Uncoupled Endothelial Nitric Oxide Synthase and Oxidative Stress in a Rat Model of Pregnancy-Induced Hypertension

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Background: Preeclampsia is a human pregnancy-associated syndrome associated with hypertension, proteinuria, and endothelial dysfunction. We tested whether increased reactive oxygen species (superoxide and peroxynitrite) production and decreased bioavailability of the endothelial nitric oxide (NO) synthase (eNOS) cofactor tetrahydrobiopterin (BH4) contributes to maternal endothelial dysfunction in rats with pregnancy-induced hypertension and several characteristics of preeclampsia.

Methods: Nonpregnant (DS) and pregnant (PDS) rats were treated with deoxycorticosterone acetate and 0.9% saline for ~3 weeks and nonpregnant (Con) and pregnant (P) rats received tap water. Blood pressure, urinary protein levels, mesenteric vascular reactivity, aortic protein expression, and aortic reactive oxygen species levels were compared between the four groups.

Results: The PDS rats had significantly decreased mesenteric endothelium-dependent relaxation responses and aortic NO production compared to Con, DS, and P rats despite increased aortic eNOS expression. Aortic superoxide and peroxynitrite levels were increased in PDS rats compared with Con, DS, and P rats. Scavenging of reactive oxygen species or increasing tetrahydrobiopterin levels normalized mesenteric endothelium-dependent relaxation responses, aortic NO production, and aortic superoxide and peroxynitrite levels in PDS rats.

Conclusions: These data suggest that increased superoxide production by NADPH oxidase, peroxynitrite degradation of BH4, and uncoupled eNOS contribute to endothelial dysfunction in a rat model of pregnancy-induced hypertension. Am J Hypertens 2007;20:1297–1304 © 2007 American Journal of Hypertension, Ltd.

Key Words: Endothelial nitric oxide synthase, NADPH oxidase, peroxynitrite, preeclampsia, superoxide.
tion and decreased antioxidant protection. The major source of the increased production of superoxide in PE is most likely NADPH oxidase, which is upregulated in placentas of women with PE. Superoxide reacts with NO to form peroxynitrite, which is also increased in placentas and systemic blood vessels of women with PE. Reduced NO bioavailability may result not only from superoxide-mediated degradation of NO, but also by peroxynitrite-mediated degradation of tetrahydrobiopterin (BH4), a major cofactor for eNOS in the production of NO. In the absence of BH4, eNOS becomes uncoupled and generates more superoxide than NO, which further contributes to endothelial dysfunction. Whether increased ROS production and eNOS uncoupling occurs in the systemic vasculature of women with PE is unknown.

We previously described a rat model in which pregnant rats are treated with deoxycorticosterone acetate (DOCA; weekly intraperitoneal injection) and 0.9% saline in the drinking water (PDS rats). These volume-expanded animals demonstrate hypertension, proteinuria, decreased plasma nitrate/nitrite levels, lower pup survival, and decreased uterine weight compared with pregnant rats (P), which mimic the major clinical characteristics of PE. Although PE is a human-specific disorder, these rats represent an animal model of pregnancy-induced hypertension in which vascular pathogenetic mechanisms can be studied.

We assessed endothelium-dependent dilation in mesenteric arteries from PDS and P rats, as well as in nonpregnant rats (Con) and nonpregnant rats treated with DOCA/0.9% saline (DS). In addition, we measured NO production, eNOS, heat shock protein 90 (HSP90) protein expression, and ROS levels in aortas from Con, DS, P, and PDS rats. We tested the hypothesis that increased NADPH oxidase-derived superoxide and peroxynitrite decreases NO and BH4 bioavailability leading to uncoupled eNOS and endothelial dysfunction in the maternal vasculature of PDS rats.

Methods

Animals and Blood Pressure Measurements

Male and female Sprague-Dawley (Harlan, Indianapolis, IN) rats weighing 200 to 250 g were used in all experiments. Female PDS rats were treated as described previously. Briefly, DS and PDS females received 12.5 mg of DOCA (intraperitoneal injection) and were given 0.9% saline to drink, followed by weekly intraperitoneal injections of DOCA (6.25 mg). Tail–cuff systolic blood pressures (BP) (ITC, Inc., Woodland Hills, CA) were measured before euthanization. Urinary protein excretion was measured as described previously. The P and PDS rats were euthanized on days 18 to 20 of pregnancy and Con and DS rats were euthanized after the same amount of time. The number of malformed fetuses was noted and the uterus was weighed both with and without pups. All procedures were approved by the respective Institutional Animal Care and Use Committees.

Organ Chamber Experiments

All rats were anesthetized with isoflurane and euthanized by exsanguination. Small mesenteric arteries (~200 μm) were immediately excised and placed in cold physiologic salt solution (PSS). Vascular reactivity of isolated endothelium-intact arterial rings (3 mm) was measured as described previously. All experiments were performed in the presence of indomethacin (10 μmol/L) to inhibit cyclooxygenase.

Some arterial rings were incubated with Nnitro-l-arginine (LNNA; 10 μmol/L, 20 min) to inhibit NOS activity, superoxide dismutase (SOD; 150 U/mL, 30 min) to scavenge superoxide, apocynin (APO; 100 μmol/L, 60 min) to inhibit NADPH oxidase activity, ebesein (Eb; 5 μmol/L, 20 min) plus uric acid (UA; 100 μmol/L, 20 min) to scavenge peroxynitrite, or sepiapterin (SEP; 100 μmol/L, 30 min) to increase BH4 levels. Concentration–response curves were obtained in a half-log, cumulative fashion to acetylcholine (ACh; 1 nmol/L to 100 μmol/L) and sodium nitroprusside (SNP; 100 pmol/L to 100 nmol/L) after contraction to an EC70 concentration of phenylephrine (1 μmol/L, 20 min).

Preparation of Aortic Homogenates

Endothelium-intact aortic rings (~4 mm) were incubated in the absence and presence of SOD (150 U/mL, 20 min), APO (100 μmol/L, 60 min), or SEP (100 μmol/L, 20 min) in 37°C PSS. The rings were then homogenized and protein concentration determined as described previously.

Nitric Oxide Oxidation

An assay using the cell-permeable dye 4-amino-5-methyl-3-difluorofluorescein diacetate (DAF-FM diacetate; Molecular Probes, Carlsbad, CA) was used to measure NO production as described previously.

Immunoblotting

Western blot analyses were performed as described previously using primary antibodies for eNOS 1:2500 (BD Transduction Labs, Franklin Lakes, NJ), nitrotyrosine 1 μg/mL (Calbiochem, San Diego, CA), and HSP90 1:1000 (BD Transduction Labs, Franklin Lakes). Densitometry is expressed as the ratio of Western blot to Coomassie blue-stained 42 kD β-actin band on the polyvinylidene fluoride (PVDF) membrane.

Superoxide and Peroxynitrite Measurements

Superoxide and peroxynitrite were measured by lucigenin- and luminol-derived chemiluminescence as described previously. In brief, endothelium-intact aortic rings (~4 mm) were incubated for 30 min in the absence and pres-
ence of SOD (150 U/mL), Eb (5 μmol/L) plus UA (100 μmol/L), LNNA (10 μmol/L), APO (100 μmol/L), or SEPIA (100 μmol/L) in PSS and then placed in scintillation vials containing PSS and either lucigenin (5 μmol/L) or luminol (500 μmol/L). Levels of superoxide and peroxynitrite are expressed as relative light units (RLU)/milligram dry tissue weight/minute.

**Statistical Analyses**

Results are presented as mean ± SEM. The Student t test was used to compare variables between groups. An analysis of variance was used for multiple comparisons followed by the Student-Newman-Keuls post hoc test when necessary. The significance level was .05.

**Results**

**Hypertension, Proteinuria, and Intrauterine Growth Restriction (IUGR) in PDS Rats**

Blood pressure, urinary protein levels, number of fetuses, and total litter weights are presented in Table 1. The P rats demonstrated a significant decrease in BP during pregnancy; however, systolic BP of PDS rats increased significantly. Importantly, systolic BP in DS rats were not significantly different compared with Con rats. The P and PDS rats exhibited significantly elevated urinary protein levels compared with Con and DS rats, but urinary protein levels were markedly higher in PDS rats compared with P rats. The PDS rats had one to three malformed fetuses in each litter, whereas P rats had none. In addition, the total litter weight and mean number of fetuses per litter (including malformed fetuses) was decreased significantly in PDS rats compared with P rats.

**Decreased Endothelium-Dependent Dilation in Blood Vessels from PDS Rats**

Maximal relaxation responses to ACh were decreased significantly in mesenteric arteries from PDS rats compared with Con, DS, and P rats (maximal relaxation from phenylephrine-induced contraction: Con = 94% ± 1%, DS = 98% ± 2%, P = 102% ± 4%, and PDS = 68% ± 2%; P < .05; Fig. 1). The NOS inhibition with LNNA abolished relaxation responses to ACh in all groups (Fig. 1). Scavenging of superoxide with SOD (Fig. 2A) and inhibition of NADPH oxidase with APO (Fig. 2B) normalized ACh-induced relaxation responses in mesenteric arteries from PDS rats but had no significant effect on relaxation responses in arteries from Con, DS, and P rats. In addition, scavenging of peroxynitrite with Eb and UA restored ACh-induced relaxation responses in mesenteric arteries from PDS rats to Con, DS, and P levels (Fig. 3A). We also tested whether uncoupled eNOS due to BH4 depletion contributes to the decreased relaxation responses of arteries from PDS rats. The SEPIA, a BH4 precursor, restored relaxation responses in mesenteric arteries from PDS rats to P levels and had no effect on relaxation of arteries from Con, DS, and P rats (Fig. 3B).

Maximal endothelium-independent relaxation responses to SNP were not different between mesenteric arteries from Con, DS, P, and PDS rats (data not shown). However, mesenteric arteries from PDS rats demonstrated a significantly decreased sensitivity to SNP after LNNA (EC50 value: PDS = −7.8 ± 0.1 [anti-log = 1.5 × 10⁻⁸] and P = −8.3 ± 0.1

**Table 1. Hypertension, proteinuria, and intrauterine growth restriction in PDS rats**

<table>
<thead>
<tr>
<th></th>
<th>Systolic BP (mm Hg)</th>
<th>Urinary Protein Excretion (mg/24 h)</th>
<th>Mean Number of Fetuses/Litter</th>
<th>Total Litter Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con (n = 14)</td>
<td>108 ± 2</td>
<td>3.0 ± 0.4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DS (n = 8)</td>
<td>105 ± 2</td>
<td>3.2 ± 0.4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>P (n = 9)</td>
<td>90 ± 4*</td>
<td>5.7 ± 0.6*</td>
<td>16 ± 1</td>
<td>73 ± 5</td>
</tr>
<tr>
<td>PDS (n = 9)</td>
<td>138 ± 4†</td>
<td>9.8 ± 0.3†</td>
<td>10 ± 1†</td>
<td>36 ± 6†</td>
</tr>
</tbody>
</table>

Con = not pregnant; DS = nonpregnant + doxycorticosterone acetate (DOCA)/saline; n = number of rats; P = pregnant; PDS = pregnant + DOCA/saline.

Values are mean ± SEM.

* P < .05 v Con; † P < .05 v P.
The EC50 values for Con and DS were 8.0 \times 10^{-8} and 8.1 \times 10^{-8}, respectively.

Decreased NO Production in Blood Vessels from PDS Rats

Peak aortic NO production in PDS rats was decreased 45%, but was increased 24% in P rats, compared to controls (LNNA-sensitive peak DAF-FM diacetate fluorescent counts: Con = 160,828 ± 2762, DS = 158,875 ± 6218, P = 199,982 ± 10,510, and PDS = 88,781 ± 8457, P and PDS, P < .05 v Con; Fig. 4). To verify that the restoration of relaxation responses in blood vessels from PDS rats by depleting ROS or increasing BH4 levels was due to increased NO bioavailability/production, we measured LNNA-sensitive DAF-FM diacetate fluorescence in P and PDS aortas after incubation with SOD, APO, and SEPIA. Superoxide dismutase, APO, and SEPIA all restored peak aortic NO production in PDS rats to P levels (Fig. 4), yet had no effect on NO production in P aortas.

**Increased eNOS and Nitrotyrosine Expression in Blood Vessels from PDS Rats**

Because NO-dependent relaxation responses and NO production were decreased in systemic arteries from PDS rats, we measured eNOS protein levels. Aortic eNOS protein expression was increased significantly in PDS rats compared to P rats (P < .05 v P; Fig. 5). The findings of decreased endothelium-dependent relaxation responses and peak NO production despite increased eNOS protein expression suggest that eNOS activity may be uncoupled or increased levels of ROS may decrease NO bioavailability. Vascular nitrotyrosine levels, a marker of peroxynitrite activity, were increased in aortas from PDS rats compared with P rats. A drastic difference was observed at ~48 kDa.
and Fig. 5 demonstrates this increased nitrotyrosine level in aortas from PDS rats compared with P rats (P < .05 v P; Fig. 5). The identity and function of this protein is being examined. Decreases in HSP90 also lead to uncoupled eNOS and superoxide generation, therefore we measured HSP90 protein expression in aortas from P and PDS rats. There were no significant differences in HSP90 expression between the two groups (Fig. 5).

**Increased ROS Levels in Blood Vessels from PDS Rats**

To confirm that ROS levels are increased in blood vessels from PDS rats, we measured superoxide and peroxynitrite levels using lucigenin-derived and luminol-derived chemiluminescence, respectively. Aortic segments from PDS rats demonstrated an almost twofold increase in superoxide levels compared with Con, DS, and P rats (P < .05 v Con; Fig. 6A). Superoxide dismutase, APO, LNNA, or SEPIA normalized superoxide levels in PDS rats suggesting that NADPH oxidase and uncoupled eNOS were sources of superoxide production (Fig. 6A). Aortic segments from PDS rats also demonstrated a twofold increase in peroxynitrite levels compared with Con, DS, and P rats (P < .05 v Con; Fig. 6B). The P rats also demonstrated increased aortic peroxynitrite levels compared with Con (P < .05 v Con, Fig. 6B). Incubation of aortas from PDS rats with Eb/UA, APO, or SEPIA normalized peroxynitrite levels (Fig. 6B). LNNA significantly decreased luminol-derived chemiluminescence in PDS rat aortas; however, the values were still significantly higher than Con but were similar to P rats (Fig. 6B).

**Discussion**

It has been established that endothelial dysfunction contributes to pregnancy-induced hypertension and PE; however, the pathogenetic mechanisms are still relatively unknown. We report that the vasculature from volume-expanded, pregnant rats exhibit increased superoxide and peroxynitrite levels and decreased NO production and endothelium-dependent relaxation responses despite increased eNOS protein expression. Endothelium-dependent relaxation and NO production were restored by scavenging ROS, inhibiting NADPH oxidase-mediated superoxide production, or by "re"-coupling eNOS in arteries from PDS rats.

Recent evidence suggests that several distinct etiologies of PE may exist. Placental PE is mediated by abnormal placentaion and placental ischemia, which ultimately lead to maternal vascular dysfunction, whereas maternal PE results from placental ischemia or maternal vascular dysfunction. To examine the mechanisms involved in the pathophysiology of this syndrome, several animal models of pregnancy-induced hypertension exist that resemble PE including rats subjected to NOS inhibition during pregnancy, inbred hypertensive mice (BPH/5), pregnant rats subjected to reduced uterine perfusion pressure (RUPP), and pregnant rats treated with soluble fms-like tyrosine kinase 1 (sFlt1) or endoglin. Like the other models, our model using rats, volume expanded with DOCA.
and saline produces many, but not all, of the characteristics of PE including hypertension, proteinuria, and IUGR. Furthermore, our nonpregnant rats treated with DOCA and 0.9% saline do not exhibit increases in systolic BP, suggesting that pregnancy alters the ability to handle the excessive volume. Other studies have induced hypertension in female rats using DOCA and saliné; however, we used a dose of ~1.2 mg/d DOCA, which is 2 to 10 times less than in other studies and we do not perform an uninephrectomy. Thus, excessive volume expansion, in this case induced by pregnancy plus low-dose exogenous mineralocorticoids, may represent a clinical form of pregnancy-induced hypertension or maternal PE in which there is a defect in water and salt handling. It should be noted that BP decline during pregnancy has been reported to occur in women with primary aldosteronism as well in other forms of preexisting hypertension. The difference in BP in these patients and the current animal model may be explained by salt intake. In addition, it is clear that in these patients the vasodilatory forces can counter their volume overload.

Maternal endothelial dysfunction occurs during pregnancy-induced hypertension and PE; however, the role of the maternal vasculature in the development of the elevated BP is still unknown. We report significantly increased NO production and slightly increased smooth muscle sensitivity to NO in arteries from P rats compared to nonpregnant controls, which explains in part why systolic BP decreased during pregnancy. In contrast, systemic arteries from PDS rats demonstrated reduced endothelium-dependent relaxation and NO production despite increased eNOS protein levels, and seemed to lose the pregnancy-induced increase in smooth muscle sensitivity to NO. These findings both support and conflict those found in the RUPP model of PE. Endothelium-dependent dilation and NO production are decreased in aortic segments from RUPP rats; however, mesenteric artery relaxation responses are not decreased. It is possible that the reduction in uterine perfusion pressure, which is initiated at day 14 of gestation, is too late to affect all vascular beds. Time course studies in both of these models are needed to delineate the role of maternal endothelial dysfunction in the development of pregnancy-induced hypertension.

Pregnancy is associated with a generalized state of increased oxidative stress. However, excessive pro-oxidant forces are evident in humans and animals with hypertension and PE. The NADPH oxidase-mediated superoxide and peroxynitrite production are increased in placenta of humans with PE; however, only one study reported increased nitrotyrosine immunostaining in systemic vessels obtained from women with PE. Our results demonstrating increased superoxide, peroxynitrite, and nitrotyrosine levels in systemic arteries of PDS rats support and extend this finding. Furthermore, inhibition of NADPH oxidase or scavenging of superoxide or peroxynitrite all restored endothelium-dependent relaxation responses and NO production to P levels. We are currently testing whether APO, SOD, or EB-UA treatment decreases the severity of PE-like symptoms in PDS rats.

The eNOS cofactor BH4 promotes eNOS dimerization and activity. Whether BH4 depletion and uncoupled eNOS occurs in the maternal vasculature of humans with hypertensive pregnancy disorders has not been examined. In the present study we demonstrate that exogenous BH4 in the form of sepiapterin restored endothelium-dependent relaxation responses of mesenteric arteries from P rats, increased vascular NO production, and reduced superoxide and peroxynitrite production. Furthermore, NOS inhibition decreased superoxide and peroxynitrite production in systemic blood vessels from PDS rats but had no effect in vessels from P rats suggesting that uncoupled eNOS contributes to the endothelial dysfunction of PDS rats. Alterations in HSP90 also affect eNOS generation of NO versus superoxide and a recent study reported decreased HSP90 protein levels in umbilical endothelial cells from women with PE. However, we did not observe any
differences in HSP90 protein expression between aortas from P and PDS rats. Although we did not directly measure BH4 levels in systemic arteries from PDS and P rats, bioavailability of BH4 or eNOS responsiveness to BH4 may explain why BH4 supplementation restored endothelial function in arteries from PDS rats. Peroxynitrite is known to degrade BH4 and the increased production of peroxynitrite in vessels from PDS rats may promote eNOS uncoupling. Reduced levels of ascorbic acid, known to stabilize BH4, have been reported in women with PE and been shown to increase myogenic tone in mesenteric arteries of pregnant rats.27–29 Alternatively, a study by Kukor and colleagues30 reported that although BH4 levels are not different between placentas from P and PE women, placental eNOS from women with PE is “resistant” to activation by physiologic levels of exogenous BH4 but normalized after supraphysiologic concentrations of exogenous BH4. Taken together, these findings suggest that exogenous BH4 may be beneficial in women with pregnancy-induced hypertension and maternal PE.

In conclusion, increased production of superoxide by NADPH oxidase and peroxynitrite, along with uncoupled eNOS, contribute to endothelial dysfunction in the vasculature of volume-expanded, pregnant rats. Recent clinical trials with antioxidants in heterogeneous populations have been disappointing thus far; however, ROS scavenging or BH4 supplementation of women with pregnancy-induced hypertensive disorders may decrease the severity of the disease.

References
