ONLINE SUPPLEMENT

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

By

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

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Materials and Methods

Human Subjects

Human materials were acquired from normal pregnant women (NT) and preeclamptic patients (PE) admitted to Memorial Hermann Hospital which were identified by the Obstetrics and Gynecology faculty of the University of Texas Medical School at Houston, or from nulligravid women (NG) with no significant past medical history in a gynecologic clinical setting. Patients were stratified to diagnoses of severe preeclampsia, mild preeclampsia, or normotensive based on blood pressure criteria set forth by the National High Blood Pressure Education Working Group (2000). Patients were classified as preeclamptic based on the presence of systolic blood pressure ≥ 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 silicone tubes were allowed to clot, meanwhile, the EDTA tubes were centrifuged at 2500x*g* for 15 minutes. After clotting, the silicon tubes were then centrifuged at 2500x*g* for 15 minutes. All plasma and sera was collected and stored at -80°C. The research protocols, consent forms were approved by the University of Texas Committee for the Protection of Human Subjects. Human subject data are summarized and included in Supplementary Table 1.

Animals

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

Measurement of Blood Pressure and Proteinuria in Mice

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

Immunohistochemistry/Immunofluorescence

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

Western Blotting

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

Placental Villus Explant Culture

Placental tissue from NT patients was obtained within 20 minutes of delivery. Placentas were transported on ice and prepared for processing. Placental tissue was isolated from the periphery of the placenta subcapsularly. 50 mg segments of placenta were perfused in DPBS, and then rinsed 2x in phenol red free DMEM (10% FBS; 1% Pen-Strep). Placenta segments were manually separated using scissors and forceps and incubated overnight at 37°C/5% 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.

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

Histologic Analysis

The kidneys and placentas of mice were formalin fixed and processed according to standard protocols. The tissues were sectioned in 5 μM serial sections and were stained via hematoxylin and eosin by standard pathology protocol. Glomeruli and placenta were examined by single-blinded study. Number and scoring of glomeruli was assessed by counting and averaging the number and score of glomeruli per 10 random high powered fields. Scoring was based on glomerular health: (1) corresponded to decreased bowman capsule space, intraglomerular loop dilation, and tubular dilation; (5) corresponded to adequate bowman capsule space, no loop dilation, and normal tubule diameter. Further description of glomerular scoring is provided in Zhang, et al., 2013.[6] Placenta calcification quantification was assessed by quantification of calcifications under 10 high powered fields. Both sets of quantification were performed via blinded analysis using Image Pro Plus software.

Statistical Analysis

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

Supplementary References

1. Wang W, Irani RA, Zhang Y, Ramin SM, Blackwell SC, Tao L, Kellems RE, Xia Y. Autoantibody-mediated complement c3a receptor activation contributes to the pathogenesis of preeclampsia. *Hypertension*. 2012;60:712-721.
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4. Irani RA, Zhang Y, Zhou CC, Blackwell SC, Hicks MJ, Ramin SM, Kellems RE, Xia Y. Autoantibody-mediated angiotensin receptor activation contributes to preeclampsia through tumor necrosis factor-alpha signaling. *Hypertension*. 2010;55:1246-1253.
5. Zhou CC, Zhang Y, Irani RA, Zhang H, Mi T, Popek EJ, Hicks MJ, Ramin SM, Kellems RE, Xia Y. Angiotensin receptor agonistic autoantibodies induce pre-eclampsia in pregnant mice. *Nat Med*. 2008;14:855-862.
6. Zhang W, Zhang Y, Wang W, et al. Elevated ecto-5'-nucleotidase-mediated increased renal adenosine signaling via a2b adenosine receptor contributes to chronic hypertension. *Circ Res*. 2013;112:1466-1478.

Supplementary 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 ± 11 mmHg	124.1 ± 11.68 mmHg	163.2 ± 25.99 mmHg
Diastolic Max	75 ± 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

Supplementary Table 2. Primers used for qRT-PCR

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

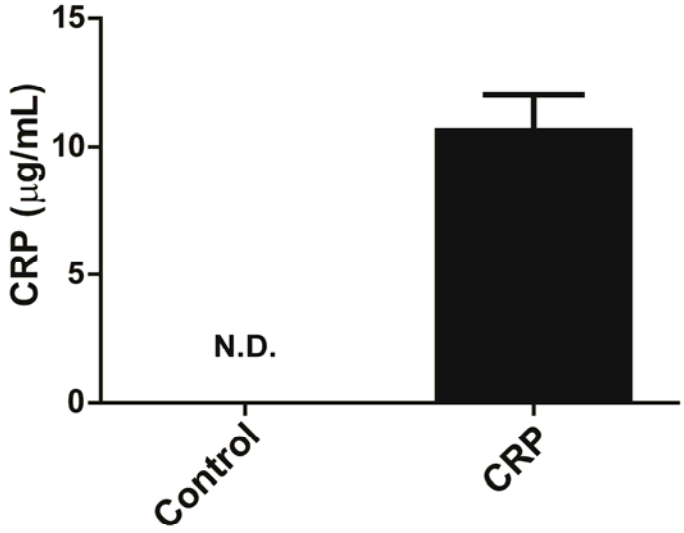

Figure S1. CRP levels verified at E18.5 timepoint. Levels of CRP in pregnant mouse sera at E18.5 timepoint were verified via ELISA. CRP injected pregnant mice were found to have a mean concentration of 11.6 μg/mL when sera was sampled at E18.5. Mice injected with control vehicle were found to have CRP levels below threshold of detection (N.D.).

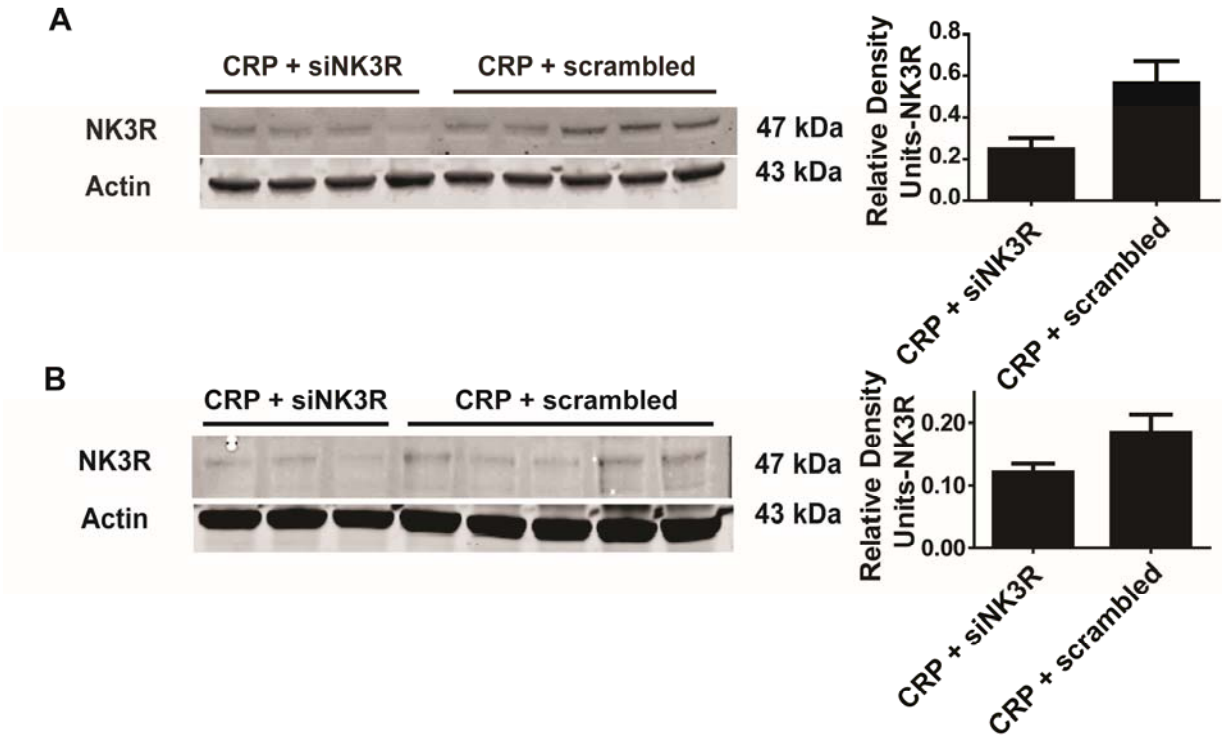

Figure S2. NK3R expression is attenuated in *in vivo* siRNA transfected mouse placental and renal tissue. (A) NK3R expression within the placenta is significantly attenuated in mice injected with CRP + siRNA specific for NK3R versus CRP + scrambled siRNA as verified by western blotting and quantified by ImageJ. Actin is shown for loading control (B) Expression of NK3R is also diminished within the kidney with CRP + siRNA specific for NK3R mouse injections, however, the difference is not as drastic due to a lower expression of NK3R within the murine renal tissue or reduced access to renal cells in contrast to placental trophoblasts. Quantification of western blotting is shown in relative density units. Actin is shown for loading control.