Evidence for Peroxynitrite Formation in the Vasculature of Women With Preeclampsia

Anne M. Roggensack, Yunlong Zhang, Sandra T. Davidge

Abstract—Preeclampsia is a multisystemic disorder of pregnancy in which the normal vascular adaptations to pregnancy are compromised. Oxidative stress as well as endothelial cell dysfunction have been implicated as pathophysiological features of preeclampsia. Endothelial cells produce the vasorelaxant nitric oxide (NO). However, NO is also known to react with superoxide anions (produced under conditions of oxidative stress), yielding peroxynitrite that may impair vascular function. Our objective was to use immunohistochemical techniques to determine whether there is evidence of peroxynitrite formation in the maternal systemic vasculature of women with preeclampsia. Vessels were obtained from a biopsy of subcutaneous fat at the time of cesarean section from normal pregnant (n = 7) and preeclamptic (n = 7) women or at the time of hysterectomy from nonpregnant women (n = 5). There were significantly more vessels staining with greater intensity for nitrotyrosine and endothelial NO synthase in the endothelium of vessels from women with preeclampsia compared with that of normal pregnant women or nonpregnant women. Both endothelial and smooth muscle cells from all vessels showed evidence for the presence of superoxide dismutase (SOD), an enzyme that scavenges superoxide anions. However, the intensity of staining for SOD in the endothelium was significantly lower in the preeclamptic and nonpregnant women than in normal pregnant women. These data of increased endothelial NO synthase, decreased SOD, and increased nitrotyrosine immunostaining in the maternal vasculature of women with preeclampsia suggest increased peroxynitrite formation. We speculate that peroxynitrite is involved in endothelial cell dysfunction in preeclamptic women and contributes to the pathophysiology of this pregnancy disorder. (Hypertension. 1999;33:83-89.)

Key Words: endothelium ■ nitric oxide synthase ■ superoxide dismutase ■ nitrotyrosine
tyrosine residues. Thus, nitrotyrosine can act as a marker for peroxynitrite. Although other nitrogen-centered oxidants may result in the formation of nitrotyrosine, peroxynitrite is the most likely source in vivo. The presence of nitrotyrosine has been shown in human atherosclerotic plaque and in Goldblatt-induced hypertension. Importantly, an elevation of nitrotyrosine in the placental villous tissue of women with preeclampsia has been reported recently. Whether there is evidence of increased peroxynitrite formation in the maternal vasculature of women with preeclampsia is not known.

Our objective was to use immunohistochemical techniques to determine whether there is evidence of peroxynitrite

### Table 1. Characteristics of Patient Groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Nonpregnant (n=5)</th>
<th>Normal Pregnant (n=7)</th>
<th>Preeclamptic (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age, y</td>
<td>33.6±1.4</td>
<td>32.7±2.0</td>
<td>30.4±2.2</td>
</tr>
<tr>
<td>Prepregnant weight, kg</td>
<td>68.4±3.7</td>
<td>58.6±6.7</td>
<td>69.6±8.4</td>
</tr>
<tr>
<td>Prepregnant blood pressure, mm Hg</td>
<td>111/76±6/2</td>
<td>111/71±3/4</td>
<td>118/72±3/3</td>
</tr>
<tr>
<td>Term blood pressure, mm Hg</td>
<td>NA</td>
<td>113/72±5/5</td>
<td>164/101±10/4*</td>
</tr>
<tr>
<td>Parity</td>
<td>ND</td>
<td>1.1±0.3</td>
<td>1.1±0.3</td>
</tr>
<tr>
<td>Proteinuria (&gt;2+ on urine testing)</td>
<td>ND</td>
<td>0/7</td>
<td>7/7</td>
</tr>
<tr>
<td>Plasma uric acid concentration, mg/dL</td>
<td>ND</td>
<td>ND</td>
<td>369.3±31.0</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>ND</td>
<td>36.0±1.1</td>
<td>33.7±1.9</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>ND</td>
<td>12.3±0.4</td>
<td>11.7±0.6</td>
</tr>
<tr>
<td>Gestational age at delivery, wk</td>
<td>NA</td>
<td>38.6±0.4</td>
<td>32.3±2.4*</td>
</tr>
<tr>
<td>Infant birth weight, kg</td>
<td>ND</td>
<td>3.38±0.20</td>
<td>1.90±0.54*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. ND indicates not determined; NA, not applicable.

*P<0.01 compared with normal pregnant.

Figure 1. Immunohistochemistry for nonspecific IgG (negative control). Representative sections of microvessels in subcutaneous fat biopsies from a normal pregnant woman at magnifications ×200 (a) and ×1000 (b) and from a woman with preeclampsia at magnifications ×200 (c) and ×1000 (d).
formation in the maternal systemic vasculature of women with preeclampsia. The hypothesis was that increased eNOS and reduced SOD would be associated with increased nitrotyrosine formation in the maternal endothelial cells of women with preeclampsia. These data would provide evidence for peroxynitrite formation that may contribute to the vascular pathophysiology of this pregnancy disorder.

**Methods**

**Subjects**

Fourteen pregnant subjects were recruited at the time of admittance to labor and delivery, and 5 nonpregnant women were recruited at the time of admittance for a hysterectomy at Royal Alexandra Hospital, Edmonton, using protocols approved by the University of Alberta Ethics Committee. Seven subjects had preeclampsia as defined using the criteria of hypertension and proteinuria. Hypertension was defined as an absolute blood pressure > 140/90 mm Hg on 2 occasions at least 6 hours apart and occurring after the 20th week of gestation. Proteinuria was defined as > 500 mg protein per 24-hour urine collection or 2+ on a voided urine Dipstix test. Seven subjects had uncomplicated pregnancies, were normotensive throughout gestation, and had no proteinuria. No subject was known to have a history of chronic hypertension or liver, renal, or metabolic disease. The 2 groups of pregnant women were best matched for maternal age, parity, and gestational age.

At the time of cesarean section or hysterectomy, biopsies of subcutaneous fat were obtained from along the incision site. The normal pregnant women were undergoing cesarean section because of either cephalopelvic disproportion or breech presentation. Biopsies were snap-frozen in liquid nitrogen and stored at –80°C.

**TABLE 2. Summary of Immunohistochemical Analysis**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>eNOS</th>
<th>SOD</th>
<th>Nitrotyrosine (peroxynitrite)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Vessels Staining†</td>
<td>Intensity of Staining</td>
<td>% Vessels Staining</td>
</tr>
<tr>
<td>Nonpregnant</td>
<td>19.9±5.5</td>
<td>1.2±0.3</td>
<td>100.0±0.0</td>
</tr>
<tr>
<td>Normal pregnant</td>
<td>29.9±6.6</td>
<td>1.5±0.2</td>
<td>100.0±0.0</td>
</tr>
<tr>
<td>Preeclampsia</td>
<td>71.0±4.3†</td>
<td>2.8±0.3†</td>
<td>90.4±9.6</td>
</tr>
</tbody>
</table>

†Percent detecting only endothelial cells expressing high levels of eNOS (see results).
‡P<0.05 compared with nonpregnant.
*P<0.05 compared with normal pregnant.
Immunohistochemistry

Frozen biopsies were cut in consecutive 8-μm sections, mounted on glass slides at −30°C, and stored at −80°C before staining. The number of blood vessels in each section as well as the presence of endothelial cells in each vessel were visualized by immunostaining with a monoclonal antibody against α-actin (1:500; Boehringer Mannheim Biochemica) and von Willebrand factor (1:100; Immunotech), respectively. All vessels in the entire section on the slide were counted. Sections were immunostained using monoclonal antibodies against eNOS (1:200; Transduction Laboratories), inducible NOS (iNOS; 1:25 to 1:200; Transduction Laboratories), SOD (1:750; Sigma Chemical Co), and nitrotyrosine residues (1:100; Upstate Biotechnology). Preliminary studies were conducted using serial dilutions of each antibody to determine optimal concentrations that allowed for a clear resolution. Negative controls were stained with nonspecific IgG (1:100; Vector Laboratories) or stained without a primary antibody. An additional control for nitrotyrosine antibody was conducted by preabsorbing antibody with 3-nitro-L-tyrosine antigen (Sigma). 3-Nitro-L-tyrosine was dissolved in blocking serum at 10 mmol/L. Nitrotyrosine antibody was diluted 1:100 in this solution overnight at 4°C. The Vectastain Elite ABC kit (Vector Laboratories) was used for the immunostaining. Slides were counterstained in a 1:1 mixture of alcian blue and methyl green.

Data Collection and Analysis

Sections were examined by 2 investigators who were blinded to the identity of the tissue. Sections were evaluated as to the percentage of vessels on each slide that showed any staining of endothelial cells, as well as a semiquantitative grading of the intensity of endothelial cell positive staining. Intensity was scored on a scale of 0 to 4 (from absent to intense). Nonparametric Kruskal-Wallis 1-way analysis was used for statistical analysis of immunohistochemistry results. Pairwise comparison was then conducted using Dunn’s test.

Results

Subjects

Data from the 3 groups of women are summarized in Table 1. Maternal age was not significantly different among the groups. As anticipated from the definition criteria, systolic and diastolic pressures at term were significantly greater in the preeclamptic group than in the normal pregnancy group (P<0.01). Gestational ages at delivery and infant birth weights were significantly lower in the preeclamptic group than in the normal pregnant control subjects (P<0.01). No other differences between the groups was found.

Immunostaining

A representative negative control using nonspecific IgG for a normal pregnant woman and a woman with preeclampsia is shown in Figure 1. There was no evidence of nonspecific immunostaining with either nonspecific IgG or without primary antibody in any of the sections. Immunostaining for actin allowed for an accurate count of the number of vessels in a section, with an average of 27.8±2.83 and 27.3±6.06 vessels on each slide from samples of women with pre-eclampsia and the normal pregnant women, respectively. For nonpregnant women, the number of vessels in each section averaged 17.0±4.12. Immunostaining for von Willebrand
factor provided evidence for intact endothelial cells in each of the samples (Figure 2).

For eNOS immunostaining, all endothelial cells from the samples immunostained at antibody concentrations of 1:100. At lower concentrations (1:200), the staining was clearer because individual cells could be differentiated, and this allowed for the detection of high eNOS expressor cells only. Under these conditions, eNOS staining in vessels from nonpregnant women was 19.9%, stained lightly, and was confined to endothelial cells. There was no significant difference in eNOS staining in the normal pregnant women compared with the nonpregnant women (Table 2). For women with preeclampsia, immunostaining for eNOS was significantly greater than in the normal pregnant or nonpregnant groups (Table 2 and Figure 3). There was no evidence of iNOS staining in the vessels, although a positive control of activated macrophages stained clearly (data not shown).

SOD immunostaining was found in virtually all cells (Table 2) and was especially intense (4 arbitrary units) in the endothelial cell layer of these microvessels for normal pregnant women (Table 2 and Figure 4). The intensity of endothelial cell staining was lighter (1.7 arbitrary units) for women with preeclampsia (Table 2 and Figure 4) and nonpregnant women (1.9 arbitrary units; Table 2).

There was no evidence of nitrotyrosine staining in the vessels from the nonpregnant group (Table 2). Evidence for nitrotyrosine was found in only 3% of the vessels from normal pregnant women (Table 2 and Figure 5). However, for women with preeclampsia, immunostaining for nitrotyrosine in endothelial cells was evident in 73% of vessels and stained strongly (2.7 arbitrary units; Table 2 and Figure 5). Furthermore, staining of nitrotyrosine was seen outside the endothelial cell layer and may reflect the diffusion of peroxynitrite from the endothelium. Control sections with preabsorbed antibody with excess nitrotyrosine antigen were devoid of any staining.

**Discussion**

In the maternal vasculature of women with preeclampsia compared with that of normal pregnant women, we found that immunostaining was increased for eNOS, decreased for SOD, and increased for nitrotyrosine. These 3 findings taken together suggest that there is increased peroxynitrite formation in women with preeclampsia compared with women with normal pregnancies.

Oxidative stress has been implicated as a pathophysiological feature of women with preeclampsia; however, the effect of oxidative stress on vascular endothelial cell function is not well defined. It is known that endothelial cells are exposed to oxygen free radicals from both intracellular sources and products in the circulation. SOD is the only intracellular enzyme that quenches superoxide anions. In women with preeclampsia, reduced SOD activity in neutrophils and

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**Figure 4.** Immunohistochemistry for SOD. Representative sections of microvessels in subcutaneous fat biopsies from a normal pregnant woman at magnifications ×200 (a) and ×1000 (b) and from a woman with preeclampsia at magnifications ×200 (c) and ×1000 (d).
placenta has been reported. In our study, SOD was observed in virtually all endothelial cells, as well as in all other cells seen in the fat biopsy, including smooth muscle cells, fibroblasts, and adipose cells. In regard to endothelial cells, we demonstrated that immunostaining for SOD in the vasculature of women with preeclampsia was similar to that in the nonpregnant group but increased in the women with uncomplicated pregnancies. Because all cells stained for SOD, we had only a semiquantitative evaluation of intensity of staining. Further studies confirming reduced SOD expression and activity in the maternal vasculature are necessary. Nonetheless, our data suggest that endothelial cells from women with preeclampsia may not have adequate protection against superoxide anions during pregnancy.

Vascular function can be affected by oxidative stress through numerous mechanisms, including an effect on the NO pathway. NO is a free radical that is synthesized by oxidation of a guanidino nitrogen of L-arginine in a reaction catalyzed by the enzyme NOS. In endothelial cells, eNOS is present as well as iNOS under certain conditions. For instance, oxidative stress can induce expression of iNOS through nuclear factor-κB. In our study, however, we were not able to detect evidence of iNOS in any of the vessels. For eNOS, there was no significant difference in the amount of immunostaining in vessels from normal pregnant women compared with the nonpregnant group; however, it is important to note that the samples were obtained at only 1 time point in the pregnancy (at the time of delivery). For women with preeclampsia, there was increased immunostaining for eNOS compared with women with uncomplicated pregnancies or nonpregnant women. In agreement with these data, we previously have shown increased eNOS expression in cultured endothelial cells after exposure to plasma from women with preeclampsia. These data provide evidence that women with preeclampsia may have an increased capacity to produce NO. An increased production of NO may be a compensatory mechanism, but it could represent a pathophysiological process.

Although NO is an important vasorelaxant, an elevation of NO in the face of oxidative stress may be damaging. NO is known to react with superoxide anions, yielding the powerful oxidant peroxynitrite, which may alter vascular function. Recently, an elevation of peroxynitrite has been demonstrated in the placenta of women with preeclampsia. Our study provides evidence that there is increased peroxynitrite in the endothelium of the maternal vasculature of women with preeclampsia compared with women with uncomplicated pregnancies or nonpregnant women.

Peroxynitrite may be indicative of a reduced availability of NO to act as a vasorelaxant. Indeed, a reduced NO-mediated vasodilation has been observed in vessels from women with preeclampsia. However, our data indicate that this may not
be due to reduced NO synthesis but rather inactivation of NO by superoxide anions. A recent study in an animal model of aortic banding–induced hypertension observed that impaired endothelium-dependent relaxation coincided with increased NOS expression and superoxide anion production. These data would suggest that the enhanced expression of eNOS, as well as evidence for nitrotyrosine residues, could coincide with endothelial cell dysfunction in women with preeclampsia.

Peroxynitrite, as a pro-oxidant, could have numerous effects on the cell. In various cell types, peroxynitrite has been shown to mediate cell necrosis and apoptosis (a form of programmed cell death). In addition, peroxynitrite may also have a role in modulating eicosanoid synthesis. A recent report has shown that exogenous peroxynitrite can activate prostaglandin endoperoxide synthase (PGHS). In animal models of oxidative stress and hypertension, PGHS-dependent vasoconstriction predominates. Perhaps in women with preeclampsia, increased peroxynitrite increases PGHS-dependent vasoconstriction. Overall, peroxynitrite could be representative of a decreased bioavailability of NO as well as an initiator of several deleterious effects on endothelial cells. However, it is possible that peroxynitrite may have a protective role. A recent study reported that peroxynitrite may be beneficial by preventing leukocyte adhesion. Ultimately, further studies are necessary to understand the vascular effects of increased peroxynitrite in women with preeclampsia.

In summary, our data provide evidence of increased eNOS and nitrotyrosine formation, as well as decreased SOD, in the maternal systemic vasculature of women with preeclampsia compared with women with uncomplicated pregnancies. From these data, we speculate that oxidative stress in the face of elevated NO may lead to vascular endothelial cell dysfunction through the scavenging of NO and the formation of peroxynitrite.

Acknowledgments

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