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PREECLAMPSIA: THE ROLES OF ACUTE INFLAMMATION AND INTRAUTERINE STRESS

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PREECLAMPSIA: THE ROLES OF ACUTE INFLAMMATION AND INTRAUTERINE STRESS

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Preeclampsia: the roles of acute inflammation and intrauterine stress

A

DISSERTATION

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for the Degree of

DOCTOR OF PHILOSOPHY

by

Nicholas Farrell Parchim, B.S.

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Dedication

My thesis is dedicated with deepest gratitude to…

My loving parents. Thank you for giving me a foundation to keep me grounded, wings to help me fly, and imagination, so I never stopped dreaming about tomorrow. To—

My Father, Mr. Nyle F. Parchim, Sr.

And

My Mother, Mrs. Elizabeth M. Parchim

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Abstract

PREECLAMPSIA: THE ROLES OF ACUTE INFLAMMATION AND INTRAUTERINE STRESS

Preeclampsia (PE) is a severe, acute disease of pregnancy affecting approximately 8% of pregnant women after week 20 of gestation. PE is characterized by hypertension and renal damage reflected by proteinuria and has significant morbidity to both mother and fetus. Maternal symptoms range from headaches, nausea, edema, to visual changes, but once maternal symptoms present, damage to the fetus has begun. Mothers who progress untreated through the disease can also experience a condition called eclampsia characterized by seizure, coma, and, ultimately, death. PE-affected newborns experience features similar to prematurity—abnormal lung and renal development, intrauterine growth retardation (IUGR), and, possibly, fetal demise. Interestingly, it has been shown that exposure to PE *in utero* can lead to many developmental problems during the progeny's life span. While intensive research has been dedicated to understanding the underlying pathogenesis of PE, no cause, predictive clinical test, nor definitive treatment has been discovered.

Although many factors are presumed as causative for PE pathogenesis, literature has indicated that acute inflammation may play a role in the development of PE. One such inflammatory factor is C-reactive protein (CRP). CRP, although regarded as a non-specific innate immune factor, is found to be elevated in PE mothers prior to onset of symptoms. The presence could indicate that CRP may play a role in the development of PE pathology. Further, CRP binds to bacterial

membranes through phosphocholine residues to trigger complement and other innate immune responses.

Similarly, phosphocholination is a cellular process wherein phosphocholine residues are added to biological molecules via an endoplasmic reticularly mediated enzyme, phosphocholine transferase. A recently identified neurally-secreted placental peptide and known pathogenic factor of PE, Neurokinin B (NKB), is phosphocholinated. This post-translational phosphocholination is thought to increase stability and binding of NKB to its Gq coupled receptor, NK3R. It is thought that this post-translational modification is bound by CRP thereby creating a molecular complex to preferentially activate NK3R.

In addition to investigating the pathogenic role of inflammation in PE, we are also curious as to how intrauterine expose to PE affects cardiovascular development in progeny. Previous literature has demonstrated that PE predisposes offspring to adult-onset hypertension; however, the mechanism of this fetal programing remains unknown. We hypothesize that hypertension in offspring exposed to PE in utero results via a two hit mechanism: 1) PE-related intrauterine stress induces epigenetic modifications in offspring, and 2) these modifications in the presence of environmental stressor lead to hypertension. Furthermore, we postulate that these epigenetic modifications are conserved across generations. Intrauterine epigenetic changes, specifically decreased global methylation of renal DNA and AT1R promoter, in offspring born to PE mothers are conserved across generations. When combined with an additional environmental stimulus, these changes result in hypertension. These finding posit novel mechanisms for the

etiology of hypertension and provide vital screening targets to assess the risk of hypertension in progeny.

These two approaches to PE study combine investigation of the maternal PE insult and pathogenesis with the investigation of transgenerational hypertensive features to progeny, we posit a global view of PE study. We not only examine potential targets for therapeutics and preclinical diagnostic exams, we also provide a mechanism for transmission of hypertensive features through generations and the possibility for hypertensive risk assessment.

Table of Contents

List of Figures

List of Tables

Background

Preeclampsia (PE) is a very severe disease of pregnancy which affects approximately 10 million mothers per year worldwide. Characterized by hypertension and proteinuria during the mid-second trimester of pregnancy, PE can result in significant fetal developmental problems and maternal morbidity. Affected mothers can experience renal and cardiac morbidity as well as a risk for life-long hypertension. Uncontrolled PE symptoms can result in seizure, coma, and death. Fetal manifestations are varied and include features of prematurity, intrauterine growth restriction, cardiac and renal malformation, and neural developmental delay.

Despite significant research efforts over the past decades to define the pathogenesis behind PE, no specific cause of the disease has been found. Further, due to the pleiotropic causes of PE, neither clinical test nor treatment has been found to halt disease pathogenesis apart from symptomatic treatment of the mother and delivery of the fetus at earliest stage of viability. There are thought to be many factors in the pathogenesis behind PE, further complicating its early detection and treatment. sFlt-1 and complement cascades, intrinsic vascular damage/malformation, and oxidative stress have all been previously posited to initiate features of PE in animal and human models.

sFlt-1 and its Role in the Initiation and Positive Feedback Cycle in PE

Soluble fms-like tyrosine kinase (sFlt-1) is an anti-angiogenic factor which regulates vascular formation during production of new vascular beds, as is seen

1

during placental formation. sFlt-1 is one of the most potent endogenous inhibitors of vascular endothelial growth factor and, thereby, inhibits placental growth factor signaling. sFlt-1 was first hypothesized as an initiator in PE after recombinant protein infusions in mouse models resulted in similar features found in clinical PE.[1-3] Increases in sFlt-1 circulating levels have been found in women who have growth-restricted fetal births as well as placental insufficiency. Early findings by the Levine group demonstrated elevations of sFlt-1 in second and third trimester of pregnancies complicated by growth restriction and PE, however, more recent studies have also show elevations of sFlt-1 as many as five weeks prior to onset of PE.[4] Evidence indicates that transcription and release in sFlt-1 is resultant from placental hypoxia and ischemia. The increases in this antiangiogenic factor result in a malicious cycle causing further placental damage.

Current literature demonstrates that balance between pro- and anti-angiogenic factors is crucial to proper placentation and fetal development. To abrogate placental damage, increases in sFlt-1 are coupled with an increase in pro-angiogenic placental growth factor (PlGF) in early placental growth stages. In PE, it has been demonstrated that this balance shifts toward anti-angiogenesis resulting in a decrease of placental growth.[5, 6] The dysfunction of placental growth leads to further placental hypoxia and increasing release of sFlt-1. This malicious anti-angiogenic factor release cycle is thought to underlie the pathogenesis of PE. However, attempts to mitigate the effects sFlt-1 have not been demonstrated to halt the progression of PE symptomology.

2

Complement Cascades and PE

The complement cascade comprises more than 50 proteins and utilizes 3 main pathways to protect against foreign pathogens—classical, alternative, and lectin pathways. The activation of the various pathways or development of split products within the pathway provides innate protection again microorganisms and assistance in the activation of intrinsic immunity. The activation of the complement cascade is regulated by inhibitors at various stages of each pathway. Both membrane and soluble inhibitors control activation at early and late stages of each pathway.[7, 8]

In pregnancy, the fetus and placenta represent foreign tissue. The maternal immune system functions to protect the feto-placental environment, however, the exact mechanism has never been elucidated. In the initial stage of pregnancy, apoptosis in the uterine wall results in decidual tissue suitable for implantation of the embryo. Activation of the adaptive immune system accompanies implantation, resulting in complement C3 activation to assist in invasion into the uterine decidua. Unique to pregnancy, decidual tissues secrete C1q, importnant for vascular formation at the decidual-embryonic interface.[9, 10] This is further shown in C1q-deficient mice, which demonstrate abnormal placentation and dysfunctional artery development within the implanted uterus.

Abnormal complement activation has been previously demonstrated to be important in the development of PE. Previous studies have demonstrated significant complement activation in PE compared to normal pregnancy

3

outcomes. Derzsy et al. reported that C3 is decreased in PE pregnancies while C3 split products and C5-C9 are increased, owing to greater complement activation within the feto-placental environment. Additionally, some studies have associated terminal complement components (C5-9) in pregnancies resulting in fetal growth restriction.[8-11]

Despite identification of complement's role in PE, large scale studies have not confirmed the diminution of PE symptoms by commercial complement inhibitors. Case reports describe eculizumab, a C5 inhibitor, could provide symptomatic relief of PE symptoms. Treatment with this inhibitor has shown efficacy of complement inhibition in prolonging the pregnancy, lowering hypertension, and resolving HELLP syndrome in one case.[8] Clinical treatment with this inhibitor is regarded as somewhat risky due to complement's role in meningococci opsonization and the potential role complement has in bacteremia. While inhibition of C5 is safer than other complement component inhibition since it is not at the beginning of the complement cascade, safety of drug therapy in pregnancy must be well-elucidated prior to human subject trials.

Vascular Damage and its Role in PE

Spiral artery formation during placentation is one of the most crucial steps in development of healthy fetuses. Normally, the process takes place over the first two trimesters in two stages—invasion into the decidua (as aforementioned) during the first trimester and myometrial invasion during the second trimester. Normal spiral artery formation is characterized by dilation during the second

4

trimester decreasing the velocity of flow into the placental bed. Failure to remodel the spiral arteries results in tonic constriction and elevated velocity of blood flow into the placenta. This elevated velocity results in placental damage, vacuolization, and shedding of the intrinsic trophoblastic tissue of the placenta. Damage to the placenta results in vasoactive cytokine release and an attempt repair of the damaged blood vessels.[12-14] If remodeling of the spiral arteries fail, it is thought that the PE cascade can occur.

Beside placental damage in PE, another hallmark of this disease is renal damage resultant from glomerular dysfunction. Proper glomerular endothelial structure is integral to effective filtration and reabsorption within the kidney. Renal biopsies of women affected by PE show a few key features of damage—endothelial edema, obliteration of the capillary spaces, and elimination of fenestrated endothelia. Interestingly, these hallmarks are also seen in other hypertensive disorders of pregnancy without proteinuria, indicating that this damage may not be intrinsically specific to PE. However, it is well-characterized that damage to the glomerulus causes renally-mediated hypertension.[3, 11, 15] Additionally, damage to glomerular endothelia is known to cause imbalances in the VEGF angiogenic cascade normally seen in PE.

Reactive Oxygen Species Cascade in PE

Related to the vascular hypothesis of PE, reactive oxygen species and nitric oxide (NO) bioavailability are related to both PE and vasodilatation, essential in normal placental perfusion. It is well-known that hypoxic

environments, as found in PE hypoperfused placentas, leads to activation of proinflammatory cytokines and activation of NADPH oxidase. It has been reported that NADPH oxidase is much more sensitive in PE patients than normal pregnant women. NADPH oxidase produces reactive oxygen species (ROS) through the catalysis of available oxygen and NADPH, an energy transport molecule. ROS production leads to vascular dysfunction through intrinsic endothelial damage. Furthermore, uncontrolled ROS leads to imbalance of nitric oxide (NO) signaling, important for regulation of vascular tone. The uncoupling of NO signaling by ROS is essential to development of hypertension and failure to rectify ROS production can lead to uncontrolled PE.[2, 16]

Despite identification of this pathway and its probable role in PE, attempts to negate ROS production by antioxidants proved unsuccessful. Antioxidant supplementation trials by the Maternal-Fetal Medicine network showed that Vitamin C and E supplementation failed to reduce PE symptom onset in at-risk mothers. Examination of other important antioxidant pathways, superoxide dismutase, catalase, and glutathione peroxidase, all have been demonstrated to be decreased in PE mothers versus normal pregnant mothers. However, neither supplementation trial nor medication has been developed to stimulate these pathways.

Role of Uterine Environment in Fetal Programming

As aforementioned, if untreated, PE has devastating effects on the mothers and fetuses. A growing body of evidence also investigates the role of

pregnancy-related stress and patterning effects on the fetus. It is hypothesized that outside of the known genetic effects on the fetus, the maternal uterine environment can developmentally pattern the fetus and predispose offspring to lifelong health problems. Data suggests that maternal uterine stress, obesity, and metabolic derangements can all have deleterious effects on the fetus including hypertension, cardiac disease, and metabolic pathway dysfunction. These patterning changes can occur on multiple levels—DNA methylation changes to alter genetic expression, RNA translation modifications, variations in hormone expression. These varied changes can all have deleterious effects within the developing organism. Moreover, epidemiologic evidence suggests that these changes can pass from generation of progeny to the next, affecting subsequent generations after initial insult.[17-22] The mechanism behind the transgenerational passage of patterning features has not been thoroughly investigated.

The body of work presented here aims to investigate the central role of innate immunity in PE and propose a mechanism behind progeny patterning in the setting of pregnancy related stress.

7

CHAPTER I:

Neurokinin 3 receptor and phosphocholine transferase: missing factors for
pathogenesis of C-reactive protein in preeclampsia

Work included from:

Parchim NF, Wang W, Iriyama T, Ashimi OA, Siddiqui AH, Blackwell S, Sibai B, Kellems
RE, Xia Y. Neurokinin 3 receptor and phosphocholine transferase: missing factors for
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Introduction

Preeclampsia (PE) Symptomology and Diagnosis

Preeclampsia (PE) is a serious disease of pregnancy affecting approximately 8% of all US pregnancies and accounts for 50,000 deaths worldwide. The key features of PE are hypertension and proteinuria after the 20th week of gestation. The increased monitoring and therapy of these women account for almost $70,000 in increased healthcare spending per case. More importantly, in spite of intensive research efforts and increased focus on prevention and treatment, neither definitive testing nor therapy has been found for PE. Currently, the only curative solution for PE symptoms is to deliver the fetus. PE presents significant acute and chronic risks for the mother—renal damage, cardiac damage, progression to seizure, coma, and death. Furthermore, PE also presents risk to fetuses exposed *in utero*—intrauterine growth restriction (IUGR), features of prematurity, abnormal heart and lung development—which predispose the fetus to cardiovascular risk later in life.[23] Therefore, understanding the mechanisms underlying PE is extremely important for early detection, as well as safe and effective therapy to treat this disease.

In recent years, increased focus has aimed toward pre-symptomatic diagnosis of PE and prevention. Currently, in high-risk patients suspected of a PE diagnosis, clinicians may analyze a PE laboratory panel—complete blood count, blood urea nitrogen, creatinine, and liver function markers. Analyzed together, these markers are sensitive for suspected diagnosis. Recent studies indicate that inflammatory, placentally-derived, and immune biomarkers may

9

serve as potential factors in better screening and pre-symptomatic diagnosis. Thus, the focus of our study was to examine various implicated in the development of PE, as well as how PE is propagated through generations of people.

Immune and Inflammatory Factors Underlie PE

Previous studies have implicated both autoimmune and inflammatory dysregulation as underlying the pathogenesis of PE. Over the past decade, literature has characterized autoimmunity as being fundamental in the pathogenesis of PE. The autoimmune factor, the activating autoantibody against the angiotensin II type I receptor (AT_1-AA), has been isolated from PE patients and extensively characterized. Pathologic activation of the angiotensin II type I receptor (AT1R) has been shown to precipitate hypertension and renal damage in patients with this circulating antibody.[10, 24-26] It is known that the renin-angiotensin-aldosterone system (RAAS) must be tightly regulated to account for homeostatic blood pressure regulation. The presence of AT_1-AA in the sera of PE patients is linked with pathologic activation of the RAAS, as well as derangement of blood pressure control mechanisms.

AT_1-AA and the RAAS have been very well characterized in both human and mouse models. AT_1-AA has been shown to bind the second extracellular loop of the AT1R, causing activation of this G_q-coupled receptor. Activation of this receptor initiates a cascade of calcium influx and secondary messaging signaling pathways within the cell. First characterized within the past decade, AT_1-AA has been shown to correlate with PE severity, IUGR, placental ischemia

and insufficiency, and maternal morbidity. Moreover, AT_1-AA is thought to lead to immune activation in both human and animal models. The reduced uterine perfusion pressure (RUPP) rat model has shown dysregulations in various proinflammatory cytokines—TNF-α, IL-6, IL-17, and CRP[27-40]—in the presence of AT_1-AA. Furthermore, an adoptive transfer mouse model involving AT1-AA precipitates PE features in a pregnant murine system. The adoptive transfer model also precipitates other features common in PE patients, immunologic dysregulation. The presence of AT_1-AA in the murine model also initiates a cascade of innate immune factors. Elevations of TNF-α and IL-6 primarily contribute to immunologic cascades and subsequent activation of the adaptive immune system.
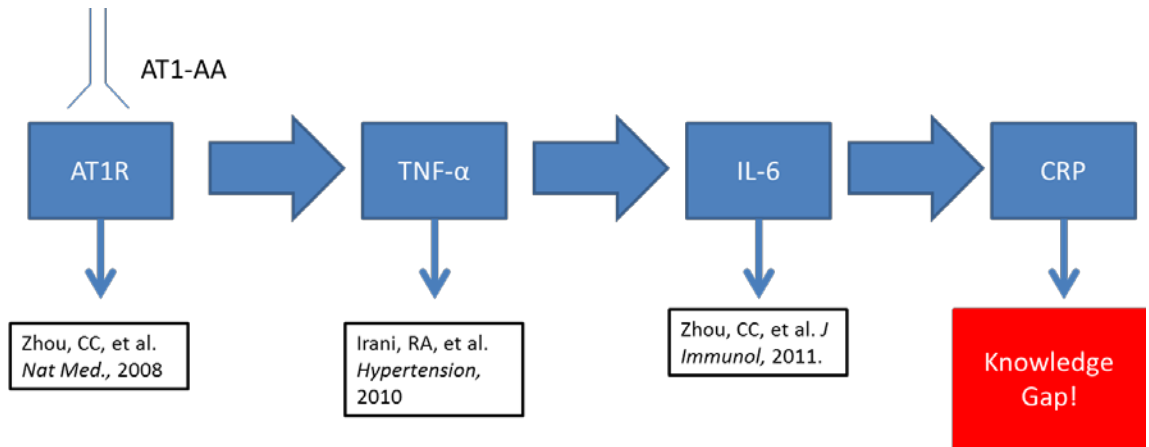
Figure 1-1. Cascade of immune factors predispose PE.

Research has demonstrated that IL-6 is a potent signaling factor for the transcription and release of CRP. Furthermore, pathologic activation of the AT1R by AT_1-AA involves TNF-α signaling and activation of the innate inflammatory signaling cascade. While this pathway has been well-elucidated in previous work, we encounter a significant knowledge gap with the action of CRP. The presence of CRP in this cascade presents a novel question in the pathogenesis of PE.

Research has demonstrated AT_1-AA and TNF-a as integral in the pathogenesis of key features of PE—hypertension, renal damage, and IUGR. These elevations also can precipitate a concurrent elevation of IL-6, a key initiator of CRP transcription and release. A significant knowledge gap exists in describing how CRP is involved in this inflammatory cascade and how it functions in the pathogenesis of PE.

C-Reactive Protein (CRP) and its role in PE

Innate immune factors have long been postulated to be important in the pathogenesis of PE. One immune factor, CRP, has been disputed as contributory to PE pathology. CRP is a pentamer joined by noncovalent bond, with each monomer sized approximately 26 kDa. Viewed axially, CRP has two faces which assist in its innate immune actions. One side binds with bacterial membranes, damaged cell membranes, foreign pathogens, while the other side communicates with complement proteins to assist in the opsonization process. Activation of the complement pathways assist in subsequent activation of adaptive immune components.[41-43] Furthermore, one of the main stimuli for synthesis and release, IL-6, is involved in signaling many other proinflammatory factors. Because of its nonspecific actions, CRP was not assumed to be important in PE pathogenesis.

Although initially assumed to be uninvolved in PE pathogenesis, CRP has recently been implicated as a pathogenic factor in many different diseases. Although mainly synthesized and secreted from hepatocytes, CRP elevations are linked to elevated cardiovascular risk and likely lead to disease pathogenesis via inappropriately targeting host tissues or recruiting immune cells to sites of injury.

Persistent elevations in CRP are associated with atherosclerosis secondary to intimal arterial damage.[42-46] This kind of damage can occur by foam cell formation and exacerbation of an atherosclerotic lesion. Of importance, CRP elevations have also been noted in PE patients and linked to symptom severity.[47-49] While studies are conflicted about the elevation of CRP early during pregnancy course,[50-53] some have indicated elevations of CRP prior to the onset of PE.[54, 55] This indicates that CRP may serve as a predictive biomarker of PE onset. However, the functional role of CRP in the pathogenesis of PE remains a mystery.

Role of the Kinin Signaling Pathway in PE

Previous research behind the pathogenesis of PE has led to the hypothesis that this disease is one involving many factors and pathways. Recently, the kinin signaling pathway has also been thought to be involved in disease progression. One member of this pathway, neurokinin B (NKB), was shown to be elevated in PE patient's sera in late diagnoses of PE. NKB is a short 13-residue neurally secreted peptide.[56-58] Earlier studies on the action of NKB have identified three primary receptors which the peptide is able to bind—the neurokinin 1 (NK1R), neurokinin 2 (NK2R), and neurokinin 3 (NK3R) receptor. These three receptors have shown to produce pleiotropic actions depending on the tissue in which they are most prevalent. Significantly, NK3R activation on vascular beds can produce vasoconstriction and increased systemic resistance.[56, 59] However, the actions of NKB on other receptors may counter the actions at the NK3R.

14

To increase specificity and preferential action at the NK3R, one study investigated a possible mechanism to explain specific activation of the NK3R. Posttranslational modification (PTM) is an indispensable mechanism to regulate cellular and systemic functions. Modifications such as phosphorylation, glycosylation, ubiqutination, methylation, and acetylation are common and necessary PTM to regulate cellular pathways. These modifications are necessary to amplify or diminish the activity of a biological molecule or alter ligand-receptor binding. One such PTM, phosphocholination, is thought to be involved in NKB preferential activation of the NK3R.[60] Interestingly, phosphocholinated NKB is thought to only be found in two tissues, placenta and testis, indicating its possible relevance to our study. Furthermore, specific activation of the NK3R is thought to be important for the development of hypertension in pregnancy via activation of the Gq-coupled transmembrane NK3R.[61]

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 ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg and presence of proteinuria ≥ 300 mg/24 hr. Further stratification for severe PE was based on the presence of systolic blood pressure ≥ 160 mmHg or diastolic ≥ 110 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 blood inside the silicone tubes was allowed to clot, meanwhile, the EDTA tubes were centrifuged at 2500xg for 15 minutes. After clotting, the silicon tubes were then centrifuged at 2500xg 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 (HSC-MS-04-384). Human subject data are summarized and included in Table 1.

Table 1. Patient Characteristics

	NG	NT	PE
Age	26.2 ± 5.6 yr	30.4 ± 7.35 yr	27.2 ± 6.66 yr
N	21	15	15
Racial Distribution (%)			
Caucasian	48	33	27
African American	10	40	47
Hispanic	33	27	19
Other	9	0	7
BMI	24.9 ± 5.6	31.352 ± 8.38	39.305 ± 8.48
Gestational Age	NA	38.4 ± 0.99 wk	36.3 ± 2.42 wk
Systolic Max	118.0 ± 11 mmHg	124.1 ± 11.68 mmHg	163.2 ± 25.99 mmHg
Diastolic Max	75.0 ± 7 mmHg	72.4 ± 9.75 mmHg	100.3 ± 22.01 mmHg
Proteinuria	<300 mg/dL	<300 mg/dL	>300 mg/dL
Fetus Weight	NA	3200.4 + 474.5 g	2140.5 ± 867.86 g

*NG, nulligravid; NT, normotensive; PE, preeclamptic; BMI, body mass index.

Animal Models

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.

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 4', 6-diamidino-2-

phenylindole (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 Polyvinylidene Fluoride (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 placental 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 Dulbecco's Modified Eagle Medium (DMEM) (10% FBS; 1% Pen-Strep). Placenta segments were manually separated using scissors and forceps and incubated overnight at 37°C/5% CO_2 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.

Enzyme-linked Immunosorbent Assay (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 albuwell and creatinine companion kits.

Quantitative Real Time-PCR

RNA was obtained from human and mouse placentas via RNeasy Mini Kit (Qiagen, Venlo, Netherlands). 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 (Table 2).

Results were calculated using 2-ΔCt method using actin as housekeeping gene

for ratio comparison.[62]

Table 2. qRT-PCR Primers

Primer	Sequence (5'→3')
hCRP For	AGG CCC TTG TAT CAC TGG CAG CA
hCRP Rev	CCA TAG CCT GGG GTG GCC CTT A
mPCYT1B For	GTC ACG CAA GGG CAC TTA TG
mPCYT1B Rev	GAG TAA GGT CAT CAC TGC AAA CT

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 (40x magnification). 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.[63, 64] 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.

Results

CRP is elevated in PE human patient samples

Aberrant innate immune factors are thought to underlie the pathogenesis of PE. Previous literature has demonstrated that aberrant inflammatory upregulation is integral in the pathogenesis of PE. To assess the inflammatory status of PE patients, we began by examining levels of CRP within our PE human patient population. Given that CRP is thought to be elevated prior to the onset of PE symptoms, we measured CRP levels to determine elevated levels up to delivery of the fetus. Using ELISA assays, we assayed CRP levels in patient sera from term delivery. We also utilized a normotensive nulligravid patient cohort as control. We observed a 3-fold increase in CRP levels in PE patients versus NT patients with term pregnancies. Additionally, CRP levels were found to be below the threshold of assay detectability in nulligravid patients (Fig. 1-2).
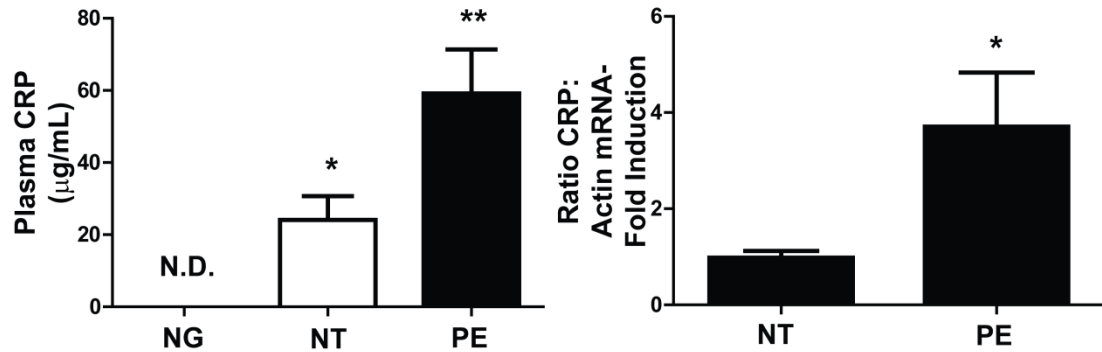
Figure 1-2. CRP is elevated in PE patient sera and is produced in PE patient trophoblastic tissue

(Left) Circulating CRP in sera was detectable on average 60 μg/mL in PE patients sera (n=15) and approximately 3-fold less in normotensive (NT) (n=15) patients. In a nulligravid cohort (n=21), levels were below the threshold of the assay. (Right) CRP mRNA transcripts were detected in patient placental samples. In samples isolated from PE patients, transcripts were elevated 4-fold than those samples isolated from NT patients. ** = $p < 0.05$ from NT; * = $p < 0.05$ from NG

While levels of CRP were detected in sera, we wanted to investigate other sources of CRP production. While hepatic tissue is the primary organ of CRP production and release, syncytiotrophoblastic tissue was analyzed using qRT-PCR techniques for the presence of mRNA for the CRP gene. Novelly, our group demonstrated that PE placental tissue does transcribe the CRP gene and could be a pathologic source of CRP production. Next, we performed western blot analysis and found that CRP protein levels were also increased in PE compared to the NT placentas (Fig. 1-3). Moreover, immunohistochemistry and immunofluorescence dual-staining demonstrated that CRP was localized in the villus syncytiotrophoblast cells, around the villus border (Fig. 1-3). Thus, we have shown that 1) syncytiotrophoblast cells in the placenta express CRP and contribute to increased circulating CRP under normal pregnancy state; 2) elevated placental CRP is an additional source responsible for substantial increased CRP under PE conditions.
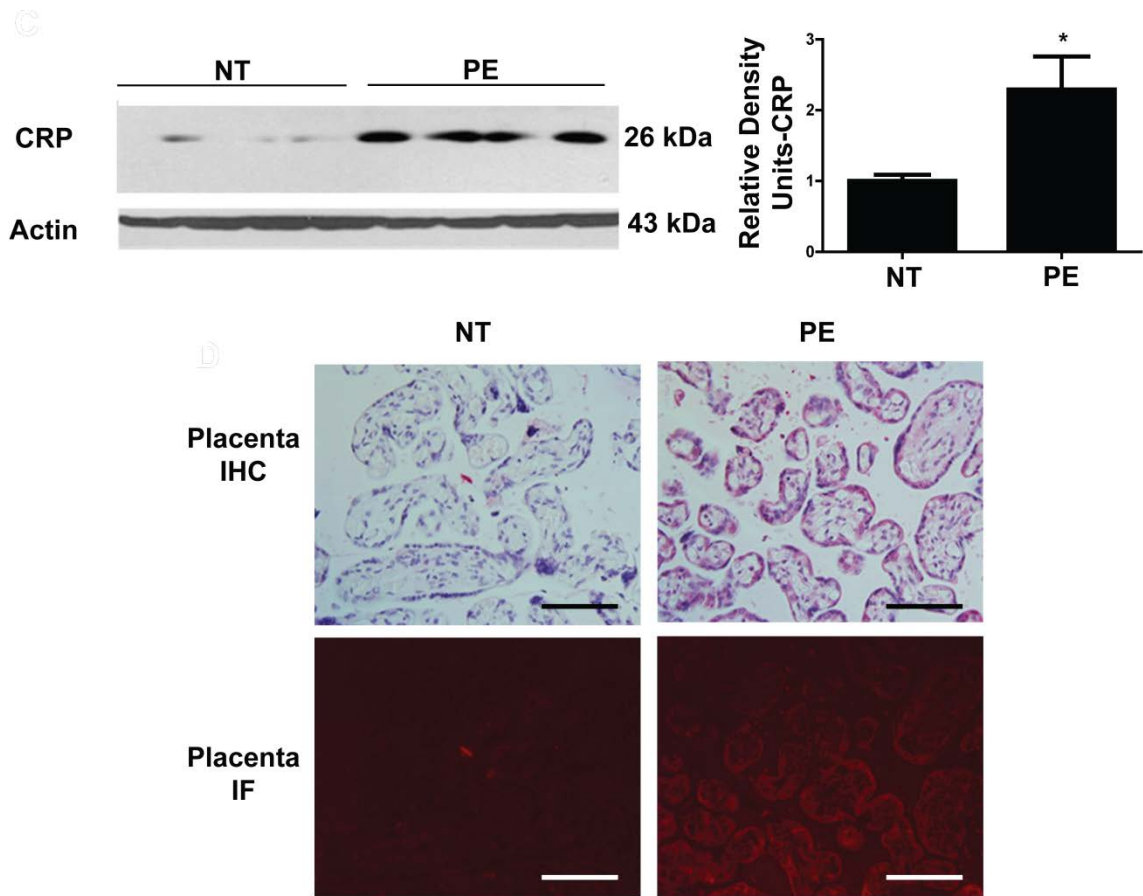
Figure 1-3. CRP is elevated in PE patient placental tissue as confirmed by immunoblotting and immunohistochemistry/immunofluorescence.

(Top panels) CRP deposition within PE placentas is significantly elevated versus deposition within NT placentas as confirmed by immunoblotting. (Bottom panel) CRP is elevated within the external villus syncytiotrophoblast cells in PE placentas as confirmed by immunohistochemistry and immunofluorescence. CRP is poorly detected in placental syncytiotrophoblastic tissue isolated from NT placentas on immunohistochemistry and immunofluorescence. 40x magnification; Scale bar = 100 μm

CRP induces pathophysiology of PE in pregnant mice but not nonpregnant mice

To examine the pathogenic role of CRP in PE, we infused CRP into pregnant C57BL/6 mice on E13 and E14 of their gestation period to achieve a similar concentration as PE patients. Our data indicate that a pathological concentration of CRP seen in PE patients at term (75 μg/mL; based on the upper standard deviation detectable in our circulating CRP ELISA; Fig. 1-2) was sufficient to cause an elevation in mean systolic pressure in CRP-infused mice (157.08 mmHg vs. 118.99 mmHg control) (Fig. 1-4). To verify the level of CRP injected into mice, sera was sampled at the endpoint 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 two injections of 75 μg/mL on E13/14. Proteinuria was also significantly elevated in CRP-infused mice (Fig. 1-4). Similar to PE patients, both hypertension and proteinuria were significantly dropped to the basal levels postpartum (Fig. 1-4).
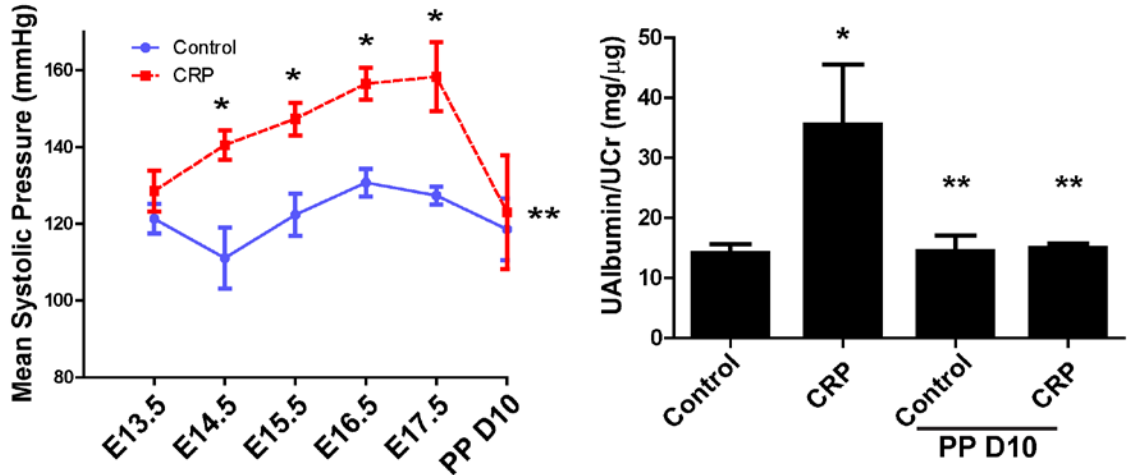
Figure 1-4. CRP injections into pregnant C57BL/6 mice result in key features of PE—hypertension and proteinuria—which resolve by post-partum day 10.

(Left) 75 ug/mL IV injections on E13/E14 induced mean systolic pressure elevation by E14.5 and dropped postpartum day 5-10. * = p < 0.05; ** = p <0.05 from CRP E17.5 (n=5-8). (Right) Microalbuminuria/creatinine ratio assayed by ELISA was significantly increased in the mice injected with CRP but diminished by postpartum D10. * = p < 0.05; ** = p < 0.05 vs. CRP peri-partum (n=5-8).

Additionally, histological studies demonstrated the typical glomerular damage featured with decreased capillary lumen space and narrowing Bowman's capsule space (Fig. 1-5). Histologic glomerular scoring, indicating an overall decrease in the health of the glomerulus in the outer renal cortical region (Fig. 1-5). Furthermore, histological studies showed that increased placental damage characterized with increased placental calcification in the CRP-infused pregnant mice (Fig. 1-5). Altogether, we provide the first evidence that CRP infusion directly induces pathophysiology of PE in pregnant mice as seen in PE patients.
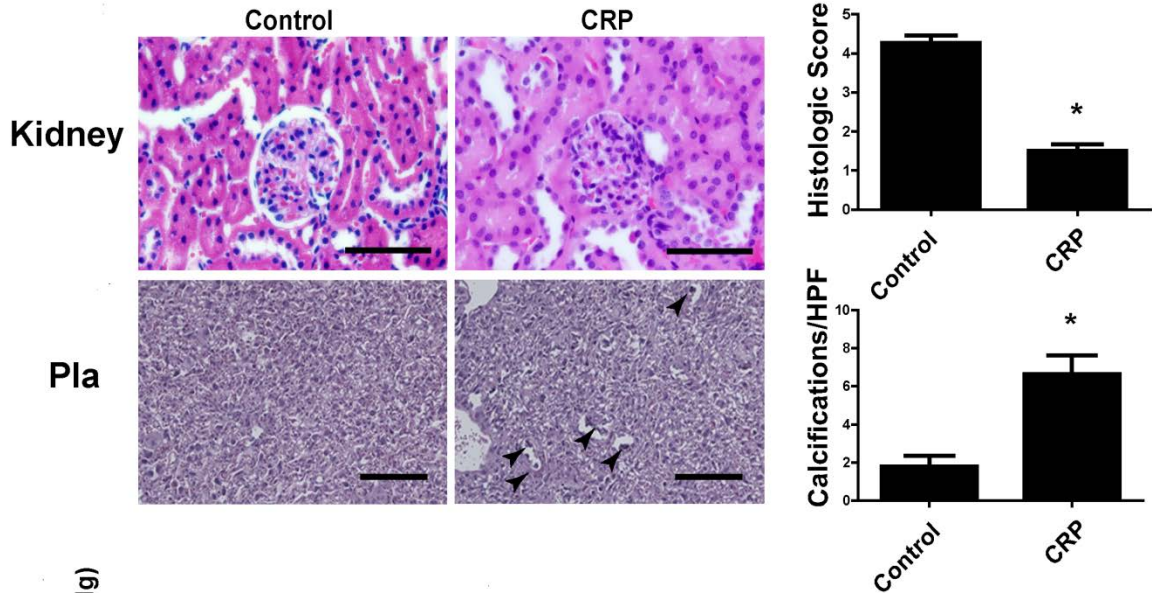
Figure 1-5. CRP induces renal and placental damage in a pregnant mouse model.

(Top Left) Oil-immersion microscopy showed CRP injection resulted in impaired glomerular histological condition. (100x magnification; scale bar = 50 μm) (Top Right) Blinded quantification of glomerular sections revealed a decrease in glomerular score of CRP injected murine renal histological sections (n=10 fields per kidney; 7 animals). * = p < 0.05 (Bottom Left) Analysis of placental sections at 20x microscopy reveals significant placental damage as reflected by an increase in placental calcification and scarring in placentas of CRP injected mice (Arrows: indicate placental calcification; scale bar = 200 μm). (Bottom Right) Confirmation by random high-powered field quantifications of placental sections (n=10 fields per placenta; 7 animals). * = p < 0.05.

Next, to determine whether CRP-induced PE pathophysiology is dependent on pregnancy, we infused similar concentrations of CRP into nonpregnant mice. The key features of PE including hypertension and proteinuria were not observed in CRP-infused nonpregnant mice (Fig 1-6). sFlt-1, a pathogenic factor, is predominantly produced in the placentas and known to be induced by inflammatory factors.[2, 6, 10, 34, 49, 64-69] Thus, CRP may be a previously unrecognized factor contributing to increased sFlt-1 production in the placentas and subsequently leading to PE. Supporting this possibility, we found that sFlt-1 levels in the sera were significantly increased in the pregnant mice with infusion of CRP compared to the control pregnant mice (Fig.1-6). In contrast, sFlt-1 levels were extremely low and no difference 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 pathophysiology of PE including hypertension, proteinuria, kidney and placental damage and increased sFlt-1 secretion.

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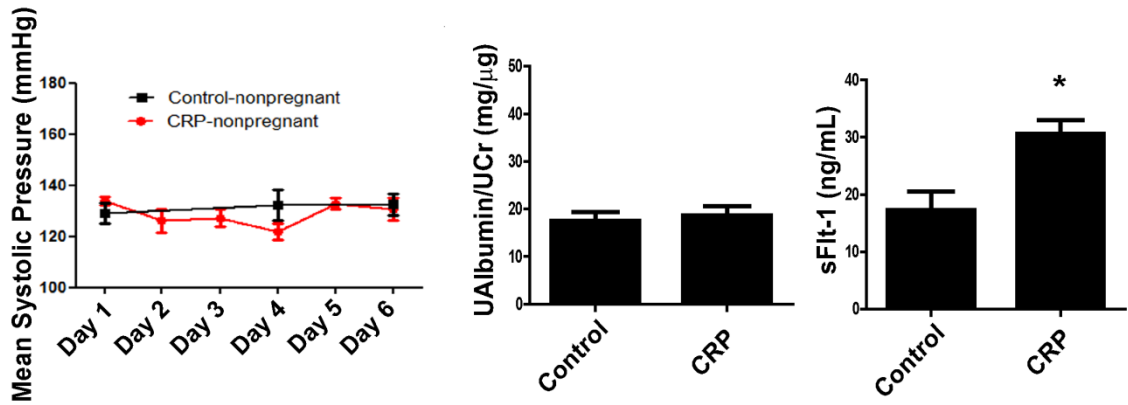
Figure 1-6. CRP injections in nonpregnant animals result in no increased hypertension and proteinuria. In pregnant animals it also induces sFlt-1 expression and release.

(Left) Nonpregnant mice injected with equivalent CRP concentrations (n=5) exhibited no elevation in mean systolic pressure versus nonpregnant mice injected with IV saline (n=10). (Middle) Microalbumin/creatinine ratio was not significantly altered in CRP injected nonpregnant mice relative to saline injected controls. (n=5-10) (Right) sFlt-1 level in the circulation was induced by CRP injection in pregnant mice. $*$ = $p < 0.05$.

Antagonism of NK3R or specific knockdown of NK3R attenuates CRP-induced pathophysiology of PE in pregnant mice

Because CRP binds with phosphocholine and phosphocholinated NKB (PC-NKB) 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 (Fig 1-7).
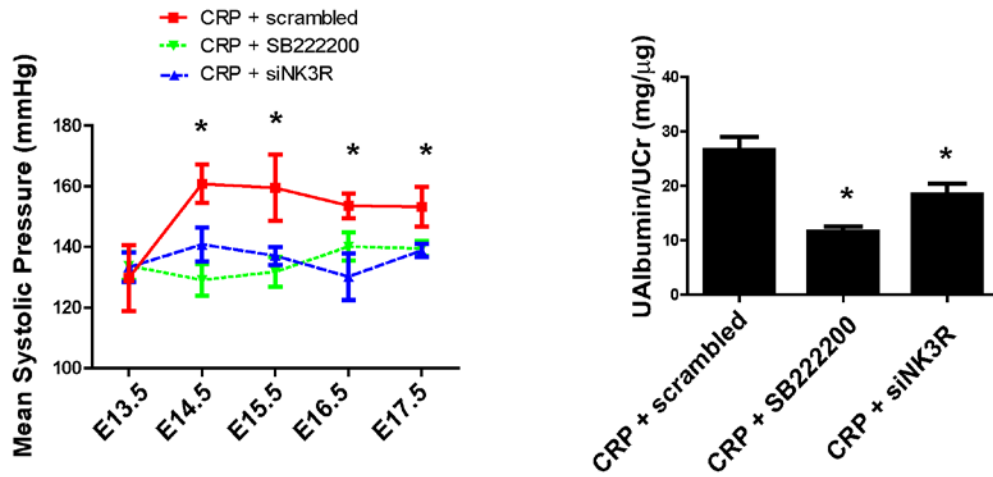
Figure 1-7. CRP-induced hypertension and proteinuria is attenuated by competitive NK3R inhibitor or *in vivo* siRNA knockdown of NK3R.

(Left) Co-injection of SB222200 prevented CRP induced mean systolic pressure of pregnant mice when administered on E13/E14. Administration of nanoparticle-encapsulated siRNA with CRP on E13/14 also reduced the CRP induced mean systolic pressure of the pregnant mice. * = $p < 0.05$ CRP + scrambled vs. CRP + siNK3R and CRP + SB222200; (n=5-8). (Right) Cotreatment with either SB222200 or NK3R siRNA reduced microalbuminuria/creatinine ratio. * = $p < 0.05$

There was a marked reduction in glomerular damage as indicated by an overall improvement tubular diameter, Bowman's capsule diameter and glomerular scoring (Fig. 1-8). We also observed a decrease in placental calcifications of CRP-infused pregnant mice with SB222200 treatment (Fig. 1-8).

To further validate our pharmacological studies, 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 compared to the scrambled siRNA in the CRP-infused pregnant mice (Fig. 1-8). In contrast, the efficiency of knockdown of NK3R in the kidneys was less evident compared to the placental tissues. 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 to the pregnant mice with nanoencapsulated scrambled RNA (Fig. 1-8). Furthermore, CRP-induced placental calcifications, kidney damage and increased circulating sFlt-1 levels were significantly attenuated by specific NK3R siRNA knockdown in pregnant mice (Fig. 1-8). Thus, both pharmacological studies using specific NK3R antagonist and quasi-genetic studies using siRNA to specific knockdown of NK3R provide strong *in vivo* evidence that CRP-induced PE pathophysiology is signaling via NK3R.
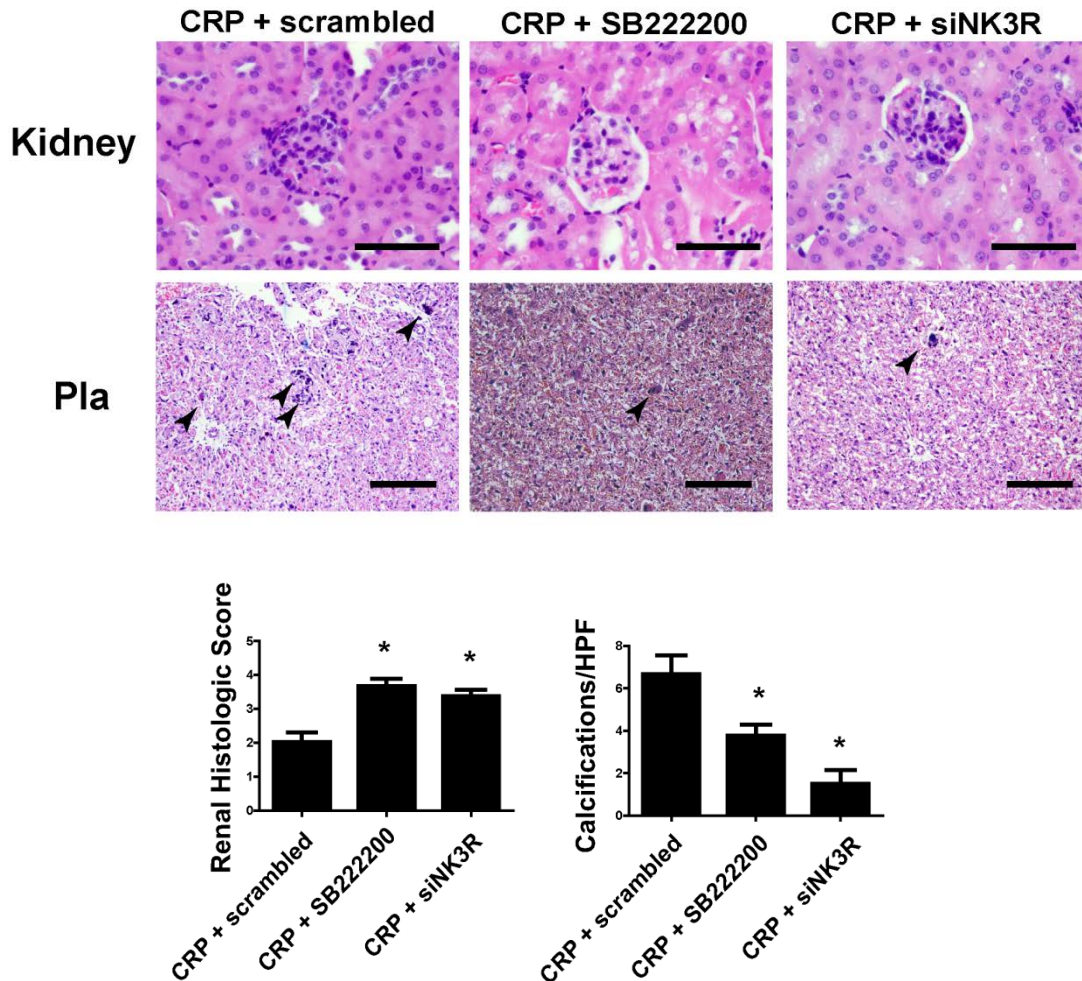
Figure 1-8. Antagonism of NK3R or siRNA knockdown for NK3R attenuates CRP-mediated renal and placental damage.

(Top panels) Glomerular damage was significantly attenuated by coadministration of SB222200 or NK3R siRNA as shown by H&E stained renal sections. (100x magnification; scale bar = 50 μm) (Middle panels) Placental damage was attenuated by cotreatment of SB222200 or NK3R siRNA, as indicated by reduction of placental calcifications and scarring shown on H&E

placental sections. (20x magnification; scale bar = 200 μm) (Bottom left panel) Histologic scoring of glomerular damage based on double-blind scoring criteria (n=10 fields per kidney; 7 animals). (Bottom right panel) Quantification of placental calcifications based on blinded image analysis (Arrows: indicate placental calcification; n=10 fields per placenta; 7 animals). * = p < 0.05

Finally, treatment with SB222200 decreased CRP-induced sFlt-1 levels in the pregnant mice (Fig. 1-9).
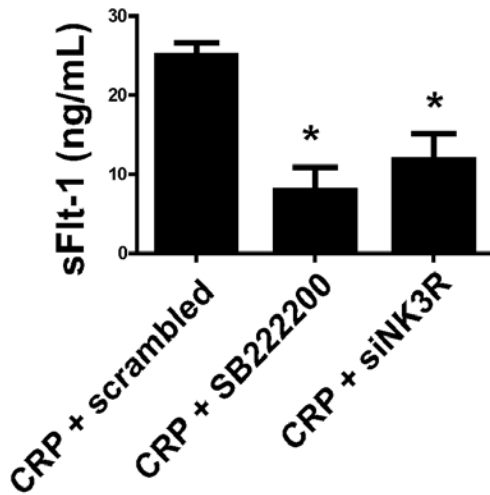
Figure 1-9. Antagonism with NK3R pharmacologic antagonist or siRNA knockdown of NK3R attenuates sera sFlt-1 levels

sFlt-1 production is significantly attenuated in pregnant mice with co-administration of SB222200 or siRNA for NK3R. $*$ = $p < 0.05$

Knockdown of phosphocholine transferase ameliorates CRP-induced PE features in pregnant mice

Because NKB is modified by placental phosphocholine transferase (PCT) (i.e. PCYT1b) and PC-NKB preferentially activates NK3R, it is possible that CRP-mediated activation of NK3R and subsequent disease development are dependent on the 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 to the scrambled siRNA (Fig. 1-10). Additionally, 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 (Fig. 1-10).
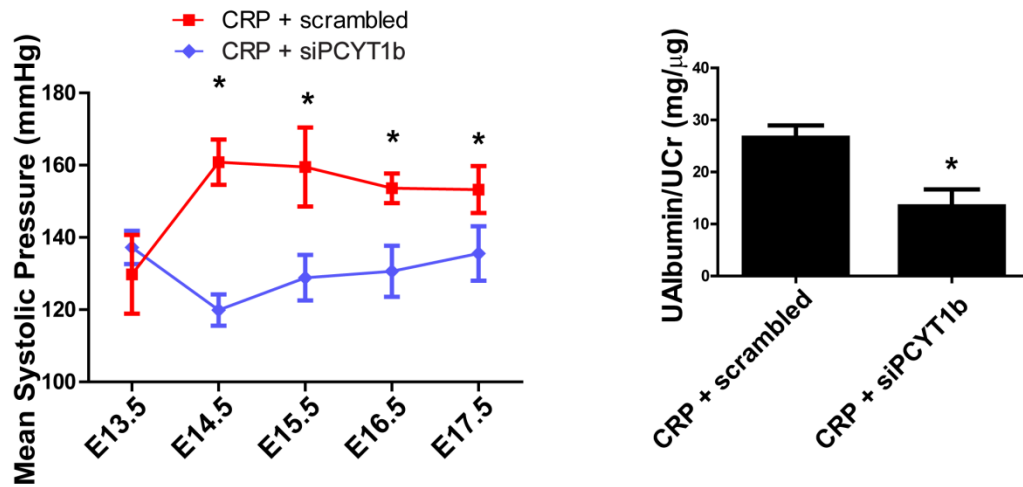
Figure 1-10. *In vivo* **siRNA knockdown of phosphocholine transferase attenuates mean systolic pressure and proteinuria.**

(Left) Confirmation of knockdown is shown by qRT-PCR on placental lysates (n=5). Administration of nanoparticle-encapsulated siRNA for PCYT1b with CRP on E13/14 reduced the mean systolic pressure of the pregnant mice. * = $p < 0.05$ (Right) Cotreatment of PCYT1b siRNA reduced microalbuminuria/creatinine ratio. * = $p < 0.05$

Furthermore, CRP-induced placental calcifications, kidney damage and increased circulating sFlt-1 levels were significantly attenuated by specific PCT siRNA knockdown in pregnant mice (Fig. 1-11). Thus, quasi-genetic studies using siRNA to specifically knockdown PCT revealed that placental PCT, which is a key enzyme responsible for NKB phosphocholination, is essential for CRP-induced PE pathophysiology.

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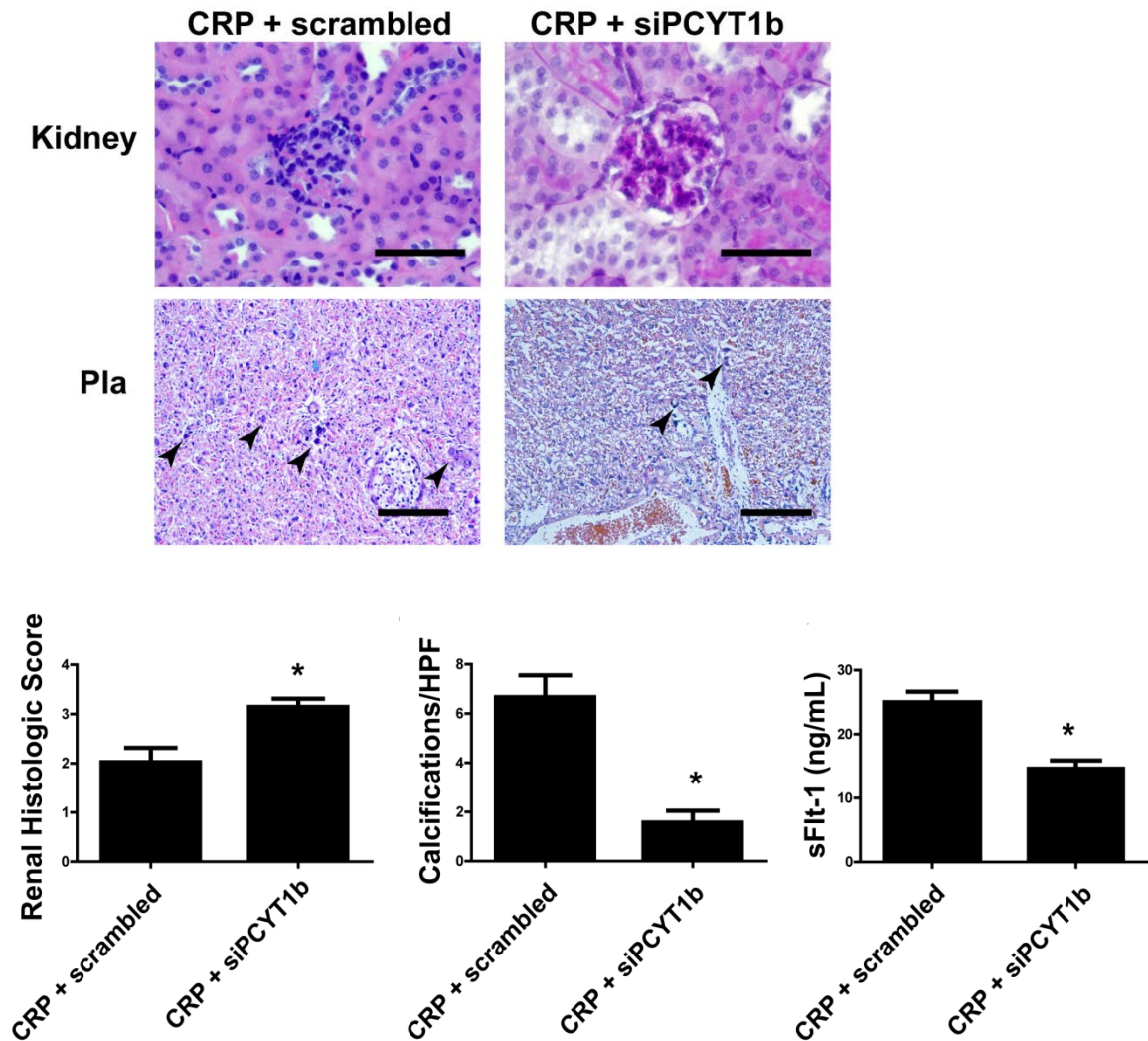
Figure 1-11. *In vivo* **siRNA knockdown of phosphocholine transferase attenuates renal and placental damage, as well as sFlt-1 production and release.**

(Top panels) Glomerular damage was significantly attenuated by coadministration of PCYT1b siRNA as shown by H&E stained renal sections. (100x magnification; scale bar = 50 μm) (Middle panels) Placental damage was attenuated by cotreatment with PCYT1b siRNA, as indicated by reduction of

placental calcifications and scarring shown on H&E placental sections. (20x magnification; scale bar = 200 μm) (Bottom left panel) Histologic scoring of glomerular damage based on double-blinded scoring criteria (n=10 fields per kidney; 7 animals). * = $p < 0.05$ (Bottom middle panel) Quantification of placental calcifications based on blinded image analysis (n=10 fields per placenta; 7 animals). * = $p < 0.05$. (Bottom right panel) CRP induced sFlt-1 production was significantly attenuated in pregnant mice with coadministration of SB222200 or siRNA for PCYT1b. * = $p < 0.05$

Elevated CRP and NKB are co-localized in syncytiotrophoblast cells of placentas of PE patients

To extend our mouse findings to human, we performed coimmunofluorescence staining to determine the localization of CRP and NKB in the term placentas from NT pregnant women and PE patients. Specifically, we found that CRP and NKB were observed in the syncytiotrophoblast cells of the maternal villi and substantially increased in the placentas of PE compared to NT pregnant women (Fig. 5A). Additionally, co-localization of CRP and NKB was visualized along the cellular membrane of the villus syncytiotrophoblast cells (Fig. 1-12). It is interesting to note that the CRP and NKB were extranuclear and mainly outside of the cytoplasm of the trophoblast cells. Thus, these translational human studies demonstrated that CRP and NKB are elevated and colocalized in the syncytiotrophoblast cells in human placentas from PE patients.
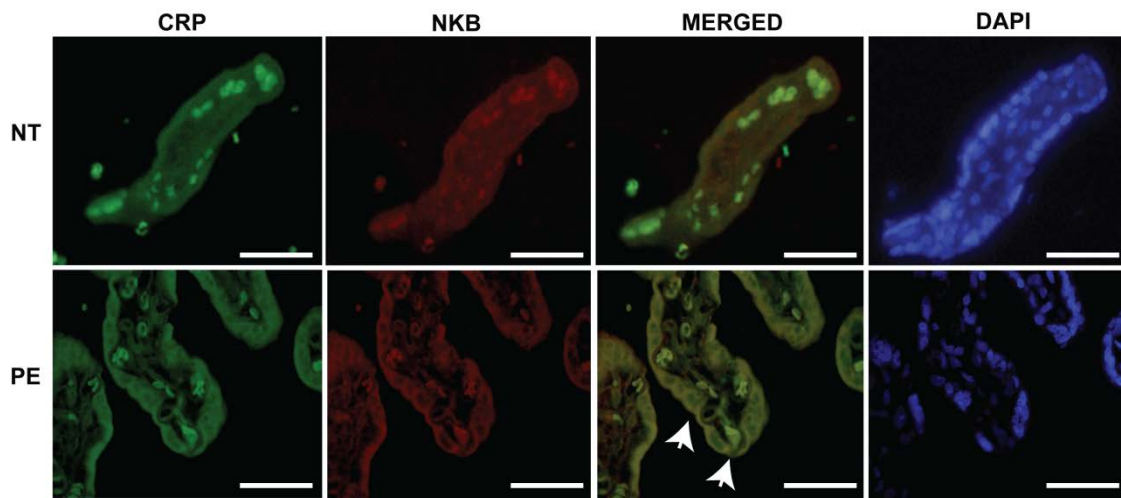
Figure 1-12. CRP and NKB colocalize within placental syncytiotrophoblast cells in PE patient placentas.

Coimmunofluorescence revealed increased CRP and NKB in syncytiotrophoblast cells in terminal villi. On the merged image, membrane colocalization was visualized along the syncytiotrophoblast cells. Some autofluorescence can be seen intravascularly due to the presence of autofluorescent RBCs. (40x magnification; scale bar = 100 µm).

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 PE in humans, we took advantage of primary cultured human villous explants to determine if CRP signaling via NK3R contributes to PE 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 (Fig. 1-13). Moreover, SB222200, a specific inhibitor of NK3R, and siRNA specific for NK3R significantly reduced CRP-induced sFlt-1 secretion from cultured human villous explants (Fig. 1-13). Finally, siRNA specific for PCT also significantly reduced CRP-induced sFlt-1 secretion from cultured human villous explants (Fig. 1-13). 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.
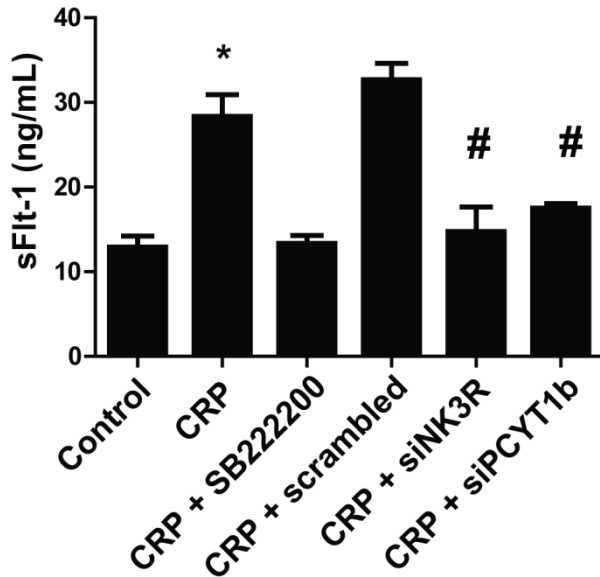
Figure 1-13. sFlt-1 production is significantly attenuated in the presence of antagonist of NK3R and knockdowns of NK3R and phosphocholine transferase in cultured placental villus explants

Placental villus explants isolated from normal patients and treated with or without CRP in the presence or absence of series of drugs or siRNA. CRP directly induced secretion of sFlt-1 from cultured human villus explants, but this increase was attenuated by treatment with SB222200 or siRNA for NK3R or PCYT1b. * = $p < 0.05$ difference from control; # = $p < 0.05$ difference from CRP + scrambled. (n = 4 wells placental villous explant culture)

Discussion

CRP is increased in the circulation at early stage prior to PE symptoms and its elevation is correlated to the disease severity. However, nothing is known about its role in PE, 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 PE placentas, indicating that syncytiotrophoblast cells in the placenta are a previously unrecognized additional source for increased circulating CRP seen in PE patients. Extending human studies we have further demonstrated the pathogenic nature of CRP in PE by showing that injection of CRP alone is sufficient to regenerate the key features of PE 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 PE pathophysiology by cross-talking with PCT and NK3R. Thus, our findings have identified the detrimental role of CRP, new sources for its production and a novel signaling cascade for its pathogenic effects in PE. These findings suggest novel therapeutic opportunities

CRP is predominantly produced by hepatocytes under nonpregnant states. Prior to our studies it was unclear what specific cell type was responsible for increased circulating CRP in PE patients. Here we demonstrated for the first

49

time that placentas contain transcripts for CRP and CRP transcript levels are significantly increased in the placentas of PE patients compared to 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 additional source contributing to circulating CRP in PE patients. Understanding how CRP gene expression is regulated under physiological and pathological pregnancy will be important questions for us to further address.

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.[42, 43, 45, 46, 70, 71] Some studies have suggested that obesity and increasing BMI, correlated with late-onset PE, are key factors in increasing CRP and those cytokines instrumental in its production and release.[72] However, multiple human studies have shown that circulating CRP is elevated at the early stage prior to clinical symptoms in PE patients and its level is correlated to the severity of the disease.[44, 47] Moreover, several studies indicate that the elevation of circulating CRP is correlated to multiple pathogenic factors including TNF-α, IL-6, excess

complement production and soluble VEGF, implicating its role in endothelial dysfunction, uterine arterial constriction, macrophage activation, and arterial plaque formation.[9] However, the pathogenic role of elevated circulating CRP in PE 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 PE patients induced hallmark features of PE including hypertension, proteinuria, kidney damage and placental impairment in pregnant mice. Because PE 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 amount of CRP into nonpregnant as pregnant mice. In contrast to pregnant mice, CRP failed to induce PE features in nonpregnant mice. These findings are consistent with reports of LaMarca and colleagues showing that TNF-α, IL-6, and IL-17 can only induce high blood pressure in pregnant rats, not in nonpregnant rats.[29, 30, 33] We further discovered that CRP induces sFlt-1 production in pregnant mice but not 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 PE including abnormal placentation, hypertension and kidney injury.[3] Thus, a possible explanation for CRP only inducing PE features in pregnant mice but not 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

51

increased sFlt-1 secretion and impaired placentas.   Without interference, CRP-sFlt-1-placental damage functions as malicious cycle leading to progression of the disease and symptom development (Fig. 1-14).
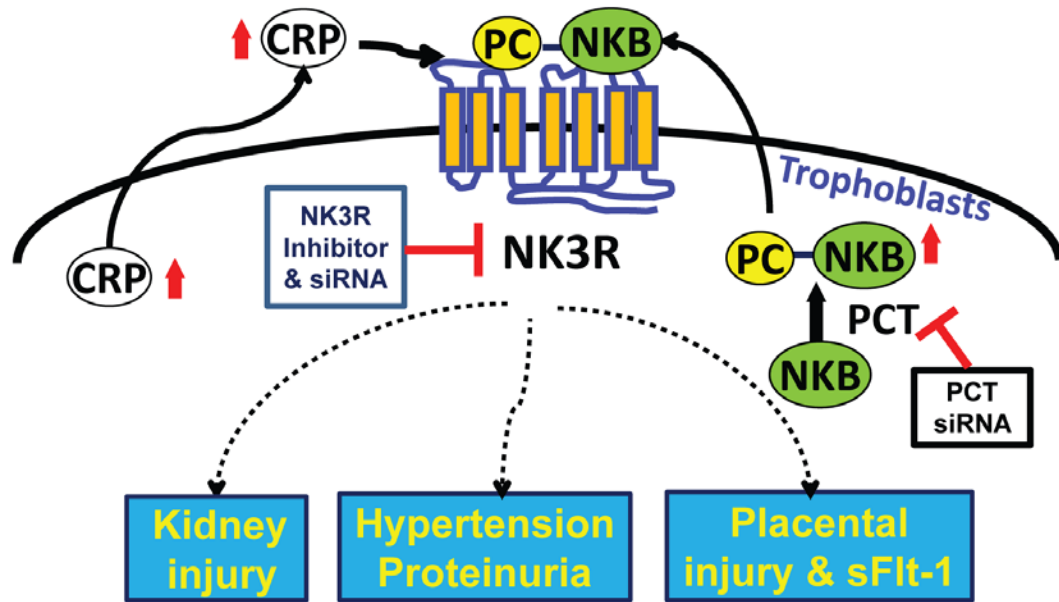
Figure 14. Working Model: CRP and PC-NKB communicate with the NK3R to elicit features of PE.

CRP is expressed in the placentas and elevated CRP in the syncytiotrophoblast cell is additional source underlying increased circulating CRP in PE patients. Locally-synthesized CRP cross-talks with post-translationally modified phosphocholinated NKB (PC-NKB) by placental-specific enzyme, PCT. Subsequently, CRP and PC-NKB work together preferentially activates NK3R leading to increased sFlt-1 production and release. Our findings reveal novel mechanisms for pathogenesis of PE and identify innovative therapeutic possibility for prevention and treatments.

NKB is mainly secreted from brain and placentas. Early human studies found that circulating NKB is significantly elevated in PE patients.[57, 58, 61] More recent studies showed that endogenous NKB is modified by phosphocholine transferase (PCT). The phosphocholinated NKB (PC-NKB) preferentially activates NK3R, a Gq coupled receptor. As such, elevated NKB 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 eventually kill foreign invaders. Here we have provided multiple lines of evidence supporting a novel but compelling molecular basis that under PE condition in the absence of infection, increased CRP signaling via NK3R contributes to PE pathophysiology in a PCT-dependent manner. First, we have provided *in vivo* animal evidence that CRP-induced PE 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 PE features in the pregnant mice are significantly ameliorated. Thus, both pharmacological and quasi-genetic studies provide strong *in vivo* functional evidence that PCT mediated NKB phosphocholination, and NK3R signaling underlies CRP-induced pathogenic effects in pregnant mice.

We have further validated our mouse finding and provided human evidence showing that NKB and CRP are co-localized in membrane of syncytiotrophoblast cells of villous human placentas and their levels were

54

significantly increased in the placentas of PE patients compared to 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 known to be expressed in two organs including placentas and testis. So far, two endogenous molecules are identified to be modified by PCT, i.e., NKB and corticotropin releasing factor (CRF).[60] PCT-mediated PTM of these two molecules is considered leading to PC-NKB preferentially activating NK3R and preventing PC-CRF degradation in circulation, respectively. Because placenta serves as the only source of PCT necessary for posttranslational phosphocholination of NKB, PE 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 posttranslational modification of NKB only occurring in the placentas, preferentially activates NK3R and subsequently promote disease development (Fig. 1-14). Interfering with these pathogenic molecules including placental specific PCT and NK3R are promising therapeutic possibilities (Fig.1-14). Although our results clearly show that PCT and NK3R signaling are functionally required for pathogenic role of CRP in PE, we have been unable to demonstrate how PCT-mediated PC-NKBs cross-talk with CRP. In view of important role of PCT and NK3R in CRP-induced pathophysiology of PE, one of the most reasonable possibilities is that CRP and PC-NKB directly interact and then this complex preferentially activates NK3B and leads to PE features. Although our human studies have showed that CRP and NKB are co-localized on the

55

membrane of syncytiotrophoblast cells of human placentas, our coimmunoprecipitation pull down assay has been unable to show a direct interaction of these two molecules. Our lack of success may reflect a weak interaction that does not persist through such experiments or low abundance of PC-NKB that does not allow us to pull down the complex. Nevertheless, lacking direct evidence for the interaction of CRP with PC-NKB does not prevent us to conclude that CRP is pathogenic for PE 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 a specific and potent PCT and NK3R inhibitors to treat PE.

In conclusion, our findings are innovative since little was known about the role of CRP and placental PCT in PE until data suggested that placental PCT and NK3R are key factors responsible for CRP-induced pathophysiology of PE. Moreover, our discovery that placentas contain CRP transcripts and syncytiotrophoblast cells contribute to increase in circulating CRP in PE patients are also novel. Finally, our findings are clinically significant since we have determined that CRP is not only an early biomarker, but it has a pathogenic role in PE Thus, our current studies have added significant new insight to the pathogenesis of PE, have revealed early pre-symptomatic pathogenic biomarker and thereby have opened up novel therapeutic possibilities for the disease prevention and treatment.

NOVELTY AND SIGNIFICANCE

What Is New?

• An acute inflammatory mediator, CRP, is elevated in the circulation and placentas of women with PE. Thus, placenta may be an additional source contributing to elevated CRP in PE

• Injection of recombinant murine CRP into pregnant mice results in increased sFlt-1 production, hypertension and proteinuria via cross-talk with NKB and subsequent activation of NK3R.

• A critical role for placenta-specific phosphocholine transferase (PCTY1b) in the modification of NKB was identified in CRP-induced pathophysiology of PE.

• CRP-NKB cross-talks with neurokinin 3 receptor (NK3R), increasing sFlt-1 production in cultured human placenta villous explants in an PCTY1b dependent manner

What is relevant?

Our data reveal a previously unrecognized pathogenic role of CRP signaling via NK3B in the pathogenesis of PE 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 to nulligravid women and further elevated in women with PE. 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 PE, including hypertension and proteinuria. Furthermore, by antagonism or knockdown of the NK3R using pharmacologic or siRNA methodology, we found that this receptor is essential for the CRP-induced features of PE including hypertension, proteinuria, placental and kidney pathology. Additionally, 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 NK3R activation in a PCTY1b-dependent manner. Altogether, our human and mouse studies reveal CRP cross talks with NKB signaling contributes to pathophysiology of PE in a PCTY1b-depenent manner, and thereby highlight potential therapeutic possibilities.

CHAPTER II:

Role of Transgenerational Epigenetic Modifications of the AT1R on the

Pathogenesis of Adult-Onset Hypertension

Introduction

Transgenerational Features of Disease

A developing field of research characterizes the role in *in utero* imprinting and transmitting disease to future generations called fetal programming. Fetal programming is encompassed into a broader field termed epigenetics. It was previously thought that only genetic factors could affect the development of a fetus. However, recent evidence points to the complex relationship between genetic and environmental factors which can affect fetal development and organogenesis.[22, 73] Epigenetics concerns all changes that can affect genetic expression above the genome. Alterations in expression occur from a variety of mechanisms—histone modifications, RNA interference, or DNA promoter methylation.[22, 74, 75] These modifications affect the overall accessibility of the DNA sequence and facilitate or inhibit replication and transcription. Of these modifications, literature indicates that DNA promoter methylation is important for transgenerational passage of epigenetic imprinting to subsequent generations.[18, 76, 77]

DNA promoter methylation involves modifications at the DNA promoter level to affect transcription of the DNA. Specifically, methyl group attachment to the C5 position of cytosine residues serves to modify individual DNA bases. The methyl group attachment primarily occurs within regulatory sequences call CpG islands, regions rich in cytosine and guanine (CG) base pairs.[21, 78] Hypermethylation of these sequences is important for transcriptional repression and inactivation of gene expression at certain developmental time points.

60

Likewise, hypomethylation can be an equally important modification to affect genetic expression. However, hypomethylation and its role in transcriptional regulation is poorly investigated. It is thought that global decreases in methylation can be precipitated by decreases in DNA methylation transferase 1 (DNMT1).[79] It is known that DNA hypomethylation plays essential roles in brain development, cancer, and cardiovascular diseases.[80, 81] However, the specific mechanisms underlying hypomethylations in this disease processes remain a mystery.

While global hypomethylation is presumed to precipitate disease processes, the mechanism underlying this process is undefined. One mechanism thought to determine methylation status on promoters is *in utero* stress. Preeclampsia is a type of intrauterine stress thought to predispose the fetus to future problems. To note, women who have one pregnancy affected by PE are more likely to have late life cardiovascular or renal disease.[21, 82-84] Likewise, progeny exposed *in utero* to PE have a higher incidence of cardiovascular disease, metabolic disease, and inflammatory conditions.[21, 22, 82-98] These features are witnessed in the absence of known genetic mutations, leading to the presumption that other mechanisms may be involved. Based on previous literature, hypomethylation and inactivation of DNMT1 could be potential factors in late-life hypertension.

Disturbance of Renin-Angiotensin-Aldosterone (RAAS) Signaling as a Pathogenic Factor in PE and Hypertension

One of the hallmarks of hypertensive disorders is disturbances in hypertensive signaling pathways and failures of the systems to counteract them.

One of the drivers of hypertension and PE is disruptions of the RAAS. The classical RAAS pathway involves signaling of pressors to counteract a depression in blood pressure sensed by the juxtaglomerular apparatus (JGA) in the kidney. JGA cells synthesize renin which catalyzes the conversion of angiotensinogen to angiotensin I (Ang I). Ang I is subsequently converted to Ang II in the lung. Ang II is a potent vasoactive peptide resulting in increased systemic resistance and blood pressure. Ang II also signals aldosterone release which acts on the kidneys to sequester fluid and sodium, thereby keeping the intravascular volume high, leading to further increases in blood pressure.[99-102] The entire system is counteracted by various mechanisms including alternative angiotensin peptide isoform signaling or compensatory signaling cascades, leading to potent vasodilation.[12, 103-105] However, in pathologic conditions, the RAAS system has been known to drive increases in systemic blood pressure with resistance to counteracting mechanisms.

Sensitivity to Ang II is a hallmark of both PE and hypertension pathology.[14, 100, 106-108] Alterations of the methyl-transferase activity within the cell leads to changes within the cytosine-rich segments of DNA within the promoter regions known as CpG islands. These alterations have been studied in hypertension. Some studies have shown alterations in the 5-methyl Cytosine (5mC) content in the promoter region of key RAAS components—Angiotensin converting enzyme and angiotensinogen.[79, 109] While hypermethylation has been shown to regulate ACE expression and is associated with hypertension, hypomethylation of the angiotensinogen promoter was associated with a greater

expression angiotensinogen and was further driven by increases in salt intake. Furthermore, it has previously been reported that the angiotensin receptor gene is hypomethylated in a salt-sensitive rat progeny as early as the first week postpartum.[79, 100, 106] Increased vascular sensitivity to Ang II has also been implicated in aortic, carotid, and small vessels in Ang II-exposed previously preeclamptic mouse and rat models.[89, 100, 110] This could suggest an exaggerated RAAS response, as well as be evidence for the importance of methylation status in hypertension.

Link between Inflammation and Intrauterine Stress

Numerous studies have linked the pathologic activation of the RAAS to presence of an autoantibody against the AT1R (AT$_1$-AA). While levels of AT$_1$-AA are positively correlated with hypertension and renal damage in pregnancy, the mechanism of development of this antibody has not been elucidated. While pathologic activation of the AT1R has been linked to elevations TNF-alpha, IL-6, ET-1[24, 25, 27, 28, 31-33, 35, 36, 111], it is unclear if inflammation or epitope presentation is the stimulus for autoantibody production. Recent data has characterized the roles of a TNF superfamily member, LIGHT, and a glutamyltransferase, tissue transglutaminase (tTG) as necessary for production of AT1-AA.[64] LIGHT-injected pregnant mice display autoantibody production via induction of a ε-(γ-glutamyl)-lysine isopeptide dimerization in the AT1R. This modification is blocked by a specific tTG inhibitor, cystamine.[112] This finding links the presence of acute inflammation via TNF activation, tTG, and the production of autoantibody in PE states (Luo R, under review, *Hypertension*).

Given these data, we present a novel mechanism for the transgenerational passage of epigenetic modifications and the role they play in the development of hypertension. We outline two necessary features integral for hypertension in a genetically homogenous mouse model. We **hypothesize** that hypertension requires:

A. **Exposure to intrauterine stress (i.e. preeclampsia stimulus)**

B. **Subsequent exposure to provasoactive substance (i.e. angiotensin II)**

Through the data presented here, we show that intrauterine stress results in initial hypomethylation of the angiotensin II type I receptor promoter within CpG islands. Hypomethylation of this promoter leads to sensitivity to angiotensin II exposure and drives further hypomethylation of the AT1R promoter. This subsequently leads to increases in further AT1R expression and the failure to rectify the hypertensive insult. We also show that the pattern of hypomethylation can be transmitted through subsequent generations of progeny, leading to similar phenotypes and epigenetic genotypes in their progeny (Fig. 2-1). The study presented leads to tighter treatment of PE during the disease course and could lead to increased prediction of the PE and hypertension based on promoter methylation status.
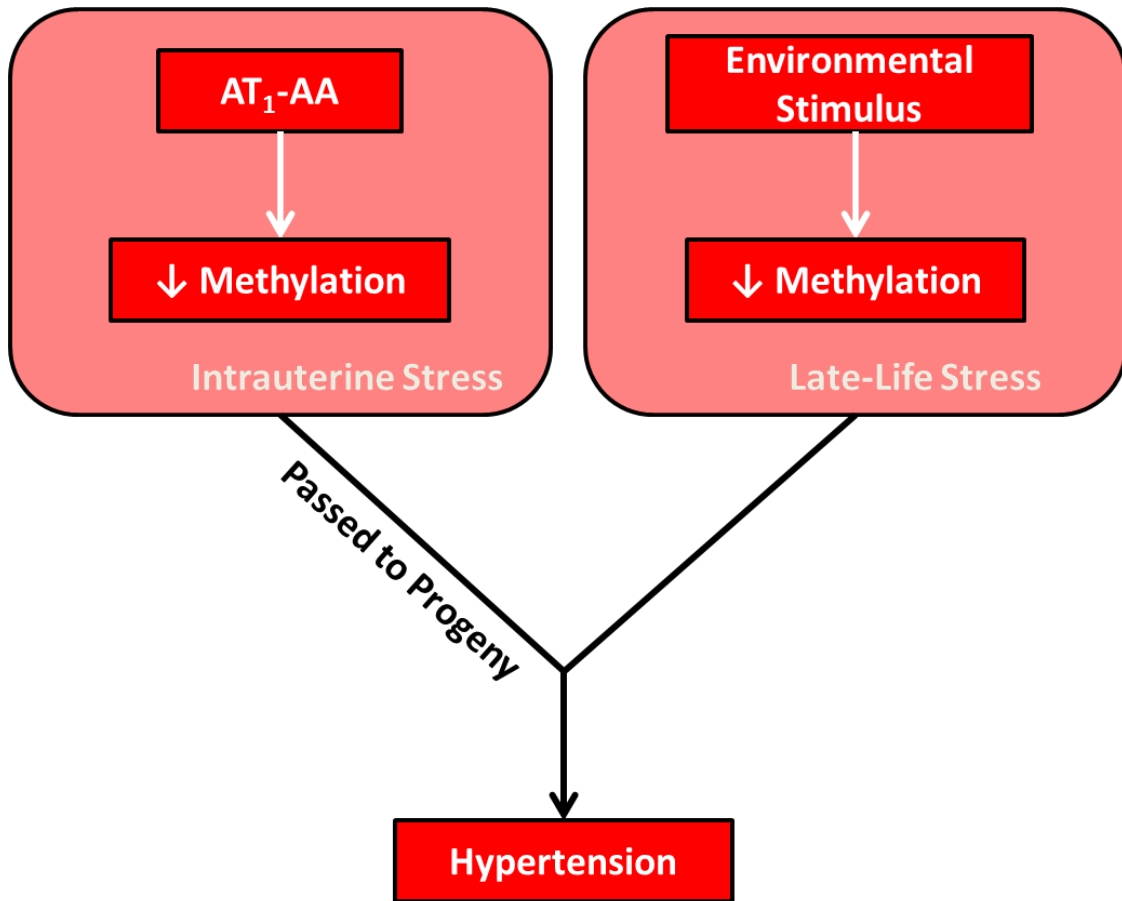
Figure 15. Hypothetical model of intrauterine stress and epigenetic modification.
We posit a two-hit hypothesis as integral to development of multigenerational A-HTN. *In utero* stress predisposes the fetus to hypomethylation of key RAAS genes, leading to their increased expression. Through mechanisms currently not understood, it has been shown that methylation patterns can be transmitted to subsequent generations. We believe that these methylation patterns, in tandem with exposure to environmental stress, will elicit a greater response, and, thus, cause features of hypertension.

Methods

Mouse Model and Microosmotic Minipump Implantation

Pregnant C57BL/6 mice were obtained on E13 of gestation (Harlan Laboratories; city). After a brief quarantine and acclimation period, baseline blood pressure was measured via tail-cuff methodology as previously described (Coda; Kent Scientific, Torrington, CT). Baseline mean systolic pressure was calculated from 20 repeated cycles of the CODA machine. After baseline blood pressure, female mice were injected retroorbitally with IgG isolated from normotensive (NT) and preeclamptic (PE) human patients as described in Irani, et al.[24, 25] Mice were injected on E13/14 of gestation. Repeat blood pressure measurements were performed diurnally until E18. Mice were allowed to deliver producing generation II. Weaning occurred at post-parturition week three. Pups were subdivided and half of the litters were bred together to produce generation III. Remaining pups were aged for 26 weeks, during which time blood pressures were measured via tail-cuff methodology. At 26 weeks, microosmotic minipumps (Alzet, Cupertino, CA) programmed to deliver 140 ng/kg/min of human angiotensin II (Sigma-Aldrich, St. Louis, MO) were implanted in the dorsal region of the mice through a 1 cm incision sub-cranially. Mice were monitored post-operatively for infection and recovery. Blood pressure was monitored via tail-cuff on minipump-implanted mice on D3, 7, 10, and 13 post-implantation. Mice were subsequently sacrificed on D14 for organ and histological analysis. Mice husbandry care was undertaken by Center for Laboratory Animal Medicine and Care (CLAMC) and Animal Welfare Protocol UT AWC-11-073/14-090.

Similar protocols were performed on generation III mice.

Quantitative Real-Time PCR

RNA was obtained from mouse renal tissue isolated and frozen in liquid nitrogen. RNA was reverse-transcribed into single-strand cDNA with use of the Quantitect 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. Results were calculated using $2^{-\Delta Ct}$ method using housekeeping gene for ratio comparison.[62]

Western Blotting

Presence of AT1R was confirmed by standard wet transfer methods. 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, Grand Island, NY). 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, Darmstadt, Germany). Membranes were probed with anti–mouse primary antibodies raised against AT1R in 1:500 dilutions (Santa Cruz Biotechnologies, Dallas, TX). An antibody to β-actin housekeeping gene(Sigma Aldrich, St. Louis, MO) was used as a loading control. Fluorescent conjugated secondary

antibodies were used for visualization of the membrane on LICOR imaging systems (LICOR, Lincoln, NE).

Bisulfite gDNA Treatment and PCR Analysis

To examine the methylation content of the renal AT1R promoter, bisulfite treatment and sequencing was utilized. gDNA was isolated from 50 mg flash-frozen kidney sections utilizing a DNeasy kit and protocol (Qiagen, Venlo, Norway). gDNA was analyzed by spectrophotometry on a multiwell Take3 plate and synergyHT plate reader (Biotek, Winooski, VT) and normalized to concentration. Normalized gDNA was subsequently treated with bisulfite compound according to the EpiJet Bisulfite protocol (Thermo Scientific, Waltham, MA). To analyze promoter regions of the AT1R, the promoter sequence of AT1R was analyzed by MethPrimer software. Primers were designed to target expected CG-rich islands, based off of designated parameters.[78, 89, 113] Primers to test the methylated and unmethylated regions were designed and subsequently used in qRT-PCR.

Sequences of designed primers used were:

5'→3' Methylated Forward AGTTTGGATTTGGAAGGGTATATC and Methylated Reverse AACTACCTAACGATCAACAACGAA

5'→3' Unmethylated Forward GAGTTTGGATTTGGAAGGGTATATT and Unmethylated Reverse CAACTACCTAACAATCAACAACAAA.

CpG island methylation ratio was taken as ratio of unmethylated amplification:methylated amplification subtracting the background of amplification from non-bisulfite treated gDNA using the $2^{-\Delta Ct}$ method.

Crosslinked Chromatin Immunoprecipitation Assay

Tissue extracted from mice treated with and without Ang II was flash-frozen in liquid nitrogen. ~50 mg sections were cut from the kidney and underwent crosslinkage reaction using 1.5% formaldehyde in MeOH. Samples were homogenized, and chromatin was isolated from the kidney sections. A transcriptional predictive analysis examining promoter sequence homology found that c-Jun (AP-1) transcription factor has a 87% sequence homology with the AT1R promoter region. Based on these results, 25 μg crosslinked chromatin isolated from the kidney sections was incubated with c-Jun antibody (Abcam, Cambridge, UK) at a ratio of 3 μg antibody:25 ug chromatin on magnetic protein A/G beads overnight at 4 C. Chromatin samples were washed, eluted and de-crosslinked according to protocol (ActiveMotif, Carlsbad, CA). Chromatin samples and input samples were analyzed by qRT-PCR as previously described above.

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 turkey's test, where appropriate. Accepted variance was

69

standardized to p < 0.05. Data were graphed on Graph Prism Pro v5.0 or SigmaPlot, where appropriate.

Results

Intrauterine stress PE mouse model

It is well accepted that intrauterine stress can have significant developmental ramifications to affected fetuses. Such developmental abnormalities can be observed within the renal, neurological, and, most significantly, cardiovascular system. Our study utilizes an established model of intrauterine and maternal stress by using an adoptive transfer model of human AT_1-AA isolated from PE patients. Previous models have demonstrated that isolated AT_1-AA injected into pregnant mice induces key features of PE including hypertension and renal damage as well as features of intrauterine growth restriction in the fetal milieu.[24, 25, 27, 28] Our model uses these features of PE to represent intrauterine stress and developmental delay.
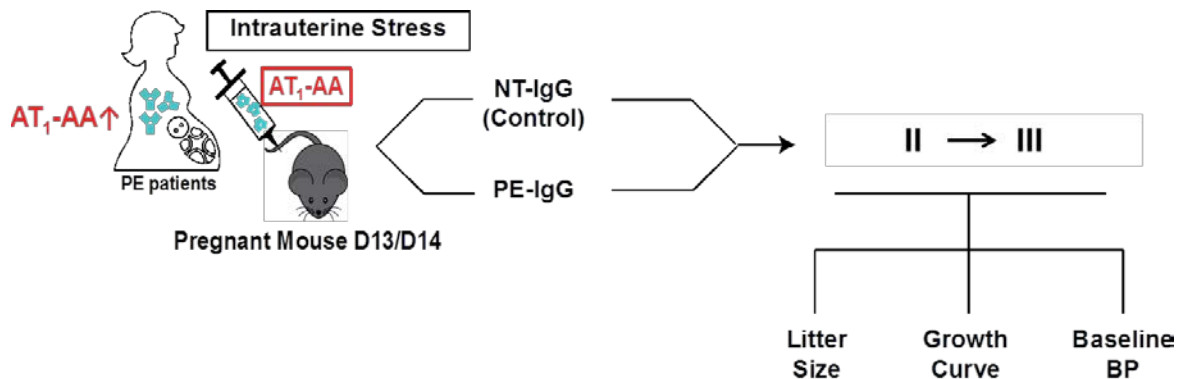
Figure 16. Model of adoptive transfer of IgG to pregnant mice on embryonic day 13/14 precipitates PE features. A transgenerational mating scheme was developed to produce Generation I, II, and III.

Our experimental model involves isolation of IgG from both normotensive mothers (NT-IgG) to serve as control and IgG from PE mothers (PE-IgG). IgG was injected into mice on embryonic day 13 and 14 of gestation. Pups (generation II) were born to affected and control mothers. Generation II mice were monitored for growth and blood pressure. Generation II mice were interbred to produce generation III mice and these were monitored for physiologic characteristics in the same manner as generation.

After parturition, baseline characteristics were measured in the pups and examined for significant phenotypic differences (Fig 2-2). To confirm the presence of true *in utero* stress and PE features, we analyzed the AT_1-AA and NT-IgG injected mice. We discovered that pregnant mice injected with AT_1-AA had significant hypertension and renal damage, as reflected by microalbuminuria, which resolved in the postpartum period (Fig. 2-3). Features of PE in the mouse model resolved by postpartum day 10, corresponding to approximately week 12 of human postpartum periods, similar to the disappearance of features in human PE pregnancies.

73
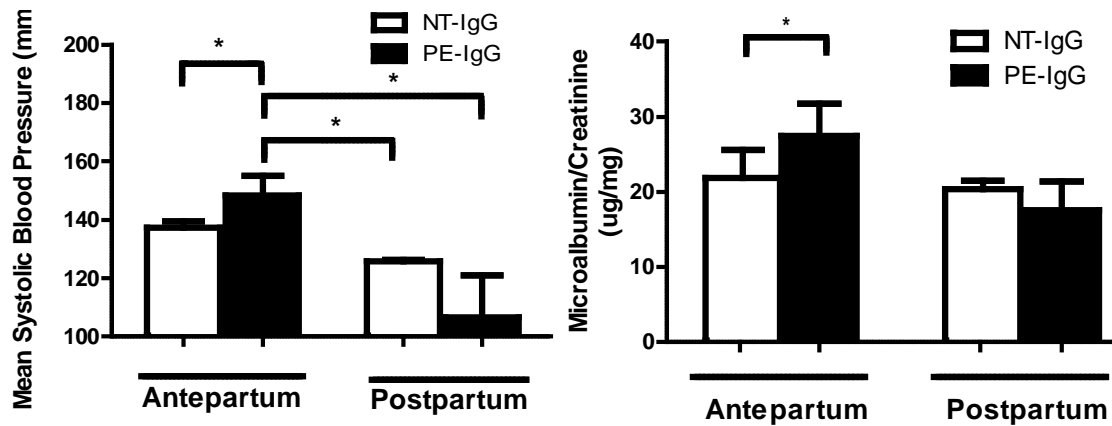
Figure 17. Mean systolic pressure and microalbuminuria are significantly increased in PE-IgG injected generation I mice but these features resolve in the postpartum period.

(Left) In PE-IgG injected dams (Generation I), mean systolic pressure was significantly increased in the antepartum period. This increased systolic pressure resolved in the 10-day postpartum period, similar to human PE chronology. n = 4-5; * = p < 0.05. (Right) Similarly, microalbuminuria was significantly increased in PE-IgG injected dams antepartum, but this increase in microalbuminuria was attenuated in the postpartum period. n = 4-5; * = p < 0.05.

74

Mice exposed to intrauterine stress demonstrate no phenotypic abnormalities

One of the key hallmark features of intrauterine stress is the presence of intrauterine growth restriction and, possibly, fetal abnormality. It is well-established that intrauterine growth restriction results in features of prematurity which often result in developmental delay organ-system specific abnormality. We observed the pups born to the *in utero* stressed pregnancy and subsequent generations. Because literature has noted a pup and litter size decrease in those pregnancies affected by AT_1-AA, we monitored the growth curve for significant difference between the AT_1-AA and control pups. Expectedly, we observed a difference in male/female weights, but no significant difference in the growth rates of pups born to AT_1-AA affected mice or to subsequent pregnancies (Table 3).

Table 3. Mouse growth curve and weight characteristics (g).

	Male				Female			
	Week 4		Week 8		Week 4		Week 8	
	NT-IgG	PE-IgG	NT-IgG	PE-IgG	NT-IgG	PE-IgG	NT-IgG	PE-IgG
Generation II	19.1 ± 1.01	18.06 ± 1.33	19.73 ± 1.17	19.60 ± 2.00	14.26 ± 1.10	17.06 ± 3.23	19.93 ± 3.38	19.91 ± 1.89
Generation III	17.76 ± 0.28	19.46 ± 1.3	21.05 ± 1.4	20.12 ± 0.57	17.1 ± 0.78	17.9 ± 1.27	18.99 ± 4.2	20.56 ± 1.05

Noting that the growth curves and litter sizes of the mice were similar, we investigated whether mice born to affected pregnant mothers had alterations of inflammatory and RAAS components as has been demonstrated in offspring of stressed pregnancies. To determine this feature, we examined mRNA values of various inflammatory markers and RAAS components via qRT-PCR from cDNA isolated from the kidney of generation II mice. Through a qRT-PCR array of kidney cDNA isolated after pup delivery, we found no difference in renin, angiotensin converting enzyme, angiotensin II type II receptor, nor aldosterone within the RAAS. We also found no difference in transcript levels of commonly deranged inflammatory and vascular signaling components such as tumor necrosis factor-alpha, endothelin-1, endothelin receptors A/B, nor endothelin converting enzyme. These data indicate pups born to *in utero* stressed environments experience no baseline derangements in terms of developmental patterning, nor do they experience alterations within their various vascular and inflammatory signaling pathways. However, these data do not completely explain the increased incidence of adult-onset hypertension seen in adult progeny of *in utero* stressed pregnancies in the absence of genetic factors. To reveal this feature, we decided to investigate each generation of progeny with a pro-vasoactive stimulus during the progeny adult period.

Low-dose Angiotensin II (Ang II) results in significant systolic pressure increase in progeny born exposed to in utero stress

While the data indicate that progeny born after exposure to *in utero* stress are phenotypically normal, observational clinical literature has noted a significant prevalence in adult-onset hypertension in the progeny which have been exposed to intrauterine stress. To test this observation in our mouse model, we aged progeny born from exposure to AT_1-AA for 24 weeks before exposing them to a low-dosage of Ang II (140 ng/kg/min)[114] (Fig. 2-4).

Figure 18. Schema of 24 week aging mouse model followed by low-dosage Ang II infusion. Mean systolic pressure was monitored at regular intervals during the experiment.

Mice born to both PE-IgG and NT-IgG injected mothers were aged for six months and implanted with an osmostic minipump containing a low-dosage of Ang II (140 ng/kg/min). Blood pressure was measured after pump implantation on days 3, 7, 10, and 13. This same schema was used in the subsequent generation to determine if this phenomena travels transgenerationally.

Low-dosage Ang II is not thought to be a model of intrinsic renal damage, however, the low-dosage is adequate to demonstrate an increased sensitivity to a provasoactive stimulus. To verify that the aging process induced no significant effect on mean systolic pressure, baseline blood pressures were taken at week 12 of aging. We demonstrated that mean systolic pressure is not affected by aging at this early timepoint (Fig. 2-5).
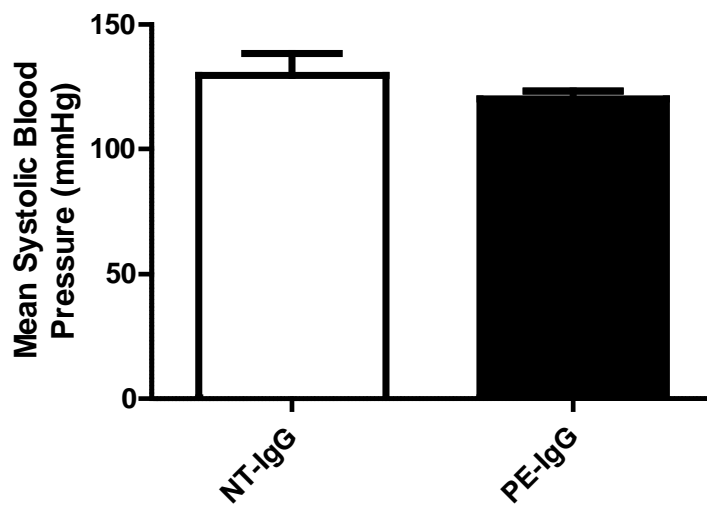
Figure19. Mean systolic blood pressure measured at week 12 indicated no significant difference in mean systolic pressure.

Generation II mice showed no significant difference in mean systolic blood pressure at week 12 after delivery. n = 6 mice

To test Ang II sensitivity, microosmotic minipumps containing infusion dosages of 140 ng/kg/min were dorsally implanted into mice born after exposure to intrauterine stress as well as control mice. During the period of implantation, mean systolic pressure was monitored. Mice born from exposure to intrauterine stressed environments show a significantly earlier elevation, as well as a sustained elevation of mean systolic blood pressure as compared to control pups (Fig. 2-6).
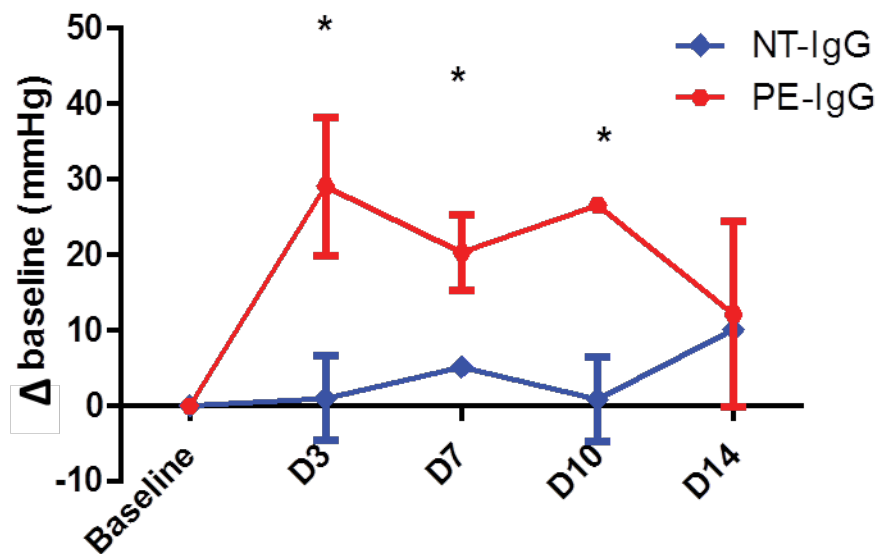
Figure 20. Low-dose Ang II resulted in more significant and earlier elevation in mean systolic pressure in generation II mice.

Implantation of a microosmotic minipump infusing a low-dosage of Ang II demonstrated that generation II mice born to PE-IgG infused dams had an earlier and larger increase in mean systolic pressure from baseline than did mice born to control dams. * = $p < 0.05$; 4-6 mice/group.

These data indicate that pups born exposed to intrauterine stress lack mechanisms to rectify increases in vascular tone. Moreover, to test that these features are also present in subsequent generations, generation II is interbred to produce generation III.

To test similar features in generation III, the same experimental plan was followed, as previously described. Generation III mice were aged and measured at midpoint through the aging period to determine if age had a significant effect on baseline blood pressure. Baseline blood pressure measurements taken at week 12 show no difference in mean systolic pressure between interbred generation III mice (Fig 2-7).
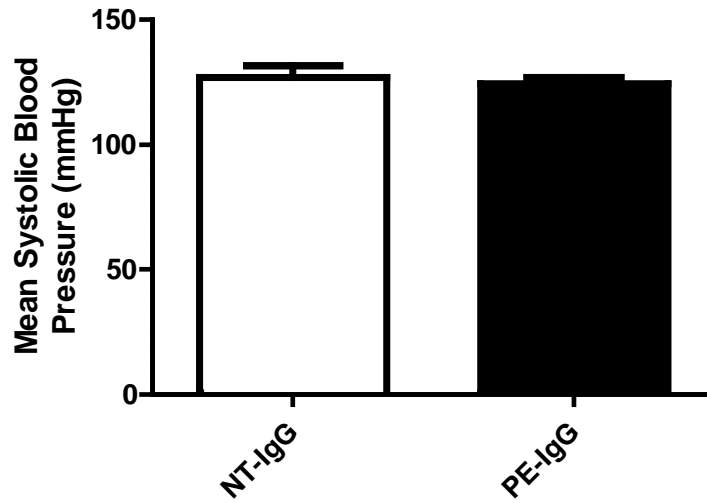
Figure 21. Generation III mice showed no significant difference in mean systolic pressure at week 12.

Similar to generation II, generation III mice show no difference in mean systolic

pressure at week 12. n = 6 mice

Thus, at week 24 mice were implanted with a microosmotic minipump with a dosage containing 140 ng/kg/min Ang II for infusion. Similar to generation II, mice exposed to intrauterine stressed environments in a prior generation experience an earlier and more significant increase in mean systolic pressure from baseline after Ang II challenge than normal control mice (Fig. 2-8).
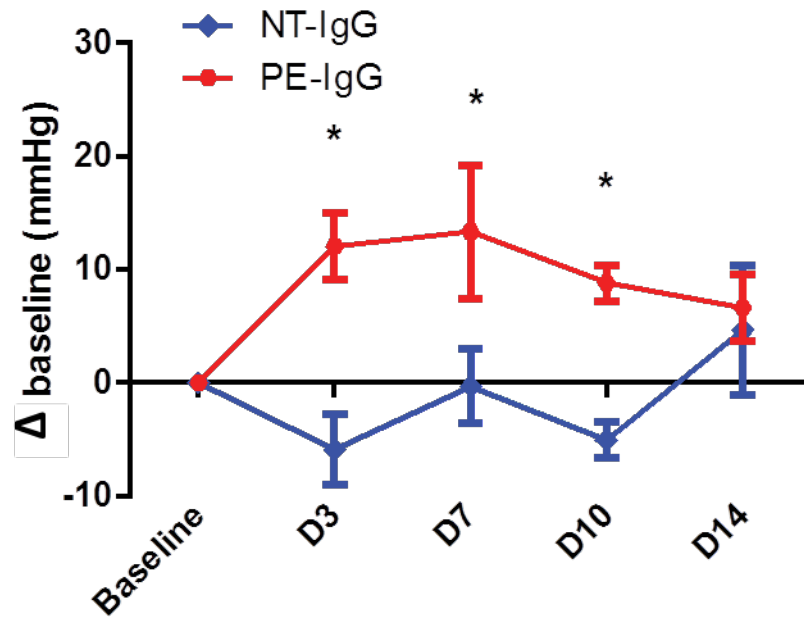
Figure 22. Generation III mice experienced a more significant and earlier increase in mean systolic pressure versus control mice.

Similar to generation II, generation III mice born to the lineage of stressed pregnancies and subsequently exposed to Ang II have a significantly earlier and sustained elevation of mean systolic pressure than control mice. * = $p < 0.05$; n = 6 mice/group

These data indicate an interesting paradox—intrauterine stress in one generation can prime future generations for increases in mean systolic pressure, given a stressor. This increase in hypertension is difficult for the organism to rectify. However, the mechanisms behind this increase in blood pressure and its presence in subsequent generations has not been characterized.

Epigenetic modifications of the AT1R predispose increased AT1R expression levels and hypertension

With a low-dosage of Ang II, we witnessed an increase in mean systolic pressure in animals exposed to intrauterine stress. We witnessed this feature propagating from the second to the third generation. However, the molecular mechanism behind this increased hypertension is poorly characterized.

It is well-accepted in other research fields that epigenetic modifications are responsible for features that are outside of the scope of genetic mutation. It is thought that epigenetic mutations are responsible for many disease processes in the fields of cancer and neurological conditions. Utilizing a genetically homogenous mouse population, we are able to characterize epigenetic changes in mice that examine changes to expression apart from genetic alteration.

To examine epigenetic changes within our mouse progeny, we utilized a bisulfite PCR screening to examine the epigenetic modifications of promoter regions of the AT1R. Because Ang II was used as the stimulus for the vasoactive challenge, and in the absence of alterations of other RAAS and inflammatory signaling factors, we looked to the AT1R as a likely candidate for modifications.

86

We designed a sequence of primer sets to test the methylation content of CG-rich sequences within the AT1R promoter region. This common technique assesses the methylation content of cytosine residues within the gene promoter region. Bisulfite sequencing revealed a significant hypomethylation of the promoter region between 597-714 base pairs upstream from the transcription start site in mice born from intrauterine stressed environments. Furthermore, this hypomethylation is exacerbated by Ang II treatment. This finding suggests that methylation patterns are imprinted during the intrauterine period and exposure to Ang II results in further hypomethylation (Fig. 2-9).
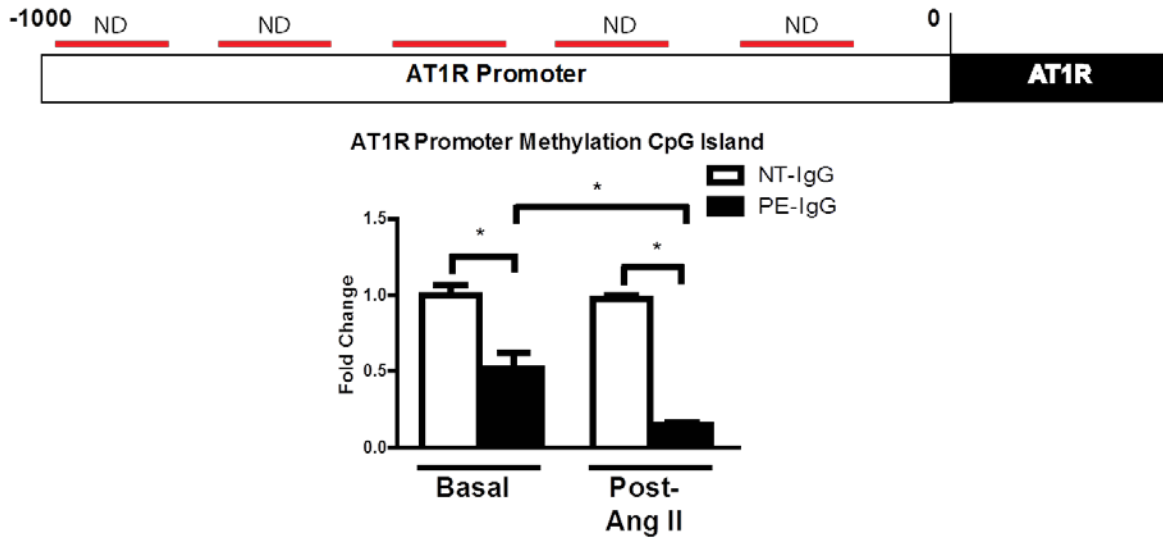
Figure 23. The AT1R promoter was hypomethylated _in utero_ and further hypomethylated with exposure to Ang II.

(Top panel) Five CpG primer pairs were designed based on promoter sequencing prediction. Only a region between 597-714 bp upstream from the transcription start site showed amplification. (Bottom panel) Bisulfite sequencing revealed that the AT1R promoter region in generation II isolated renal tissue is initially hypomethylated _in utero_ prior to Ang II challenge. Hypomethylation is further exacerbated by Ang II challenge. * = $p < 0.05$; 3-4 renal samples/group

This pattern of hypomethylation has significant effects on AT1R expression. AT1R expression is increased in pups exposed to intrauterine stress, but, this difference is exacerbated by treatment with Ang II. This expression data was confirmed by qRT-PCR and immunoblotting data from kidney cDNA and membrane receptor isolation, respectively (Fig. 2-10).

89
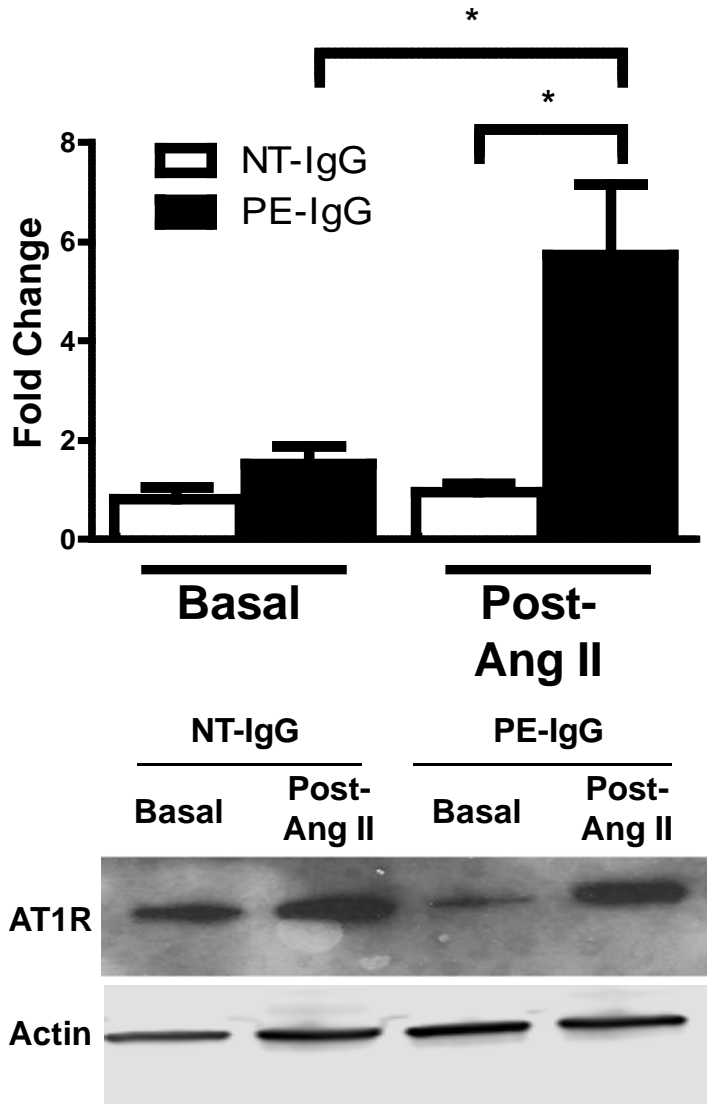
Figure 24. AT1R expression increased with Ang II exposure as confirmed by qRT-PCR and immunoblotting.

(Top panel) AT1R mRNA expression in generation II indicated that expression of AT1R initially is elevated *in utero* but is exacerbated by treatment with Ang II. This expression level is confirmed by qRT-PCR. (Bottom panel) Representative immunoblotting revealed that Ang II treatment increased expression levels of AT1R from basal levels. * = $p < 0.05$; n = 3-4 renal sections/group

Extending this hypothesis to the subsequent generations, we investigated whether exposure to intrauterine stress had an effect the third generation. Thus, we performed similar experiments on the third generation to test if the animals had the same methylation patterns as the second generation. Bisulfite sequencing showed that the AT1R promoter region was hypomethylated in the same region as the second generation animals. Furthermore, in the presence of Ang II stimulus, generation III animals exposed in their previous generation experienced more significant hypomethylation in a key CG rich region as opposed to control animals. Examining expression of AT1R, we witnessed a higher expression of AT1R in generation III animals exposed to *in utero* stress. This was exacerbated once again by exposure to Ang II stimulus (Fig. 2-11).
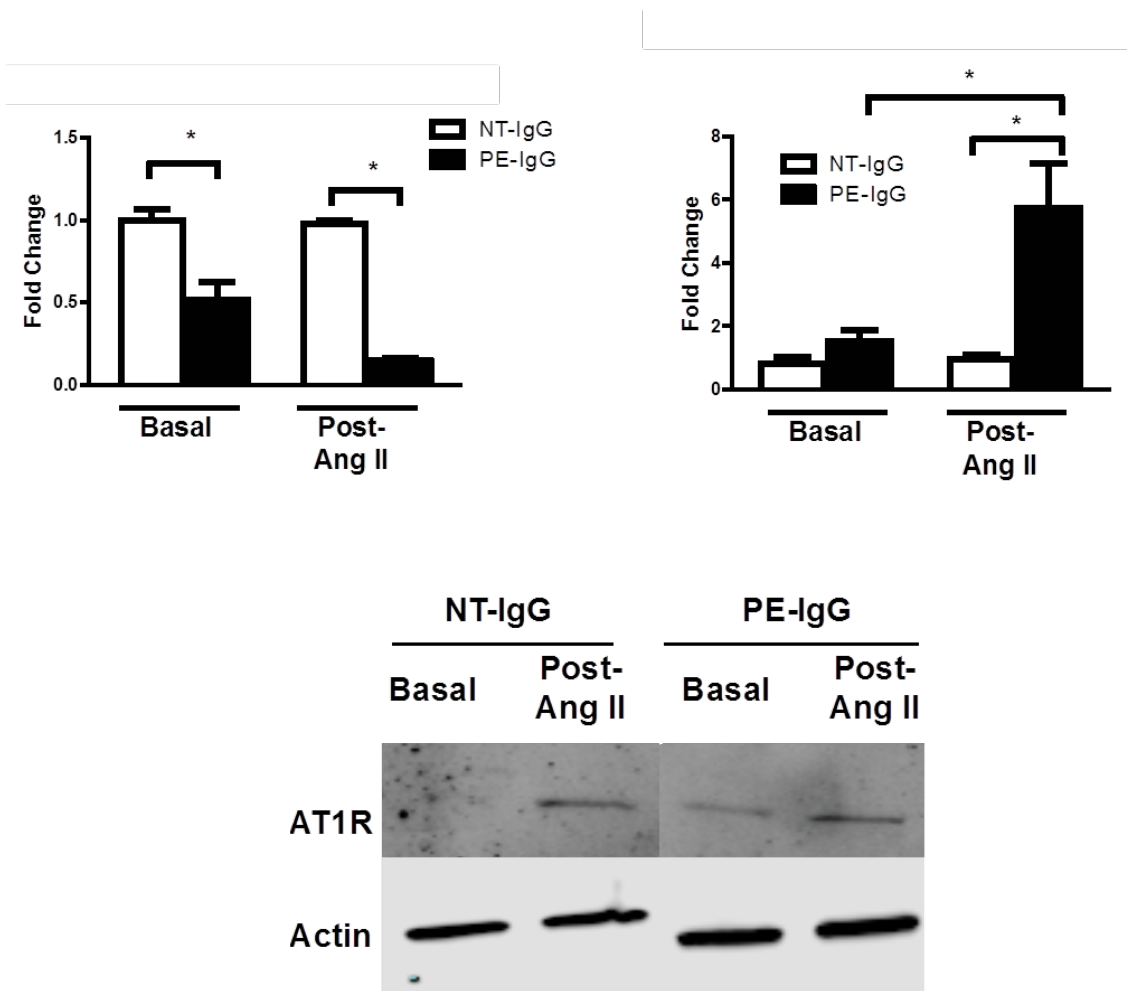
Figure 25. AT1R specific methylation was decreased and AT1R expression is increased as confirmed by qRT-PCR and immunoblotting in generation II mice.

(Top left panel) Mice born to a lineage of *in utero* stress (generation III) display a

similar pattern of methylation as generation II. CpG island methylation is

decreased *in utero* prior to Ang II challenge, but displays a further decrease with

low-dose Ang II treatment. (Top right panel) Decreases in methylation precipitate

an increase in AT1R expression evident after treatment with Ang II as confirmed

by qRT-PCR. (Bottom panel) AT1R expression is elevated in mice born to *in*

utero stressed lineages. The AT1R expression is further exacerbated by

treatment with Ang II as confirmed by immunoblotting. * = p < 0.05

These data suggest a key finding in the pathogenesis of vascular disorder and the heritability of these features. We noted that sensitivity to a stimulus is determined by amount of hypomethylation of the AT1R promoter region. Furthermore, provasoactive stimulus causes significant hypomethylation of the AT1R promoter. This hypomethylation activity has significant effects on gene expression. Finally, these data suggest that all of these features can pass through generations, implying that *in utero* insults in one generation can affect development of future generations.

Hypomethylation of AT1R promoter region results in AP-1 transcriptional activation

As reported in other literature, promoter modification can affect transcriptional gene activation or repression by influencing the binding affinity of transcription factors at key sites. To assess the degree of transcriptional binding affinity, chromatin binding immunoprecipitation assays (ChIP) is frequently used. These assays determine the binding efficiency of transcription factors in a promoter sequence. Because of the observation of differential promoter methylation in our mouse model, we decided to test the binding efficiency of transcription factors in the hypomethylated promoter region.

Prior to designing targets for ChIP assay, we performed a blinded sequence analysis on the hypomethylated promoter region. By BLAST analysis, we determined that the c-Jun (AP-1) transcription factor had a predicted 88% sequence homology with the hypomethylated AT1R promoter region.

93

Interestingly, AP-1 is extensively involved in growth factor, vascular factor, and inflammatory factor signaling. Therefore, we decided to test the transcriptional affinity of AP-1 with hypomethylated AT1R promoter sequences. Utilizing crosslinking ChIP assays with chromatin isolated from the kidney of the generation I mice, we found an increase in AP-1 binding to the hypomethylated region of the AT1R prior to Ang II treatment. However, after Ang II treatment, we observed a 2-fold increase in AP-1 activation and binding in the AT1R promoter region. This indicates significant activation of AP-1 and a potential explanation behind the increased expression of AT1R both *in utero* and after provasoactive stimulus. We further extend these findings to generation III. Chromatin was isolated from kidney tissue of interbred animals prior to treatment with Ang II and subsequent to treatment.
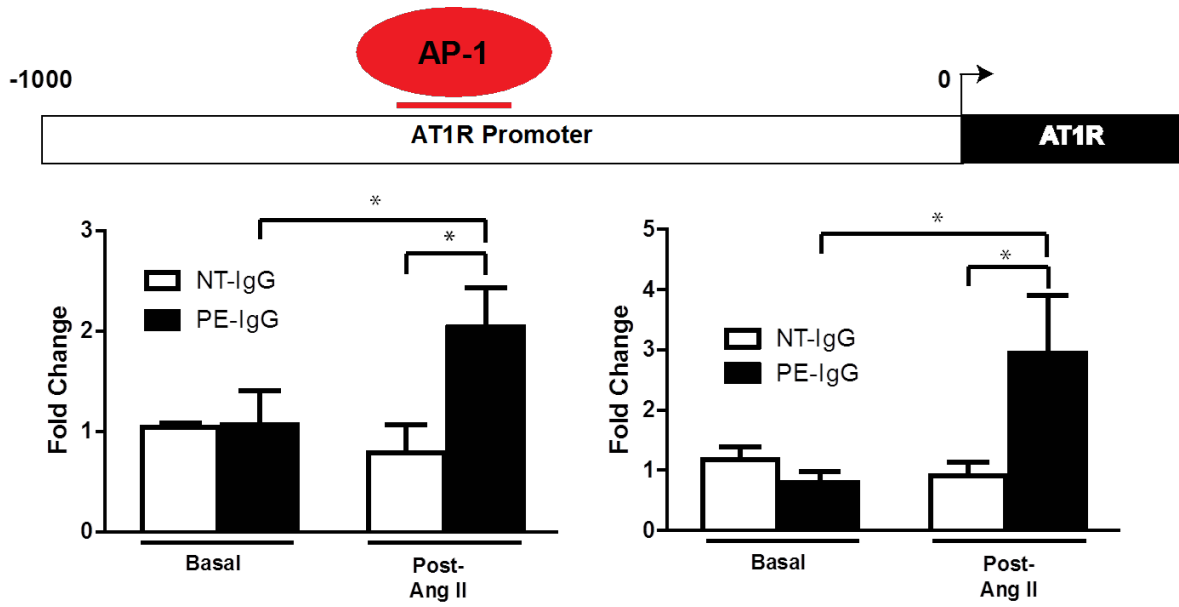
Figure 26. AP-1 transcription factor binding affinity is increased in hypomethylated AT1R promoter regions.

(Top panel) Transcription factor prediction by PROMO analysis showed an 88% sequence homology with a hypomethylated promoter sequence in the AT1R promoter. (Left panel) XChIP assays crosslinking AP-1 transcription factor to chromatin segments found greater AP-1 binding and amplification of hypomethylation AT1R promoter regions in generation II after Ang II treatment in pups exposed to *in utero* stress. (Right panel) In pups born from lineages of *in utero* stress (generation III), exposure to Ang II increases the binding of AP-1 transcription factor and subsequent amplification of specific hypomethylated sequences of the AT1R promoter.

Discussion

Presented here is the first mouse model to recapitulate the features of adult-onset hypertension when primed with an *in utero* stress. Genetically homogenous cohorts of mice were given IgG isolated from human patient cohorts to represent a PE pregnant model or normal pregnancy. PE is associated with hypertension and renal damage both in patients and in generation I of this mouse model. Similar to patients, PE features of hypertension and microalbuminuria resolved in the murine postpartum period. In generation II, after the mice were aged for 24 weeks and exposed to a vasoactive peptide (Ang II), mice born from intrauterine stressed pregnancies were more susceptible to hypertensive features. Mechanistically, consistent with current literature in hypertensive disorders, we witnessed an activation of the RAAS with increased sensitivity to Ang II stimuli. Interestingly, these data presented here offer a unique two-hit mechanism to demonstrate how *in utero* stress precipitates hypertensive feature in progeny. We established that aberrant promoter methylation patterns occurring during intrauterine stressed environments can predispose an organism to increased transcription of the AT1R gene and receptor. This aberrant methylation pattern and increased transcription of RAAS components increases sensitivity to environmental stimuli, and thus, increases the chance for the organism to develop adult-onset hypertension. This new paradigm stresses the importance of the intrauterine milieu as causative for future development of disease in progeny. While the mechanism underlying promoter methylation pattern changes is not addressed in this work, our data is

96

consistent with other studies which also demonstrate decreased total methylation in PE states.

In addition to methylation changes in direct progeny born from *in utero* stressed environments, we also show that subsequent generations exhibit the same aberrant methylation patterns within the RAAS. Previous studies have demonstrated that epigenetic promoter methylation patterns can be transmitted between generations and imprinted onto progeny DNA. Thus, methylation patterns can affect subsequent generations genetic expression outside of genetic modifications. Our data demonstrate these similar features. Generation III, exhibits similarly decreased promoter methylation as the previous generation. Therefore, similar features of hypertension exist within this generation upon challenge with a vasoactive peptide.

97
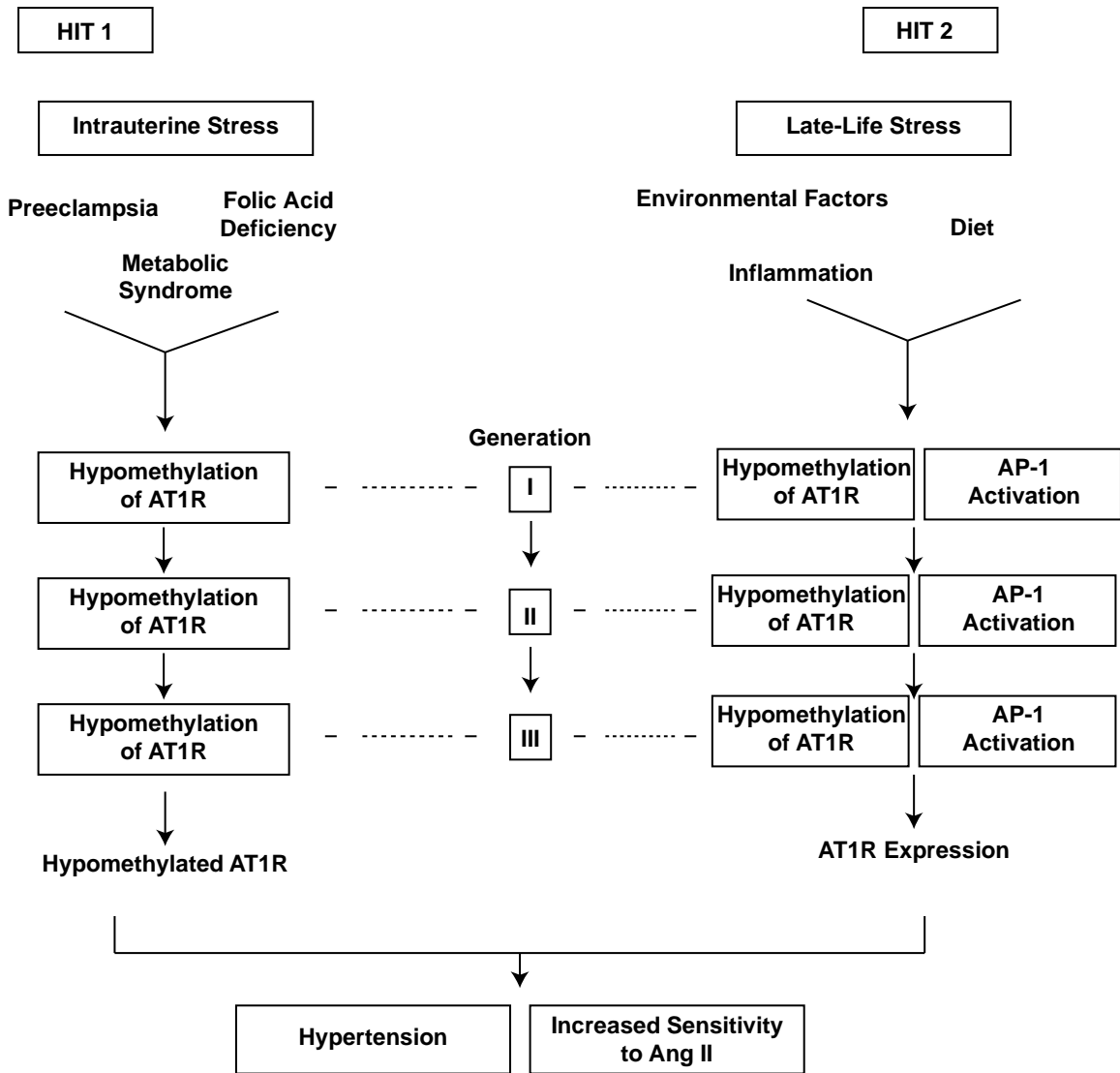
Figure 27. Working model of transgenerational passage of epigenetic features.

While we observe this two-hit hypothesis in our mouse model, we have not investigated the mechanism behind the alteration of epigenetic patterning in progeny nor the passage from one generation to the next. Some studies have demonstrated the importance of S-adenosyl methionine and methyl transferases as necessary for epigenetic alterations on the histone or promoter transcriptional level. Studies have indicated that intracellular stress modulates the activity of methyl transferase and the recycling of S-adenosyl homocysteine to S-adenosyl methionine. Therefore, stressed conditions have the ability to decrease methylated DNA within the promoter. Depending on the modification, this can have activating or inhibiting consequences for genetic transcription. Future studies will address how this pathway is modulated in times of intracellular or *in utero* stress. These studies will have important ramifications for risk stratification based on pregnancy status.

This study investigates the role of PE in the pathogenesis of adult-onset hypertension. We posit a unique combination of decreased AT1R methylation combined with increased expression of AT1R as instrumental in the activation of the RAAS. Previous clinical data has implicated PE as an important risk factor future cardiovascular risk. We also know that progeny born from intrauterine stressed pregnancies also have increased cardiovascular risk. Based off of these data, asking for familial incidence of PE may help us stratify a patient's risk to develop hypertension, stroke, or cardiovascular death. Furthermore, our study also investigates a specific mechanism of upregulation of the AT1R. By

99

assessing a patient's promoter methylation patterns, clinicians would have a picture of which messengers within the RAAS are altered. With further clinical and epidemiological study, our data may lead to more personalized treatment of hypertensive disorder and proper cardiovascular risk stratification.

CHAPTER III: CONCLUSIONS AND FUTURE DIRECTIONS

Disorders of hypertension during pregnancy present a unique challenge as a healthcare issue. Decades of research have provided insights into the pathogenesis of the disease; however, no definitive molecular process has outlined the disease in totality. Furthermore, the lack of definitive mechanism has also led to a lack of appropriate diagnostic tests to assess a mother's chance to develop preeclampsia or other hypertensive disorder of pregnancy. Finally, due to the length of prospective studies, we are unaware of the effect that *in utero* stress has on generations of progeny. Because of these problems, research in the field of preeclampsia is vital to our understanding of the disease pathogenesis and pregnancy, as a whole.

The studies presented here provide valuable insight into the pathogenesis of PE as well as posit potential therapeutic targets and diagnostic exams to treat and estimate a mother's risk for disease development. The data confirming the interplay between CRP, NKB and phosphocholine transferase, being well-known possible factors inciting vascular damage, examines a new pathogenic mechanism for PE. Moreover, the CRP-NKB-phosphocholine complex provides a novel therapeutic target and potential diagnostic panel to predict and diagnose PE prior to onset. Furthermore, the transgenerational mouse model is one of the first studies to address the influence of *in utero* stress as a potential mechanism of vascular genetic expression in a homogenous genetic population. The implication for PE priming vascular damage through subsequent generations provides an opportunity for progeny testing for hypertension risk.

The roles of CRP and NKB in the pathogenesis of PE have been extensively hypothesized in earlier literature. However, this report was the first to indicate that CRP acts as more than a nonspecific inflammatory mediator. We present evidence that CRP can combine with a posttranslationally modified and phosphocholinated NKB to activate the NK3R, a receptor instrumental in hypertension. These data novelly indicate that the placenta was a contributory source of CRP, and this excess production was instrumental in PE symptomology. The placenta is vital in the production of PE features and key to therapeutics.

In recent decades, the placental-fetal environment has become a potential target for study in the heritability of PE and hypertensive disorders. While much research has been dedicated to investigating genetic causes behind hypertension, other mechanisms have been less studied. Epigenetic mechanisms of disease are thought to underlie many pathogenic processes. Elucidation of epigenetic modifications could serve as prediction for adult-onset hypertension. Additionally, epigenetic screening could lead to targeted therapy for hypertensive disorder. By characterizing the epigenetic modification on various promoters, we may be better to able screen for upregulated genes and develop a targeted therapeutic to treat refractory hypertensive patients. Furthermore, by studying *in utero* stress as it propagates through generations, we can counsel women to avoid potential stressors, as they may affect their intrauterine environment, and, ultimately their progeny.

The novel data presented here posit a holistic view of preeclampsia, hypertension, and hypertensive disorders of pregnancy. First, we have demonstrated that acute inflammation and kinin signaling are integral in the pathogenesis of PE. The molecular complex formation of CRP joining with post-translationally modified NKB is essential to activation of the NK3R and production of PE features. More importantly, the placenta is required for CRP-induced PE features, as CRP has no effect in non-pregnant animals in the absence of inflammatory stimulus. Inflammatory stress and activation of the kinin signaling system leads to intrauterine stress, creating a malicious cycle harming the mother and fetus. The fact that intrauterine stress has been noted as a potential cause of developmental patterning dysfunction is an important clinical finding that we have translated into our mouse model. We have demonstrated that acute inflammation in the form of TNF-alpha superfamily members can induce elevations of pathogenic autoantibodies against the AT1R. The presence of AT_1-AA in maternal circulation is positively correlated with PE features, IUGR, and, thus, intrauterine stress. Through a multigenerational mouse model, we have shown that intrauterine stress can initiate hypomethylation in the AT1R promoter, and prime the developing fetus to development of hypertension. Further exposure to a stress increases risk of hypertension development. Without compensatory mechanisms, progeny are more sensitive to stimuli, and, thus, develop hypertension more than progeny born to normal pregnancies. More significantly, demehtyalted promoter regions are passed to future generations, thus, predisposing a fetus in the absence of a direct fetal stress. These findings,

in tandem, present numerous opportunities for preclinical diagnostics and targeted therapy (Fig. 3-1).
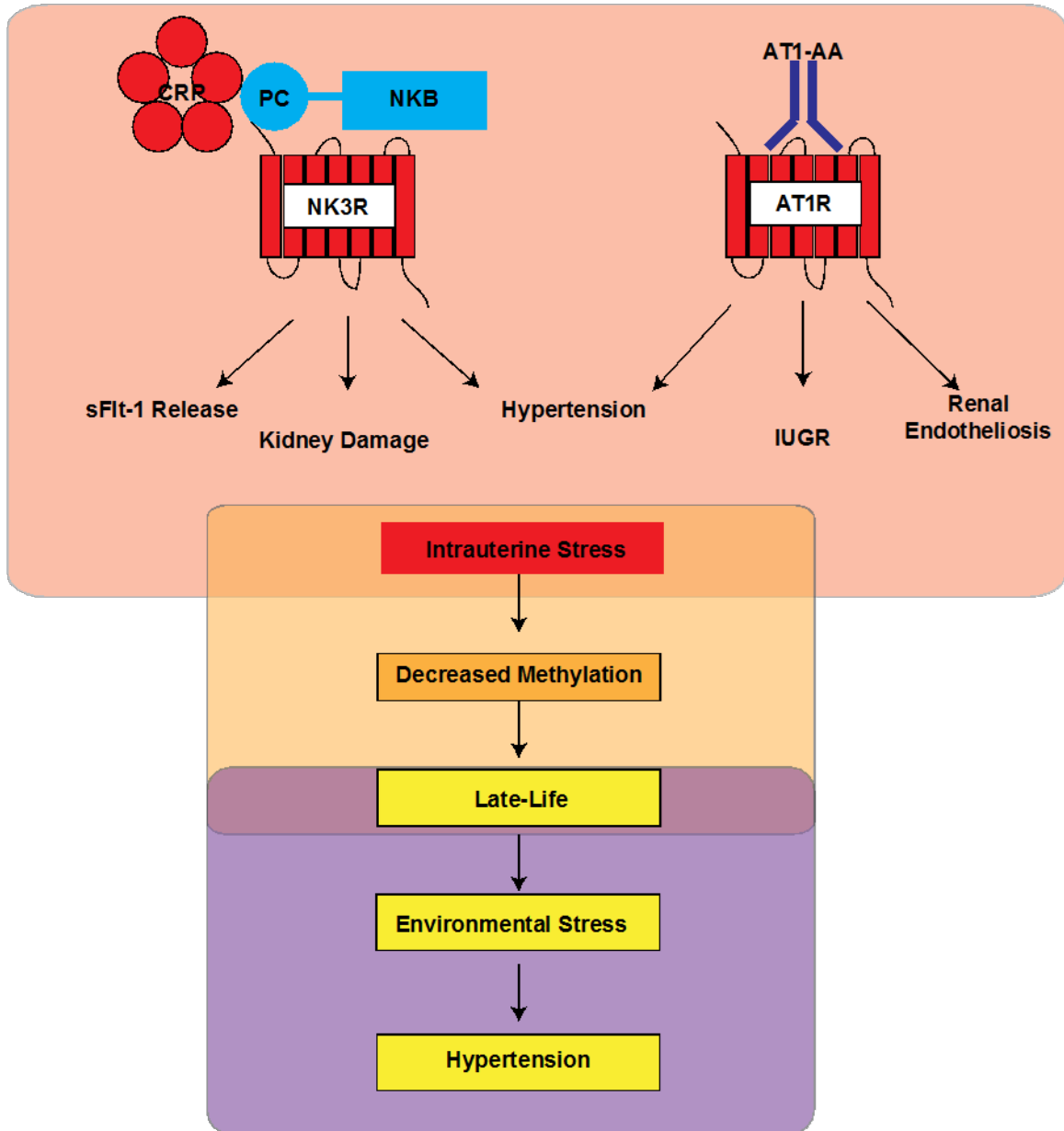
Figure 28. Working model linking intrauterine stress to transgenerational programming.

CRP-PC-NKB activation of the NK3R leads to pathogenic features of PE—hypertension, renal damage, and sFlt-1 expression. The contribution of these to

intrauterine stress can prime a developing fetus with hypomethylation of the RAAS system and risk of hypertension after a late life exposure to environmental stimulus.

Future Directions

The data presented here reveal a comprehensive picture of the pathogenesis of PE as well as offer explanations for transgenerational feature propagation. However, even with intensive research efforts, neither the totality of PE pathogenesis has not been elucidated, nor is research closer to finding effective therapeutics and diagnostic assays. One significant finding to further investigate is the value of assessing CRP and NKB prior to 20 weeks of gestation in our pregnant cohort. Currently, human sera and plasma samples from the **C**ombined **A**ntioxidant and **P**reeclampsia **P**rediction **S**tudy are available in various time points both ante- and post-partum. These samples would be a valuable tool to develop and test a diagnostic predictive clinical panel to predict the onset of PE. Furthermore, this panel could become part of a woman's routine clinical prenatal exam.

In addition to PE prediction, our study also addressed fetal developmental outcomes. We have demonstrated that epigenetic promoter modifications are conserved throughout generation. According to available literature, peripheral blood mononuclear cells provide an excellent tool for examining the methylation/epigenetic portrait of a patient's cells. By further characterizing the methylation aberration within the patient's cells, we would better be able to see the effect of intrauterine stress on progeny, and estimate a conferred risk to future generations. Furthermore, utilizing identified epigenetic markers, targeted treatment options are more effective.

107

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Vita

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