**Neurokinin 3 Receptor and Phosphocholine Transferase**

**Missing Factors for Pathogenesis of C-Reactive Protein in Preeclampsia**

Nicholas F. Parchim, Wei Wang, Takayuki Iriyama, Olaide A. Ashimi, Athar H. Siddiqui, Sean Blackwell, Baha Sibai, Rodney E. Kellems, Yang Xia

**Abstract**—C-reactive protein (CRP), an innate immune mediator, is elevated in the circulation before symptoms in patients with preeclampsia, a severe hypertensive pregnancy disorder with high mortality and morbidity. However, the specific sources underlying increased CRP and the role of elevated CRP in preeclampsia are undefined. Here, we report that circulating CRP levels are significantly increased in a large cohort of normotensive pregnant individuals when compared with nulligravid women and is further increased in patients with preeclampsia. These findings led us to discover further that placental syncytiotrophoblasts are previously unrecognized cellular sources of CRP and underlie elevated CRP in normotensive pregnant women and the additional increase in patients with preeclampsia. Next, we demonstrated that injection of CRP induces preeclampsia features, including hypertension (157 mm Hg CRP treated versus 119 mm Hg control), proteinuria (35.0 mg/μg CRP treated versus 14.1 mg/μg control), kidney, and placental damage and increased levels of sFlt-1 in pregnant mice but not in nonpregnant mice. Our study implicates that phosphocholine transferase, a placental-specific enzyme post-translationally modifying neurokinin B, is essential for the pathogenic role of CRP in preeclampsia through activation of the neurokinin 3 receptor. Overall, our studies have provided significant new insight on the pathogenic role of CRP in preeclampsia and highlighted innovative therapeutic strategies. *(Hypertension. 2015;65:00-00. DOI: 10.1161/HYPERTENSIONAHA.114.04439) ● Online Data Supplement*

**Key Words:** C-reactive protein ■ inflammation ■ neurokinin B ■ phosphocholine transferase

**P**reeclampsia is a serious disease of pregnancy affecting ≈8% of pregnant women and accounts for >50,000 maternal deaths worldwide per year. The key symptoms of preeclampsia are hypertension and proteinuria, and the disease is frequently associated with intrauterine growth restriction, a condition that puts the fetus at risk for numerous cardiovascular disorders later in life. Thus, preeclampsia is a leading cause of maternal and neonatal morbidity and mortality and has both acute and long-term effect on moms and babies. Despite substantial research efforts, the pathogenesis of preeclampsia remains poorly understood, and the clinical management of preeclampsia is hampered because of lack of presymptomatic screening, reliable diagnostic tests, and effective therapy. The only effective treatment is delivery of the fetus, often resulting in serious complications of prematurity for the neonate. Thus, understanding the mechanisms involved in the pathogenesis of preeclampsia is extremely urgent and important for early detection and safe and effective therapeutic strategies for disease.

Of the prevailing hypotheses underlying preeclampsia, inflammatory mediators have been prominently implicated in the pathophysiology of this disease. An acute innate immune mediator, C-reactive protein (CRP), is a pentamer with =26 kDa monomeric subunits noncovalently bound to each other. CRP production is stimulated by the potent cytokines interleukin-6/8, as well as tumor necrosis factor-α,1,2 and is predominately produced and secreted by liver. Although CRP is largely thought to work with complement components to assist in the removal of foreign pathogens by binding with phosphocholine on the membrane of the pathogen, a growing body of studies suggests that prolonged elevated CRP links with cardiovascular risk and likely plays a detrimental role in disease development by targeting on host tissues or signaling inflammatory cells to the site of insult.3–5 For example, recent studies have demonstrated that elevated CRP is associated with renal dysfunction and atherosclerosis secondary to intimal arterial damage.6–9 Moreover, persistently elevated CRP is correlated to progressive decline of kidney function in patients with chronic kidney disease likely because of deposition of CRP in glomeruli and subsequent increase in oxidative stress. Of note, early studies showed that CRP directly induces endothelial damage by increasing foam cell formation and subsequently leading to atherosclerosis.10,11
Notably, previous human studies report that circulating CRP is elevated in patients with preeclampsia, and its elevation is correlated to the clinical symptoms. Moreover, plasma CRP is found to be elevated before maternal disease development in patients with preeclampsia, implicating that CRP is likely a presymptomatic biomarker for early detection. However, the functional role of circulating CRP in preeclampsia remains unknown.

Intriguingly, recent studies show that neurokinin B, a known pathogenic molecule of preeclampsia, is post-translationally modified by a phosphocholine transferase (PCT; ie, PCYT1b). Post-translational modification is an essential biological mechanism to control fundamental cellular and systemic functions. Modifications of biological molecules typically serve to amplify or diminish activity of the molecule or alter their ligand–receptor relationship. Phosphorylation, glycosylation, ubiquitination, methylation, and acetylation are common and well-recognized post-translational modification. However, phosphocholination modification has only recently begun to be studied and is poorly understood. At this moment, phosphocholination modification carried by PCT is only detected in 2 tissues: placenta and testis. One of the endogenous phosphocholinated molecules is neurokinin B and phosphocholinated neurokinin B preferentially activates the neurokinin 3 receptor (NK3R), a Gq-coupled transmembrane factor contributing to preeclampsia by binding to phosphocholine, we hypothesize that elevated PCT, and (4) CRP is elevated in preeclampsia and known to bind with phosphocholine, we hypothesize that elevated CRP is not just an early biomarker but likely a pathogenic factor contributing to preeclampsia by binding to phosphocholinated neurokinin B and preferentially activating NK3R.

Methods

For detailed description see Methods in the online-only Data Supplement.

Results

Placenta Is an Additional Source for Increased Circulating CRP in Patients With Preeclampsia

Although CRP is increased in the circulation under preeclampsia conditions, the specific cell type responsible for its elevation remains largely unknown. In an effort to determine the potential source for increased circulating CRP under preeclampsia conditions, we first measured circulating CRP in blood samples from nulligravid women (n=21), normotensive pregnant women (NT; n=15), and patients with preeclampsia (n=15; Table S1 in the online-only Data Supplement). Intriguingly, the CRP level in sera of the nulligravid women was below the standard detectable threshold provided by the assay. However, circulating CRP level was significantly increased in the normal pregnant women when compared with that in nulligravid individuals and further increased in the women with preeclampsia compared with that in NT (Figure 1A). Although CRP is predominantly produced by the liver under nonpregnant state, our studies suggest that the placenta is likely an additional source for increased circulating CRP seen in NT and further increased CRP seen in preeclampsia setting.

To test this intriguing possibility, we first conducted quantitative reverse-transcriptase-polymerase chain reaction to determine whether CRP transcripts are expressed in the placentas. We found that CRP mRNA was present in the placentas of NT individuals, and that CRP mRNA levels significantly increased ≈4- to 5-fold in placentas from women with preeclampsia (Figure 1B). Next, we performed Western blot analysis and found that CRP protein levels were also increased in preeclampsia when compared with those in the NT placentas (Figure 1C). Moreover, immunohistochemistry and immunofluorescence dual-staining demonstrated that CRP was localized in the villus syncytiotrophoblast cells, around the villus border (Figure 1D).

CRP Induces Pathophysiology of Preeclampsia in Pregnant Mice But Not in Nonpregnant Mice

To examine the pathogenic role of CRP in preeclampsia, we infused CRP into pregnant C57BL/6 mice on E13 and E14 of their gestation period to achieve a similar concentration as patients with preeclampsia. Our data indicate that a pathological concentration of CRP seen in patients with preeclampsia at term (75 μg/mL; based on the upper SD detectable in our circulating CRP ELISA; Figure 1A) was sufficient to cause an elevation in mean systolic pressure in CRP-infused mice (157.08 versus 118.99 mm Hg control; Figure 2A). To verify the level of CRP injected into mice, sera were sampled at the end point of the experiment on E18 and measured by ELISA. We observed a mean level of 11.6 μg/mL of circulating CRP in our mice. The level of CRP on E18 is consistent with extrapolated predictions of 2 injections of 75 μg/mL on E13/14 (Figure S1). Proteinuria was also significantly elevated in CRP-infused mice (Figure 2B). Similar to patients with preeclampsia, both blood pressure and urinary protein returned to basal levels postpartum (Figure 2A and 2B). In addition, histological studies demonstrated the typical glomerular damage featured with decreased capillary lumen space and narrowing Bowman capsule space (Figure 2C). Histological glomerular scoring indicated an overall decrease in the health of the glomerulus in the outer renal cortical region (Figure 2D). Furthermore, histological studies showed increased placental damage characterized with increased placental calcification in the CRP-infused pregnant mice (Figure 2E–2F).

Altogether, we provide the first evidence that CRP infusion directly induces pathophysiology of preeclampsia in pregnant mice as seen in patients with preeclampsia.

Next, to determine whether CRP-induced preeclampsia pathophysiology is dependent on pregnancy, we infused similar concentrations of CRP into nonpregnant mice. The key features of preeclampsia, including hypertension and
proteinuria, were not observed in CRP-infused nonpregnant mice (Figure 2G and 2H). sFlt-1, a pathogenic factor, is predominantly produced in the placentas and known to be induced by inflammatory factors. Thus, CRP may be a previously unrecognized factor contributing to increased sFlt-1 production in the placentas and subsequently leading to preeclampsia. Supporting this possibility, we found that sFlt-1 levels in the sera were significantly increased in the pregnant mice with infusion of CRP when compared with that in the control pregnant mice (Figure 2I). In contrast, sFlt-1 levels were extremely low, and no difference was observed in the nonpregnant mice with or without CRP infusion (data not shown). Thus, we demonstrated that CRP is a novel pathogenic factor contributing to the pathophysiology of preeclampsia, including hypertension, proteinuria, kidney, and placental damage, and increased sFlt-1 secretion.

Antagonism of NK3R or Specific Knockdown of NK3R Attenuates CRP-Induced Pathophysiology of Preeclampsia in Pregnant Mice

Because CRP binds with phosphocholine and phosphocholine-ligated neurokinin B preferentially activates NK3R, it is possible that CRP-induced pathophysiology is dependent on NK3R activation. To test this intriguing possibility, we treated CRP-infused mice with or without NK3R-specific inhibitor, SB222200. We found that SB222200 treatment significantly reduced mean systolic pressure and proteinuria in CRP-infused pregnant mice (Figure 3A and 3B). There was a marked reduction in glomerular damage as indicated by an overall improvement tubular diameter, Bowman capsule diameter, and glomerular scoring (Figure 3C–3E). We also observed a decrease in placental calcifications of CRP-infused pregnant mice with SB222200 treatment (Figure 3D–3F). Finally, treatment with SB222200 decreased CRP-induced sFlt-1 levels in the pregnant mice (Figure 3G).

To validate our pharmacological studies further, we performed an in vivo knockdown of the NK3R via encapsulation of siRNA specific for the NK3R by a nanoparticle delivery system (Altogen). First, we demonstrated that siRNA specific for NK3R significantly reduced more than half of NK3R protein levels in the placentas when compared with the scrambled siRNA in the CRP-infused pregnant mice (Figure S2A). In contrast, the efficiency of knockdown of NK3R in the kidneys was less evident when compared with the placental tissues (Figure S2B). Thus, we concluded from these results that siRNA specifically for NK3R successfully reduced NK3R in the placentas but not kidneys in the CRP-infused pregnant mice. Next, we found that knockdown of NK3R more than half by specific siRNA was sufficient to attenuate mean systolic pressure and proteinuria in CRP-infused pregnant mice compared with the pregnant mice with nanoencapsulated scrambled RNA (Figure S3A). Furthermore, CRP-induced placental calcifications, kidney damage, and increased circulating sFlt-1 levels were significantly attenuated by specific NK3R siRNA knockdown in pregnant mice (Figure S3C–S3G). Thus, both pharmacological studies using specific
NK3R antagonist and quasi-genetic studies using siRNA to specifically knockdown NK3R provide strong in vivo evidence that CRP-induced preeclampsia pathophysiology is signaling via NK3R.

Knockdown of PCT Ameliorates CRP-Induced Preeclampsia Features in Pregnant Mice

Because neurokinin B is modified by placental PCT (ie, PCYT1b) and phosphocholinated neurokinin B preferentially activates NK3R, it is possible that CRP-mediated activation of NK3R and subsequent disease development are dependent on placental PCT. To overcome the difficulty of lack of a potent and specific inhibitor for PCT, we performed quasi-genetic studies using nanoparticle-encapsulated siRNA specifically to knockdown the synthesis of this important enzyme in CRP-infused pregnant mice. First, we confirmed that siRNA specific for PCT significantly reduced mRNA of this enzyme in the placentas of CRP-infused mice compared with the scrambled siRNA (Figure 4A). In addition, knockdown of PCYT1b by specific siRNA for PCT significantly attenuated mean systolic pressure and proteinuria in the CRP-infused pregnant mice versus the CRP-infused pregnant mice injected with scrambled siRNA (Figure 4A and 4B). Furthermore, CRP-induced placental calcifications,
kidney damage, and increased circulating sFlt-1 levels were significantly attenuated by specific PCT siRNA knockdown in pregnant mice (Figure 4C–4G). Thus, quasi-genetic studies using siRNA to knockdown PCT specifically revealed that placental PCT, which is a key enzyme responsible for neurokinin B phosphocholination, is essential for CRP-induced preeclampsia pathophysiology.

**Elevated CRP and Neurokinin B Are Colocalized in Syncytiotrophoblast Cells of Placentas of Patients With Preeclampsia**

To extend our mouse findings to human, we performed coimmunofluorescence staining to determine the localization of CRP and neurokinin B in the term placentas from NT pregnant women and patients with preeclampsia. Specifically, we found that CRP and neurokinin B were observed in the syncytiotrophoblast cells of the maternal villi and substantially increased in the placentas of preeclampsia compared with that in the NT pregnant women (Figure 5A). In addition, colocalization of CRP and neurokinin B was visualized along the cellular membrane of the villus syncytiotrophoblast cells (Figure 5A). Thus, these translational human studies demonstrated that CRP and neurokinin B are elevated and colocalized in the syncytiotrophoblast cells in human placentas from patients with preeclampsia.

**CRP Signaling via NK3R Directly Induces sFlt-1 Secretion From Cultured Human Placental Villus Explants in a PCT-Dependent Manner**

Although we showed that CRP-induced hallmark features, including hypertension, proteinuria, and increased circulating sFlt-1 levels in an intact animal by activating NK3R and in a PCT-dependent manner, the pathological role of CRP in humans remains unidentified. To test the significance of CRP in preeclampsia in humans, we took advantage of primary cultured human villous explants to determine whether CRP signaling via NK3R contributes to preeclampsia by directly inducing sFlt-1 secretion in a PCT-dependent manner. Similar to mouse finding, we found that CRP treatment directly induced sFlt-1 secretion from cultured human villous explants from NT pregnant women at term (Figure 5B). Moreover, SB222200, a specific inhibitor of NK3R, and siRNA specific for NK3R significantly reduced CRP-induced sFlt-1 secretion from cultured human villous explants (Figure 5B). Finally, siRNA specific for PCT also significantly reduced CRP-induced sFlt-1 secretion from cultured human villous explants (Figure 5B). Taken together, these results translate our mouse finding to human pregnancy by showing that CRP signaling via NK3R contributes to sFlt-1 secretion in a PCT-dependent manner from cultured human villous explants.
CRP is increased in the circulation at early stage before preeclampsia symptoms, and its elevation is correlated to the disease severity. However, nothing is known about its role in preeclampsia, the specific cell types responsible for its increased production and the molecular basis for its function. Here, we have provided human evidence showing that CRP transcripts are present in syncytiotrophoblast cells of normal placentas and further increased in the placentas with preeclampsia, indicating that syncytiotrophoblast cells in the placenta are a previously unrecognized additional source for increased circulating CRP seen in patients with preeclampsia. Extending human studies, we have further demonstrated the pathogenic nature of CRP in preeclampsia by showing that injection of CRP alone is sufficient to regenerate the key features of preeclampsia, including hypertension, proteinuria, kidney damage, and impaired placentas in pregnant mice. Mechanistically, we revealed CRP signaling via NK3R contributes to sFlt-1 secretion and disease development in PCT-dependent manner in intact animals and in cultured human villous explants. Overall, we have provided both human and mouse evidence that increased CRP contributes to preeclampsia pathophysiology by crosstalk with PCT and NK3R. Thus, our findings have identified a detrimental role of CRP, new sources for its production and a novel signaling cascade for its pathogenic effects in preeclampsia and immediately suggest novel therapeutic opportunities.
CRP is predominantly produced by hepatocytes under non-pregnant states. Before our studies, it was unclear what specific cell type was responsible for increased circulating CRP in patients with preeclampsia. Here, we demonstrated for the first time that placentas contain transcripts for CRP and CRP transcript levels are significantly increased in the placentas of patients with preeclampsia when compared with NT pregnant individuals. However, CRP levels are virtually undetectable in nulligravid women. Using immunohistological studies, we further found that CRP is predominantly expressed in syncytiotrophoblast cells of human placentas. Thus, we provide human evidence that placental syncytiotrophoblast cell is a previously unrecognized cell type expressing CRP and it is an additional source contributing to circulating CRP in patients with preeclampsia. Understanding how CRP gene expression is regulated under physiological and pathological pregnancy will be important questions for us to address further.

CRP is an innate immune factor and works with complement components to kill the bacteria and virus or remove damaged tissues. Because of its early rise in the inflammatory process, CRP is often considered as an important early predictor for the immune response. Although the transient elevation of CRP in response to the presence of pathogens is beneficial, the prolonged and persistent elevation of CRP is likely harmful resulting in damage to host tissues. Some studies have suggested that obesity and increasing body mass index, correlated with late-onset preeclampsia, are key factors in increasing CRP and those cytokines instrumental in its production and release. However, multiple human studies have shown that circulating CRP is elevated at the early stage before clinical symptoms in patients with preeclampsia and its level is correlated to the severity of the disease. Moreover, several studies indicate that the elevation of circulating CRP is correlated to multiple pathogenic factors, including tumor necrosis factor-α, interleukin-6, excess complement production and sFlt-1, implicating its role in endothelial dysfunction, uterine arterial constriction, macrophage activation, and arterial plaque formation. However, the pathogenic role of elevated circulating CRP in preeclampsia has not been previously examined in vivo. Here, we demonstrated for the first time that injection of CRP to achieve similar concentrations to those seen in patients with preeclampsia induced hallmark features of preeclampsia, including hypertension, proteinuria, kidney damage, and placental impairment in pregnant mice. Because preeclampsia is a pregnancy-related disease, the placenta has been long considered to play an important role in
the disease development. To examine the role of placentas in CRP-induced hypertension and proteinuria, we injected similar amounts of CRP into nonpregnant mice as pregnant mice. In contrast to pregnant mice, CRP failed to induce preeclampsia features in nonpregnant mice. These findings are consistent with reports of LaMarca et al., showing that tumor necrosis factor-α, interleukin-6, and interleukin-17 can only induce high blood pressure in pregnant rats and not in nonpregnant rats. We further discovered that CRP induces sFlt-1 production in pregnant mice but not in nonpregnant mice, suggesting the placenta as the source of increased sFlt-1. Extending our mouse studies, we have demonstrated that CRP directly induces sFlt-1 secretion from cultured human villous explants. Elevated sFlt-1 contributes to pathophysiology of preeclampsia, including abnormal placentation, hypertension, and kidney injury. Thus, a possible explanation for CRP only inducing preeclampsia features in pregnant mice but not in nonpregnant mice is that CRP only induces secretion of placental-derived toxic factor, sFlt-1, during pregnancy. Taken together, our studies revealed that elevated CRP is a potent immune mediator responsible for increased sFlt-1 secretion and impaired placentas. Without interference, CRP-sFlt-1-placental damage functions as a malicious cycle leading to the progression of the disease and symptom development (Figure 5C).

Neurokinin B is mainly secreted from brain and placentas. Early human studies found that circulating neurokinin B is significantly elevated in patients with preeclampsia. More recent studies showed that endogenous neurokinin B is modified by PCT. The phosphocholinated neurokinin B preferentially activates NK3R, a Gq-coupled receptor. As such, elevated neurokinin B activating NK3R induces calcium influx and subsequent vascular hypertension and kidney damage. During infection, CRP binds with phosphocholine on the membrane of bacteria and virus and works with complement systems and inflammatory cells together to kill foreign invaders eventually. Here, we have provided multiple lines of evidence supporting a novel but compelling molecular basis that under preeclampsia condition in the absence of infection, increased CRP signaling via NK3R contributes to preeclampsia pathophysiology in a PCT-dependent manner. First, we have provided in vivo animal evidence that CRP-induced preeclampsia pathophysiology is significantly reduced by blocking NK3R signaling by its specific antagonist or by lowering its protein levels by its specific siRNA. Subsequently, using quasi-genetic studies to specific knockdown PCT, CRP-induced preeclampsia features in the pregnant mice are significantly ameliorated. Thus, both pharmacological and quasi-genetic studies provide strong in vivo functional evidence that PCT mediated neurokinin B phosphocholination, and NK3R signaling underlies CRP-induced pathogenic effects in pregnant mice.

We have further validated our mouse findings and provided human evidence, showing that neurokinin B and CRP are colococalized in the membrane of syncytiotrophoblast cells of villous human placentas and their levels were significantly increased in the placentas of patients with preeclampsia compared with the NT. Similar to our mouse finding, we have demonstrated that CRP signaling via NK3R directly induces sFlt-1 secretion from cultured human villous explants in a PCT-dependent manner. PCT is only expressed in 2 organs, placenta and testis. To date, 2 endogenous molecules are identified to be modified by PCT (ie, neurokinin B and corticotropin releasing factor). PCT-mediated post-translational modification of these 2 molecules is considered leading to phosphocholinated neurokinin B preferentially activating NK3R and preventing phosphocholinated-corticotropin releasing factor degradation in circulation, respectively. Because placenta serves as the only source of PCT necessary for post-translational phosphocholination of neurokinin B, preeclampsia features were not observed in nonpregnant CRP-injected mice. Thus, our findings support a novel working model: increased CRP functionally coupled with PCT, a key enzyme carrying post-translational modification of neurokinin B only occurring in the placentas, preferentially activates NK3R and prevents phosphocholinated neurokinin B crosstalks with CRP. In view of the important role of PCT and NK3R in CRP-induced pathophysiology of preeclampsia, one of the most reasonable possibilities is that CRP and phosphocholinated neurokinin B directly interact, and then this complex preferentially activates NK3R and leads to preeclampsia features. Although our human studies have showed that CRP and neurokinin B are colocalized in the membrane of syncytiotrophoblast cells of human placentas, our communoprecipitation pull-down assay has been unable to show a direct interaction of these 2 molecules. Our lack of success may reflect a weak interaction that does not persist through such experiments or low abundance of phosphocholinated neurokinin B that does not allow us to pull down the complex. Nevertheless, lacking direct evidence for the interaction of CRP with phosphocholinated neurokinin B does not prevent us to conclude that CRP is pathogenic for preeclampsia functioning through NK3R signaling in a PCT-dependent manner. Thus, the important and clear evidence of CRP functionally dependent on NK3R and PCT leads to several exciting new directions including determining the molecular mechanisms by which CRP pathogenesis requires PCT and activates NK3R signaling and developing specific and potent PCT and NK3R inhibitors to treat preeclampsia.

In conclusion, our findings are extremely innovative because nothing was known about the role of CRP and placental PCT in preeclampsia until we revealed that placental PCT and NK3R are key factors responsible for CRP-induced pathophysiology of preeclampsia. Moreover, our discoveries that placentas contain CRP transcripts and syncytiotrophoblast cells contribute to the increase in circulating CRP in patients with preeclampsia are also novel. Finally, our findings are clinically significant because we have determined that CRP not only is an early biomarker but also has a pathogenic role in preeclampsia. Thus, our current studies have added significant new insight to the pathogenesis of preeclampsia, have revealed early...
presymptomatic pathogenic biomarkers, and thereby have opened up novel therapeutic possibilities for the disease prevention and treatment.

**Perspectives**

The study is the first to show a pathogenic role for elevated CRP in preeclampsia via activation of NK3R. In addition, our work also revealed a critical role for placenta-specific PCT (PCTY1b) in CRP-induced preeclampsia features. Finally, we have also demonstrated that the placenta is an additional source contributing to elevated circulating CRP in preeclampsia. Significantly, we validate our mouse studies and demonstrated that CRP activates NK3R coupled with PCTY1b contributing to increased production of sFlt-1 by human placental villous explants. Thus, our findings have revealed that CRP coupled with PCTY1b contributes to features of preeclampsia in pregnant mice and increased production of sFlt-1 by human placental villous explants by activating NK3R. As such, our work has revealed neurokinin B, PCTY1b, and NK3R as potential therapeutic targets for preeclampsia.

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**Disclosures**

None.

**References**


What Is New?
- An acute inflammatory mediator, C-reactive protein (CRP), is elevated in the circulation and placentas of women with preeclampsia. Thus, the placenta is an additional source contributing to elevated CRP in preeclampsia.
- Injection of recombinant murine CRP into pregnant mice results in increased sFlt-1 production, hypertension, and proteinuria via crosstalk with neurokinin B and subsequent activation of neurokinin 3 receptors.
- A critical role for placenta-specific phosphocholine transferase (PCTY1b) in the modification of neurokinin B was identified in CRP-induced pathophysiology of preeclampsia.
- CRP-neurokinin B crosstalks with neurokinin 3 receptor, increasing sFlt-1 production in cultured human placenta villous explants in a PCTY1b-dependent manner.

What Is Relevant?
- Our data reveal a previously unrecognized pathogenic role of CRP signaling via NK3B in the pathogenesis of preeclampsia, dependent on placental-specific enzyme, phosphocholine transferase, highlighting potential therapeutic possibilities.

Summary
We report that CRP, previously thought to be a nonspecific inflammatory mediator, is elevated in circulation of normotensive pregnant women compared with nulligravid women and further elevated in women with preeclampsia. Additional experiments determined that CRP is produced by the syncytiotrophoblasts of the placenta. We demonstrated the pathogenic role of CRP by infusion of recombinant CRP into pregnant mice to show features of preeclampsia, including hypertension and proteinuria. Furthermore, by antagonism or knockdown of the neurokinin 3 receptor using pharmacological or siRNA methodology, we found that this receptor is essential for the CRP-induced features of preeclampsia, including hypertension, proteinuria, placental and kidney pathology. In addition, we found that the placenta-specific enzyme, phosphocholine transferase (PCTY1b) was required for the CRP-induced pathophysiology in pregnant mice. Finally, we validated our mouse studies in human and showed that CRP induced sFlt-1 secretion by isolated human placental villus explants via neurokinin 3 receptor activation in a PCTY1b-dependent manner. Altogether, our human and mouse studies reveal that CRP crosstalks with neurokinin B and signaling contributes to pathophysiology of preeclampsia in a PCTY1b-dependent manner. Our findings highlight potential therapeutic possibilities.
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Neurokinin 3 receptor and phosphocholine transferase: missing factors for pathogenesis of C-reactive protein in preeclampsia

By

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Running title: CRP cross-talks with NK3R and PCT in preeclampsia

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**Materials and Methods**

**Human Subjects**

Human materials were acquired from normal pregnant women (NT) and preeclamptic patients (PE) admitted to Memorial Hermann Hospital which were identified by the Obstetrics and Gynecology faculty of the University of Texas Medical School at Houston, or from nulligravid women (NG) with no significant past medical history in a gynecologic clinical setting. Patients were stratified to diagnoses of severe preeclampsia, mild preeclampsia, or normotensive based on blood pressure criteria set forth by the National High Blood Pressure Education Working Group (2000). Patients were classified as preeclamptic based on the presence of systolic blood pressure $\geq 140 \text{ mmHg}$, diastolic blood pressure $\geq 90 \text{ mmHg}$ and presence of proteinuria $\geq 300 \text{ mg/24 hr}$. Further stratification for severe PE was based on the presence of systolic blood pressure $\geq 160 \text{ mmHg}$ or diastolic $\geq 110 \text{ mmHg}$ (NHBPEWG; 2000). Patients identified by research staff were consented and enrolled in a collection study. Patient blood was collected in EDTA and silicone collection tubes. The silicone tubes were allowed to clot, meanwhile, the EDTA tubes were centrifuged at 2500x$g$ for 15 minutes. After clotting, the silicone tubes were then centrifuged at 2500x$g$ for 15 minutes. All plasma and sera was collected and stored at -80°C. The research protocols, consent forms were approved by the University of Texas Committee for the Protection of Human Subjects. Human subject data are summarized and included in Supplementary Table 1.

**Animals**

Pregnant C57BL/6 mice were obtained from Harlan Laboratories on embryonic day 13 of gestation (E13) and injected with 75 μg/mL of recombinant CRP (R&D/Tocris, Bristol, UK) on E13/E14. Eight week old nonpregnant mice were also injected with 75 μg/mL of recombinant CRP. In addition to CRP, pharmacologic and siRNA knockdowns of the kinin system were used in the model. The specific NK3R antagonist SB222200 (2.5 μM; R&D/Tocris, Bristol, UK) was coinjected with CRP. All injection volumes were normalized to 100 μL total volume. Alternatively, an *in vivo* siRNA knockdown method (Altogen Biosystems, Las Vegas, NV) using nanoparticles was used to surround ~150 μg of specific NK3R and/or PCT siRNA constructs (Sigma; Santa Cruz Biosciences, St. Louis, MO; Dallas, TX, respectively). siRNA constructs were validated by companies in terms of knockdown efficiency and multiple constructs were pooled to achieve the most efficient knockdown strategy. Injections were prepared w/v 5% glucose to 100 μL volume according to the Altogen protocol. Mice were injected IV via retro-orbital injection methodology with ≤ 100 μL injection volume per inferior orbital vein. Mice husbandry care was undertaken by Center for Laboratory Animal Medicine and Care (CLAMC) and Animal Welfare Protocol UT AWC-11-073/14-090.

**Measurement of Blood Pressure and Proteinuria in Mice**

The systolic blood pressure of all mice was measured at the same time daily by a carotid catheter-calibrated tail-cuff system (CODA, Kent Scientific, Torrington, CT), and the mice were kept warm using a warming pad (AD Instruments Co, Colorado Springs, CO). Blood pressure was recorded and averaged over a 20-min period. For the measurement of proteinuria, urine was collected for analysis using metabolic cages (Nalgene, Rochester, NY). Total microalbumin and creatinine in the urine were determined by using ELISA kit (Exocell, Philadelphia, PA) and then the ratio of urinary albumin to creatinine was calculated as an index of proteinuria as previously described.1-5
**Immunohistochemistry/Immunofluorescence**

Tissues were paraffin fixed and sectioned using standard 5 µM sections and mounted onto glass slides. To begin immunohistochemistry/immunofluorescence protocol, all slides were deparaffinized using a standard deparaffinizing protocol. Tissue sections were marked with wax pens and blocked for 30 minutes using a standard blocking reagent (Vector Labs, Burlingame, CA). Anti-human and anti-mouse primary antibodies were diluted 1:50-1:200 in blocking buffer and incubated in a humidified chamber overnight at 4°C. Slides were washed in phosphate buffered saline and subsequently incubated in secondary antibody raised against the primary antibody host at room temperature for 30 minutes (Vector Labs, Burlingame, CA). Slides were then incubated with an alkaline phosphatase substrate mixture for 45 minutes (Vector Labs, Burlingame, CA). Slides were developed using a dual alkaline phosphatase/fluorescent development solution (Vector Labs, Burlingame, CA). Slides were counterstained with hematoxylin and differentiation solution. All slides were mounted with a DAPI antifade solution (Life Technologies, Grand Island, NY) and sealed. Images were taken with Olympus BX-60 microscope and camera. All fluorescent images were digitally aperture standardized.

**Western Blotting**

Presence of CRP was analyzed by previously described western blotting procedures. To confirm expression within the tissue, all tissues were cut to ~50 mg and homogenized in RIPA buffer with protease inhibitors (Santa Cruz Biotechnologies, Dallas, TX). Protein concentrations of tissue lysate were measured by BCA assay (Pierce). To analyze by western blotting, 20-40 ug of protein were loaded onto 4-20% stacked SDS-PAGE gels (Bio-rad, Hercules, CA) and transferred onto PVDF-FL membranes (Millipore, Billerica, MA). Membranes were probed with anti-human or – mouse primary antibodies raised against CRP in 1:500 dilutions (Abcam, Abbiotec; Cambridge, UK, San Diego, CA, respectively). A 43-kDa actin (Sigma, St. Louis, MO) housekeeping antibody was used as a loading control. Fluorescent conjugated secondary antibodies were used for visualization of the membrane on LICOR imaging systems (LICOR, Lincoln, NE).

**Placental Villus Explant Culture**

Placental tissue from NT patients was obtained within 20 minutes of delivery. Placentas were transported on ice and prepared for processing. Placental tissue was isolated from the periphery of the placenta subcapsularly. 50 mg segments of placenta were perfused in DPBS, and then rinsed 2x in phenol red free DMEM (10% FBS; 1% Pen-Strep). Placenta segments were manually separated using scissors and forceps and incubated overnight at 37°C/5% CO₂ in phenol red free DMEM in 12-well plates. Villi were then treated with CRP or various antagonists or siRNA treatment via nanoparticle encapsulated siRNA constructs prepared as previously described in the “animals” method section. Placental villus explants and tissue culture media was then harvested after a 48 hour period and stored at -80°C.

**ELISA**

Determination of CRP and sFlt-1 levels were quantified by commercially available ELISA kits. Human sera and/or plasma were diluted 100-fold (human CRP, Life Technologies, Grand Island, NY; murine CRP, Exocell, Philadelphia, PA) or 4-fold (NKB; RayBiotech, Norcross, GA). sFlt-1 (R&D Tocris, Bristol, UK) was detected in either murine sera or placental villus explant culture.
media. For microalbumin/creatinine quantifications, murine urine was diluted 13-fold (albumin) or 20-fold (creatinine) for quantification by Exocell albumin and creatinine companion kits.

**Quantitative Real Time-PCR**

RNA was obtained from human and mouse placentas. RNA was transcribed into single-strand cDNA with use of a reverse transcription kit (Qiagen, Venlo, Netherlands). cDNA was analyzed by qRT-PCR using SYBR green (Qiagen, Venlo, Netherlands) via specific primers to assess mRNA relative copy counts in the sample (Supplementary Table 2). Results were calculated using $2^{-\Delta C_t}$ method using actin as housekeeping gene for ratio comparison.

**Histologic Analysis**

The kidneys and placentas of mice were formalin fixed and processed according to standard protocols. The tissues were sectioned in 5 µM serial sections and were stained via hematoxylin and eosin by standard pathology protocol. Glomeruli and placenta were examined by single-blinded study. Number and scoring of glomeruli was assessed by counting and averaging the number and score of glomeruli per 10 random high powered fields. Scoring was based on glomerular health: (1) corresponded to decreased bowman capsule space, intraglomerular loop dilation, and tubular dilation; (5) corresponded to adequate bowman capsule space, no loop dilation, and normal tubule diameter. Further description of glomerular scoring is provided in Zhang, et al., 2013. Placenta calcification quantification was assessed by quantification of calcifications under 10 high powered fields. Both sets of quantification were performed via blinded analysis using Image Pro Plus software.

**Statistical Analysis**

All data were statistically analyzed with use of Graph Prism Pro v5.0, and the data were subjected to student’s t-test (paired and unpaired), two-way ANOVA, and Tukey’s test, where appropriate. Accepted variance was standardized to p < 0.05. Data were graphed on Graph Prism Pro v5.0 or SigmaPlot, where appropriate.
Supplementary References


**Supplementary Table 1. Patient Characteristics**

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<th>NT</th>
<th>PE</th>
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<td><strong>Racial Distribution (%)</strong></td>
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<tr>
<td>Other</td>
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<td>0</td>
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<tr>
<td><strong>BMI</strong></td>
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<td>39.305 ± 8.48</td>
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<td><strong>Gestational Age</strong></td>
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<td>Systolic Max</td>
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<td>124.1 ± 11.68 mmHg</td>
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<td>Diastolic Max</td>
<td>75 ± 7 mmHg</td>
<td>72.4 ± 9.75 mmHg</td>
<td>100.3 ± 22.01 mmHg</td>
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<td>Proteinuria</td>
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<td>&lt;300 mg/dL</td>
<td>&gt;300 mg/dL</td>
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<td>Fetus Weight</td>
<td>NA</td>
<td>3200.4 ± 474.5 g</td>
<td>2140.5 ± 867.86 g</td>
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**Supplementary Table 2. Primers used for qRT-PCR**

<table>
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<tr>
<td>hCRP Rev</td>
<td>CCA TAG CCT GGG GTG GCC CTT A</td>
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<tr>
<td>mPCYT1B For</td>
<td>GTC ACG CAA GGG CAC TTA TG</td>
</tr>
<tr>
<td>mPCYT1B Rev</td>
<td>GAG TAA GGT CAT CAC TGC AAA CT</td>
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**Figure S1. CRP levels verified at E18.5 timepoint.** Levels of CRP in pregnant mouse sera at E18.5 timepoint were verified via ELISA. CRP injected pregnant mice were found to have a mean concentration of 11.6 μg/mL when sera was sampled at E18.5. Mice injected with control vehicle were found to have CRP levels below threshold of detection (N.D.).
Figure S2. NK3R expression is attenuated in in vivo siRNA transfected mouse placental and renal tissue. (A) NK3R expression within the placenta is significantly attenuated in mice injected with CRP + siRNA specific for NK3R versus CRP + scrambled siRNA as verified by western blotting and quantified by ImageJ. Actin is shown for loading control (B) Expression of NK3R is also diminished within the kidney with CRP + siRNA specific for NK3R mouse injections, however, the difference is not as drastic due to a lower expression of NK3R within the murine renal tissue or reduced access to renal cells in contrast to placental trophoblasts. Quantification of western blotting is shown in relative density units. Actin is shown for loading control.