ONLINE SUPPLEMENT

MECHANISMS OF ENHANCED VASCULAR REACTIVTIY IN PREECLAMPSIA

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EXPANDED MATERIALS and METHODS

Study Subjects

Omental fat biopsies (approximately 2 cm x 4 cm x 2 cm) were collected from 40 normal pregnant and 9 preeclamptic women undergoing C-section at MCV Hospitals, Virginia Commonwealth University Medical Center. Preeclamptic patients had blood pressures of ≥140/90 mmHg on 2 separate readings 6 hours apart and proteinuria (0.3 gm/24 hours or ≥1+ urine dipstick). Blood pressure was recorded for each subject at pre-operation admittance by surgery registered nurses using a Drager Medical Infinity Delta Sphygmomanometer placed on the left arm with the subject supine and the sphygmomanometer at heart level. Cuff size varied depending on the size of the patient's arm. The first phase was used for systolic blood pressure and the fifth phase for diastolic blood pressure. Neutrophils were isolated from whole blood of 11 female subjects using dual Histopaque (Sigma, St. Louis, MO) density gradient centrifugation as previously described [1]. The Office of Research Subjects Protection of Virginia Commonwealth University approved this study, all subjects gave informed consent, and the procedures followed were in accordance with institutional guidelines. Clinical characteristics of the patient groups are given in Table S1.

Myograph Experiments

An omental fat biopsy was placed in Dulbecco's phosphate buffered saline (D-PBS, Invitrogen, Carlsbad, CA) on a silicone dissection dish pre-cooled to 4˚C. The formulation for D-PBS was calcium chloride (anhydrous) 100 mg/L, magnesium chloride 100 mg/L, potassium chloride 200 mg/L, potassium phosphate monobasic 200 mg/L, sodium chloride 8000 mg/L, sodium phosphate dibasic 2160 mg/L, D-glucose 1000 mg/L, sodium pyruvate 36 mg/L. An artery was identified under a dissecting microscope; a 1 cm length was dissected and mounted on glass micro-cannulae of a pressure myograph system (Model 110P, Danish Myo Technologies (DMT), Denmark, Netherlands). Omental fat arteries were studied because they are representative of systemic blood vessels and they contribute to total peripheral vascular resistance. Mean lumen diameter of the arteries studied was 287 ± 99 µ. Vessels were studied with endothelium intact and some with endothelium denuded. Endothelium was denuded by passing a fine glass micro-cannula through the vessel lumen. The vessel was immersed in 10 ml of D-PBS in the myograph chamber and secured at both ends using two 11-O silk suture ties at each end. The myograph chamber temperature was maintained at 37˚C and the vessel pressures maintained at constant inlet (45 mmHg) and outlet (42 mmHg) pressures to achieve flow through the vessel. The vessel chamber was aerated with compressed air. Changes in lumen diameter were continually recorded and used to calculate changes in resistance.

<u>Comparison of vascular reactivity between arteries of normal and preeclamptic pregnancy</u>: After a period of stabilization, endothelium intact arteries from normal pregnant and preeclamptic women were challenged with 60 µmol/L potassium chloride to assess vessel reactivity and viability. Angiotensin II (Ang II) dose response was then run in 10-fold increments for 10 min periods in concentrations of 0.001-10 µmol/L in the vessel chamber. Ang II doses were based on those previously reported by other

investigators studying in vitro vascular effects of Ang II in omental and pregnancy vessels [2-6]. Fresh buffer was placed in the vessel chamber and the vessel allowed to recover. Ang II dose response was repeated in the presence of superoxide dismutase (SOD, 5000 IU/ml, Sigma, St. Louis, MO) and catalase (150 U/ml, Sigma, St. Louis, MO) to quench reactive oxygen species (ROS) or Y-27632 (a specific RhoA kinase (ROK) inhibitor, 3 μmol/L, Calbiochem, Gibbstown, NJ) to specifically inhibit RhoA kinase. Catalase was used with SOD because superoxide quickly dismutates to hydrogen peroxide, which is the signaling molecule [7]. The vessel was contracted with potassium chloride after each treatment to assess viability and recharge intracellular calcium stores.

Effect of neutrophil products, ROS and tumor necrosis factor-alpha (TNFα): To assess the role of ROS to enhance vessel reactivity, arteries from normal pregnant women were used. Both endothelium-intact and endothelium-denuded vessels were used to assess the role of the endothelium. Ang II dose response was run alone and in the presence of a ROS generating solution composed of 0.36 mmol/L hypoxanthine (HX, Sigma, St. Louis, MO) and 0.003 U/ml xanthine oxidase (XO, Calbiochem, Gibbstown, NJ) added to the vessel chamber. After a 10-minute stabilization period to record the response to ROS alone, the Ang II dose response was repeated. After recording Ang II dose response in the presence of ROS, fresh buffer was added to the vessel chamber and Ang II dose response plus ROS was repeated in the presence of SOD/catalase or ROK inhibitor. Endothelium-denuded vessels were used to assess the effect of SOD/catalase and ROK inhibitor to ROS in order to obtain clean responses because endothelium-intact vessels displayed tachyphylaxis to Ang II. Ang II was also tested with TNFα (1 ng/ml), another neutrophil product.

Effect of neutrophils: The experimental design for neutrophils was similar to ROS except neutrophils were substituted for ROS. Endothelium denuded vessels were used. Isolated neutrophils were re-suspended in 1 ml of D-PBS and counted using a hemocytometer. Neutrophils were activated using human recombinant interleukin-8 (0.01 μmol/L, final concentration, R&D Systems, Minneapolis, MN) and perfused through the vessel lumen in a physiologic concentration of approximately 2000/mm^3. Ang II dose response was also tested with perfusion of un-activated neutrophils or interleukin-8 alone through the vessel lumen as controls.

Effect of ROS on norepinephrine (NE) dose response:
NE dose response was run in 2-fold increments, 0.15 to 5 μmol/L, and repeated in the presence of ROS generating solution. NE dose was based on that previously reported for omental arteries [8]. Response to ROK inhibitor and ROS was tested at a single dose of 1.25 μmol/L NE. Due to the phenomenon of tachyphylaxis with NE, the vessel was exposed to each dose of NE for only 2 minutes with a 20-minute recovery period in fresh buffer in between doses.

Immunohistochemistry
Omental fat samples were formalin-fixed, paraffin embedded and cut into 8 μm sections. Tissues were stained for CD66b, a neutrophil antigen, CD14, a monocyte/macrophage

antigen, and CD99, a lymphocyte antigen, as previously described [9-11]. Tissues were stained with the following antibodies: 1) mouse monoclonal IgM anti-human CD66b (1:50, BD BioSciences, San Diego, CA), 2) rabbit polyclonal IgG anti-human CD14 (titer 1:100, ProteinTech, Chicago, IL), 3) mouse monoclonal IgG1 anti-human CD99 (titer 1:400, Serotec, Oxford, UK). Negative control for CD66b was stained with a mouse isotype control (pre-diluted, Invitrogen, Carlsbad, CA). Images were captured with cellSens Imaging Software, Olympus America.

Western Blot

Primary human vascular smooth muscle cells (VSMC) were isolated from placental chorionic plate arteries and cultured as described previously [12]. VSMC were grown to confluence in T-25 flasks in M-199 with 10% FBS. Cells were treated for 5 minutes with 10 µmol/L Ang II alone or with Ang II plus activated neutrophils (50,000 neutrophils/ml) or Ang II plus ROS with and without ROK inhibitor, 3 µmol/L. The treatments and 5-minute time period were chosen to correlate with vessel myograph experiments. ROS generating solution was composed of 0.05 mmol/L HX and 0.003 U/ml XO. Western blot was performed using phospho-myosin light chain 2 (Ser19) antibody (pMLC, Cell Signaling Technologies, 1:2000, Beverly, MA), total myosin light chain 2 antibody (MLC, Cell Signaling Technologies, 1:300), phospho-myosin phosphatase target subunit 1 (Thr696) antibody (pMYPT1, Santa Cruz, 1:1000), total myosin phosphatase target subunit 1 antibody (MYPT1, Cell Signaling Technologies, 1:1000) and β-actin antibody (Sigma, 1:10,000). Blots were scanned and quantified using the Licor Odyssey system. β-Actin was used to normalize data.

RhoA Kinase Activity Assay

Equal amounts of omental vessels were dissected and weighed into two 0.5 ml centrifuge tubes and treated with D-PBS alone (control) or D-PBS with a ROS generating solution composed of 0.36 mmol/L HX and 0.003 U/ml XO for 5 minutes. At the end of 5 minutes the vessels were flash frozen in liquid nitrogen and stored at -80˚C until the RhoA Kinase activity assay was run as described previously [13]. Briefly, 20 µl of Rho kinase immunoprecipitates were added to the reaction mixture containing 1 mmol/L adenosine triphosphate (ATP) and 10 µCi of [γ-^{32}P] ATP (3000 Ci/mol) along with 5 µg of myelin basic protein, followed by incubation for 15 min at 37˚C. Phosphorylation of myelin basic protein was absorbed onto phosphocellulose disks, and free radioactivity was removed by washing 3 times with 75 mmol/L phosphoric acid (H_3PO_4). The amount of radioactivity on the disks was measured using liquid scintillation. The results are expressed as counts per minute per milligram protein.

Data Analysis

The myograph experimental data were analyzed by repeated measures two-way ANOVA with Bonferroni post-hoc test for treatment effects. Immunohistochemical staining of vessels was analyzed by visual score ranging from 0 to 4 (absent to intense vessel staining) and % of vessels with staining as previously described [9-11]. Vessels between 25 – 150 microns were evaluated. An average of 68 vessels were analyzed per patient. Staining data were analyzed by the t-test. Western blot data were quantified using the average intensity measurements normalized to β-actin and then calculated as

a percentage of the control and analyzed using Kruskal-Wallis and Dunnett's Multiple Comparison Test. The RhoA kinase immunoassay data were analyzed using Mann Whitney test. Patient clinical characteristic data were analyzed by t-test. A statistical software program was used (Prism 4, GraphPad software, San Diego, CA). A probability of <0.05 was considered significant. All data are presented as mean ± SE.

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phosphorylation via Rho-associated kinase/myosin phosphatase targeting subunit 1 and protein kinase C/CPI-17 pathway. *Biochem J.* 2003;374:145-155.

Table S1. Clinical Characteristics of Patient Groups

Variable	Normal Pregnant (n = 40)	Preeclamptic* (n = 9)
Maternal age (y)	27.5 ± 1.0	24.6 ± 1.4
Pre-pregnancy BMI (kg/m^2)	31.9 ± 1.6	36.1 ± 2.5
Systolic blood pressure (mmHg)	126.6 ± 2.5	164.8 ± 4.2†‡
Diastolic blood pressure (mmHg)	73.8 ± 1.8	92.6 ± 33.3†‡
Proteinuria (mg/24 h)	ND	186 ± 47 (n = 5)
Dipstick	ND	3.0 ± 0.7 (n = 4)
Parity		
Primiparous	21	7
Multiparous	19	2
Gestational age (wk)	39.1 ± 0.1	35.8 ± 1.5‡
Infant birth weight (g)	3387 ± 80	2623 ± 394§
Uric acid (mg/dL)	ND	6.0 ± 0.4

Values are expressed as mean ± SE.

* Liver enzymes were within the normal range except for one preeclamptic patient with elevated levels of alanine transaminase of 398 unit/L and aspartate transaminase of 240 units/L.

† 1^{st} trimester blood pressures for preeclamptic patients were: systolic 119 ± 5.1 mmHg and diastolic 74.9 ± 3.7 mmHg.

‡ $P < 0.001$, § $P < 0.01$ by t-test

ND, not determined.

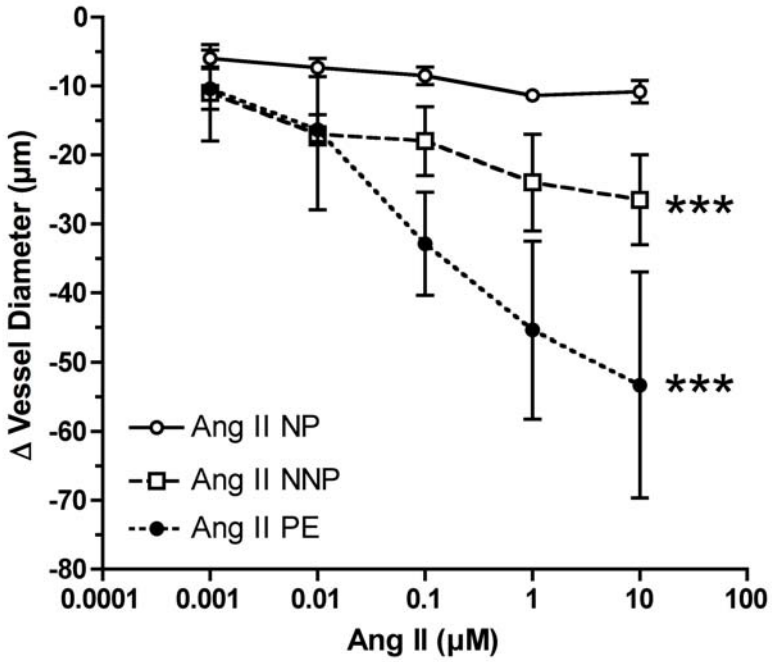

Figure S1. Comparison of vessel reactivity to angiotensin II (Ang II) of non-pregnant patients (NNP, n = 2) with normal pregnant (NP, n = 6) and preeclamptic patients (PE, n = 9). Vessel reactivity of NNP was between that of NP and PE.

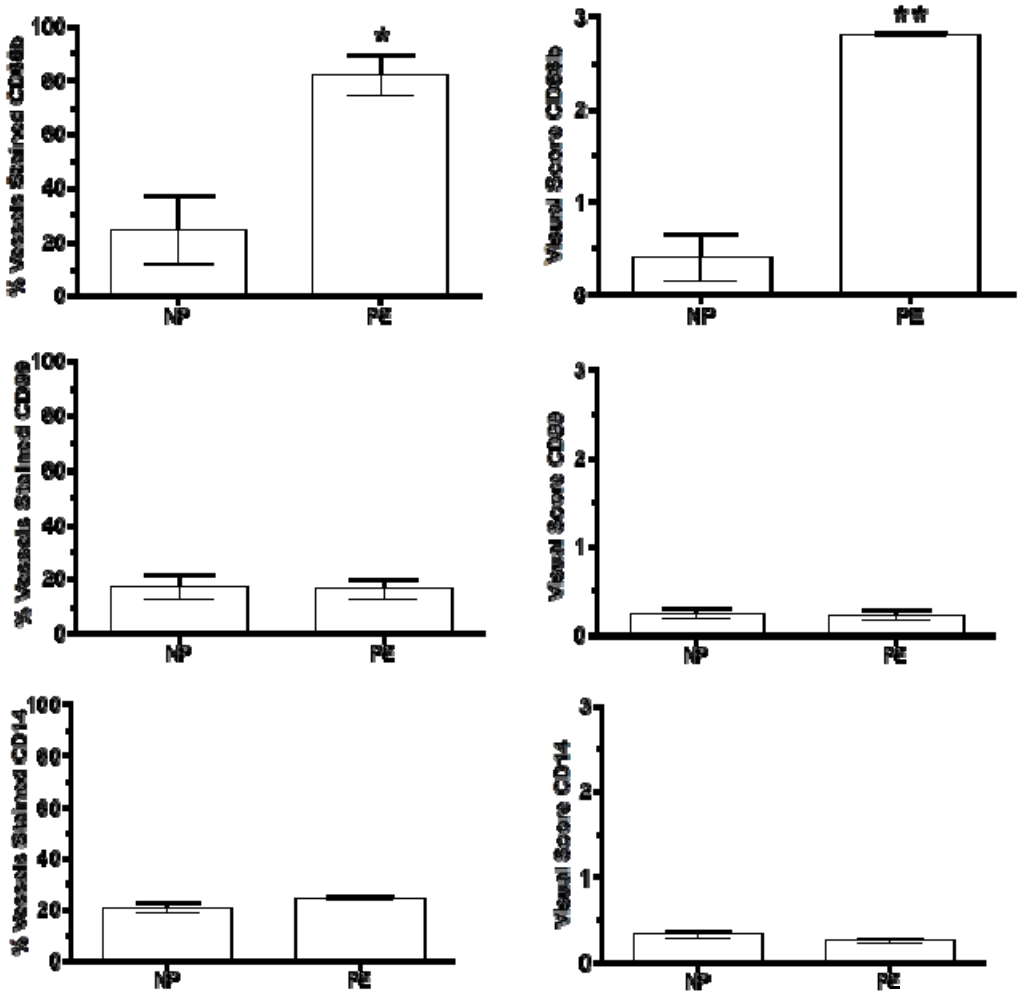

Figure S2. Percentage of vessels stained and visual staining score for CD66b, a neutrophil antigen, CD99, a lymphocyte antigen, and CD14, a monocyte/macrophage antigen in omental fat tissue of normal pregnant (n = 3) and preeclamptic (n = 3) women. Percentage of vessels with staining and their visual score were significantly higher in preeclampsia only for CD66b. There were no differences in staining for CD99 or CD14 between NP and PE. Approximately 80% of vessels stained for neutrophils in preeclamptic tissue as compared to only 20% of vessels for lymphocytes or monocytes/macrophages. * $P < 0.05$, ** $P < 0.01$, NP - normal pregnancy, PE - preeclamptic pregnancy.

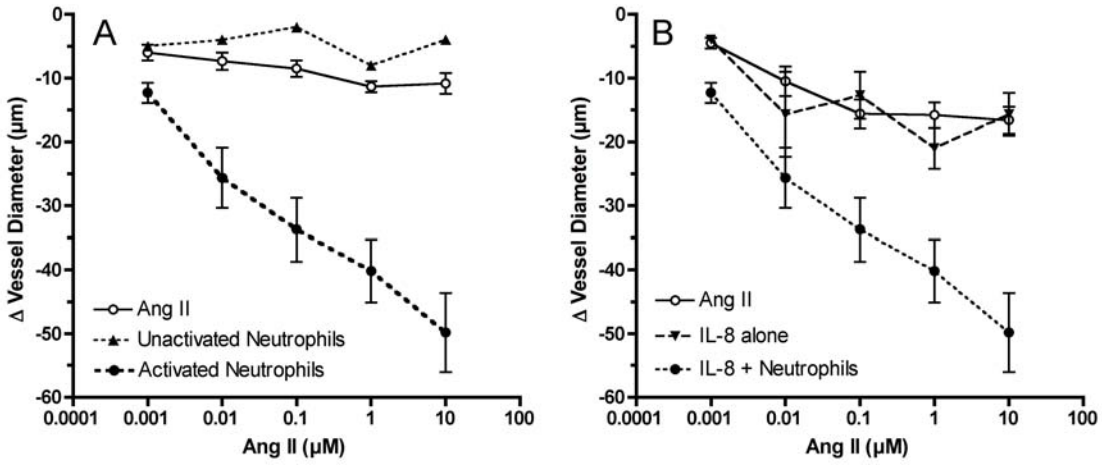

Figure S3. Control data for comparison of vessel reactivity to angiotensin II (Ang II). A) As compared to activated neutrophils, perfusion of the vessel lumen with un-activated neutrophils (n = 1) did not enhance vessel reactivity to Ang II. B) As compared to neutrophils activated with interleukin-8 (IL-8), perfusion of IL-8 alone through the vessel lumen (n = 3) did not enhance vessel reactivity to Ang II.