Title Page

Differential Effects of Complement Activation Products C3a and C5a on Cardiovascular Function in Hypertensive Pregnant Rats

Kathryn E. Lillegard, Alex C. Loeks-Johnson, Jonathan W. Opacich, Jenna M. Peterson, Ashley J. Bauer, Barbara J. Elmquist, Ronald R. Regal, Jeffrey S. Gilbert, Jean F. Regal

Department of Biomedical Sciences, University of Minnesota Medical School Duluth (K.E.L., A.C.L., J.W.O., J.M.P., A.J.B., B.J.E., J.S.G., J.F.R.) and Department of Mathematics and Statistics (R.R.R.), University of Minnesota Duluth, Duluth, Minnesota
Running title

Running title: C3a, C5a and pregnancy-induced hypertension

Corresponding author:

Jean F. Regal, Ph.D.
Department of Biomedical Sciences
University of Minnesota Medical School Duluth
1035 University Dr.
Duluth, MN  55812

Tel: 218 726 8950
Fax: 218 726 7906
Email: jregal@d.umn.edu

Number of text pages: 16
Number of tables: 0
Number of figures: 7
Number of references: 48
Number of words in Abstract: 246
Number of words in Introduction: 619
Number of words in Discussion: 1683

Nonstandard Abbreviations:

C3aRA, C3a receptor antagonist SB290157, N²-[(2,2-Diphenylethoxy)acetyl]-L-arginine
C5aRA, C5a receptor antagonist PMX53, acetyl-F-[Orn-P-(D-Cha)-WR]
GD, gestation day
RUPP, reduced uterine perfusion pressure
VEGF, vascular endothelial growth factor

Recommended Section Assignment:

Cardiovascular or Inflammation, Immunopharmacology, and Asthma
ABSTRACT

Early onset preeclampsia is characterized by decreased placental perfusion, new-onset hypertension, angiogenic imbalance and endothelial dysfunction associated with excessive activation of the innate immune complement system. While our previous studies demonstrated that inhibition of complement activation attenuates placental ischemia-induced hypertension using the rat reduced uterine perfusion pressure (RUPP) model, the important product(s) of complement activation has yet to be identified. We hypothesized that antagonism of receptors for complement activation products C3a and C5a would improve vascular function and attenuate RUPP hypertension. On gestation day (GD) 14, rats underwent sham surgery or vascular clip placement on ovarian arteries and abdominal aorta (RUPP). Rats were treated once-daily with C5a receptor antagonist acetyl-F-[Orn-P-(D-Cha)-WR] (C5aRA), C3a receptor antagonist N²-[(2,2-Diphenylethoxy)acetyl]-L-arginine (C3aRA) or vehicle from GD14-18. Both C3aRA and C5aRA attenuated placental ischemia-induced hypertension without affecting the decreased fetal weight or decreased concentration of free circulating vascular endothelial growth factor (VEGF) also present in this model. C5aRA, but not C3aRA, attenuated placental ischemia-induced increase in heart rate and impaired endothelial-dependent relaxation. C3aRA abrogated the acute pressor response to C3a peptide injection, but it also unexpectedly attenuated the placental ischemia-induced increase in C3a suggesting non-receptor mediated effects. Overall, these results indicate that both C3a and C5a are important products of complement activation that mediate the hypertension regardless of the reduction in free plasma VEGF. The mechanism by which C3a contributes to placental ischemia-induced hypertension appears to be distinct from C5a, and management of pregnancy-induced hypertension is likely to require a broad anti-inflammatory approach.
Introduction

Preeclampsia is a pregnancy-specific condition characterized by decreased placental perfusion, new-onset hypertension, intrauterine growth restriction, and endothelial dysfunction. Affecting up to 10% of pregnancies in the United States, preeclampsia and hypertensive disorders of pregnancy are a leading cause of maternal and perinatal morbidity and mortality with few effective treatment options (Ananth et al., 2013; Roberts, 2013). The complement system, part of the innate immune response, is an enzymatic amplification system composed of plasma proteins essential for host defense and inflammation. The complement system can be activated by multiple pathways with a central event being formation of the enzyme C3 convertase that cleaves C3 to generate fluid phase C3a as well as C3b that covalently binds to an invader or self, i.e. C3 deposition (Figure 1). C3b serves as a nucleus for formation of C5 convertase that cleaves C5 into two fragments: C5a and the larger fragment C5b that inserts into membranes. Continuation of the cascade results in formation of membrane attack complex C5b-9 that either forms a pore in the membrane or is released as a soluble complex into circulation. Complement activation products include C3a, C5a and C5b-9. While the complement system is essential for fetal survival (Usami et al., 2010) and normal placental development (Singh et al., 2011), excessive complement activation is associated with recurrent miscarriages, preterm labor and preeclampsia (Lynch and Salmon, 2010). During pregnancies complicated with preeclampsia there is strong evidence of excessive complement activation in the circulation with a decrease in C3 concentration and an increase in C5a, sC5b-9 and the ratio of C3a/C3 (Derzsy et al., 2010; Soto et al., 2010), as well as excessive complement activation in placenta (Buurma et al., 2012; Gilbert et al., 2012c) and urine (Burwick et al., 2013). A recent case report suggests that complement contributes to preeclamptic symptoms since depletion of complement component
C₅ in a severely preeclamptic woman was able to prolong pregnancy by more than two weeks (Burwick and Feinberg, 2013).

Mechanistic studies in clinically relevant animal models are clearly needed to identify critical products of complement activation contributing to preeclamptic symptoms. Using the reduced uterine perfusion pressure (RUPP) rat model of placental ischemia-induced hypertension and soluble Complement Receptor 1 (sCR1) as an inhibitor of complement activation, our studies were the first to describe a link between complement system activation and the hypertension resulting from third trimester placental ischemia in pregnancy (Lillegard et al., 2013). We also demonstrated that angiogenic imbalance as well as impaired endothelial-dependent relaxation in isolated blood vessels occurs in this model (Gilbert et al., 2010). Inhibiting complement activation attenuated hypertension in the RUPP model, but angiogenic imbalance, i.e. decreased vascular endothelial growth factor (VEGF), was still evident, suggesting that the role of complement in placental ischemia-induced hypertension was independent of VEGF. Complement activation products C₃a and C₅a are potent ∼10kDa fluid phase inflammatory mediators that interact with G protein coupled receptors on many cell types to elicit biological activities including smooth muscle contraction and changes in blood pressure (Regal et al., 1980; Regal, 1982; Proctor et al., 2009), activation and chemotaxis of immune cells, and increased vascular permeability (Klos et al., 2013). In addition, excessive complement activation can adversely affect endothelial function (Collard et al., 2000). With the realization of the importance of immune inflammatory pathways in numerous disease states, C₃a and C₅a receptor antagonists are being developed for potential clinical use and have also been used in numerous animal models to investigate the role of C₃a and C₅a in disease models (Ricklin and Lambris, 2013). Using antagonists of the C₃a and C₅a receptor, the present study is the first to
test the hypothesis that C3a and C5a are the complement activation products that mediate endothelial dysfunction and hypertension following placental ischemia.
Materials and Methods

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

RUPP procedure was performed on gestational day 14 (GD14) to achieve chronic placental ischemia and hypertension in pregnant rat on GD19 (See supplemental material for details) (Lillegard et al., 2013). All animal experiments were approved by University of Minnesota Institutional Animal Care and Use Committee and conformed to NIH Guide for Care and Use of Laboratory Animals. Animals were treated with either C3a receptor antagonist SB290157 (Ames et al., 2001) at 5 mg/kg iv (C3aRA; N²-{(2,2-Diphenylethoxy)acetyl]-L-arginine, Merck KGaA, Darmstadt, Germany), the C5a receptor antagonist PMX53 (Finch et al., 1999) at 3 mg/kg subcutaneously (C5aRA; cyclic acetyl-F-[Orn-P-(D-Cha)-WR]; synthesized by Atlantic Peptides, Lewisburg, PA) or 10% ethanol/saline vehicle (Veh). Once-daily treatments began 10-15 min before RUPP or Sham surgery on GD14 and ended GD18. Animals were randomly assigned to the following treatment groups: 1). RUPP surgery with iv Veh; 2). RUPP surgery with subcutaneous Veh; 3). RUPP surgery with C3aRA (RUPP C3aRA); 4). RUPP surgery with C5aRA (RUPP C5aRA); 5). RUPP surgery with C3aRA and C5aRA; 6). Sham surgery with iv Veh; 7). Sham surgery with subcutaneous Veh; 8). Sham surgery with C3aRA (Sham C3aRA); 9). Sham surgery with C5aRA (Sham C5aRA). The vehicle for both antagonists was 10% ethanol/saline and was administered either intravenously or subcutaneously. With statistical analysis, no differences in outcomes were detected with administration of Veh by either the subcutaneous or iv route so groups 1 (n=10) and 2 (n=13) were combined as RUPP Veh and groups 6 (n=10) and 7 (n=9) combined as Sham Veh in final data presentation and analysis.
Mean arterial pressure measurements and tissue collection

Mean arterial pressure (MAP) and heart rate were measured via intra-arterial catheter in unanesthetized restrained animals on GD19 and serum, plasma, fetal and placental tissue harvested as described previously (Lillegard et al., 2013). Circulating white blood cells in EDTA blood were counted by standard methods in a hemacytometer. Blood smears were stained with a modified Wrights’ stain (Diff Quik, American Scientific Products, McGraw Park, IL) and at least 400 cells were counted and categorized as neutrophils, eosinohils, monocytes or lymphocytes as determined by their morphology. Myeloperoxidase in homogenized lung was determined as an indicator of the number of neutrophils in the lung (Greene et al., 2005) (Details in supplemental methods). Circulating free VEGF in EDTA plasma collected GD19 was measured using commercially available kit for mouse VEGF (R&D Systems; Minneapolis, MN).

C3a pressor response in nonpregnant and pregnant rats

To test efficacy of C3aRA we used C3a peptide, an analog of C3a described by Ember et al (Ember et al., 1991) (WWGKKYRASKLGLAR, AnaSpec, Fremont, CA), to acutely increase blood pressure. Pregnant and nonpregnant female rats were anesthetized ip with 90 mg/kg ketamine plus 2.5 mg/kg xylazine for placement of jugular catheter (used for iv administration of C3a peptide and C3aRA) and a carotid catheter (MAP measurements). Blood pressure was allowed to stabilize for 15 minutes and either 100 µg/kg C3aRA or 10% ethanol/saline vehicle administered. After 15 min, C3a peptide (30 µg/kg) was delivered and increase in MAP monitored until it returned to baseline within 10 min. Norepinephrine (80 µg/kg; Sigma-Aldrich, St. Louis, MO) was then administered to verify responsiveness.
Complement measurements

C3a serum concentration was measured by Western immunoblot as described previously (Lillegard et al., 2013) and is expressed as C3a units/µl relative to the signal intensity of 1 µl of standard pool of yeast activated rat serum. Total complement hemolytic activity was measured as previously described and expressed as inverse dilution of serum causing 50% hemolysis of sensitized sheep erythrocytes (CH50) (Lillegard et al., 2013). The ability of C3aRA or C5aRA to inhibit complement activation in vitro was also assessed by incubation of antagonist or Veh with a pool of non-pregnant rat serum prior to determination of CH50.

Mesenteric artery myography

Third order mesenteric arteries were cleaned of adipose tissue and 2 mm segments mounted on 40 µm thick wires for myography as previously described (Gilbert et al., 2012a). Buffers and vessel normalization were performed according to DMT Multi Wire Myography System User Manual (Model 610M, Danish Myo Technology, Aarhus, Denmark). Vessels were normalized to mimic a transmural pressure of 100 mm Hg and equilibrated 30-60 min with 3-4 buffer washes. Diameters ranged from 200-350 µm, and vessels were pre-contracted with thromboxane-mimetic U46619 (5.7 X 10^-7 mol/L) followed by half log increments of acetylcholine (Ach; 0.03 to 33 X 10^-6 mol/L) to assess endothelial-dependent relaxation. After washing, vessels were contracted again with U46619 and relaxation to increasing concentrations of sodium nitroprusside (SNP; 0.01 to 333 X 10^-6 mol/L) was measured as an indicator of endothelial-independent vasodilation. Finally, a cumulative concentration response curve to U46619 confirmed vessel reactivity.

Statistical analyses
Data were analyzed using three-way ANOVA (JMP and SAS software; Cary, North Carolina) and presented as geometric mean or mean ± SE. Differences were considered significant when p<0.05. Specific individual contrasts evaluated and presented in figures were: 1). Sham Veh vs RUPP Veh, 2). RUPP vs RUPP C3aRA, 3). RUPP vs RUPP C5aRA, 4). Sham vs Sham C3aRA, 5). Sham vs Sham C5aRA.
Results

Receptor antagonists attenuate placental ischemia-induced hypertension

To determine if complement activation products C3a and/or C5a were important in mediating placental ischemia-induced hypertension, we evaluated the effect of treatment with C3aRA or C5aRA. Chronic placental ischemia caused a significant increase in MAP by GD19 (Figure 2A). Clearly, treatment with either C3aRA or C5aRA significantly inhibited RUPP-induced increase in MAP without altering MAP in Sham animals. Treatment of animals with a combination of C3aRA and C5aRA did not result in greater attenuation of MAP than treatment with either antagonist alone (104±3 mm Hg, n=9; data not shown). As seen in Figure 2B, heart rate in RUPP rats was increased as previously described (Gilbert et al., 2012e) and was significantly decreased by treatment with C5aRA (p<0.05) but not C3aRA (p=0.11).

Placental ischemia-induced decrease in free plasma VEGF, fetal weight and resorptions is unaffected by receptor antagonists

As previously shown, placental ischemia resulted in decreased free plasma VEGF (Figure 3A) and intrauterine growth restriction in RUPP compared to Sham controls (Figure 3B). Treatment with either C3aRA or C5aRA did not alter VEGF or fetal weight in RUPP rats (Figure 3). Combined treatment with both antagonists (n=9) also did not affect RUPP-induced decrease in either outcome (2.1±0.8 g, 1679±220 pg/ml VEGF). Antagonist treatment of Sham animals did not significantly alter circulating free plasma VEGF or fetal weight compared to Sham Veh animals. As previously reported, no increase in soluble fms-like tyrosine kinase-1 (sFlt-1 or soluble VEGF R1; as measured by the commercially available mouse kit from R&D Systems; Minneapolis, MN) was detected in EDTA plasma collected GD19 from RUPP vs Sham in our
experiments (Lillegard et al., 2013). In addition, a significant increase in protein in urine was also not detected between RUPP and Sham in these studies, consistent with previous reports regarding inconsistency of this measure in the RUPP model (Granger et al., 2006). Fetal resorptions were also assessed, and as previously shown (Lillegard et al., 2013), RUPP surgery increased fetal resorptions (Figure S1). Treatment of RUPP or Sham animals with C3aRA, C5aRA or in combination did not change the number of resorptions (Figure S1).

C3aRA but not C5aRA inhibits increased C3a in vivo following placental ischemia

Relative serum C3a concentrations were used as an indicator of complement activation. Rat C5a was not detectable by current assay methods. C3aRA and C5aRA were not expected to inhibit complement activation, but only antagonize actions of cleavage fragments C3a and C5a at their receptors. As seen in Figure 4, our data confirm our previous studies (Lillegard et al., 2013) demonstrating that RUPP significantly increases C3a in the circulation. Treatment with C5aRA altered neither RUPP-induced increase in C3a, nor baseline C3a concentration in Sham. Surprisingly, treatment with C3aRA in vivo significantly inhibited C3a generation in both RUPP and Sham groups. Hemolysis of sensitized sheep erythrocytes, termed the CH₅₀, measures the ability of the classical complement pathway components in serum to react sequentially and lyse the red blood cell coated with antibody. The receptor antagonists are not expected to affect the CH₅₀ because they prevent the action of C3a and C5a on their receptors without affecting activation of the complement cascade. Neither C3aRA nor C5aRA treatment in vivo, either alone or in combination, significantly affected total hemolytic complement activity (CH₅₀; Figure S2) indicating they were not inhibiting classical pathway complement activation or appreciably depleting total complement activity in vivo. In addition, in vitro studies demonstrated that
incubating the C3aRA (≤ 390 µg/ml) or C5aRA (≤ 100 µg/ml) with normal rat serum did not inhibit the CH50 of the serum (data not shown) indicating that the antagonists were not altering classical hemolytic complement pathway activity in vitro.

C3aRA inhibits C3a-induced pressor response

Previous studies of others indicated differential vascular reactivity in pregnant vs non-pregnant rats (Paller, 1987). Thus, the ability of C3a to cause a pressor response in the pregnant rat was evaluated. An iv injection of 30 µg/kg C3a peptide in anesthetized pregnant dams on GD19 resulted in a rapid transient pressor response within minutes (Figure 5), similar to the response in non-pregnant rats of a comparable age and size. Injection of the C3aRA (100 µg/kg) itself did not significantly alter MAP in non-pregnant or pregnant rats at GD19 (Figure 5), but it significantly inhibited the pressor response to 30 µg/kg C3a peptide in both non-pregnant and pregnant rats.

C5aRA reduces circulating neutrophils

Since studies of Proctor et al (Proctor et al., 2004) indicated that C3aRA exhibits partial agonist activity and affects circulating neutrophils in the non-pregnant rat, we determined the effect of both C3aRA and C5aRA on circulating white blood cells (WBC). Although RUPP did not alter total WBC and neutrophils (PMN) in circulation, analysis of variance indicated C5aRA decreased circulating WBC and PMN, regardless of Sham or RUPP surgery (Figure 6). Chronic C3aRA administration had no significant effect on circulating WBC or neutrophils. Total lymphocytes were not affected by either RUPP surgery, C3aRA or C5aRA (data not shown).

Previous studies of others have clearly demonstrated an important role for lymphocytes in the
RUPP model (LaMarca et al., 2013). However, our studies were not designed to detect changes in lymphocyte subpopulations. Analysis of neutrophils in the lung demonstrated that neutrophils were not being sequestered in the lung by any of the treatments (Figure S3).

C5aRA but not C3aRA attenuates endothelial dysfunction following placental ischemia

As previously demonstrated in this model, vasodilation in response to Ach was attenuated in mesenteric arteries from rats with placental ischemia (Figure 7). In contrast, endothelial-independent vasodilation in response to SNP was not affected by placental ischemia (Figure S4). The C5aRA, but not the C3aRA, preserved endothelial function following placental ischemia as indicated by Ach-induced vasodilation equivalent to that seen in rats undergoing Sham Veh treatment. Combination treatment with both C3aRA and C5aRA affected Ach and SNP relaxation in the same way as C5aRA alone (data not shown). Both C3a and C5a have been reported to contract isolated blood vessels (Regal, 1982; Bjork et al., 1985) including microvessels in the hamster cheek pouch (Bjork et al., 1985), but neither C3a peptide nor recombinant human C5a contracted the isolated rat mesenteric arteries (data not shown). In addition, neither C3aRA nor C5aRA significantly relaxed isolated mesenteric arteries whether at normal resting tension or when pre-contracted with the thromboxane mimetic U46619.
Discussion

Using sCR1, an established inhibitor of complement activation, our previous studies demonstrated a critical role for complement activation in placental ischemia-induced hypertension in rat (Lillegard et al., 2013) suggesting that manipulation of the complement system is a viable therapeutic strategy for management of pregnancy-induced hypertension. Given the importance of complement in host defense and immune complex clearance, defining specific product(s) of complement activation responsible for placental ischemia-induced hypertension is an important step towards defining therapeutic targets that minimally compromise the protective role of complement. Since complement activation products C3a and C5a are well known for their role as inflammatory mediators through interaction with G protein coupled receptors on multiple cell types (Klos et al., 2013), we used receptor antagonists to assess the contribution of C3a and C5a to placental ischemia-induced hypertension. Our studies are the first to demonstrate both C3a and C5a contribute to placental ischemia-induced hypertension, and inhibiting both receptors was no more effective than inhibiting each receptor alone.

By design, multiple inflammatory mediators can be evoked by any given perturbation, thus promoting survival of the organism but also contributing to difficulty in treating inflammatory disease. If the action or synthesis of one mediator (i.e., C3a, C5a) is prevented, another mediator may assume a primary role in uncontrolled inflammation. Our studies, and those of others, suggest that increased inflammation in preeclampsia involves cytokines, T cells, autoantibodies and complement along with pro-and anti-angiogenic factors (LaMarca et al., 2013). This complex interplay of mediators suggests that therapy of pregnancy-induced hypertension will be a challenge similar to treatment of general inflammatory disease.
Increasing evidence supports a role for angiogenic imbalance in pregnancy-induced hypertension (Gilbert et al., 2012d). Our current studies demonstrated a decrease in circulating free VEGF but no increase in sFlt-1 in the RUPP model. Further, neither C3aRA nor C5aRA impacted the decrease in circulating VEGF concentrations suggesting this effect mechanistically precedes increased C3a and/or C5a action, or VEGF mediates hypertension via a parallel pathway. In the RUPP model, decreased VEGF could also potentially result in decreased complement control proteins allowing increased complement activation as demonstrated in kidney endothelium (Mason et al., 2004; Kerr and Richards, 2012). Although a recent study by Weissgerber et al (Weissgerber et al., 2014) questions the validity of the ELISA assay for measurement of VEGF in rat plasma and serum, our previous studies have reported decreased angiogenic potential via endothelial tube formation assays in RUPP compared to normal pregnant controls (Gilbert et al., 2012b; Bauer et al., 2013). Clearly a re-evaluation of the methodology for assessing angiogenic factors in the rat circulation is needed with additional studies to understand the relationship between angiogenic imbalance and complement activation/control proteins following placental ischemia.

Both endothelial dysfunction and increased heart rate may contribute to cardiovascular abnormalities observed in preeclampsia and the RUPP rat model (Gilbert et al., 2012e). The C5aRA prevented endothelial dysfunction and increased heart rate associated with RUPP hypertension suggesting that C5a substantially contributes to both in the RUPP model. We have previously shown that C5a has positive chronotropic effects on isolated male guinea pig atria (Regal, 1985). Thus, it is possible that C5aRA prevents a direct action of C5a on cardiomyocytes to increase heart rate in the RUPP. Alternatively, C5aRA treatment may decrease sympathetic tone reportedly elevated in RUPP rat (Hines et al., 2007). However, the
relevance of this to cardiovascular function in preeclampsia remains unclear (Greenwood et al., 2001). C5aRA also decreased circulating neutrophils in our study consistent with recent data indicating C5a stimulates production of granulocyte colony stimulating factor (Bosmann et al., 2013), a substance known to play a role in regulating number of neutrophils in circulation. C5a is also a potent neutrophil activator. The difficulty in detecting increases in C5a in circulation may be due to binding of any C5a generated in the circulation to the very high affinity receptor for C5a on neutrophils. However, our data demonstrate no evidence that the RUPP procedure, nor the C3aRA or C5aRA result in neutrophil activation with resultant pulmonary sequestration. Our preliminary studies indicate that depletion of neutrophils also attenuates placental ischemia-induced hypertension (Lillegard KE, 2013) suggesting that C5aRA could be limiting placental ischemia-induced hypertension by a neutrophil-dependent effect.

Clearly a limitation of our study lies in inherent limitations of receptor antagonists. PMX53, the C5aRA chosen for this study, has been used in numerous animal models in both mice and rats to assess the importance of C5a in mediating a response through the C5a receptor (CD88; C5aR; C5aR1). It is important to recognize that C5a can interact with another receptor (C5L2; C5aR2), but PMX53 does not alter C5a action at this second C5a receptor (Klos et al., 2013). Both the C5aR and C5L2 mediate production of granulocyte colony stimulating factor (G-CSF) by C5a (Bosmann et al., 2013), and C5aR and C5L2 can form heteromers to regulate C5a function with heteromer formation being inhibited by C5aRA (Croker et al., 2013). Our studies do not provide information regarding the importance of C5L2 or the cell or tissue location of the important C5aR for mediating hypertension. Further studies are needed to investigate these possibilities.
Both C3a and C5a have distinct acute hemodynamic effects in the anesthetized rat, with C3a increasing blood pressure transiently and C5a causing a more prolonged decrease in blood pressure (Proctor et al., 2009). In the guinea pig, we previously demonstrated that C5a increases blood pressure via thromboxane, and the acute increase in blood pressure is dependent on white blood cells and platelets (Fraser and Regal, 1990). Vascular reactivity to agonists can vary during pregnancy when compared to the non-pregnant state (Paller, 1987), but our current studies indicate that a pregnant rat reacts to C3a peptide with a similar acute pressor response as a non-pregnant rat (Proctor et al., 2006). C3aRA prevents the increase in blood pressure indicating that it is an effective C3aR antagonist in the pregnant and non-pregnant rat. The reduction in circulating C3a following administration of the C3aRA SB290157 suggests that it has other effects and may exert its inhibitory effect on placental ischemia-induced hypertension by non-C3aR actions such as interfering with generation of C3a or complement activation in general. SB290157 in vitro has demonstrated high efficacy and specificity for the C3a receptor in rat and human cells and does not disrupt C5a-mediated cellular responses in vitro (Ames et al., 2001). However, Proctor et al noted that SB290157 in vivo caused an acute transient increase in blood pressure and decrease in circulating neutrophils with iv injection suggesting it was also acting as a C3a receptor agonist (Proctor et al., 2004). They concluded that the favorable effect of SB290157 in intestinal ischemia/reperfusion was likely due to its agonist action on neutrophils rather than its antagonism of C3aR (Wu et al., 2013). Mathieu et al (Mathieu et al., 2005) also described 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 study, the most perplexing limitation of C3aRA was the fact that at 5 mg/kg it inhibited the RUPP-induced increase in C3a itself and an explanation for this is not readily
apparent. These data temper conclusions regarding the importance of C3a in our studies and suggest that the C3aRA is affecting hypertension by both C3a receptor and non-receptor pathways. In addition, our studies temper a conclusive role for C3aR in hypertension and proteinuria in pregnancy following adoptive transfer of AT1-AA into third trimester mice (Wang et al., 2012) where doses up to 30 mg/kg/day SB290157 were used to provide evidence for an important role of C3a. In our studies, the C3aRA SB290157 definitely inhibits increases in blood pressure following either C3a administration or placental ischemia suggesting that C3a is an important mediator of placental ischemia-induced hypertension. However, non-C3aR effects of SB290157 may also be operating \textit{in vivo} to reduce placental ischemia-induced hypertension.

In a recently published case report by Burwick et al (Burwick and Feinberg, 2013), eculizumab, an antibody to C5, favorably extended pregnancy in a woman with severe preeclampsia. Whether eculizumab was effective in prolonging pregnancy because of its ability to limit C5a or C5b-9 formation or both is unknown. However, our studies in the rat model suggest that eculizumab may attenuate the severity of preeclampsia in part through its ability to limit formation of C5a and further studies are underway to evaluate this possibility. Our studies to date have also not addressed how complement is activated following placental ischemia or which pathway(s) of complement activation is leading to the increase in circulating C3a. In a pregnant mouse model, studies of Wang et al (Wang et al., 2012) indicate that infusion of autoantibodies to the angiotensin II Type 1 receptor lead to complement activation and hypertension so a role for autoantibodies in RUPP-induced complement activation is possible. In addition, future immunohistochemistry studies are warranted to determine the site of complement activation by determining if C3 deposition is occurring in the vasculature and/or placenta and is associated with the increased blood pressure seen following placental ischemia.
Overall, our studies suggest an important role for both C3aR and C5aR in mediating placental ischemia-induced hypertension. Differential effects of the C3a and C5a receptor antagonists on endothelial cell dependent vascular relaxation, cardiovascular function and circulating neutrophil count suggest that C5a contributes to hypertension by increasing heart rate and impairing endothelial-dependent relaxation of blood vessels, whereas C3aRA likely impacts placental ischemia-induced hypertension by both C3aR and non-C3aR pathways. Previous studies of others have also demonstrated an important role for angiotensin II Type 1 receptor agonistic autoantibodies, lymphocytes, endothelin, IL10, IL6, TNF, and angiogenic imbalance in placental ischemia-induced hypertension, with partial attenuation of the hypertension as each of these pathways are manipulated. The interrelationship of complement activation with these mediator pathways is unexplored as is the mechanism of complement activation following placental ischemia. Clearly a complex array of mediators and pathways including the complement activation products C3a and C5a are involved and data suggest that therapeutic strategies for management of placental ischemia-induced hypertension will require a broad anti-inflammatory approach including therapy targeted at limiting the production or activity of complement activation products.
Acknowledgments

We gratefully acknowledge the excellent technical assistance of Megan Strehlke and Courtney Bauer for completion of these studies.
Authorship Contributions

Participated in research design: Lillegard, R. Regal, Gilbert, J. Regal

Conducted experiments: Lillegard, Loeks-Johnson, Opacich, Peterson, Bauer, Elmquist, Gilbert, J. Regal

Performed data analysis: R. Regal, J. Regal

Wrote or contributed to the writing of the manuscript: Lillegard, Loeks-Johnson, Opacich, Peterson, Bauer, Elmquist, R. Regal, Gilbert, J. Regal
References


Mason JC, Steinberg R, Lidington EA, Kinderlerer AR, Ohba M and Haskard DO (2004) Decay-accelerating factor induction on vascular endothelium by vascular endothelial growth factor (VEGF) is mediated via a VEGF receptor-2 (VEGF-R2)- and protein kinase C-


Footnotes

This work was supported by National Institute of Health National Heart Lung and Blood Institute (HL109843 to JFR and JSG, HL114096 to JSG).


Address all correspondence to: Jean F. Regal, Ph.D., Department of Biomedical Sciences, University of Minnesota Medical School Duluth, 1035 University Dr., Duluth, MN 55812. Email Jean F. Regal: jregal@d.umn.edu
Figure Legends

Figure 1. Major pathways of complement activation leading to generation of anaphylatoxins C3a and C5a.

Figure 2. C3a and C5a receptor antagonists differentially attenuate placental ischemia-induced hypertension and heart rate. Sham or RUPP animals were treated with vehicle (Veh), C3a receptor antagonist (C3aRA) or C5a receptor antagonist (C5aRA) from GD14-18. Values represent mean ± SE of MAP or heart rate measured on GD19. *p<0.05 for indicated comparisons. A. Increase in mean arterial pressure (MAP) in RUPP Veh (n=23) was significantly inhibited by C3aRA (n=12) or C5aRA (n=11). MAP did not differ between Sham animals treated with Veh (n=19), C3aRA (n=6) or C5aRA (n=5). B. Increased heart rate in RUPP Veh (n=23) vs Sham Veh (n=19) was significantly inhibited by C5aRA (n=11; p<0.05) but not C3aRA (n=12; p=0.11). Heart rate did not differ between Sham animals treated with Veh, C3aRA (n=6) or C5aRA (n=5).

Figure 3. C3a and C5a receptor antagonists do not reverse decreased free plasma VEGF or average fetal weight following placental ischemia. Sham or RUPP animals were treated with vehicle (Veh), C3a receptor antagonist (C3aRA) or C5a receptor antagonist (C5aRA) from GD14-18. A. Decrease in VEGF in RUPP (n=23) was not affected by C3aRA (n=12) or C5aRA (n=11). Free plasma VEGF at GD19 did not differ between Sham animals (n=19) treated with Veh (n=19), C3aRA (n=6) or C5aRA (n=5). Values represent geometric mean ± SE of VEGF measured GD19. *p<0.05 for indicated comparisons. B. The decrease in fetal weight in RUPP Veh (n=22) was not affected by C3aRA (n=11) or C5aRA (n=10). Fetal weight did not differ
between Sham animals treated with Veh (n=19), C3aRA (n=6) or C5aRA (n=5). *p<0.05 for indicated comparisons.

Figure 4. C3a receptor antagonist (C3aRA) but not C5a receptor antagonist (C5aRA) attenuates increased serum C3a following placental ischemia. Sham or RUPP animals were treated with vehicle (Veh), C3aRA or C5aRA from GD14-18. The increase in C3a in RUPP Veh (n=23) was significantly inhibited by C3aRA (n=12) but not C5aRA (n=11). Compared to Sham Veh animals (n=19), the C3a concentration was significantly decreased by C3aRA (n=6) but not C5aRA (n=5). Values represent mean ± SE of serum C3a measured on GD19. *p<0.05 for indicated comparisons.

Figure 5. C3a receptor antagonist (C3aRA) prevents increased MAP induced by C3a peptide in pregnant (GD19) and non-pregnant females. C3a peptide increased MAP in GD19 animals or non-pregnant females. C3a receptor antagonist (C3aRA) did not increase MAP but inhibited the C3a peptide-induced increase in MAP. Values represent mean ± SE of % increase in MAP in 4-8 animals per treatment group; *p<0.05 vs C3a peptide injection alone.

Figure 6. C5a receptor antagonist (C5aRA) but not C3a receptor antagonist (C3aRA) attenuates numbers of circulating neutrophils. Sham or RUPP animals were treated with vehicle (Veh), C3aRA or C5aRA from GD14-18. A significant decrease in circulating neutrophils occurred with C5aRA treatment. Values represent mean ± SE of circulating neutrophils measured on GD19. **p<0.05 for C5aRA effect by ANOVA.
Figure 7. C5a receptor antagonist (C5aRA) but not C3a receptor antagonist (C3aRA) prevents placental ischemia-induced endothelial dysfunction. Sham or RUPP animals were treated with vehicle (Veh), C3aRA or C5aRA from GD14-18. Mesenteric arteries were isolated on GD19, pre-contracted with the thromboxane mimetic U46619 and relaxation to acetylcholine (Ach; endothelial-dependent vasodilation) assessed. Relaxation to Ach was reduced in RUPP Veh (n=14) compared to Sham Veh (n=10). C5aRA (n=9), but not C3aRA (n=6) prevented the RUPP-induced change in Ach vasodilation. Values represent mean ± SE of fractional relaxation in pre-contracted arteries. *p<0.05 comparing RUPP to Sham. #p<0.05 comparing RUPP Veh to RUPP C3aRA or RUPP C5aRA. No difference was detected comparing Sham Veh to Sham C5aRA.
Figure 1

Classical Pathway

Mannose-binding Lectin Pathway

Alternative Pathway

C1 → C4 → C4b
C2 → C2a

C3 convertase

C3b

C3 deposition

C3a

C3a Receptor

C5 convertase

C5b
C6
C7
C8
C9

C5b-9

Membrane Attack Complex ➔ Lysis

C5a

C5a Receptor
Figure 2

A

Mean arterial pressure (mm Hg)

B

Heart Rate (bpm)
Figure 4
Figure 5

The figure shows a bar graph comparing the mean arterial pressure (% increase) in pregnant and non-pregnant states with the presence (+) or absence (-) of C3a peptide and C3sRA. The graph indicates that C3a peptide and C3sRA have different effects on mean arterial pressure in pregnant versus non-pregnant states, with significant differences marked by asterisks (*) and double asterisks (**) indicating statistical significance.
Figure 6

![Bar chart showing neutrophil counts for different groups: Veh Sham, Veh RUPP, C3aRA RUPP, C5aRA RUPP, C3aRA Sham, C5aRA Sham. The y-axis represents Neutrophils (x 10^7) ranging from 0 to 0.8. The x-axis lists the different groups. Bars are accompanied by error bars, and some bars have asterisks indicating statistical significance.](chart_url)
Figure 7

Endothelial-dependent vasodilation

Fractional Relaxation

Log Ach x 10^{-6}