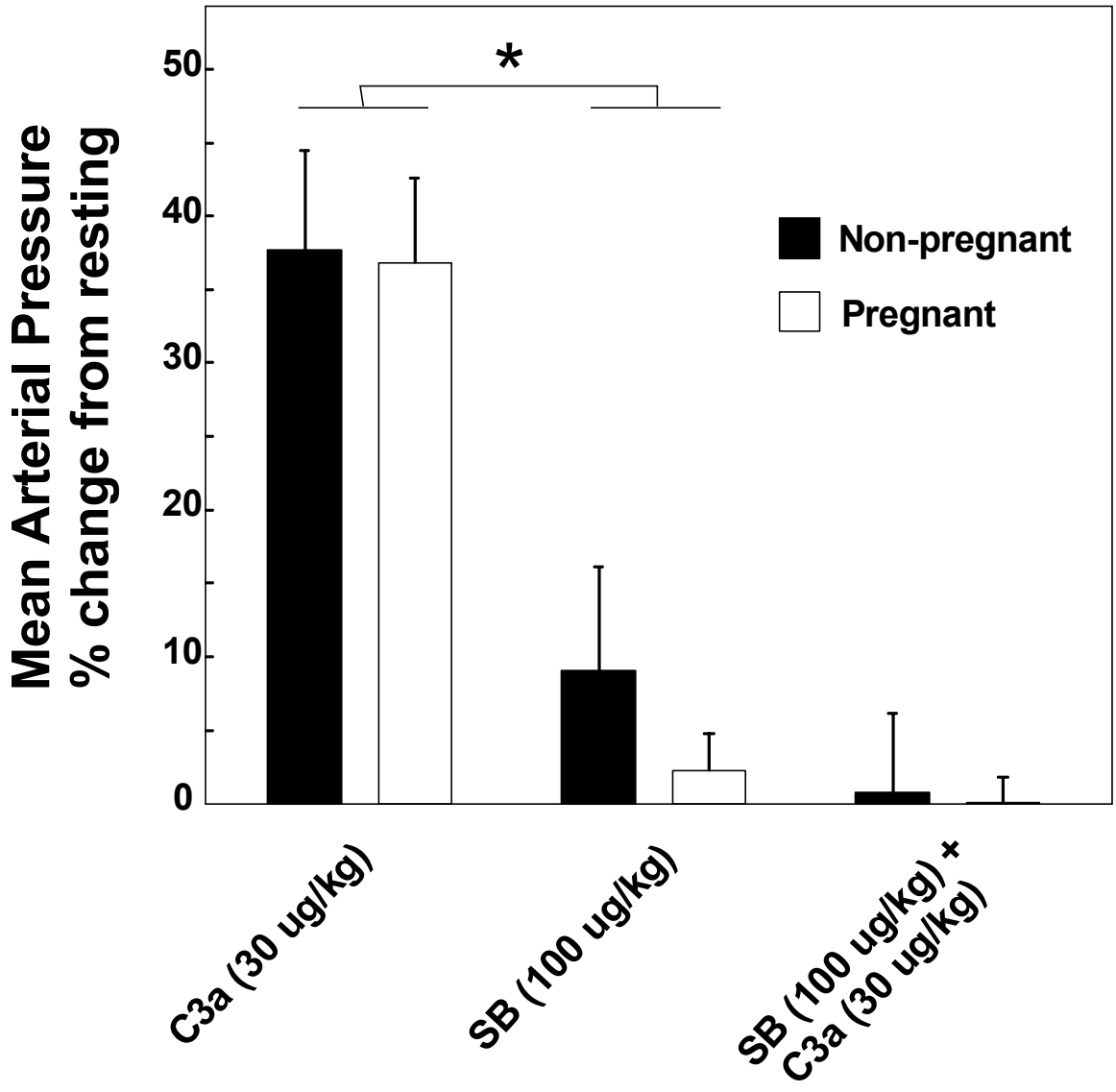

Figure 19. Unrestrained anesthetized rats given 30 ug/kg C3a peptide exhibited an increase in mean arterial pressure (MAP) from resting (non-pregnant 37.67±6.8% increase in MAP, n=7; pregnant 36.87±5.7% increase in MAP, n=6). SB290157 (100 ug/kg) itself did not have an effect on MAP (9.09±7.0% increase from resting in non-pregnant, n=5; 2.25±2.51% increase from resting in pregnant, n=4) and completely abrogated the 30 ug/kg C3a analog-induced pressor response (0.74±5.4% change from resting in non-pregnant, n=5; 0.07±1.69% change from resting in pregnant, n=4). Data is presented as mean ± SE, *$p<0.05$ for indicated comparisons.

SB290157 has no effect on CH_{50} in vivo *or complement activation* in vitro

To determine if SB290157 altered complement levels *in vivo*, we measured total hemolytic complement activity in plasma that uses antibody as an initiating event (Figure 20). There was no difference among any of the treatment groups (Sham Veh 2.40±0.23, n=6; RUPP Veh 2.59±0.67, n=7; RUPP SB 2.49±0.18, n=9; Sham SB 2.16±0.18, n=6). *In vitro*, we added SB290157 or 10% EtOH/saline vehicle (volume equivalent to 5 mg/kg based on total blood volume of a 300 g rat) to a standard rat serum pool. Neither the presence of SB290157 nor vehicle had any impact on classical pathway activation *in vitro* in the CH_{50} assay (data not shown).

We exposed the same volume of SB290157 or 10% EtOH/saline to the same rat serum pool, this time in the presence of yeast to test alternative pathway complement activation measured by Western immunoblot. Serum samples were taken at multiple timepoints to monitor the rate and extent of complement activation as it occurred. There was no difference in complement activation in the presence of SB290157 or the vehicle in this assay (data not shown).

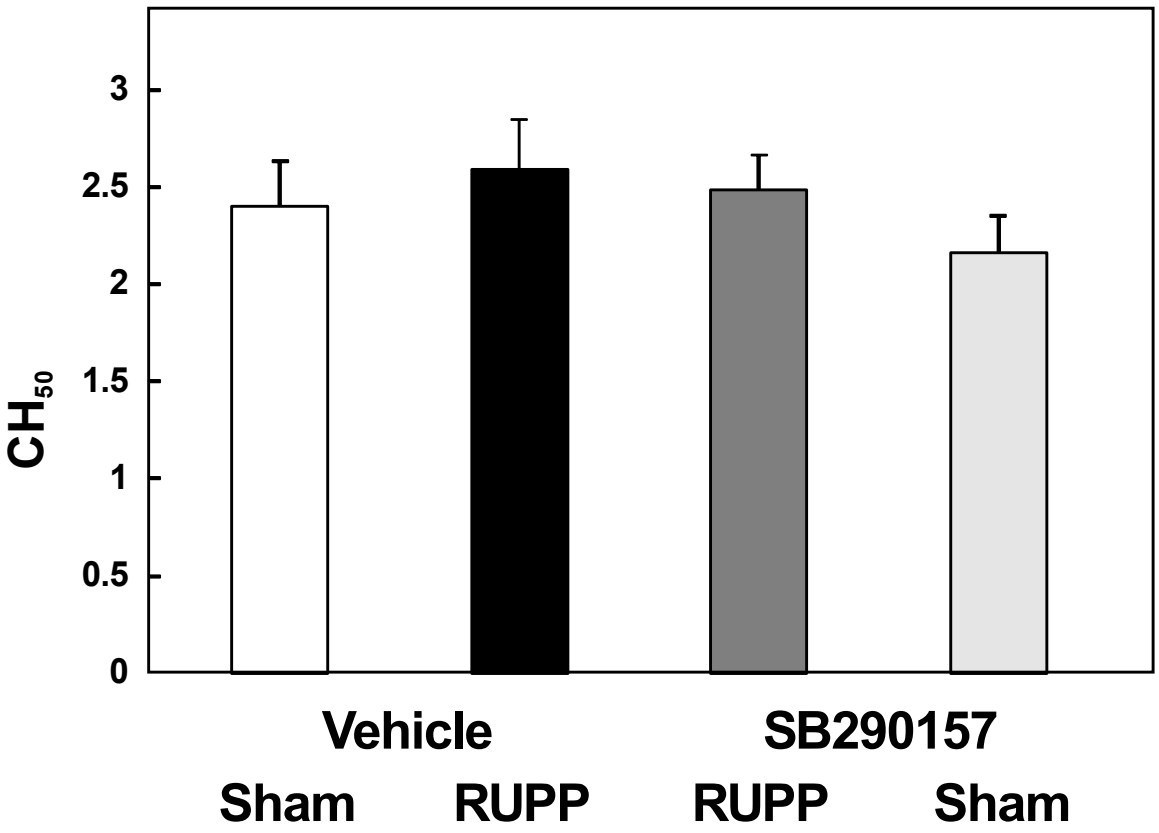

Figure 20. The complement activity of serum was assessed by the ability of the plasma to lyse antibody-sensitized sheep erythrocytes. No differences were observed between any of the treatment groups (Sham Veh 2.40±0.23, n=6; RUPP Veh 2.59±0.67, n=7; RUPP SB 2.49±0.18, n=9; Sham SB 2.16±0.18, n=6). Data is presented as mean ± SE.

SB290157 had no effect on circulating neutrophil concentrations in plasma

Some studies have shown partial complement agonist activity in that acute administration of SB290157 caused a transient increase in blood pressure and neutropenia. Therefore, circulating neutrophils (PMN) in the plasma from gestational day 19 were measured (Figure 21). Though RUPP Veh tended to be decreased compared to Sham Veh, the difference did not reach statistical significance (0.21±0.08 x 10^7 PMN/ml, n=6 vs 0.41±0.07 x 10^7 PMN/ml, n=7; p=0.06). No difference in circulating

neutrophils was evident with SB290157 in either the RUPP or Sham group (0.34±0.08 x 10^7 PMN/ml, n=9, 0.40±0.08 x 10^7 PMN/ml, n=6, respectively).

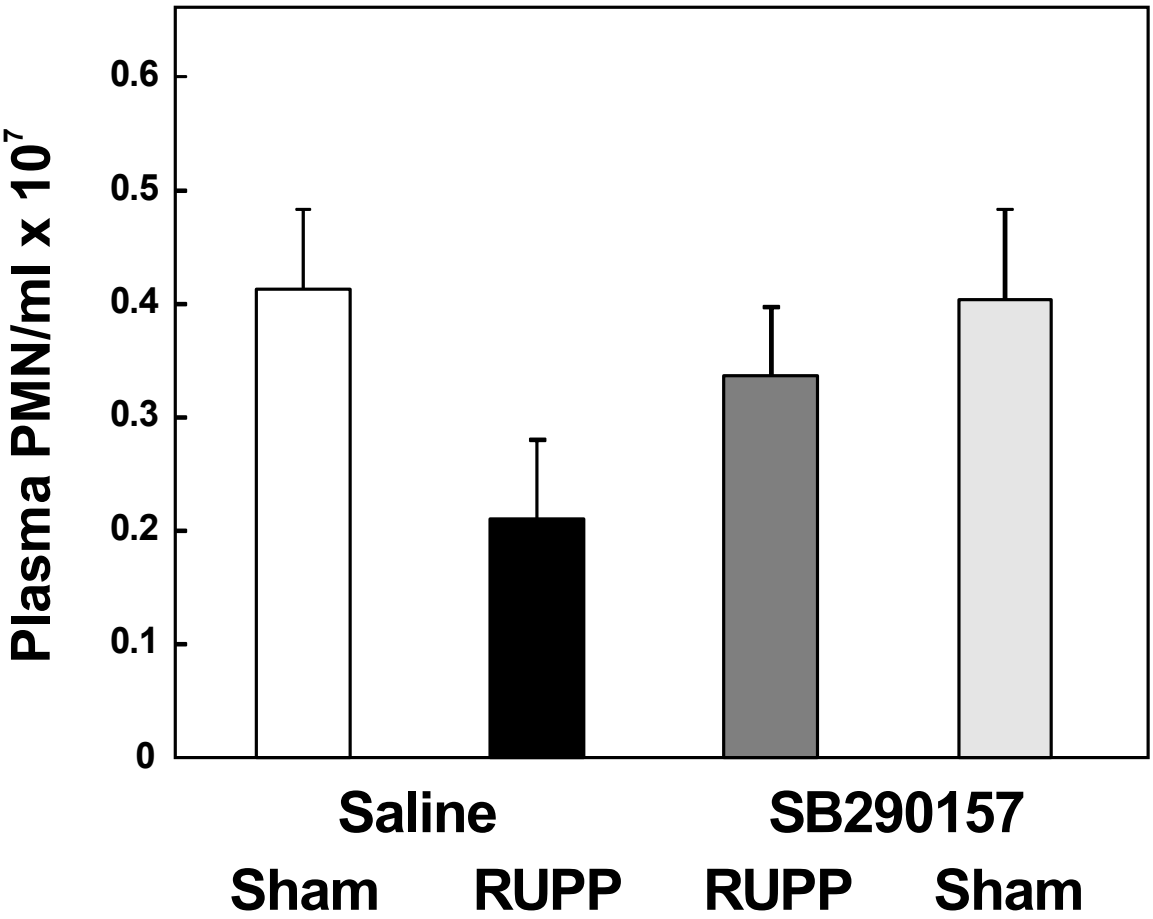

Figure 21. Neutrophil concentrations did not change significantly between any of the treatment groups (Sham Veh 0.41±0.07 x 10^7 PMN/ml, n=7; RUPP Veh 0.21±0.08 x 10^7 PMN/ml, n=6; RUPP SB 0.34±0.08 x 10^7 PMN/ml, n=9; Sham SB 0.40±0.08 x 10^7 PMN/ml). Data is presented as mean ± SE.

Discussion

Complement activation is heightened in preeclampsia (Denny, 2012). The complement activation product C3a is anaphylatoxic (along with C4a and C5a) and plays numerous roles in cellular activation of certain leukocytes. C3a is known to have direct

microvascular effects and modulate inflammatory factors, and C3a is increased in preeclamptic patients (Lynch et al., 2011). Though C5a is a more potent chemoattractant, C3a and its precursor C3 are approximately 15 times more concentrated in the plasma as compared to C5a if complement is activated (Feinberg, 2006), making C3a-mediated activity a likely contributor to the development of hypertension. We therefore hypothesized that C3a is the critical complement activation product involved in placental ischemia-induced hypertension. To this end, we examined both acute and chronic *in vivo* responses to a competitive nonpeptide antagonist to the C3a receptor. We hypothesized that this antagonist, SB290157, would reduce C3a-mediated cellular responses that may be important in hypertension and other damage associated with placental ischemia.

Our data demonstrate that the C3aR antagonist SB290157 is an effective agent to ameliorate both chronic placental ischemia-induced hypertension and acute C3a-mediated pressor responses. SB290157 has a demonstrated high efficacy and specificity for the C3a receptor in rat cells and does not disrupt C5a-mediated cellular responses *in vitro* (Ames et al., 2001). However, Mathieu et al (2005) described some agonist activity of SB290157 in systems with high C3aR density, and others observed similar agonist properties in Chinese hamster ovary cells and human U937 cells (Scully et al., 2010).

In our studies, mean arterial pressure (MAP) significantly increased in vehicle-treated animals following the RUPP procedure, and this hypertension decreased in RUPP rats treated with SB290157 with no effect on the Sham animals. No change occurred in plasma sFlt-1 concentrations with the RUPP procedure (data not shown) and free plasma VEGF tended to be decreased with the RUPP procedure in these experiments, but the

difference was not significant. Antagonism of the C3a receptor did not affect concentrations of plasma sFlt-1 (data not shown) or free plasma VEGF. Therefore, these data indicate that the decrease in free VEGF may not be due to increased plasma sFlt-1 and alterations in free VEGF may occur prior to complement activation or acts as a parallel mechanism to contribute to hypertension.

Though both fetal and placental weights were lowered in the RUPP SB group, the interaction of placental ischemia and C3aR antagonism may be involved in improving placental efficiency. Lim & Lappas (2012) provided immunohistochemical evidence that C3aR expression was decreased in the placentae of women with preeclampsia as compared to healthy pregnancies. As Mathieu et al (2005) indicated, tissues with low C3aR expression do not experience the agonistic effects of SB290157. Perhaps the beneficial effects of SB290157 in the RUPP model are due to a decrease in receptor expression or increase in receptor degradation in the placentae following placental ischemia. Immunohistochemical assessment of receptor density in the placenta following the RUPP procedure may shed some light on the validity of this process.

Unexpectedly, circulating serum C3a levels decreased in the RUPP SB treatment group. A true antagonist to the C3aR should not have any impact on complement activation, but should only inhibit cellular responses that result from C3a binding to its receptor. Therefore, we speculate that SB290157 may be responsible for some additional activity *in vivo* due to its L-arginine moiety. Qu et al (2009) noted that the L-arginine in SB290157 was essential for binding strength, but it also caused a shortened half-life and decreased bioavailability of whole-molecule SB290157. If unbound SB290157 is

metabolized and the L-arginine group is freed, it is possible that nitric oxide (NO) synthesis is potentiated and hypertension is attenuated. Thus, NO should be measured in future experiments using this compound.

The possibility for degradation of SB290157 resulting in free L-arginine yields some potentially intriguing insights to understanding its pathway of action *in vivo*, especially during pregnancy. Nitric oxide synthase (NOS) can be produced and expressed in the syncitiotrophoblast and catalyzes the reaction of L-arginine and NADPH to produce NO, a contributor to uterine vasodilation. The NOS pathway has been inhibited by acutely administered L-arginine analogs to result in a substantial decrease in glomerular filtration rate and renal plasma flow and increase in renal vascular resistance in the pregnant rat (Molnar & Hertelendy, 1992). The state of pregnancy enhances the response to L-arginine-induced changes in renal hemodynamics; changes in GFR, RPF, and vascular resistance are more robust in pregnant rats as compared to non-pregnant (Conrad et al., 2009). L-arginine analogs (i.e. L-NG-nitroarginine methyl ester (L-NAME)) hinder NO production by inhibiting NOS, resulting in hypertension in animal models (Molnar & Hertelendy, 1992). L-arginine, a necessary component for NO synthesis, has been shown to attenuate hypertension in sFlt-1-infused Harlan Sprague Dawley rats (Murphy et al., 2011) and in RUPP rats (Alexander et al., 2004). It is possible, then, that the attenuation of RUPP-induced hypertension by SB290157 is partly due to the presence of free L-arginine.

Future studies should assess whether complement is activated to result in an increase in C3a that is depleted by SB290157, or if complement activation is inhibited by

SB290157 itself *in vivo*. SB290157 did not have inhibitory effects on alternative complement activation when exposed to yeast in a standard pool of rat serum, nor did it affect classical pathway activation when incubated with antibody-sensitized sheep erythrocytes (data not shown). Thus, serum samples for C3a quantification should be collected to follow the time course of chronic administration.

Our acute pressor response experiments support the observation of Proctor et al. (2006) that SB290157 blocked the increase in blood pressure following complement activation. However, in contrast, our studies presented no evidence for neutropenia in the presence of SB290157. SB290157 has been shown to block C3aR internalization in human neutrophils (PMN) (Ames et al., 2001), and PMN have been implicated as important in generating reactive oxygen species that contribute to oxidative stress, endothelial damage, and inflammation (Ishihara et al., 2004). Additionally, PMN infiltration to ischemic tissues has been shown to lead to reactive oxygen species activation of the RhoA kinase pathway, which ultimately causes enhanced vascular reactivity (Mishra et al., 2011). Activated monocytes and PMN are known to be present in preeclampsia (Powers et al., 2001; Vinatier et al., 1995; Haeger et al., 1992), and activation may occur via oxidative stress (Roberts et al., 2001). Activation of PMN results in degranulation of the cell and release of various substances that act on the vasculature, such as elastase, which damages endothelial cell integrity, and toxic substances that also damage the endothelium and potentiate the inflammatory response (Greer et al., 1991). PMN are known to infiltrate ischemic tissue and antibodies to C5 reduce tissue damage due to decreased PMN infiltration and activation in

ischemia/reperfusion injury (Vakeva et al., 1998). Since our RUPP Veh dams had neutrophil counts trending downward, we suspect neutrophils may be sequestered in the placental tissue. SB treatment in the RUPP group seemed to trend toward partial restoration of absolute circulating neutrophil count, so perhaps SB290157 is able to displace sequestered neutrophils. A reasonable next step would be to check for neutrophil activation and/or sequestration in the placenta.

Our studies are the first to examine the effects of the C3a receptor antagonist SB290157 in the context of placental ischemia. SB290157 effectively attenuated hypertension in the RUPP model of placental ischemia-induced hypertension and did not alter free plasma VEGF concentrations, indicating an important role for C3a and/or its receptor in hypertension in a pathway distinct from VEGF. These data may be useful in developing specific and clinically feasible management strategies for gestational hypertension, and they also suggest further investigation of other complement activation products (i.e., C5a, C5b-9) and their possible role(s) in placental ischemia-induced hypertension.

Chapter 4

Discussion

Recently, we demonstrated that complement activation is important for placental ischemia-induced hypertension (Lillegard et al., 2013). We used a complement activation inhibitor (sCR1) to interrupt the complement cascade in a rat model of preeclampsia known as the reduced utero-placental perfusion pressure (RUPP) model, which is well known to increase blood pressure in a pregnant rat on day 19 of gestation (Vest et al., 2012). We found that RUPP rats that were given a saline vehicle had increased mean arterial pressure, increased serum concentrations of the complement activation product C3a, and reduced vascular endothelial growth factor (VEGF) on gestational day 19 when compared to their Sham counterparts. sCR1 successfully reduced complement activation, evidenced by a marked decrease in circulating serum C3a levels. Moreover, sCR1 attenuated the elevated mean arterial pressure in the RUPP animals with no effect on the Sham group. These data indicate that complement inhibition is important in placental ischemia-induced hypertension, and that pharmacological manipulation of the complement system may be a new strategy for treatment of pregnancy-induced hypertension. It is important to note that general complement inhibition is not a viable treatment route for preeclampsia because it potentiates maternal and fetal risk of infection, so it is necessary to explore which particular components of complement activation are associated with pregnancy-induced hypertension.

Because the complement activation product C3a is vasoactive and highly concentrated in plasma following complement activation, we chose to target the C3a receptor to inhibit C3a-mediated cellular responses that may be key to placental ischemia-induced hypertension. We induced placental ischemia-induced hypertension with the RUPP procedure in the rat on day 14 of a 21-day gestation. RUPP dams had increased blood pressure and decreased pup weights on gestational day 19 when compared to Sham dams. Antagonism of the C3a receptor with the competitive non-peptide SB290157 ameliorated the increased blood pressure and did not restore VEGF, but it did improve placental efficiency in the RUPP dams. These data indicate that actions involving the C3a receptor contribute to placental ischemia-induced hypertension, and antagonism at this receptor suggests a potentially viable method to attenuate hypertension and thus prolong gestation.

Between these experiments, we observed a few variances in the cohorts that may warrant further consideration. In the sCR1 study, intrauterine growth restriction with the RUPP procedure was not significant, perhaps due to an unusually small Sham cohort. We did see a significant decrease in concentrations of free plasma VEGF but no increase in circulating sFlt-1, presumably due to the absence of heparin in these animals. In the SB290157 study, we had a reduction in average fetal weight in RUPP dams and no significant decrease in free plasma VEGF concentrations. These inconsistencies complicate translation of our data across studies. Further, evidence exists for several key differences between distributors of Sprague Dawley rats (i.e. Harlan vs Charles River) in the severity of endothelial-dependent artery relaxation after RUPP surgery (Crews et al.,

2000; Gilbert et al., 2010) and hypertensive response to NO inhibition (Buhimschi et al., 2001; Pollock & Rekito, 1998). Studies using NO inhibition have recently elucidated differences between distributors in nephropathy susceptibility and systemic and renal hemodynamic responses (Griffin et al., 2012).

Figure 22 outlines the current working model of preeclampsia with placental ischemia as the initiating event that incorporates the data from these studies. Based on our findings, we hypothesize that placental ischemia causes increased complement activation and decreased free VEGF to result in hypertension. Antagonism of the C3aR attenuated the hypertension and had no effect on free VEGF concentrations, indicating that the cellular actions mediated by the interaction of C3a and C3aR are important in placental ischemia-induced hypertension. Because C5a stimulates similar cellular actions at its receptor, we include the possibility that C5a may also impact blood pressure. The mechanism by which free VEGF decreases remains unclear, but we hypothesize that this change may occur prior to complement activation, indicated by the dashed arrow. As these variables are tested, we may expand the scope of this hypothesis to examine effects on fetal health, how sFlt-1 is involved, or other possible mediators or mechanisms related to complement system activation that may lead to hypertension.

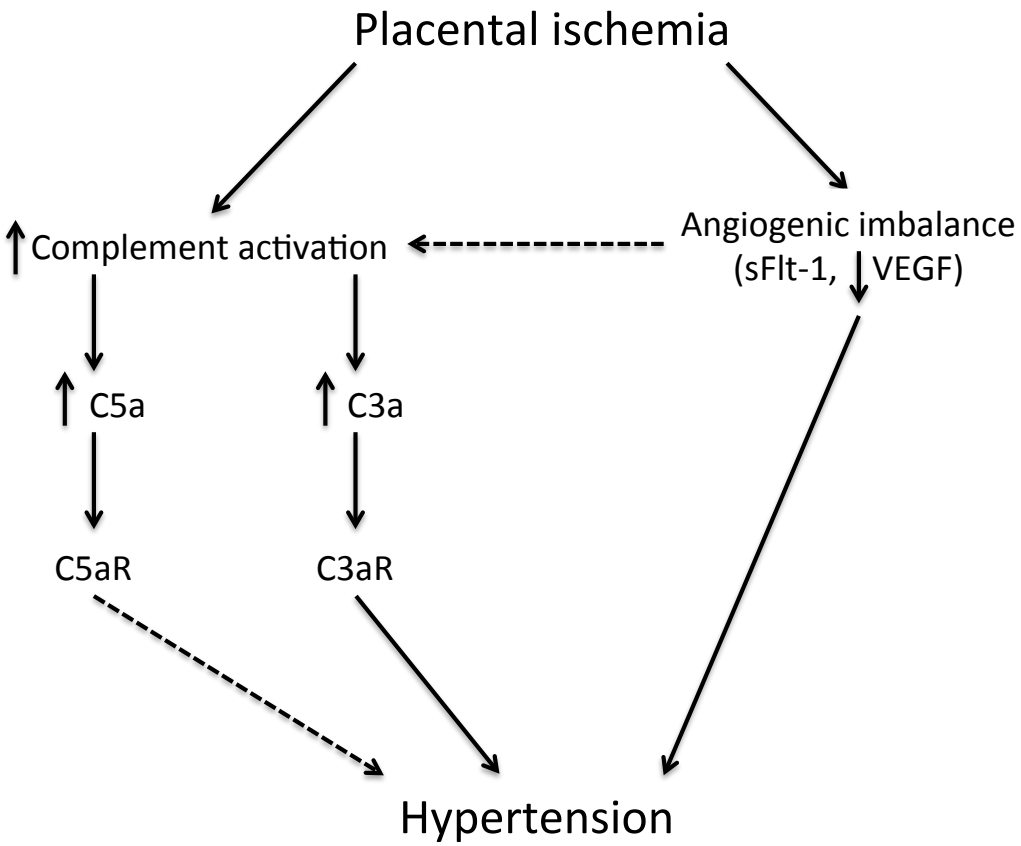

Figure 22. Working hypothesis. Our working hypothesis states that placental ischemia causes increased complement activation and decreased free VEGF to result in hypertension. Cellular actions mediated by C3a at the C3aR contribute to hypertension.

Several experiments are necessary given the current working hypothesis. First, it is important to examine the relationship between VEGF and complement. We have shown that the RUPP procedure results in hypertension and increased complement activation and previously, VEGF infusion has been shown to attenuate RUPP-induced hypertension (Gilbert et al., 2010). However, it is not known if VEGF infusion affects complement activation, and we did not observe any change in free plasma VEGF following complement inhibition. Therefore, we can infuse VEGF into rats receiving the RUPP surgery and take serum to measure complement activation (i.e., serum C3a) over

the course of the infusion. If VEGF infusion prevents excessive complement activation, this data would suggest the RUPP-induced decrease in free VEGF leads to activation of the complement cascade to contribute to hypertension. Because we observed a decrease in serum C3a in the RUPP rats with SB290157 treatment, future experiments should include taking serum samples every 24 hours over the course of the experiment. It is important to identify if SB290157 is somehow inhibiting complement activation immediately following the RUPP procedure or if complement activation occurs and then is blocked. The mechanism by which SB290157 works *in vivo* must be more carefully examined if C3aR antagonism is to be pursued as a management strategy for maternal symptoms.

The working hypothesis leads to some logical future directions. Effective management strategies for preeclampsia must consider both mother and fetus. C3aR antagonism decreased hypertension in the RUPP rats and increased placental efficiency, indicating the placenta was able to efficiently transfer resources to the fetus. It would be interesting to examine changes in nutrient exchange with C3aR antagonism. Extensive long-term fetal studies must take place to identify any potential adverse developmental effects in these pups or in future generations. Also, because C3a and C5a act similarly, it is important to explore C5a and its interaction at its receptor. Antagonism of the C5aR may attenuate placental ischemia-induced hypertension, and perhaps antagonism of both the C3aR and C5aR would attenuate the hypertension more than either receptor alone.

Clinically, our data support the concept that the immune system plays a key role in placental ischemia-induced hypertension. Elevated concentrations of complement

activation products are associated with preeclampsia and are present before its onset. Therefore, carefully controlled manipulation of the complement system holds promise as a therapeutic strategy to manage the symptoms of preeclampsia. A recent case study described the effect of the C5a receptor antagonist eculizumab in a woman with HELLP syndrome. Antagonism of the receptor improved symptoms to lengthen gestation by a critical 17 days, allowing the fetus to mature and thus lessen neonatal health complications. Certainly, our data add to the rationale to target complement activation products and/or receptors and offer compelling evidence to pursue these factors as a management strategy for preeclamptic patients.

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