Mechanisms of Enhanced Vascular Reactivity in Preeclampsia

Nikita Mishra, William H. Nugent, Sunila Mahavadi, Scott W. Walsh

Abstract—Preeclamptic women have enhanced blood pressure response to angiotensin II and extensive systemic vascular infiltration of neutrophils. Neutrophils release reactive oxygen species that might activate the RhoA kinase pathway to enhance vascular reactivity. We hypothesized that enhanced vascular reactivity in preeclampsia is attributed to neutrophil-mediated reactive oxygen species activation of the RhoA kinase pathway. Omental arteries were obtained at cesarean section and studied using a myograph system. We found that arteries of preeclamptic women had extensive infiltration of neutrophils and enhanced reactivity to angiotensin II. Treatment of arteries of normal pregnant women with reactive oxygen species or activated neutrophils enhanced vessel reactivity to angiotensin II mimicking preeclamptic vessels. Pretreatment with superoxide dismutase/catalase to quench reactive oxygen species or RhoA kinase inhibitor blocked enhanced responses in preeclamptic and normal vessels. Reactive oxygen species also enhanced vessel reactivity to norepinephrine, which was blocked by RhoA kinase inhibition. Treatment of arteries with reactive oxygen species increased RhoA kinase activity 3-fold, whereas culture of human vascular smooth muscle cells with angiotensin II and activated neutrophils or reactive oxygen species resulted in phosphorylation of key proteins in the RhoA kinase pathway. We conclude that enhanced vascular reactivity of omental arteries in preeclampsia is attributed to reactive oxygen species activation of the RhoA kinase pathway and that enhanced vascular reactivity is likely attributed to the infiltration of neutrophils. We speculate that neutrophil infiltration into systemic vasculature of preeclamptic women is an important mechanism for hypertension. (Hypertension. 2011;58:867-873.) • Online Data Supplement

Key Words: preeclampsia • neutrophils • reactive oxygen species • RhoA kinase • angiotensin II • hypertension

Preeclampsia is a hypertensive disorder of pregnancy that complicates 5% to 7% of all pregnancies, resulting in significant maternal and fetal morbidity and mortality.1 The cause of hypertension in preeclampsia has never been fully explained. In 1973, Gant et al2 described enhanced blood pressure response to angiotensin II (Ang II) in women who went on to develop preeclampsia. However, mechanisms underlying this increased vascular reactivity remained elusive. Increased blood pressure in preeclampsia is not attributed to elevated levels of Ang II, because Ang II levels are not elevated,3–5 nor are the levels of other vasoconstrictive hormones, such as epinephrine or norepinephrine.6

Recently, we showed significant infiltration of neutrophils into systemic vasculature of women with preeclampsia, which was associated with marked vascular inflammation.7,8 Neutrophils release reactive oxygen species (ROS) that may enhance vascular reactivity via the RhoA kinase pathway. When RhoA kinase is activated, it phosphorylates myosin phosphatase target subunit 1 (MYPT1), which inhibits myosin light chain phosphatase, so myosin light chains (MLCs) remain phosphorylated, which enhances calcium sensitization.9,10 ROS have been shown to activate this pathway in rat aorta and rat pulmonary arteries,11,12 and ROS are mediators of Ang II signaling.13

Neutrophils are usually thought of as part of the innate immune system and the first line of defense against infection at the site of a wound.14,15 A role for neutrophils in the control of blood pressure is not commonly considered; however, given the extensive infiltration of neutrophils into the systemic vasculature of women with preeclampsia, neutrophil release of ROS might activate the RhoA kinase pathway to enhance vessel reactivity. In this study we used human omental arteries obtained from normal pregnant and preeclamptic women to test the hypothesis that enhanced vascular reactivity in preeclampsia is attributed to neutrophil-mediated ROS activation of the RhoA kinase pathway.

Materials and Methods

Omental fat biopsies (≈2 cm × 4 cm × 2 cm) were collected from 40 normal pregnant and 9 preeclamptic women undergoing cesarean section. Arteries were dissected, cleared of fat, and used for the myograph experiments. The Virginia Commonwealth University Office of Research Subjects Protection approved this study, all of the subjects gave informed consent, and the procedures followed were in accordance with institutional guidelines. Please see the online Data
Comparison of Vascular Reactivity Between Arteries of Normal and Preeclamptic Pregnancy

Figure 1A shows that vessel reactivity to Ang II was modest in omental arteries from normal pregnant women. In contrast, arteries from preeclamptic women showed significantly enhanced vessel reactivity as compared with arteries of normal pregnant women. Figure 1B shows that the enhanced reactivity of preeclamptic arteries was blocked by pretreatment with a combination of superoxide dismutase and catalase (SOD/Cat) or RhoA kinase (ROK) inhibitor (n=4). (**P<0.001 for treatment effects).

Results

Comparison of Vascular Reactivity Between Arteries of Normal and Preeclamptic Pregnancy

Figure 1A shows that vessel reactivity to Ang II was modest in omental arteries from normal pregnant women. In contrast, arteries from preeclamptic women showed significantly enhanced vessel reactivity as compared with arteries of normal pregnant women. Figure 1B shows that the enhanced reactivity of preeclamptic arteries was blocked by pretreatment with either superoxide dismutase (SOD)/catalase or RhoA kinase (ROK) inhibitor. Neither SOD/catalase nor ROK inhibitor alone significantly affected vessel diameter. Vascular reactivity of 2 nonpregnant subjects was also evaluated for comparison with pregnancy. Reactivity was between normal pregnant and preeclamptic patients (Figure S1).

Neutrophil Infiltration Into Omental Fat Vessels

Figure 2 shows immunostaining for CD66b, a neutrophil antigen (Figure 2A through 2F); CD99, a lymphocyte antigen (Figure 2G); and CD14, a monocyte/macrophage antigen (Figure 2H) in omental fat vessels of normal pregnant (n=3) and preeclamptic patients (n=3). Visual staining scores and percentage of vessels stained data are shown in Figure S2. The visual staining score (0–4) for CD66b was significantly greater for preeclamptic patients than for normal pregnant patients (2.8±0.03 versus 0.39±0.26; P<0.01). Neutrophil staining was present in 82±8% of vessels of preeclamptic patients as compared with 25±13% of vessels in normal pregnant patients (P<0.05). Vessels of preeclamptic patients showed extensive staining of neutrophils in the lumen, adhered and flattened along the endothelium, and infiltrated to the vascular smooth muscle (Figure 2C, 2D, and 2F). Few vessels stained for CD99 or CD14, and there were no differences in staining between preeclamptic and normal pregnancy (CD99: visual score, 0.23±0.06 versus 0.25±0.06 and percentage vessels stained, 16.6±3.4% versus 17.3±4.7%; CD14: visual score, 0.26±0.03 versus 0.34±0.05 and percentage of vessels stained, 24.7±1.0% versus 20.7±2.2%, respectively).

Effect of Neutrophil Products, ROS, and Tumor Necrosis Factor-α

To determine whether neutrophils or neutrophil products could enhance vessel reactivity, normal pregnant arteries were used. The effect of ROS was studied in both endothelium-intact and endothelium-denuded arteries to determine the role of the endothelium in enhanced vascular reactivity. ROS alone did not cause significant vessel contraction; however, ROS significantly enhanced vascular reactivity to Ang II in endothelium-intact vessels (Figure 3A). When ROS was tested in endothelium-denuded vessels (Figure 3B), reactivity to Ang II was much greater than in endothelium-intact vessels demonstrating that enhanced vascular reactivity was independent of the endothelium. Pretreatment with SOD/catalase to quench ROS or ROK inhibitor significantly inhibited ROS-induced enhancement of vascular reactivity to Ang II (Figure 3B). Removal of the endothelium resulted in a significant increase in vessel reactivity to Ang II (Figure 3C) but less than that induced by ROS. In contrast to the enhancing effect of ROS, tumor necrosis factor-α at a concentration higher than circulating levels in preeclampsia did not alter vascular reactivity to Ang II (Figure 3D).

Effect of Neutrophils

Perfusion of neutrophils activated with interleukin 8 through the vessel lumen did not significantly affect vessel contractility; however, when the Ang II dose response was repeated in the presence of activated neutrophils, vascular reactivity was significantly enhanced similar to ROS (Figure 4). Pretreatment with SOD/catalase significantly blocked enhanced vascular reactivity to Ang II. ROK inhibitor also abolished enhanced vascular reactivity to Ang II in the presence of activated neutrophils. Perfusion through the vessel lumen of either unactivated neutrophils or interleukin 8 alone did not affect the Ang II dose response (Figure S3).
Change in Resistance

Resistance is inversely proportional to the fourth power of the radius, so changes in vessel diameter induced by Ang II resulted in large increases in resistance in preeclamptic vessels and normal vessels treated with ROS or neutrophils. The highest dose of Ang II increased resistance 7.4-fold in preeclamptic vessels as compared with normal vessels. Similarly, treatment of normal vessels with ROS or neutrophils resulted in 3.6-fold and 4.1-fold increases, respectively.

Effect of Neutrophils and ROS on ROK Activity and Phosphorylation of MYPT1 and MLC

To study the direct effect of ROS on ROK activity in human vessels, omental arteries were treated with ROS. ROS resulted in a 3-fold increase in ROK activity as compared with untreated arteries (Figure 5A). To study phosphorylation of key proteins in the ROK pathway, human vascular smooth muscle cells were used and exposed to the treatments used for vascular reactivity. Ang II alone slightly increased phosphorylation of MYPT1 and MLC but not significantly (Figures 5B and 5C and 6). However, when Ang II was combined with either ROS or neutrophils, phosphorylation of MYPT1 and MLC was significantly increased as compared with control. The increased phosphorylation of MLC is consistent with inhibition of myosin light chain phosphatase by phosphorylated MYPT1.

Norepinephrine Dose Response

To determine whether enhanced vascular reactivity was specific to Ang II, we examined another vasoconstrictor, norepinephrine (NE). Because ROS was identified as the stimulator of the ROK pathway in the studies above, we evaluated vascular responsiveness to NE in the presence of ROS. As shown in Figure 7A, ROS significantly enhanced the vasoconstrictive response to NE. To test the role of the ROK pathway, a midrange dose for NE of 1.25 μmol/L was chosen. The increase in vasoconstrictive response to NE by ROS was significantly inhibited by ROK inhibitor (Figure 7B).

Figure 2. Representative sections of omental fat vessels immunostained for CD66b, a neutrophil antigen; CD99, a lymphocyte antigen; and CD14, a monocyte/macrophage antigen. A, Isotype negative control for CD66b (preeclamptic). B, Normal pregnancy for CD66b. Vessels of normal pregnant patients had some brown staining for neutrophils primarily in the lumen. C and D, Preeclamptic pregnancy for CD66b. Vessels of preeclamptic patients showed extensive brown staining of neutrophils in the lumen, adhered and flattened along the endothelium, and infiltrated to the vascular smooth muscle. E and F, Lower magnification of CD66b staining showing little to no staining of vessels in normal pregnancy (E) as compared with almost all of the vessels showing extensive staining in preeclampsia (F). G, CD99 staining and (H) CD14 staining in preeclamptic vessels. Few vessels showed staining for CD99 or CD14, and, when they did, there were only 1 or 2 lymphocytes or monocytes/macrophages per vessel (arrows). A, adipocyte; VL, vessel lumen; NP, normal pregnancy; PE, preeclamptic pregnancy. All of the images were taken with a ×40 lens except E and F taken with a ×10 lens.

Mishra et al Enhanced Vascular Reactivity in Preeclampsia 869

Downloaded from http://hyper.ahajournals.org/ by guest on June 25, 2017
Discussion

In this study we demonstrated enhanced vascular reactivity to Ang II in omental arteries obtained from preeclamptic women as compared with omental arteries obtained from normal pregnant women. Enhanced vessel reactivity was attributed to ROS activation of the ROK pathway, because it was abolished by pretreatment with either SOD/catalase or ROK inhibitor. Normal pregnant vessels were relatively resistant to the vasoconstrictor effect of Ang II, which is consistent with a previous report using a wire myograph system and omental vessel rings.17 If neutrophil infiltration begins before clinical symptoms, our study may explain the enhanced blood pressure response to Ang II observed by Gant et al2 in women who went on to develop preeclampsia.

We also demonstrate extensive infiltration of neutrophils, but not monocytes or lymphocytes, into omental fat vessels in preeclamptic women, which is consistent with our previous reports for subcutaneous fat vessels.7,8,18 We provide evidence that neutrophils are the likely source of ROS for enhanced vascular reactivity, because enhanced reactivity of preeclamptic vessels could be mimicked in arteries of normal pregnant women by exposing them to ROS or perfusing them with activated neutrophils. In both cases, quenching ROS or inhibiting ROK blocked enhanced vessel reactivity. Neither ROS alone nor neutrophils alone caused a significant change in vessel diameter. Their effect was only manifest in the presence of a vasoconstrictor hormone. Tumor necrosis factor-α (TNF-α), another neutrophil product, was ineffective in acutely enhancing vessel reactivity; however, we cannot rule out an effect of a longer exposure that would affect gene expression.

The number of neutrophils present in preeclamptic vessels is remarkable. Normal pregnancy is associated with a leukocytosis that is attributed to an increase in neutrophils. Their number increases 2.5-fold by 30 weeks of gestation19 and increases significantly more in preeclampsia.20 Our data demonstrate that the increased number of neutrophils in preeclampsia represents activated neutrophils, because CD66b is expressed on the cell surface of activated neutrophils.21 CD66b is thought to play a role in cell adhesion

Figure 3. Vessel reactivity of omental arteries of normal pregnant women to angiotensin II (Ang II) in response to neutrophil products and the presence or absence of endothelium. A, A reactive oxygen species (ROS)-generating solution significantly enhanced vessel reactivity to Ang II in endothelium-intact vessels (n=6). B, ROS enhanced vessel reactivity to Ang II to a greater extent when endothelium was removed (endo denuded) as compared with when endothelium was intact (n=19). Pretreatment with superoxide dismutase and catalase (SOD/catalase) to quench ROS (n=5) or RhoA kinase (ROK) inhibitor (n=4) abolished enhanced vessel reactivity to Ang II in response to ROS. C, Removal of the endothelium enhanced vessel reactivity to Ang II (n=21) as compared with when endothelium was intact (n=6), but the enhancement was much less than that induced by ROS. D, Tumor necrosis factor-α (TNF-α), another neutrophil product, did not enhance arterial reactivity to Ang II (n=4). (**P<0.001 for treatment effects; NS, nonsignificant as compared with Ang II alone).
during extravasation. The fact that preeclampsia is most commonly diagnosed in the third trimester when neutrophil numbers are increased lends credence to the notion that neutrophils are causative of clinical symptoms.

To confirm the role of ROS activation of the ROK pathway, we measured ROK activity in omental arteries and phosphorylation of key proteins of the pathway in cultured human vascular smooth muscle cells. Treatment of omental arteries with ROS resulted in a 3-fold increase in the activity of ROK. Treatment of cultured cells with the treatments used for the vascular reactivity myograph studies demonstrated that Ang II in the presence of ROS or activated neutrophils significantly increased phosphorylation of MLC and MYPT1.

ROS activation of the ROK pathway may not be the only mechanism for enhanced vascular reactivity in preeclampsia. Mechanisms may also involve deficiencies in endothelial vasodilators, such as prostacyclin and NO. Decreased prostacyclin in preeclampsia is well documented, and endothelial NO may also be decreased, because peroxynitrite is increased in maternal endothelium in preeclampsia. Superoxide reacts with NO to form peroxynitrite; so increased peroxynitrite suggests an endothelial deficiency of NO. Both of these deficiencies could be attributed to the infiltration of neutrophils, because ROS inhibit prostacyclin synthase, and superoxide specifically reacts with NO to form peroxynitrite. To evaluate the role of endothelial factors, we compared vascular response in endothelium-intact and endothelium-denuded vessels. Vascular reactivity to Ang II was enhanced when the endothelium was removed, but the enhancement was only 21% to 31% of that induced by ROS, neutrophils, or those present in preeclamptic vessels. This suggests that enhanced vascular reactivity in preeclampsia is primarily attributed to ROS activation of the ROK pathway, although deficiency of endothelial vasodilators also contributes.

The ability of ROS to enhance vascular reactivity via the ROK pathway was not specific to Ang II, because ROS also enhanced vascular reactivity to NE. This finding is pertinent to preeclampsia, because NE is induced by stress and sympathetic activation, factors reported to be associated with
preeclampsia, and previous work has shown that in vitro vascular reactivity to NE is increased in omental arteries of preeclamptic women as compared with normal pregnant women.

**Perspectives**

In this study we demonstrated that enhanced vascular reactivity of omental arteries of preeclamptic women is primarily attributed to ROS activation of the ROK pathway and provide support for the idea that enhanced vascular reactivity is likely attributed to the infiltration of activated neutrophils. We proposed previously that neutrophils are activated as they circulate through the intervillous space and are exposed to increased levels of oxidized lipids secreted by the placenta. This contention is supported by the observation that neutrophils in the uterine vein express significantly higher levels of integrins than neutrophils in the antecubital vein in preeclamptic, but not normal pregnant, women. Other possible mechanisms for neutrophil activation include exposure to elevated plasma levels of matrix metalloproteinase 1 or activating autoantibodies against Ang II receptor 1 in preeclamptic women. Matrix metalloproteinase 1 activates neutrophils, and activating autoantibodies against Ang II receptor 1 stimulate NADPH oxidase in vascular smooth muscle, and, so, might also stimulate NADPH oxidase in neutrophils.

This study could provide novel avenues for the treatment of hypertension based on regulation of neutrophils, their products, or their cellular effects. Potential treatments that are becoming available for clinical studies are neutralizing antibodies against adhesion molecules necessary for neutrophil infiltration and selective ROK inhibitors. Antioxidant therapy with vitamins C and E has been tried but was ineffective in preventing preeclampsia in clinical trials. The high doses of vitamin C used in these trials may have been counterproductive, because vitamin C is a potent oxidant in the presence of free iron, which is elevated in the blood of women with preeclampsia. On the other hand, meta-analysis of low-dose aspirin trials demonstrates that aspirin is effective in preventing preeclampsia, the effectiveness of which is related to patient compliance. The ability of aspirin to reduce preeclampsia may be because of its ability to inhibit neutrophil superoxide production, which would be consistent with the findings of our study.

**Acknowledgments**

We thank Dr Srinivasan Karnam for his help and support with the ROK activity assay funded by National Institutes of Health grant RO1 DK15564, Department of Physiology and Biophysics, Virginia Commonwealth University Medical Center, Richmond, VA.
Enhanced Vascular Reactivity in Preeclampsia

Mishra et al

Sources of Funding
This work was supported by a grant to S.W.W. from the National Institutes of Health, National Heart, Lung, and Blood Institute (RO1 HL069851).

Disclosures
None.

References
Mechanisms of Enhanced Vascular Reactivity in Preeclampsia
Nikita Mishra, William H. Nugent, Sunila Mahavadi and Scott W. Walsh

Hypertension. 2011;58:867-873; originally published online September 26, 2011;
doi: 10.1161/HYPERTENSIONAHA.111.176602

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://hyper.ahajournals.org/content/58/5/867

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2011/09/23/HYPERTENSIONAHA.111.176602.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
MECHANISMS OF ENHANCED VASCULAR REACTIVITY IN PREECLAMPSIA

Nikita Mishra\textsuperscript{1,2}, Ph.D., M.B.B.S, William H. Nugent\textsuperscript{1}, Ph.D., Sunila Mahavadi\textsuperscript{2}, M.S, Scott W. Walsh\textsuperscript{1,2}, Ph.D.

\textsuperscript{1}Departments of Obstetrics and Gynecology, and \textsuperscript{2}Physiology and Biophysics, Virginia Commonwealth University, Richmond, VA

Corresponding Author: Scott W. Walsh, Ph.D.
Virginia Commonwealth University Medical Center
Department of Obstetrics and Gynecology
P.O. Box 980034
Richmond, VA 23298-0034
(804) 828-8468-Phone; (804) 828-0573-Fax
swwalsh@vcu.edu
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.

Comparison of vascular reactivity between arteries of normal and preeclamptic pregnancy: 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. 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. 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 (TNF-α): 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 TNF-α (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/mm³. 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. 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-32P] 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 (H₃PO₄). 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.

REFERENCES

Table S1. Clinical Characteristics of Patient Groups

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

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.
† 1st 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.