Generation of Reactive Oxygen Species by Neutrophils and Endothelial Cell Injury in Normal and Preeclamptic Pregnancies

Kiyomi Tsukimori, Kotaro Fukushima, Akitoshi Tsushima, Hitoo Nakano

Abstract—The aim of this study was to investigate the role of neutrophil-derived reactive oxygen species on endothelial cell dysfunction in preeclampsia. We first assessed the correlation between nitrite and superoxide anion production in normal nonpregnant (n=10), normal pregnant (n=15), and preeclamptic women (n=12). We then examined neutrophil-mediated oxygen radical damage to human umbilical vein endothelial cells in vitro. Neutrophil superoxide release was measured by cytochrome C reduction; nitrite release was measured by the modified Griess reaction, and endothelial cell injury was measured by $^{51}$Cr release. N-formyl-methionyl-leucyl-phenylalanine–stimulated superoxide release by neutrophils was significantly increased in women with preeclampsia compared with the other 2 groups. Nitrite release by neutrophils was significantly decreased in preeclampsia compared with normal pregnancy. When neutrophils were pretreated with superoxide dismutase, nitrite release by neutrophils did not differ between normal pregnancy and preeclampsia, suggesting that excess superoxide anion in preeclampsia could reduce bioavailability of nitric oxide through neutrophil autocrine function. Neutrophil-mediated endothelial cell injury was significantly greater in women with preeclampsia than in the other 2 groups. Hydrogen peroxide was important in neutrophil-mediated endothelial cell injury in preeclampsia as catalase inhibited endothelial cell injury. When neutrophils were pretreated with N$\text{G}$-nitro-L-arginine methyl ester, neutrophil-mediated endothelial cell injury in preeclampsia was decreased, indicating a role for peroxynitrite formation as a mechanism of endothelial cell injury. In conclusion, the modulation of neutrophils causing superoxide release to dominate over nitrite release provides a reasonable explanation for endothelial cell dysfunction in preeclampsia. (Hypertension. 2005;46:696-700.)

Key Words: endothelium ■ free radicals ■ neutrophils ■ nitric oxide ■ preeclampsia ■ pregnancy

Preeclampsia is a hypertensive disorder of human pregnancy and a leading cause of maternal and fetal morbidity and mortality. There is increasing evidence to suggest endothelial cell damage and dysfunction in the pathogenesis of preeclampsia. Although the actual cause of this endothelial damage is not well-known, neutrophils, through their ability to produce reactive oxygen species (ROS), have been implicated as likely candidates. Superoxide anions ($O_2^-$) have been shown to influence vascular tone either indirectly, by inactivating NO and reducing the release of prostacyclin, or by directly contracting smooth muscle. High concentrations of superoxide have been found to reorient the arachidonic acid pathway in cells toward the production of thromboxane A2, which is a potent stimulator of vasoconstriction and platelet aggregation. The imbalance between prostacyclin and thromboxane A2 is well-documented in preeclampsia. Furthermore, neutrophil-derived superoxide anions can damage vascular integrity and endothelial cell function. In preeclampsia, we and other investigators have demonstrated a significant increase in $O_2^-$ production by neutrophils. Thus, it seems plausible that the increased ROS produced by neutrophils may be important in mediating endothelial damage in preeclampsia.

Neutrophils synthesize NO in addition to ROS from L-arginine by the enzyme NO synthase (NOS). The NO released by neutrophils has been documented to prevent neutrophil adhesion to the vascular endothelium, to control aggregation of neighboring platelets and, in the absence of endothelium, to produce a vasodilatory effect. NO also reacts with $O_2^-$ to yield the powerful peroxynitrite radical, which may alter vascular function. In contrast, NO inhibits nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and scavenges free radicals. Thus, NO can either scavenge $O_2^-$ or be transformed by $O_2^-$ into highly reactive nitrogen intermediates. In addition, NO is metabolized by a variety of other pathways. One is oxidation to nitrite and nitrate, and the other is reduction by S-nitrosogluthathione reductase to ammonia.
in vivo is usually measured by the concentrations of nitrite and nitrate in plasma, serum, or urine. In preeclampsia, the data on nitrite levels in blood are controversial, with studies reporting reduced,2 normal,23 or elevated24 nitrite levels. The circulating levels of nitrite and nitrate are affected by elimination, most of which occurs through the kidney, so renal changes in preeclampsia could affect circulating nitrite and nitrate levels.

In the present study, we investigated the role of neutrophil-derived ROS on endothelial cell dysfunction in preeclampsia. We first assessed the correlation of nitrite production with O$_2^-$ production, in normal nonpregnant, normal pregnant, and preeclamptic women. We then examined neutrophil-mediated oxygen radical damage of human umbilical vein endothelial cells in vitro.

Materials and Methods

Study Population

The study subjects included 12 pregnant women with preeclampsia, 15 women with normal pregnancies, and 10 nonpregnant women. Preeclampsia was diagnosed according to criteria of the International Society for the Study of Hypertension in Pregnancy,25 which includes a blood pressure $>140$/90 mm Hg and $>300$ mg of protein in a 24-hour urine collection. Women in active labor were excluded. All subjects were nonsmokers without a history of hypertension or of diabetes mellitus. Control subjects were matched for age and body mass index. The 2 groups of pregnant women were also matched for parity and gestational age at time of sampling. Mothers were cared for in the Maternity and Perinatal Care Unit, Kyushu University Hospital, and the nonpregnant subjects were volunteers. The Institutional Ethics Committee approved the study, and all subjects gave informed consent before participation. Procedures were in accordance with institutional guidelines.

Variable measurements included superoxide release by neutrophils, nitrite release by neutrophils, correlation between superoxide and nitrite production, neutrophil-mediated endothelial cell injury, and the effect of oxygen radical scavengers on endothelial cell injury.

Details on chemicals, isolation of human umbilical vein endothelial cells, preparation of neutrophils, variable measurements, and statistical analysis are available in an online supplement (http://hypertension.aha.org).

Results

Clinical data for normal nonpregnant women, women with normal pregnancies, and women with preeclampsia are summarized in Table 1.

$N$-formyl-methionyl-leucyl-phenylalanine (FMLP)-induced O$_2^-$ release by neutrophils in women with preeclampsia ($6.20 \pm 0.92$ nmol/10$^6$ cells per 5 minutes) was significantly higher than by cells from women with normal pregnancy or who were not pregnant ($3.63 \pm 0.91$ in normal pregnancy, $3.70 \pm 0.68$ in normal nonpregnancy) ($P<0.01$) (Figure 1).

Although neutrophils were observed to produce nitrite in all 3 groups, L-NAME was found to significantly inhibit nitrite release by neutrophils obtained from normal pregnant and preeclamptic women, but not from nonpregnant subjects (Figure 2). The L-NAME–inhibitable nitrite release by neutrophils in preeclampsia ($1.34 \pm 0.49$ mmol/2 $\times$ 10$^6$ cells/L) was significantly lower than in normal pregnancy ($2.46 \pm 0.64$) ($P<0.01$). The formula was L-NAME–inhibitable nitrite release= nitrite concentrations of neutrophils pretreated without L-NAME minus nitrite concentrations of neutrophils pretreated with L-NAME.

**Table 1. Clinical Profile of Women in the Study**

<table>
<thead>
<tr>
<th>Clinical Data for Subjects</th>
<th>NNP (n=10)</th>
<th>NP (n=15)</th>
<th>PE (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>29.1±4.4</td>
<td>28.1±4.7</td>
<td>30.9±5.6</td>
</tr>
<tr>
<td>Parity</td>
<td>NA</td>
<td>1.1±0.4</td>
<td>1.1±0.4</td>
</tr>
<tr>
<td>GA at sampling, wk</td>
<td>NA</td>
<td>34.6±2.9</td>
<td>34.2±2.8</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>124.2±5.4</td>
<td>118.3±5.8</td>
<td>172.4±15.2*</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>83.4±4.8</td>
<td>76.2±3.8</td>
<td>111.6±12.4*</td>
</tr>
<tr>
<td>Proteinuria, g/24h</td>
<td>ND</td>
<td>ND</td>
<td>3.2±1.5</td>
</tr>
<tr>
<td>GA at delivery, wk</td>
<td>NA</td>
<td>38.2±1.2</td>
<td>35.6±1.4*</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>NA</td>
<td>3050±320</td>
<td>2314±510*</td>
</tr>
<tr>
<td>Severe preeclampsia, N</td>
<td>NA</td>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>

Data are presented as the mean values (SD), or N, as appropriate. BMI indicates body mass index; BP, blood pressure; GA, gestational age; NA, not applicable; ND, not determined; NNP, normal nonpregnant; NP, normal pregnant; PE, preeclamptic. *P<0.01 compared to NNP and NP

When neutrophils were pretreated with L-NAME and compared with untreated neutrophils from each subject group, FMLP-induced O$_2^-$ release by neutrophils was significantly increased in normal pregnancy, but not in either preeclampsia or nonpregnancy (Figure 3). Compared with the different groups after L-NAME treatment, FMLP-induced O$_2^-$ release by neutrophils was also significantly higher in normal pregnancy than nonpregnancy and was further increased in preeclampsia (Figure 3).

When neutrophils were pretreated with SOD and compared with untreated neutrophils in each group, nitrite release by neutrophils in preeclampsia was significantly increased, but not in normal pregnancy (Table 2). In addition, the levels of nitrite release by neutrophils did not differ between normal pregnancy and preeclampsia after pretreatment with SOD (Table 2).

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**Figure 1.** FMLP-induced O$_2^-$ release of neutrophils compared among 3 groups: nonpregnant, normal pregnant, and preeclamptic subjects. Data are expressed as the mean O$_2^-$ release ±1 SD in each group. *P<0.01, compared with the other 2 groups.
Neutrophils from women with preeclampsia significantly increased the %Cr release compared with those from normal pregnant and normal nonpregnant women (21.8 ± 3.1% in pre-eclampsia, 8.4 ± 1.4 in normal pregnancy, 8.1 ± 1.2 in normal nonpregnancy; \( P < 0.01 \)) (Figure 4). Catalase significantly decreased the %Cr release values in all groups, but SOD did not (Table 3). When neutrophils were pretreated with L-NAME, the %Cr release in preeclampsia was significantly decreased; in contrast, the value in normal pregnancy was significantly elevated. The combination of L-NAME and SOD significantly decreased %Cr release value compared with neutrophils pretreated with L-NAME in preeclampsia (\( P < 0.05 \)), but not in normal pregnancy.

**Discussion**

In the present study, FMLP-induced \( O_2^- \) release by neutrophils was significantly enhanced in preeclampsia. This enhancement seems to have been achieved by modulating the signal transduction pathway for \( O_2^- \) production at the level between the FMLP receptors and protein kinase C, because enhanced \( O_2^- \) release was induced by FMLP, a receptor-mediated agonist, but not by phorbol 12-myristate 13-acetate, an agonist which bypasses the receptors and directly activates the protein kinase.\(^{10}\) NADPH oxidase is a major source of \( O_2^- \) in neutrophils and vascular endothelial cells.\(^{26}\) Lee et al reported increased sensitivity of the NADPH oxidase enzyme to agonist stimulation in preeclampsia.\(^{27}\) These findings suggest that preeclampsia is associated with an increased agonist-stimulated NADPH oxidase-mediated ROS production.

L-NAME inhibited nitrite release in both normal pregnant and preeclamptic women, but not in nonpregnant subjects. The amount of nitrite release by neutrophils in pregnancy in this experimental system was within the biological activity range (100 nmol/L to 1 \( \mu \)mol/L) previously reported.\(^{28}\) Therefore, these findings suggest that neutrophil NO release is increased in pregnancy. Garcia-Duran et al demonstrated that 17\( \beta \)-estradiol stimulated expression of the neuronal NOS isoform in a dose-dependent manner in human neutrophils.\(^{29}\) This finding suggests that increased neutrophil nitrite release in pregnant women is associated with enhanced NO production by estrogen-induced NOS expression because serum levels of 17\( \beta \)-estradiol are significantly increased during normal pregnancy.\(^{30}\) Other factors include systemic inflammatory response-induced NOS expression and enhanced NO production by neutrophils because normal pregnancy itself is a state of systemic inflammation.\(^{3} \) In contrast, neutrophil nitrite release in preeclampsia was signifi-

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**TABLE 2. Effect of Oxygen Radical Scavengers on Nitrite Release by Neutrophils in Normal and Preeclamptic Women**

<table>
<thead>
<tr>
<th>Scavengers</th>
<th>Nitrite Concentration (( \mu )mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.54 ± 0.64</td>
</tr>
<tr>
<td>SOD (10 ( \mu )mol/mL)</td>
<td>2.73 ± 0.56*</td>
</tr>
<tr>
<td>Catalase (200 IU/mL)</td>
<td>2.61 ± 0.72</td>
</tr>
</tbody>
</table>

\*\( P < 0.01 \), compared with no scavengers

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**Figure 2.** Nitrite concentration of neutrophils in nonpregnant, normal pregnant, and preeclamptic women. Data are expressed as the mean nitrite concentration ±1 SD in each group. Open bars: culture in RPMI1640. Black bars: culture in RPMI1640 containing L-NAME. \*\( P < 0.01 \), compared with culture in RPMI1640.

**Figure 3.** The FMLP-induced \( O_2^- \) release of neutrophils pretreated with or without L-NAME in nonpregnant, normal pregnant, and preeclamptic women. Data are expressed as the mean \( O_2^- \) release ±1 SD in each group. White bars: culture in RPMI1640. Black bars: culture in RPMI1640 containing L-NAME. \*\( P < 0.05 \), compared with the other 2 groups. \#\( P < 0.05 \), compared with nonpregnancy.

**Figure 4.** Neutrophil-mediated endothelial cell injury compared in nonpregnant, normal pregnant, and preeclamptic women. Data are expressed as the mean %Cr release ±1 SD in each group. \*\( P < 0.01 \), compared with the other 2 groups.
cantly decreased compared with normal pregnancy. In our preliminary study, serum levels of estradiol did not differ between normal pregnancy and preeclampsia (data not shown). Circulating levels of pro-inflammatory cytokines have been reported to be increased in preeclampsia compared with normal pregnancy.3 These findings suggest that excess O$_2^-$ could account for the reduced bioavailability of NO in neutrophils from women with preeclampsia because NO can be scavenged by O$_2^-$ to form peroxynitrite (ONOO$^-$), effectively reducing the bioavailability of NO.31

With regard to the correlation between O$_2^-$ and NO syntheses, low concentrations (nmol/L) of NO enhance O$_2^-$ generation in neutrophils, whereas higher concentrations (μmol/L) inhibit it.19,32,33 NO in higher concentrations inhibits NADPH oxidase activity and scavenges free radicals.32-34 In the present study, inhibition of NOS led to the enhancement of O$_2^-$ release in neutrophils from normal pregnant women. This phenomenon suggests that neutrophil-derived NO in normal pregnancy can be considered as a scavenger for suppressing O$_2^-$ activity because neutrophil NO release was enhanced in normal pregnancy. In preeclampsia, l-NAME administration was not effective in neutrophils regardless of nitrite in higher concentrations (μmol/L). However, O$_2^-$ release by neutrophils after pretreatment with l-NAME in preeclampsia was significantly enhanced compared with the other 2 groups. These findings indicate that O$_2^-$ production by neutrophils is increased by normal pregnancy and further enhanced in preeclampsia. This phenomenon is consistent with a previous report demonstrating that intracellular ROS are increased in normal pregnancy and further increased in preeclampsia.11 The mechanism for the increase in ROS may involve syncytiotrophoblast microvil- lous membranes, which are shed into the maternal circulation in increased amounts in preeclampsia.35 and which induce neutrophil to generate superoxide radicals.36

SOD administration, which dismutates O$_2^-$ to hydrogen peroxide (H$_2$O$_2$), led to the enhancement of nitrite release in neutrophils from preeclamptic women, but not in neutrophils from normal pregnant women when compared with untreated neutrophils from each group. In addition, the levels of nitrite release by neutrophils after pretreatment with SOD did not differ between normal pregnancy and preeclampsia. These findings indicate that neutrophil nitrite production was enhanced in normal pregnancy and suggest that excess O$_2^-$ in preeclampsia could reduce bioavailability of NO through neutrophil autocrine function. In addition, modulation of oxygen radical formation in neutrophils in preeclampsia results in the dominance of O$_2^-$ production over nitrite release.

The generation of ROS from activated neutrophils has been shown to result in significant endothelial cell injury in a variety of disease states.37 Several in vitro studies have been performed to determine the mechanism of neutrophil-mediated cell injury.28,38,39 In those studies, H$_2$O$_2$ or the hydroxyl radical were implicated as the oxidant responsible for cell injury. In the present study, neutrophils from women with preeclampsia produced significantly greater endothelial cell injury than did neutrophils from the other 2 groups. This finding indicates that neutrophils from women with preeclampsia damage endothelial cells directly. Catalase, which catalyzes the conversion of H$_2$O$_2$ to oxygen and water, inhibited neutrophil-mediated endothelial cell injury in preeclampsia, suggesting that H$_2$O$_2$ may serve as a mechanism of neutrophil-mediated endothelial cell injury in preeclampsia. In contrast, SOD, which dismutates O$_2^-$ to H$_2$O$_2$ did not affect neutrophil-mediated endothelial cell injury. Two possible explanations should be considered. First, H$_2$O$_2$ is converted by endogenous catalase to water, and regulation of any of these endogenous enzyme systems may modulate H$_2$O$_2$ levels. Second, the decline in O$_2^-$ is expected to reduce the generation of ONOO$^-$, which has also been found to mediate cytotoxicity,33 and consequently to reduce endothelial cell injury mediated by ONOO$^-$. Under normal circumstances, the relatively high abundance of the SOD enzyme dismutates O$_2^-$ to H$_2$O$_2$; however, when NO is produced in large quantities, a significant amount of O$_2^-$ reacts with NO to produce ONOO$^-$.40 The production of ONOO$^-$ in neutrophils has also been found to mediate cytotoxicity.33 In the present study, when neutrophils were pretreated with l-NAME, neutrophil-mediated endothelial cell injury was significantly decreased in preeclampsia. These findings suggest that ONOO$^-$, formed by the interaction of excess neutrophil-derived O$_2^-$ with neutrophil and/or endothelial derived NO, may also serve as a mechanism of neutrophil-mediated endothelial cell injury in preeclampsia. This phenomenon is consistent with a previous report demonstrating increased endothelial NO synthase and immunostaining of nitrotyrosine, which acts as a marker for ONOO$^-$ in the maternal vasculature of women with preeclampsia.41 In addition, the combination of l-NAME and SOD significantly decreased neutrophil-mediated endothelial cell injury compared with neutrophils pretreated with l-NAME alone in preeclampsia. This finding suggests that neutrophil-derived ROS could also have an ONOO$^-$-independent cytotoxic effect on endothelial cells because NO can react with H$_2$O$_2$ to produce yet another deleterious radical, singlet oxygen.42 Interestingly, the neutrophil-induced endothelial cell injury from women with normal pregnancy was significantly enhanced by pretreatment with l-NAME. This may have been caused by neutrophil-mediated O$_2^-$ injury to endothelial cells because l-NAME led to the enhancement of O$_2^-$ release in the neutrophils of normal pregnant women.

In conclusion, neutrophil-derived NO acts as a scavenger that protects cytotoxic O$_2^-$ activity during a normal pregnancy. Neutrophils are modulated in preeclampsia so that there is a dominance of O$_2^-$ production over NO release, causing endo- thelial cell injury via the products of O$_2^-$, H$_2$O$_2$, and ONOO$^-$. 

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**TABLE 3. Effect of Oxygen Radical Scavengers on Neutrophil-Mediated Endothelial Cell Damage**

<table>
<thead>
<tr>
<th>Scavengers</th>
<th>%Cr Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (n=6)</td>
<td>6.8±1.4</td>
</tr>
<tr>
<td>SOD (10 μmol/mL)</td>
<td>6.7±1.5</td>
</tr>
<tr>
<td>Catalase (200 IU/mL)</td>
<td>3.4±0.7*</td>
</tr>
<tr>
<td>L-NAME (10 mmol/L)</td>
<td>7.1±1.2</td>
</tr>
<tr>
<td>L-NAME (10 mmol/L) + SOD (10 μmol/mL)</td>
<td>5.8±1.4</td>
</tr>
</tbody>
</table>

l-NAME indicates N G nitro-L-arginine methyl ester.

*P<0.01, †P<0.05, compared to no scavengers, ‡P<0.05, compared to l-NAME pretreated.
Neutrophil ROS generation provides a reasonable explanation for endothelial cell dysfunction in preeclampsia.

Perspectives

The present study is the first, to our knowledge, to assess the correlation of neutrophil nitrite production with superoxide anion production in preeclampsia. Neutrophil ROS generation in preeclampsia is characterized by the dominance of $O_2^-$ production over nitrite release. Neutrophil ROS generation could explain the mechanism of endothelial dysfunction, which has been implicated as the central pathophysiological feature of preeclampsia. The newly recognized role for neutrophil ROS generation in preeclampsia represents a significant advance in our understanding of this disease.

Acknowledgments

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