

Interactions between the complement and endothelin systems in normal pregnancy and following placental ischemia

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ABSTRACT

Preeclampsia is characterized by new onset hypertension and fetal growth restriction and is associated with aberrant activation of the innate immune complement system and stressed or ischemic placenta. Previous studies have suggested a role for both endothelin and complement system activation products in new onset hypertension in pregnancy, but inter-relationships of the pathways are unclear. We hypothesized that complement activation following placental ischemia stimulates the endothelin pathway to cause hypertension and impair fetal growth. The Reduced Uterine Perfusion Pressure (RUPP) model results in hypertension and fetal growth restriction in a pregnant rat due to placental ischemia caused by mechanical obstruction of blood flow to uterus and placenta. The effect of inhibitor of complement activation soluble Complement Receptor 1 (sCR1) and endothelin A receptor (ET_A) antagonist atrasentan on hypertension, fetal weight, complement activation (systemic circulating C3a and local C3 placental deposition) and endothelin [circulating endothelin and message for preproendothelin (PPE), ET_A and endothelin B receptor (ET_B) in placenta] in the RUPP rat model were determined. Following placental ischemia, sCR1 attenuated hypertension but increased message for PPE and ET_A in placenta, suggesting complement activation causes hypertension via an endothelin independent pathway. With ET_A antagonism the placental ischemia-induced increase in circulating C3a was unaffected despite inhibition of hypertension, indicating systemic C3a alone is not sufficient. In normal pregnancy, inhibiting complement activation increased plasma endothelin but not placental PPE message. Atrasentan treatment increased fetal weight, circulating endothelin and placental ET_A message, and unexpectedly increased local complement activation in placenta (C3 deposition) but not C3a in circulation, suggesting endothelin controls local placental complement activation in normal pregnancy. Atrasentan also significantly decreased message for endogenous complement regulators Crry and CD55 in placenta and kidney in normal pregnancy. Results of our study indicate that complement/endothelin interactions differ in pregnancies complicated with placental ischemia vs normal pregnancy, as well as locally vs systemically. These data clearly illustrate the complex interplay between complement and endothelin indicating that perturbations of either pathway may affect pregnancy outcomes.

1. Introduction

Control of the immune system is very important in maintaining a normal pregnancy. Consequently, dysregulation of the immune system can play a role in the pathogenesis of adverse pregnancy outcomes including pregnancy-induced hypertension and fetal growth restriction. Both adaptive and innate immune mechanisms have been implicated in

abnormal spiral artery remodeling in the developing placenta, resulting in placental ischemia, hypertension and intrauterine growth restriction. Our studies focus on events following placental ischemia, where exacerbated immune activation has a demonstrated role in the pathophysiology. In particular, our studies are designed to define inter-relationships of the innate immune complement system and the endothelin system in normal pregnancy in the rat, as well as in the

Abbreviations: ET-1, endothelin-1; ET_A, Endothelin A receptor; ET_B, Endothelin B receptor; GD, gestation day; PPE, preproendothelin; RUPP, reduced uterine perfusion pressure; sCR1, soluble complement receptor 1; VEGF, vascular endothelial growth factor

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stressed placenta following third trimester placental ischemia using the Reduced Uterine Perfusion Pressure (RUPP) model to cause gestational hypertension and fetal growth restriction.

The complement system is composed of more than 30 different proteins operating extracellularly and intracellularly to protect from invaders, both in concert with the adaptive immune response as well as independent of cellular and humoral immunity (Kolev et al., 2014). The complement system is essential for a normal pregnancy and for protection of both mother and child from infection. The innate immune complement system is activated in a normal pregnancy (Regal et al., 2017; Derzsy et al., 2010), and numerous studies have demonstrated more pronounced activation in preeclamptic pregnancies (Regal et al., 2015a), with excessive activation of the complement system having deleterious effects. In fact, a case study provided evidence that treatment of a preeclamptic pregnancy with antibody to complement component C5 prolonged the pregnancy by more than 2 weeks, indicating the clinical relevance of excessive complement activation in the pathology of preeclampsia (Burwick and Feinberg, 2013).

Chronic placental ischemia in the RUPP model in the rat results in increased blood pressure in the mother, fetal growth restriction and excessive complement activation as indicated by generation of C3a (Lillegard et al., 2013, 2014) with increased C3 deposition in placenta (Regal et al., 2016). Inhibiting complement activation with soluble Complement Receptor 1 (sCR1), as well as antagonism of C3a or C5a receptors, attenuates placental ischemia-induced hypertension in the RUPP rat model (Lillegard et al., 2013, 2014) but does not reverse fetal growth restriction. In addition to complement system activation, placental ischemia in the RUPP model results in activation of endothelin 1 (ET-1) pathway (LaMarca et al., 2013) with clinical evidence to indicate that endothelin is increased in preeclampsia (Lu et al., 2017). ET-1 is produced by the endothelium abluminally and acts on the endothelin A receptor (ET_A) in smooth muscle to cause vasoconstriction (Davenport et al., 2016). It can also act via the endothelin B receptor (ET_B) to cause vasodilation and/or to clear endothelin, minimizing entry into the circulation. Evidence suggests that ET_B on smooth muscle plays a minor role in blood pressure regulation (Miller et al., 2017) with the most significant contribution from ET_A. In a rat model of placental ischemia-induced hypertension, antagonism of ET_A with the selective ET_A antagonist, atrasentan, prevents the increase in blood pressure (Alexander et al., 2001; LaMarca et al., 2008a), but does not reverse the intrauterine growth restriction. An ET-1 dependent mechanism is also responsible for hypertension in the pregnant rat induced by TNF (LaMarca et al., 2008b), sFlt-1 (Murphy et al., 2010) and autoantibodies to the angiotensin receptor (LaMarca et al., 2009), providing supportive evidence that ET-1 is a central downstream mediator of placental ischemia-induced hypertension (George and Granger, 2012). However, not all mediators of placental ischemia-induced hypertension are ET-1 dependent as data indicates that IL-17 causes hypertension in pregnancy but acts independently of ET-1 (Cornelius et al., 2013). Whether complement activation leads to placental ischemia-induced hypertension by an endothelin dependent mechanism is unknown. Though complement activation has been linked with endothelin activation in a number of other experimental model systems (Gombos et al., 2012; Howell et al., 2011, 2014; Kajdacs et al., 2016; Soop et al., 2004), connections of the two pathways have not been investigated following placental ischemia.

Multiple interventions in the RUPP model result in attenuation of the hypertension, but without favorable effects on fetal growth. In our previous studies using sCR1 to inhibit complement activation, we noted that sCR1 treatment exacerbated the placental ischemia-induced fetal growth restriction, suggesting a role for complement activation in maintaining normal fetal growth following placental ischemia. In addition, evidence indicates that endothelin acting through the ET_A receptor results in fetal growth restriction following hypoxia in the pregnant rat (Thaete et al., 1997; Zhou et al., 2013). In the present study, we hypothesized that complement activation following placental

ischemia results in activation of endothelin pathway to cause hypertension and impact fetal growth. To test this hypothesis, we determined if an inhibitor of complement activation (soluble complement receptor 1; sCR1) that attenuates placental ischemia-induced hypertension would affect changes in the ET-1 system following placental ischemia, or whether ET_A antagonist atrasentan altered placental ischemia-induced complement activation systemically or locally. Our results reveal important interactions between the endothelin and complement systems that may impact fetal growth and pregnancy outcomes, with different interactions identified following placental ischemia compared to normal pregnancy, either locally or systemically.

2. Materials and methods

2.1. Reduced uterine perfusion pressure (RUPP) procedure

The RUPP procedure was used as a model of placental ischemia-induced hypertension in the third trimester pregnant rat as previously described (Lillegard et al., 2013, 2014; Regal et al., 2016, 2015b). All studies in animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and were approved by the University of Minnesota Institutional Animal Care and Use Committee. Timed pregnant Sprague Dawley dams (CrI:CD IGS, Charles River Laboratories, Raleigh NC) were anesthetized with isoflurane on gestation day (GD)14 with the date of vaginal plug designated as 0. A ventral midline incision was made, the lower abdominal aorta isolated, and a sterile silver clip (0.203 mm inner diameter) placed around the aorta above the iliac bifurcation. Both right and left uterine arcades were also clipped at the ovarian end, directly before the first segmental artery, using a silver clip (0.100 mm inner diameter) to prevent compensatory blood flow to the placenta. For the comparison group, a Sham surgery differing only in absence of the clips was also conducted. On GD18, the carotid artery was cannulated under isoflurane anesthesia using a 25% dextrose lock solution in sterile pyrogen free saline to maintain cannula patency. On GD19, mean arterial pressure (MAP) was measured from the arterial catheter in an unanesthetized restrained rat. Following measurement of blood pressure, the serum, plasma and tissues were collected in necropsy. The uterus was exteriorized, the total number of viable and resorbed pups counted and the pups and placentae weighed. From the right horn, select placenta in atrasentan experiments were frozen in OCT for immunohistochemistry and measurement of C3 deposition. From the left horn, select placenta were flash frozen in liquid nitrogen for isolation of RNA for qRT PCR.

2.2. Experimental design and treatments

sCR1. We previously reported that daily intravenous treatment with 15 mg/kg sCR1 from GD14 to 18 significantly attenuated placental ischemia-induced hypertension (Lillegard et al., 2013). sCR1 (Celldex Therapeutics, Inc., Needham, MA) is a soluble form of the endogenous complement regulator CR1 (CD35) with demonstrated ability to inhibit complement activation at the C3 and C5 convertases of the complement pathways, and demonstrated efficacy in numerous rat models of autoimmune and inflammatory diseases. Flash frozen placenta and plasma archived from those published studies were used to determine if sCR1 treatment significantly altered message for preproendothelin (PPE), ET_A or ET_B or plasma endothelin. Control injections in Sham and RUPP animals consisted of daily intravenous treatment with saline (Veh).

Atrasentan. Atrasentan hydrochloride was purchased from ChemScene (CAS No 195733-43-8; Monmouth Junction, NJ) and was 99.64% pure by LCMS. Rats were provided with either 50 or 75 ug/ml atrasentan in drinking water beginning GD13 until necropsy. Total water consumption was measured over the 6 day period and body weight monitored daily. Rats were randomly assigned to one of six experimental groups based on surgical procedure and water treatment:

1) RUPP surgery with water ad lib (RUPP Water, n = 8); 2) RUPP surgery with 50 ug/ml atrasentan in water (RUPP 50 ug/ml atrasentan, n = 6); 3) RUPP surgery with 75 ug/ml atrasentan in water (RUPP 75 ug/ml atrasentan, n = 6); 4) sham surgery with water ad lib (Sham Water, n = 10); 5) sham surgery with 50 ug/ml atrasentan in water (Sham 50 ug/ml atrasentan, n = 7); 5) sham surgery with 75 ug/ml atrasentan in water (Sham 75 ug/ml atrasentan, n = 5).

2.3. Complement system measurements

2.3.1. C3a

The circulating complement activation product C3a was measured in serum by Western blot as previously described (Lillegard et al., 2013) using the IgG fraction of a rabbit polyclonal antibody to the 9 carboxy terminal amino acids of rat C3a (Research Genetics, Huntsville, AL) as primary antibody. Modifications included use of NuPAGE Novex 10% Bis-Tris gels with MES SDS Running buffer and the secondary antibody IRDye 800CW Goat anti-Rabbit IgG (H + L) at 1/10,000 dilution and LiCor Odyssey Fc for imaging. A standard curve of rat serum activated by yeast was included on each gel. Relative amounts of C3a were expressed as C3a units/ul based on signal intensity of 1 ul of standard pool of rat serum activated by yeast.

2.3.2. CH50

Total hemolytic complement activity was determined as previously described (Larsen et al., 2001). Briefly, the assay involves serial dilution of serum and incubation with antibody-coated sheep red blood cells for one hour at 37 C. The antibody coated cells are lysed by complement, releasing hemoglobin. The inverse dilution of serum that results in 50% hemolysis of sensitized sheep erythrocytes (CH50) was determined in the presence or absence of atrasentan. CH50 is a general measure of the overall function of the classical pathway of complement activation.

2.3.3. Immunohistochemistry for C3 and IgM

Placenta were frozen in OCT freezing medium and 8 um sections cut and placed on slides for immunohistochemistry as previously described (Regal et al., 2016) using polyclonal goat anti-rat C3 (MP Biomedical 55,713; Santa Ana, CA) and appropriate isotype control antibody followed by appropriate secondary antibodies. This polyclonal antibody will detect C3 and its fragments but cannot distinguish between C3b or C3c/d to provide an indication of the acute vs chronic nature of the complement activation. The images were scored by two blinded observers in comparison to the isotype control as negative (0), weakly positive (1), positive (2) or strongly positive (3).

2.4. qRT PCR

RNA isolation and cDNA synthesis from flash frozen tissues was as previously described (Regal et al., 2016). Primers for assessment of rat Vascular endothelial growth factor A (VEGF) were obtained from Bio-Rad (Prime PCR SYBR Green Assay; Vegfa, Rat) and reaction efficiency confirmed. For all other primers, expected product size was confirmed by gel electrophoresis, and qRT PCR products were sequenced to verify that the product was of the expected size and sequence for each primer set (University of Minnesota Genomics Center). Equal primer efficiencies for β -actin and each of the targets were validated. Real time PCR reactions for all primers were performed in duplicate and the Delta-delta Ct method of relative quantification was used to determine fold change in mRNA expression compared to β -actin with the change in Sham animals with plain drinking water defined as 1. Primers for rat ET_A and ET_B receptors were as published (Mazzuca et al., 2014). For other targets, custom primers were obtained from Integrated DNA technologies (Coralville, Iowa). For rat complement regulators Crry, CD55 and rat β -actin we used previously published and validated primers (Regal et al., 2016). Primers for preproendothelin (PPE) were; forward 5-GAACTCCGAGCCCAAAGTACCATG-3, reverse 5-TTAGTTTT

CTTCCCTCCACCAGCTG-3. In addition, selected experiments used previously published primers for PPE (Santiago-Font et al., 2019) to confirm that qRT PCR results with either primer set were equivalent.

2.5. Endothelin measurements

Endothelin-1 was quantitated in EDTA plasma using R&D Endothelin Quantikine ELISA kit (DET100, Minneapolis, MN).

2.6. Effect of atrasentan on cells in culture

IEC-6 (Intestinal epithelial cells) cells were obtained from ATCC and cultured as described by ATCC and previously published (Quaroni et al., 1979). RBE4 cells were a kind gift from Dr. Les Drewes and were cultured as originally described by Roux et al (Roux and Couraud, 2005). Atrasentan at 0, 10, 20 and 40 ug/ml was added to the culture media and cells cultured in duplicate for 24 h. Cells were harvested and RNA isolated for qRT PCR as for tissues. Values presented represent the mean \pm standard error for 4–5 separate experiments (each with duplicates) and are expressed as the change from control media (0 atrasentan), paired for the same cell date. With the analysis paired for cell date, all values in media alone are defined as 1.

2.7. Statistical analysis

Data were expressed as mean \pm standard error of the mean. Differences were considered significant when $p < 0.05$; p values 0.05–0.1 were considered trends. Two way ANOVA was conducted to determine if either the surgery (RUPP vs Sham) or the treatment (atrasentan vs water; sCR1 vs Vehicle) had a significant effect. In addition, post hoc individual contrasts using JMP and SAS software (SAS Institute, Cary, NC) were considered. For atrasentan treatment, the following comparisons were considered: RUPP water vs Sham water; RUPP water vs RUPP atrasentan 50; RUPP water vs RUPP atrasentan 75; Sham water vs Sham atrasentan 50; Sham water vs Sham atrasentan 75. For sCR1 or saline (Veh) treatment, the following comparisons were considered: Sham Veh vs RUPP Veh; Sham Veh vs Sham sCR1; RUPP Veh vs RUPP sCR1; Sham sCR1 vs RUPP sCR1.

3. Results

3.1. Effect of inhibiting complement activation on placental ischemia-induced events

Our previous studies in the RUPP model demonstrated that inhibiting complement activation with sCR1 significantly attenuated placental ischemia-induced hypertension (Lillegard et al., 2013) and did not reverse RUPP-induced fetal growth restriction. In our previous study, a comparison of Sham Veh to RUPP Veh demonstrated a statistically significant increase in both mean arterial pressure and C3a. A more comprehensive analysis of the fetal and placental weights from that study are shown in Fig. 1A. A significant difference between Sham Veh and RUPP Veh was not detected with an individual contrast for fetal and placental weight. However, as expected in the model, ANOVA indicated a significant main effect with a decrease in fetal and placental weights with RUPP surgery (** $p < 0.05$). Strikingly, post hoc comparisons showed both fetal and placental weight were significantly lower in RUPP animals treated with sCR1 compared to vehicle, suggesting a role for complement activation differs in normal vs ischemic pregnancy. To determine if inhibiting complement activation altered indicators of the endothelin pathway activation (PPE, ET_A and ET_B receptor message; plasma endothelin), archived plasma and placenta from Sham and RUPP animals treated with 15 mg/kg sCR1 or Veh were evaluated (Lillegard et al., 2013). Synthesis of the potent 21 amino acid peptide endothelin in endothelial cells is a 3 step process starting with translation of preproendothelin (PPE) mRNA into preproendothelin

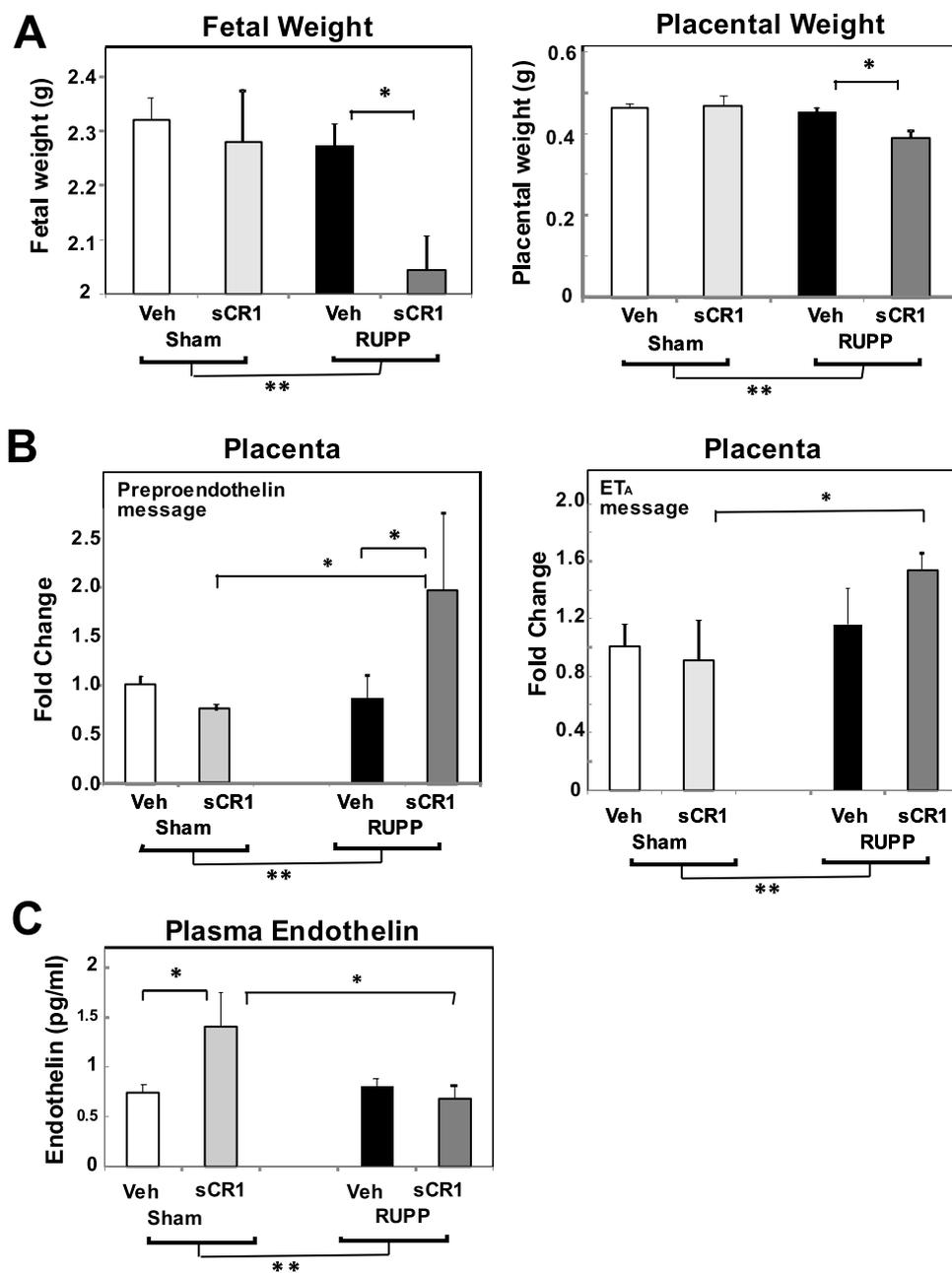


Fig. 1. Effect of inhibitor of complement activation, sCR1, on average fetal and placental weight and the endothelin system. Animals were treated daily with 15 mg/kg sCR1 or saline (Veh) iv from GD14-18 and plasma endothelin measured by ELISA in plasma collected from the abdominal aorta at GD19. mRNA was isolated from GD19 placenta and the Delta delta Ct method of relative quantification was used to determine fold change in mRNA expression compared to β actin with change in Sham Veh defined as 1. ** $p < 0.05$ for main surgery effect by ANOVA, * $p < 0.05$ for indicated post hoc comparisons. Values represent the mean \pm SE in Veh ($n = 10-20$) or sCR1 treated animals ($n = 5-11$). A. RUPP surgery significantly decreased fetal and placental weight and sCR1 exacerbated the effect following placental ischemia. B. sCR1 treatment increased PPE and ET_A message following placental ischemia. C. Post hoc comparisons demonstrated a significant increase in plasma endothelin in Sham animals treated with sCR1.

followed by sequential cleavage of PPE by furin and endothelin converting enzymes into Big ET-1 and ET-1, respectively. ET-1 released abluinally from the endothelial cell acts on the local ET_A receptor on smooth muscle to cause vasoconstriction. Previous studies by others have demonstrated marked increase in PPE message in kidney and placenta with RUPP-induced increases in blood pressure in the rat (Alexander et al., 2001; Mazzuca et al., 2014; George et al., 2011; Santiago-Font et al., 2016), no increase in circulating ET-1, with a decrease in ET_B but not ET_A reported in aorta (Mazzuca et al., 2014). Evaluating message for placental preproendothelin, no change was evident in normal pregnant Sham animals treated with sCR1, nor comparing Sham Veh to RUPP Veh animals (Fig. 1B). However, following placental ischemia, sCR1 treatment surprisingly increased message for placental PPE and ET_A (Fig. 1B) with no changes noted in ET_B (data not shown). This increased PPE and ET_A message in placenta paralleled the decreases in fetal and placental weight seen in RUPP animals treated with sCR1 (Fig. 1A). Given these changes, we also evaluated circulating endothelin (Fig. 1C) with increased circulating

endothelin noted in Sham animals treated with sCR1. These data suggest that following placental ischemia, complement activation is important in the hypertension (Lillegard et al., 2013) as well as in favoring normal placental and fetal growth and counteracting placental ischemia-induced fetal growth restriction.

3.2. Effect of inhibiting ET_A receptor on placental ischemia-induced events

Pregnant dams were treated with 2 different concentrations of atrasentan in drinking water, 50 or 75 μ g/ml. The delivered dose of atrasentan was estimated by measuring total water consumption over the 6 days (GD13 to 19; average ml of water or mg atrasentan consumed/day) and daily monitoring of body weight. The highest and lowest body weight recorded over the six day period were used to estimate the range of delivered dose of atrasentan to each animal (mg atrasentan consumed/highest body weight in kg/day to mg atrasentan consumed/lowest body weight in kg/day). Because of RUPP surgery and fetal resorptions, RUPP animals weigh less and also consume

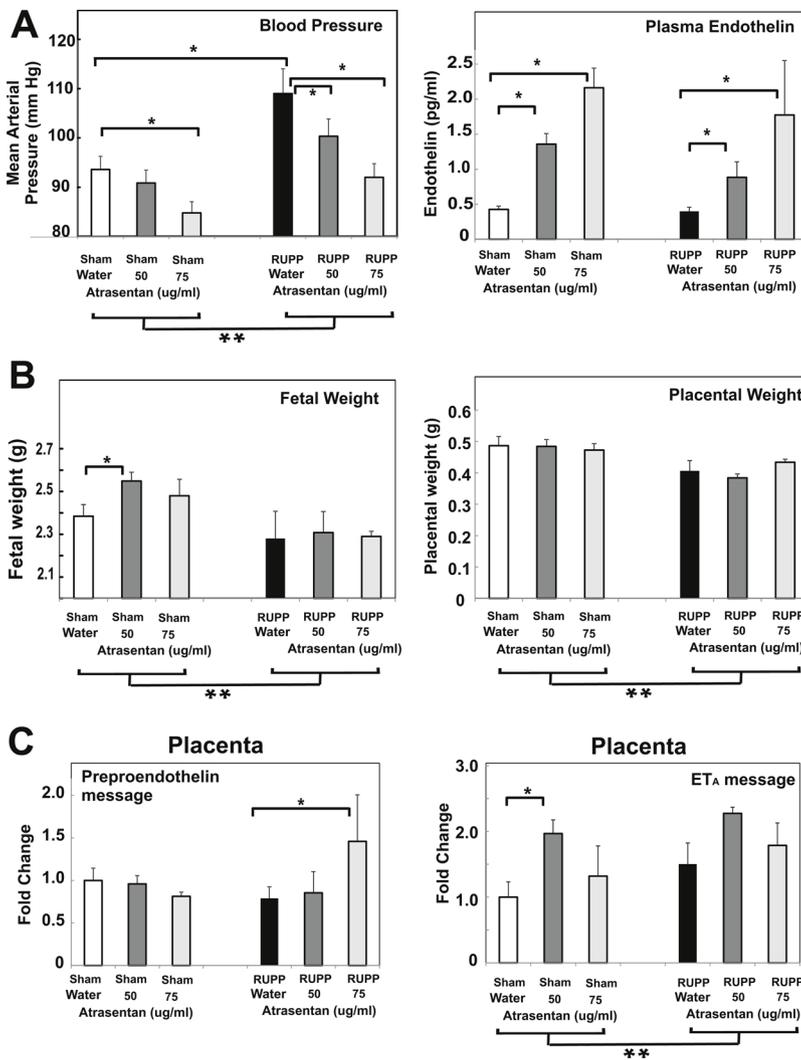


Fig. 2. ET_A antagonist atrasentan inhibits placental ischemia-induced increase in mean arterial pressure, increases circulating endothelin and increases ET_A message. Animals received drinking water ad lib either with or without 50 or 75 ug/ml atrasentan from GD13-19 and mean arterial pressure and average fetal and placental weight determined on GD19. Plasma endothelin was measured by ELISA in plasma collected from the abdominal aorta at GD19. mRNA was isolated from GD19 placenta and the Delta delta Ct method of relative quantification was used to determine fold change in mRNA expression compared to β actin with change in Sham Water defined as 1. ***p* < 0.05 for main surgery effect by ANOVA, **p* < 0.05 for indicated post hoc comparisons. Values represent the mean \pm SE of mean arterial pressure or average fetal weight measured GD19 in water (*n* = 7–10) or atrasentan treated animals (*n* = 5–7). A. The increase in mean arterial pressure in RUPP animals was decreased by treatment with atrasentan. Atrasentan also significantly decreased the mean arterial pressure in Sham animals. B. RUPP surgery significantly decreased average fetal and placental weight as determined by ANOVA analysis. Post hoc comparison indicated increased fetal weight in Sham animals. C. By ANOVA RUPP surgery did not significantly increase PPE message in placenta. Post hoc comparisons indicated that PPE message was significantly increased in RUPP animals treated with atrasentan. For ET_A, ANOVA analysis demonstrated a significant surgery and treatment effect, with increased ET_A in Sham animals detected post hoc.

slightly less water than Sham animals (152.0 ± 6.4 vs 125.2 ± 6.6 ml water/kg/day; *p* < 0.05). The amount of water consumed did not differ when adding atrasentan to the water. Animals receiving 50 ug/ml atrasentan in drinking water received 5.4 mg/kg/day at a minimum (estimated range 5.4–5.7 mg/kg/day). Animals drinking 75 ug/ml atrasentan received 7 mg/kg/day atrasentan at a minimum (estimated range 7.0–9.2 mg/kg/day). Previous studies of others reported a dose of 5 mg/kg/day delivered in drinking water attenuated placental ischemia-induced hypertension in the rat RUPP model (Alexander et al., 2001).

As seen in Fig. 2A, RUPP surgery significantly increased mean arterial pressure (MAP) that was attenuated by the ET_A antagonist atrasentan as previously reported by others. Atrasentan significantly reduced MAP in Sham animals as well. Also consistent with previous reports, RUPP surgery did not increase circulating ET-1 concentrations in animals with no atrasentan in drinking water, and atrasentan treatment significantly increased circulating plasma endothelin (Oggenorth et al., 2000). Considering fetal and placental weight of the treatment groups (Fig. 2B), ANOVA demonstrated a significant RUPP surgery effect with a decrease in both fetal and placental weights. Atrasentan also significantly increased fetal weight in Sham animals, similarly to a previous report (Thaete et al., 2004).

As seen in Fig. 2C, no significant change in PPE message was detected in placenta comparing RUPP vs Sham animals with no atrasentan in the drinking water. Post hoc analysis demonstrated a significant increase in PPE message in placenta of RUPP animals treated with the

higher concentration of atrasentan. ET_A message significantly increased with RUPP surgery (***p* < 0.05 by ANOVA) and post hoc analysis revealed a significant atrasentan effect as well in the Sham. No changes in ET_B in placenta was noted (data not shown). In addition, PPE message in kidney cortex did not change with RUPP surgery (data not shown). We also used PPE primers published by Santiago-Font (Santiago-Font et al., 2019) and were unable to detect any changes in PPE message in placenta comparing RUPP to Sham animals, indicating that a lack of detectable change was not due to different primers.

3.3. Effect of ET_A antagonism on complement activation systemically and locally

Our previous studies demonstrated that placental ischemia increased complement activation product C3a in circulation, as well as local complement deposition of C3b in the placenta (C3 deposition) and IgM deposition (Regal et al., 2016). In addition, sCR1 significantly inhibited the increased C3a in the circulation coincident with attenuation of placental ischemia-induced hypertension. As seen in Fig. 3, placental ischemia increased circulating complement activation product C3a, with no significant change due to atrasentan treatment. Unexpectedly, atrasentan treatment of Sham animals resulted in increased C3 deposition in placenta (Fig. 3), a method that detects both C3 and C3b. This is the first demonstration of an effect of ET_A antagonism on local placental complement activation in normal pregnancy. This increased complement deposition in placenta in Sham animals treated with

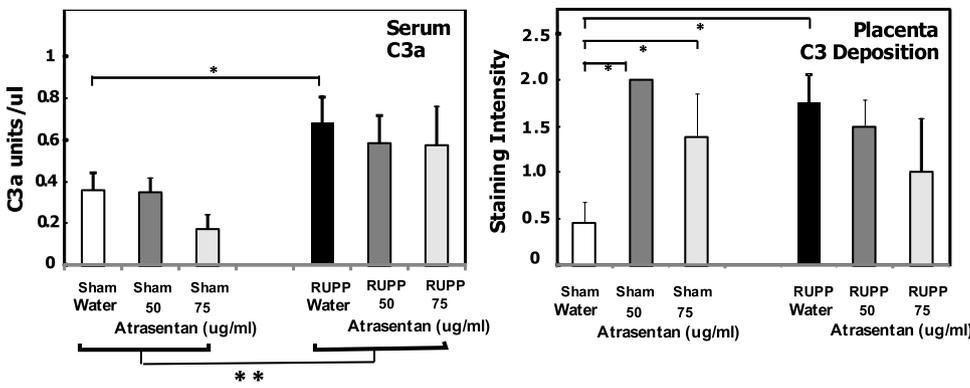


Fig. 3. Effect of the ET_A antagonist atrasentan on complement activation. Animals were treated with either 50 or 75 ug/ml atrasentan in drinking water from GD13-19 and placenta and serum obtained at GD19. Values represent the mean ± SE in water (n = 7–12) or atrasentan treated animals (n = 5–6). Serum C3a was determined by Western blot with units of C3a relative to a standard pool of yeast activated rat serum as described in Methods. C3 deposition in GD19 placenta was determined by immunohistochemistry with staining graded by two blinded observers from 0 to 3, negative to strongly positive vs isotype control. **p < 0.05 for main surgery effect. *p < 0.05 for indicated comparisons. The

RUPP-induced increase in C3a was not significantly altered by atrasentan. Atrasentan treatment significantly increased C3 deposition in Sham animals, and C3 deposition was significantly increased comparing Sham Water to RUPP Water animals.

atrasentan was not associated with adverse effects on fetal and placental growth in Sham animals (Fig. 2B).

Uncontrolled and excessive complement activation can result in depletion of complement components because activation exceeds new synthesis of complement proteins. Total hemolytic complement activity (CH50) of rat serum in the 6 treatment groups depicted in Fig. 3 did not differ (data not shown), indicating that complement components were not being depleted in RUPP vs Sham. Treatment of normal rat serum with atrasentan *in vitro* also did not affect total hemolytic complement activity (data not shown).

3.4. Effect of ET_A antagonism on complement regulators in normal pregnancy

In normal pregnant Sham animals, 50 ug/ml atrasentan had no effect on MAP or circulating C3a, yet still significantly increased circulating endothelin and C3 deposition in placenta (Fig. 2A and Fig. 3). Increased complement activation can be due to increased activation of the pathway(s) and/or a decrease in endogenous integral membrane complement regulators that protect our own cells. Membrane bound regulators Crry and CD55 control C3 activation in rodents. We considered that increased C3 deposition in the placenta of Sham normal

pregnant animals treated with atrasentan (Fig. 3) was due to a reduction in expression of complement regulators Crry or CD55. As seen in Fig. 4A, atrasentan treatment *in vivo* significantly decreased the message for Crry and CD55 in placenta and kidney, with different doses resulting in inhibition in the two tissues. These data suggest that in normal pregnancy, endothelin maintains adequate complement regulators to prevent excessive complement activation locally. When ET_A is blocked, complement regulators may decline and excessive complement deposition occurs.

To further assess whether atrasentan directly affects complement regulators at the cell level, we assessed the effect of atrasentan *in vitro* on cultured rat cells, using a rat intestinal epithelial cell line (IEC-6) and rat brain endothelial cell line (RBE4). As seen in Fig. 4B, incubation of IEC-6 cells with 10–40 ug/ml atrasentan for 24 h resulted in a significant increase in message for the regulators. In RBE4 cells, no significant changes in CD55 and Crry were noted (data not shown). Comparing message for regulators indicated that RBE4 cells had 2.6 times higher expression of CD55 and 1.8 times higher Crry than IEC cells.

An imbalance in angiogenic (vascular endothelial growth factor; VEGF) and anti-angiogenic (s-Flt-1) factors has been implicated in the pathophysiology of placental ischemia-induced hypertension. VEGF has

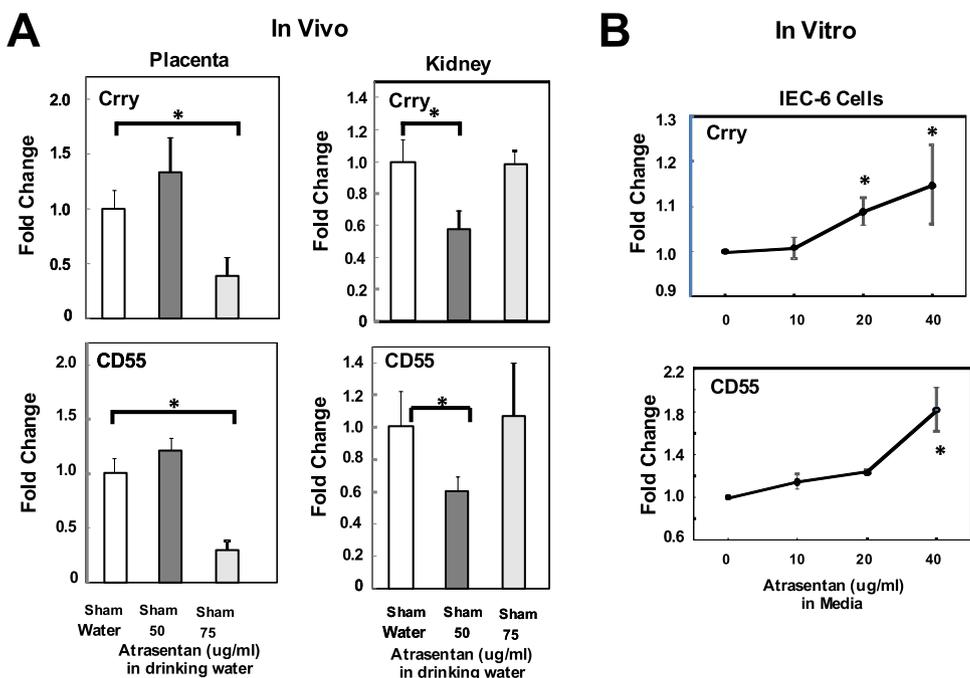


Fig. 4. Effect of the ET_A antagonist atrasentan on message for complement regulators. A. Animals were treated with either 50 or 75 ug/ml atrasentan in drinking water from GD13-19 and placenta and kidney cortex obtained at GD19. Values represent the mean ± SE in water (n = 7–12) or atrasentan treated animals (n = 5–6). Atrasentan treatment *in vivo* significantly decreased message for complement regulators Crry and CD55 in placenta and kidney cortex of Sham animals. B. IEC-6 cells were treated with either 0, 10, 20 or 40 ug/ml atrasentan in media for 24 h and the change in message for complement regulators Crry and CD55 determined in cells harvested after 24 h of treatment. mRNA was isolated from cells and the Delta-delta Ct method of relative quantification used to determine fold change in mRNA expression compared to β actin with change in media alone defined as 1. Values represent the mean ± SE of the change from media alone in 4–5 different experiments. *p < 0.05 for indicated comparisons.

also been demonstrated to regulate local inhibitory effect of the complement regulator Factor H in the eye and kidney (Keir et al., 2017). Podocytes produce VEGF that crosses the basement membrane and maintains the fenestrated epithelium. Disruption of VEGF in kidney decreases local Factor H and other complement regulators, leading to increased complement deposition in kidney. Thus, we hypothesized that the decrease in regulators in placenta following atrasentan treatment was associated with decreased message for VEGF in placenta. However, evaluation of VEGF message in placenta of Sham animals revealed no significant change with atrasentan treatment (data not shown). The concentration of VEGF in plasma and placental extracts could not be measured accurately since it was below the level of reliable detection by rat ELISA (R&D VEGF Quantikine kit for rat VEGF, RRV00, Minneapolis, MN), consistent with published limitations regarding measurement of rat VEGF (Weissgerber et al., 2014) by commercial ELISA. sFlt-1 was not measured in this study, but our previous study (Lillegard et al., 2013) demonstrated no increase in sFlt-1 with RUPP surgery with concentrations of sFlt-1 being measured near the level of reliable detection.

4. Discussion

The role of endothelin in preeclampsia has not been thoroughly tested, though evidence clearly indicates that endothelin is increased in plasma of women with preeclampsia compared to a normal pregnancy, particularly in women with severe preeclampsia and proteinuria (Lu et al., 2017). Using animal models, ET_A is of demonstrated importance in placental ischemia-induced hypertension in the rat RUPP model, as previously published (Alexander et al., 2001) and confirmed in the present study. However, direct therapeutic modulation of the endothelin pathway during pregnancy is contraindicated due to the potential of adverse effects on fetal development (Luscher and Barton, 2000). Thus, it is increasingly important to understand interactions of the endothelin pathway and other mediator pathways activated following placental ischemia. Increased activation of one such pathway, the complement system, is evident in preeclampsia compared to normal pregnancy (Regal et al., 2015a). Our previous studies in the RUPP model demonstrated that inhibiting complement activation attenuates placental ischemia-induced hypertension attesting to its importance in the mechanism leading to hypertension (Lillegard et al., 2013). We simplistically hypothesized that complement activation results in activation of the endothelin pathway with ET_A activation leading to placental ischemia-induced hypertension. If our hypothesis was correct, placental ischemia would cause increased complement activation in the presence of ET_A antagonism as noted. Placental ischemia would also result in a decrease in PPE message in the presence of an inhibitor of complement activation. However, PPE message increased followed sCR1 treatment, suggesting that hypertension induced by placental ischemia-induced complement activation was independent of the endothelin system. Thus, a complex interplay between endothelin and complement system activation is operating to result in placental ischemia-induced hypertension.

Results of our current study also demonstrate a previously unrecognized role for endothelin in normal pregnancy; endothelin via the ET_A receptor influences the level of local placental C3 deposition in normal pregnancy (Fig. 3). This endothelin influence in the presence of ET_A blockade in the placenta could be due to increased circulating endothelin acting through ET_B or due to a decrease in local placental complement regulators or both. Thus, we evaluated both ET_B and complement regulators following atrasentan treatment in the placenta. Clearly, placental complement regulators were decreased *in vivo* in the presence of ET_A antagonism which is consistent with increased placental C3 deposition. We extended these studies to cultured cells and found that intestinal epithelial cells up-regulated CD55 and Crry message with atrasentan treatment, but no change was detected in RBE-4 cells. The placenta is composed of numerous cell types including

trophoblasts, endothelial cells, and multiple immune cells, with evidence for endothelin receptors on each. Thus, effect of endothelin antagonism on complement regulators differs with the cell type and the tissue, so complex *in vivo* effects cannot be attributed solely to a direct effect on a single cell type.

In normal pregnancy, ET_A antagonism increased complement activation in the placenta, but not the circulation. If complement activation is important for the blood pressure increase following placental ischemia, why doesn't blood pressure increase with atrasentan treatment in the Sham animals? Our previously published studies suggest it is the circulating complement activation products C3a and/or C5a acting in the maternal vasculature to increase the blood pressure. The evidence for this lies in our previous studies where C3a and C5a receptor antagonists attenuated placental ischemia-induced hypertension and/or increased heart rate and C5a receptor antagonists reversed endothelial dysfunction in mesentery of rats following placental ischemia, suggesting that C3a/C5a generation and endothelial function is critical. Taken together, these data indicate that the site and extent of complement activation is important in determining whether complement activation is positively or negatively impacting pregnancy outcomes. In our study, placental C3 deposition following atrasentan treatment in normal pregnancy did not adversely affect blood pressure or fetal weight. However, in conditions of placental ischemia, with increases in circulating C3a and C3b deposition, fetal and placental weight are decreased with inhibition of complement activation exacerbating the reduced fetal weight (Fig. 1A).

In our study, sCR1 is used as a tool to assess the role of complement activation. sCR1 (TP10, CDX1135) was one of the first complement drugs to reach clinical trials but its development has been halted in lieu of more promising and smaller molecules targeted to endothelial cells in transplantation to inhibit complement activation (Ricklin et al., 2018). The effectiveness of sCR1 in inhibiting complement activation in the pregnant rat is evidenced by significant decrease of circulating C3a following placental ischemia, as well as a significant reduction in the CH50 (Lillegard et al., 2013) at doses that have been used in previous studies in the rat.

Limitations of this study primarily lie in the use of an animal model to mimic a pregnancy disorder manifested by a variety of symptoms with widely varying severity and timeline. The RUPP model is a model of hypertension in pregnancy with fetal growth restriction due to placental insufficiency induced mechanically. The RUPP model most closely mimics early-onset preeclampsia (symptoms present < 34 weeks of pregnancy) rather than the late-onset (> 34 weeks of pregnancy) type that is not usually associated with fetal growth restriction or placental ischemic injury (Stanek, 2019). Despite the many similarities between the RUPP model and early onset preeclampsia, it is clearly not a spontaneous model of preeclampsia. Preeclampsia is far more complex and varied than simply placental ischemia, with a variety of genetic and individual risk factors contributing to the preeclampsia pathology. Another limitation is placental structure. While the rat and human share a hemochorial placental structure, differences in trophoblast invasion and placental structure limit translation of findings in a rat placenta to the human condition. A comparison of rat and human placenta by Soares et al (Soares et al., 2012) indicate that both human and rat undergo similar spiral artery remodeling but the uterine and vascular structure clearly differ. Hence, our studies focus on the events that occur after placental ischemia. Moreover, any mechanistic findings in rat need to be grounded in findings in human preeclamptic placenta. Another limitation of this study is the limited evidence of the importance of endothelin in human preeclampsia. Our studies of endothelin in placenta do not localize changes in PPE message to placental regions where changes in turbulence and flow due to ischemia may significantly alter a role for endothelin in different placental regions.

Our studies clearly confirmed the effectiveness of an ET_A antagonist on placental ischemia induced hypertension, and atrasentan clearly increased circulating endothelin as expected from previous studies.

Previous studies have also seen increases in PPE message in the placenta, but not circulating endothelin following placental ischemia, presumably because endothelin is released abuminally from the endothelial cells and does not readily enter the circulation. However, we were unable to detect changes in PPE message in placenta or kidney that have been previously reported following placental ischemia (Alexander et al., 2001; Mazzuca et al., 2014; George et al., 2011; Santiago-Font et al., 2016; Harmon et al., 2015). Besides validating our PCR product, we also used published primers (Santiago-Font et al., 2016) and were still unable to detect any change in PPE message in RUPP compared to Sham animals. Our published studies have consistently used Sprague Dawley rats from Charles River comparing a Sham surgery to RUPP surgery, whereas the majority of studies published compare RUPP surgery on Harlan Sprague Dawley rats to a normal pregnant animal with no surgery. As the surgery itself alters the immune response, appropriate comparisons are critical, but Sprague Dawley rats also appear to differ based on the supplier and strain (CD vs Sasco). A significant body of literature has demonstrated mechanistic differences in cardiovascular responses in rats from these two different suppliers (Buhimschi et al., 2001; Griffin et al., 2012; Pollock and Rekito, 1998; Wehrwein et al., 2014), but neither has an obvious advantage for modeling pregnancy disorders.

Atrasentan is a highly selective ET_A antagonist with minimal ET_B antagonistic activity; a 28,000 fold higher affinity for ET_A than ET_B (Luo et al., 2016). In our study, consistent with results of others, plasma endothelin did not increase following placental ischemia in the rat RUPP model, and atrasentan itself significantly increased circulating endothelin in both RUPP and Sham animals. In experimental animals following treatment with endothelin antagonists, particularly ET_B antagonists (Opgenorth et al., 2000) but also ET_A antagonists (LaMarca et al., 2005), increased plasma endothelin has been reported. With ET_B antagonists, the increase of endothelin in plasma may be due to blockade of ET_B and impaired clearance of endothelin. Mazzuca reported a decrease in ET_B receptor in aorta following placental ischemia with a concomitant increase in endothelin ET_A vasoconstriction (Mazzuca et al., 2014). Atrasentan treatment may also result in partial ET_A receptor internalization (Chiou et al., 2000), potentially changing the ET_A/ET_B ratio and the ultimate effect of endothelin in placenta or vasculature. Opgenorth (Opgenorth et al., 2000) suggested that a feedback mechanism increased PPE message following ET_A receptor blockade. Our studies measuring PPE message also suggested it increases with atrasentan treatment in placenta of RUPP animals.

Both complement activation and endothelin production have been reported in attacks of hereditary angioedema due to C1-inhibitor deficiency (Kajdacs et al., 2016) as well as in endotoxin induced systemic inflammation in healthy volunteers (Soop et al., 2004). In addition, activation of both systems has been documented in mouse models of glaucoma (Howell et al., 2011, 2014) and heart failure (Gombos et al., 2012). Literature indicating that complement activation recruits the endothelin pathway is quite limited. In a study of nonocclusive mesenteric ischemia using partial aortic occlusion in the rat, a C5a receptor antagonist prevented the increase in plasma endothelin along with increased mean arterial pressure, heart rate and cardiac output resulting from mesenteric hypoperfusion (Erces et al., 2016). In a hemorrhagic model of shock in rat, preventing complement activation using sCR1 prevented the observed increase in endothelin (Chen et al., 2016). In addition, C5b-9 causes increased endothelin production by glomerular epithelial cells (Cybulsky et al., 1993). None of these studies determined the effect of complement inhibition on endothelin production or message in a control animal or in pregnancy.

Our results reveal a complex interaction of the endothelin and complement system in pregnancy with significant downregulation of normal complement regulators *in vivo* in placenta following ET_A receptor antagonism. Thus, our data suggest that activation of the ET_A receptor *in vivo* is important in maintaining optimal levels of complement regulators to protect tissues from excessive complement

activation during normal pregnancy. With blockade of the ET_A receptor, excessive tissue complement activation may occur. Our data also suggest that inhibition of complement activation may compromise control of the endothelin pathway. Thus, therapeutic strategies for pregnancy-induced hypertension should optimally target upstream events leading to activation of the complement and endothelin systems to minimize unintended complications of therapy. Our data provide evidence for a strikingly important role of endothelin and the ET_A receptor in maintaining adequate complement regulators in placenta to prevent excessive and potentially damaging complement activation.

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Declaration of Competing Interest

The authors have no conflicts to report

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