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

Preeclampsia is a common (~7% of all pregnancies) disorder of human pregnancy in which the normal hemodynamic response to pregnancy is compromised. It remains a leading cause of maternal morbidity and mortality and is associated with a significant increase in perinatal mortality.1 It is diagnosed primarily by the onset of hypertension and proteinuria in the latter half of gestation. Other manifestations of preeclampsia include generalized vasoconstriction, increased vasoactivity, reduced perfusion to organs, and platelet activation.1 Both the etiology and pathophysiology of preeclampsia are poorly understood.

There is accumulating evidence that a major component of the pathophysiology of preeclampsia is endothelial cell dysfunction.2 Endothelial cells produce a number of vasoactive substances to modulate vascular function, including the potent vasorelaxant nitric oxide (NO). Although pregnancy is a state of vasodilatation mediated in part by NO,3 the role of NO in preeclampsia is not clear. Evidence for NO production in women with preeclampsia has been in conflict with reports of reduced,4 unchanged,5 or elevated6 NO metabolites in the circulation. However, when the effects of circulating factors are studied, the levels of NO metabolites as well as endothelial NO synthase (eNOS) protein were greater in endothelial cells exposed to plasma from women with preeclampsia than in those exposed to plasma from women with normal pregnancies.7 These data suggest that endothelium-derived NO may be increased in women with preeclampsia.

Oxidative stress has also been implicated in the pathophysiology of preeclampsia. Oxidative stress is an imbalance between pro-oxidant and antioxidant forces. In pregnancies complicated with preeclampsia, there is a marked decrease in serum antioxidant protection compared with uncomplicated pregnancies.8 One specific example of decreased antioxidant protection in preeclampsia is the enzyme superoxide dismutase (SOD). SOD, an intracellular enzyme that scavenges the free radical superoxide anion, has been shown to be decreased in neutrophils9 and placentas10 of preeclamptic women.

In women with preeclampsia, an elevation of endothelium-derived NO in the face of oxidative stress may be damaging. NO is known to react with superoxide anions (produced under conditions of oxidative stress), yielding the powerful oxidant peroxynitrite, which may alter vascular function.11 Peroxynitrite modifies tyrosine in proteins, resulting in nitro-
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 formation in the placental vasculature of women with preeclampsia. The presence 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.

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>% Vessels Staining†</th>
<th>Intensity of Staining</th>
<th>% Vessels Staining</th>
<th>Intensity of Staining</th>
<th>% Vessels Staining</th>
<th>Intensity of Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonpregnant</td>
<td>19.9±5.5</td>
<td>1.2±0.3</td>
<td>100.0±0.0</td>
<td>1.9±0.3</td>
<td>0.0±0.0</td>
<td>0.0±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>
<td>4.0±0.0†</td>
<td>3.2±1.4</td>
<td>0.0±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>
<td>1.7±0.2*</td>
<td>72.8±3.0†</td>
<td>2.7±0.3†</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 preeclampsia 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

Figure 3. Immunohistochemistry for eNOS. 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).
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 preg-
nant 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). Further-
more, staining of nitrotyrosine was seen outside the endothe-
lial 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 forma-
tion in women with preeclampsia compared with women with
normal pregnancies.

Oxidative stress has been implicated as a pathophysiolog-
tical 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
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

This work was supported by the Heart and Stroke Foundation of Alberta and N.W.T. and the Medical Research Council of Canada. S.T. Davidge is a scholar of the Alberta Heritage Foundation for Medical Research and the Heart and Stroke Foundation of Canada. A.M. Roggensack was supported by a Medical Research Council of Canada Burroughs Wellcome scholarship.

References

Evidence for Peroxynitrite Formation in the Vasculature of Women With Preeclampsia
Anne M. Roggensack, Yunlong Zhang and Sandra T. Davidge

Hypertension. 1999;33:83-89
doi: 10.1161/01.HYP.33.1.83

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 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/33/1/83

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/