Abstract—This study investigated whether production of reactive oxygen species by neutrophils differs between women with preeclampsia and those with essential hypertension. First, we assessed superoxide production by neutrophils during pregnancy and 4 weeks after delivery in 15 healthy pregnant women, 12 women with preeclampsia, and 7 pregnant women with essential hypertension. We then examined effects of serum from each subject on superoxide production by neutrophils obtained from healthy nonpregnant women. Neutrophil superoxide production was measured by cytochrome C reduction. N-formyl-methionyl-leucyl-phenylalanine-stimulated superoxide production was significantly increased in neutrophils from women with preeclampsia and women with essential hypertension compared with normal pregnant women. Four weeks postpartum, the level of superoxide production was significantly decreased in women with preeclampsia but not in women with either normal pregnancy or essential hypertension. When neutrophils obtained from nonpregnant women were preincubated with predelivery sera from each group, sera from women with preeclampsia significantly enhanced superoxide production compared with sera from the other 2 groups. When postpartum serum was used, enhancement of neutrophil superoxide production by serum from women with preeclampsia was significantly decreased compared with that by predelivery serum. In conclusion, increased neutrophil superoxide production resolved after delivery in preeclampsia, whereas activation persisted postpartum in women with essential hypertension. The different transition of neutrophil superoxide production in response to pregnancy appears to be that preeclampsia is characterized by the presence of serum factors that enhance neutrophil superoxide production. Thus, in preeclampsia, serum factors bear a more essential role producing superoxide than a behavior of neutrophils. (Hypertension. 2007;49:1436-1441.)

Key Words: preeclampsia ■ essential hypertension ■ neutrophil ■ free radical ■ superoxide

Preeclampsia is a hypertensive disorder of unknown etiology affecting 5% to 10% of all pregnancies. Pathophysiological changes include elevated systemic vascular resistance, generalized vasoconstriction, and activation of the coagulation cascade, all of which may be explained by disruption of normal maternal endothelial function. Recent evidence suggests that neutrophils, through their ability to produce reactive oxygen species (ROS), play a role in mediating endothelial damage in women with preeclampsia. We and other investigators have demonstrated a significant increase in superoxide anion \( \text{O}_2^- \) production by neutrophils in patients with preeclampsia. Recently, we demonstrated that enhanced \( \text{O}_2^- \) production by neutrophils from women with preeclampsia damages cultured human umbilical vein endothelial cells directly in vitro. In addition to damaging endothelial cells directly, ROS inhibit vascular relaxation by NO in vivo and in vitro. Furthermore, ROS may contribute to the prostaglandin imbalances characteristic of preeclampsia, because oxidative stress increases prostaglandin H synthesis activity. Thus, it seems plausible that the increased level of ROS produced by neutrophils may be implicated in the pathophysiology of preeclampsia. However, the mechanisms leading to increased ROS production by neutrophils in preeclampsia are not well known.

Like preeclampsia, essential hypertension is characterized by increased \( \text{O}_2^- \) production by neutrophils compared with the \( \text{O}_2^- \) production in healthy adults. In the Sabra rat model of experimental hypertension, increased \( \text{O}_2^- \) production by neutrophils precedes the development of hypertension. In addition, p22phox polymorphisms may be important in altered reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase-generated \( \text{O}_2^- \) production in human essential hypertension. These findings implicate a genetic predisposition to neutrophil activation and subsequent ROS production in essential hypertension. The mechanisms leading to increased ROS production by neutrophils in preeclampsia may not be the same as those for women with essential hypertension, because blood pressure returns to

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normal after delivery in women with preeclampsia, whereas hypertension persists after delivery in women with essential hypertension.

In the present study, we initially studied $O_2^\cdot$ production by neutrophils during pregnancy and 4 weeks postpartum in women with preeclampsia and women with essential hypertension to investigate whether ROS production by neutrophils differs between these 2 conditions. In addition, to examine whether circulating factors in women with preeclampsia or essential hypertension cause neutrophil activation, we studied the effect of sera collected during pregnancy and 4 weeks postpartum from women with preeclampsia and from women with essential hypertension on $O_2^\cdot$ production by neutrophils obtained from healthy, nonpregnant women.

### Methods

#### Study Population

The study subjects included 12 pregnant women with preeclampsia, 7 pregnant women with essential hypertension, and 15 healthy, pregnant women. Preeclampsia was diagnosed according to the criteria of the International Society for the Study of Hypertension in Pregnancy,\(^1\) which include a blood pressure $>$ 140/90 mm Hg and $>$ 300 mg of protein in a 24-hour urine collection. The women with essential hypertension did not have superimposed preeclampsia. Women in active labor were excluded. All of the subjects were nonsmokers without a history 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 women were volunteers. All of the pregnant women were studied before delivery and at 4 weeks postpartum. The Institutional Ethics Committee approved the study design, and all of the subjects gave informed consent before participation. Procedures were in accordance with institutional guidelines.

#### Preparation of Neutrophils

At each sampling point, heparinized blood samples (25 to 30 mL) were obtained from 9 pregnant women with preeclampsia (7 with severe preeclampsia and 2 with mild preeclampsia), 5 pregnant women with essential hypertension, and 15 healthy pregnant women, which are listed in the Table. Neutrophils were isolated according to the following procedure: dextran sedimentation, hypotonic lysis of erythrocytes, and use of the Conray–Ficoll method.\(^3\)\(^4\) The cell population used for subsequent analysis had $>$ 98% neutrophils and $<$ 2% combined of lymphocytes, monocytes, and eosinophils.

#### Superoxide Production by Neutrophils

Neutrophils ($1 \times 10^6$ cells/mL) were suspended in a HEPES buffer solution containing 131 mmol/L of NaCl, 4.7 mmol/L of KCl, 1 mmol/L of CaCl\(_2\), 5 mmol/L of glucose, and 20 mmol/L of HEPES (pH 7.4) for 5 minutes at 37°C before the addition of $1 \times 10^{-7}$ mol/L of N-formyl-methionyl-leucyl-phenylalanine (FMLP) (Sigma Chemical Co). Production of $O_2^\cdot$ was measured by determining the rate of superoxide dismutase–inhibitable reduction of ferricytochrome $c$ (Sigma Chemical Co) at 550 to 540 nm with use of a dual-wavelength spectrophotometer (model 557, Hitachi Co).\(^3\)\(^4\) The mean $O_2^\cdot$ value for each subject obtained from 3 independent measurements was used.

#### Effect of Circulating Factors on Neutrophil Superoxide Production

To assess whether circulating factors contribute to neutrophil activation in pregnant women with preeclampsia or essential hypertension, we studied the effect of sera from pregnant women with preeclampsia and those with essential hypertension on $O_2^\cdot$ generation by neutrophils obtained from healthy, nonpregnant subjects. The serum fraction obtained from each subject was separated by centrifugation at 1500 rpm for 10 minutes and then heated to 56°C for 30 minutes to inactivate the complement system, which can affect neutrophil function.\(^3\) Neutrophils ($1 \times 10^6$ cells/mL) from healthy, nonpregnant women were preincubated with sera from study subjects at a concentration of 10 mg/mL in HEPES buffer for 1 hour at 37°C. $O_2^\cdot$ generation was then assessed as described above. An incubation time of 1 hour was chosen because it has been reported previously that the serum-enhancing effect depends on preincubation time, with maximal effect achieved after 1 hour.\(^3\) Protein concentrations of the samples were quantified by the method of Lowry et al.\(^1\)

#### Statistical Analysis

Results are expressed as the mean±SD. Differences in $O_2^\cdot$ production by neutrophils were first statistically evaluated using the Kruskal–Wallis $H$ test and then analyzed by means of the Mann–Whitney $U$ test to compare subgroups using the computer software programs StatView and ANOVA (Abacus Concepts Inc). The difference in $O_2^\cdot$ production by neutrophils before delivery and at 4

### Clinical Profile of Women in the Study

<table>
<thead>
<tr>
<th>Variable</th>
<th>NP (n=15)</th>
<th>PE (n=12)</th>
<th>EHT (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>29.5±2.3</td>
<td>31.4±2.7</td>
<td>34.3±3.8</td>
</tr>
<tr>
<td>Prepregnancy BMI</td>
<td>24.8±5.0</td>
<td>27.1±6.4</td>
<td>27.9±4.2</td>
</tr>
<tr>
<td>Primigravida, No. (%)</td>
<td>10 (66.7)</td>
<td>9 (75)</td>
<td>4 (57.2)</td>
</tr>
<tr>
<td>GA at sampling, weeks</td>
<td>37.6±1.5</td>
<td>34.5±2.8</td>
<td>36.5±3.0</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>112.6±5.8</td>
<td>162.2±18.2*</td>
<td>154.8±8.2*</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>71.2±5.8</td>
<td>114.0±9.4*</td>
<td>105.3±7.8*</td>
</tr>
<tr>
<td>Proteinuria, g/24 h</td>
<td>ND</td>
<td>3.8±1.2*</td>
<td>ND</td>
</tr>
<tr>
<td>Severe preeclampsia, No.</td>
<td>NA</td>
<td>8</td>
<td>NA</td>
</tr>
<tr>
<td>GA at delivery, weeks</td>
<td>37.6±1.5</td>
<td>35.1±3.2</td>
<td>37.0±3.0</td>
</tr>
<tr>
<td>Cesarean section, No.</td>
<td>10</td>
<td>8</td>
<td>5</td>
</tr>
</tbody>
</table>

Data are presented as mean (SD) or as number as appropriate. NP indicates normal pregnant women; PE, women with preeclampsia; EHT, women with essential hypertension; BMI, body mass index; GA, gestational age; BP, blood pressure; NA, not applicable; ND, not determined.

*P<0.01 vs NP.
weeks postpartum was analyzed by a paired, 2-tailed t test. A $P<0.05$ was considered to be significant.

**Results**

Clinical data for women with normal pregnancies, women with preeclampsia, and women with essential hypertension are summarized in the Table. Blood pressures during pregnancy did not differ between women with preeclampsia and those with essential hypertension. At 4 weeks postpartum, blood pressures had returned to normal in all of the women with preeclampsia, whereas blood pressures were the same as in pregnancy for women with essential hypertension.

During pregnancy, FMLP-induced $O_2^-$ production by neutrophils in women with preeclampsia ($6.14\pm0.90$ nmol/10$^6$ cells per 5 minutes) and women with essential hypertension ($5.05\pm1.10$ nmol/10$^6$ cells per 5 minutes) were significantly higher than by cells from women with normal pregnancies ($2.82\pm0.44$ nmol/10$^6$ cells per 5 minutes; $P<0.01$ and $P<0.05$, respectively; Figure 1). There was no significant difference in FMLP-induced $O_2^-$ production by neutrophils between women with preeclampsia and women with essential hypertension. At 4 weeks postpartum, FMLP-induced $O_2^-$ production by neutrophils in women with preeclampsia was significantly decreased ($2.74\pm0.35$ nmol/10$^6$ cells per 5 minutes; $P<0.01$) compared with pregnancy levels, but this was not observed in women with normal pregnancies or essential hypertension ($2.52\pm0.48$ nmol/10$^6$ cells per 5 minutes in normal pregnancy and $4.98\pm0.97$ nmol/10$^6$ cells per 5 minutes in essential hypertension). FMLP-induced $O_2^-$ production by postpartum neutrophils in women with essential hypertension was significantly higher than by postpartum cells from women with either preeclampsia or normal pregnancies.

When neutrophils obtained from healthy, nonpregnant women were preincubated with sera obtained during pregnancy, sera from women with preeclampsia significantly enhanced FMLP-induced $O_2^-$ production by neutrophils ($5.55\pm0.82$ nmol/10$^6$ cells per 5 minutes) compared with sera from the other 2 groups ($2.65\pm0.71$ nmol/10$^6$ cells per 5 minutes for normal pregnancy and $2.97\pm0.55$ nmol/10$^6$ cells per 5 minutes for essential hypertension; $P<0.01$; Figure 2). At 4 weeks postpartum, the enhancement effect of sera from women with preeclampsia on FMLP-induced $O_2^-$ production by neutrophils was significantly decreased ($2.68\pm0.61$ nmol/10$^6$ cells per 5 minutes) compared with enhancement seen with sera obtained during pregnancy ($P<0.01$); furthermore, enhancement with postpartum sera did not differ from that seen for the other 2 groups of postpartum women ($2.49\pm0.69$ nmol/10$^6$ cells per 5 minutes for normal pregnancy and $2.72\pm0.52$ nmol/10$^6$ cells per 5 minutes for essential hypertension).

**Discussion**

In the present study, we showed that FMLP-induced $O_2^-$ production by neutrophils during pregnancy was increased in women with preeclampsia compared with women with normal pregnancies. At 4 weeks postpartum, the levels of neutrophil $O_2^-$ production in women with preeclampsia did not differ from those of women with normal pregnancies. These results indicate that, in preeclampsia, neutrophil $O_2^-$ production is enhanced during pregnancy but returns to baseline levels after delivery. This finding is consistent with a previous report demonstrating that neutrophil activation resolves after delivery in preeclampsia based on the expression of adhesion molecules CD11b and CD18 on the surface of neutrophils.16

In women with essential hypertension, neutrophil $O_2^-$ production increased during pregnancy and remained at an increased level 4 weeks postpartum. These findings indicate that chronological changes in neutrophil $O_2^-$ production throughout pregnancy differ between women with preeclampsia and those with essential hypertension. The main
clinical difference between preeclampsia and essential hypertension is that the blood pressure returns to normal in the postpartum period in preeclamptic women but remains elevated in the women with essential hypertension. In the spontaneously hypertensive rat model, in which neutrophil $O_2^-$ production is increased, administration of superoxide dismutase normalizes blood pressure. In the Sabra rat model of experimental hypertension, the increase in $O_2^-$ production by neutrophils precedes the development of hypertension. These findings implicate increased neutrophil $O_2^-$ production in the development of hypertension both in women with preeclampsia, as well as those with essential hypertension.

The mechanisms leading to increased ROS production by neutrophils are not well known. In the present study, sera from women with preeclampsia enhanced $O_2^-$ production by neutrophils from healthy nonpregnant women. This enhancing effect diminished in the postpartum period. These findings indicate that serum factors that enhance $O_2^-$ production by neutrophils are present during pregnancy in preeclampsia. Evidence of a serum factor in the plasma from women with preeclampsia is not, however, a universal finding. The discrepancies in the serum-enhancing effect between what we documented and the findings of a previous report may partly be the result of different experimental conditions, because we have reported previously that serum-enhancing effect depends on preincubation time, with maximal effect achieved after 1 hour, whereas an incubation time of 20 minutes was chosen in the other report.

In contrast, in women with essential hypertension, whose blood pressures during pregnancy were the same as those of women with preeclampsia, serum-enhancing effects on neutrophil $O_2^-$ production did not differ from the effects of sera obtained from women with normal pregnancies. NADPH oxidases are one of the most important sources of $O_2^-$ in phagocytic and vascular cells. Recently, several polymorphisms of CYBA, the gene that encodes the essential subunit of the NADPH oxidase p22phox, have been found to be functionally associated with NADPH oxidase activity in essential hypertension. Hypertensive subjects carrying the GG genotype of the p22phox polymorphism exhibit increased phagocytic p22phox mRNA expression and enhanced NADPH oxidase activity. These findings implicate a genetic predisposition in the increased ROS production in essential hypertension. However, no difference in the abundance of any of the NADPH oxidase phox subunits between preeclampsia and normal pregnancy has been reported. In the present study, none of our patients with preeclampsia had a family history of hypertension. In addition, the presence of a serum-enhancing effect on neutrophil $O_2^-$ production is associated with increased $O_2^-$ production by neutrophils from preeclampsia. These findings suggest that increased ROS production by neutrophils in preeclampsia is at least in part because of an extrinsic influence rather than genetic predisposition. Thus, the mechanisms leading to increased ROS production by neutrophils during pregnancy may differ for women with preeclampsia and women with essential hypertension.

Clinically, a major difference between groups is that the extent and degree of ischemic damage in the placentas of women with essential hypertension is significantly less than the damage in placentas of women with preeclampsia. In vivo and in vitro hypoxia and ischemia have been shown to increase production of proinflammatory cytokines such as tumor necrosis factor-$\alpha$ and interleukin (IL)-1$\beta$ and IL-6 by placental tissue. These cytokines activate human neutrophils. Circulating levels of IL-6 and tumor necrosis factor-$\alpha$ and expression of tumor necrosis factor-$\alpha$ and IL-1$\beta$ by the placenta were shown to be increased in some but not all studies of preeclampsia. Furthermore, in vitro hypoxia–reoxygenation stimulates apoptotic changes within syncytiotrophoblasts in normal third-trimester placentas. These
aponecrotic processes can lead to deportation of syncytiotrophoblast microvesicles into the maternal circulation,35 circulating syncytiotrophoblast microvesicles, which also lead to enhanced \( \text{O}_2^- \) production by maternal neutrophils,36 are significantly elevated in women with preeclampsia.37 Although we previously reported no significant differences in the serum concentrations of proinflammatory cytokines, such as tumor necrosis factor-\( \alpha \), IL-1\( \beta \), and IL-6 between normal pregnant and preeclamptic women,3 the findings from the former studies suggest that placental ischemia may be important in initiating the systemic neutrophil activation seen in preeclampsia. The present results demonstrating the postpartum disappearance of the serum-enhancing effect on neutrophil \( \text{ROS} \) production in preeclampsia are compatible with these findings.

In conclusion, chronological changes of neutrophil \( \text{O}_2^- \) production throughout pregnancy differ between women with preeclampsia and those with essential hypertension; increased \( \text{O}_2^- \) production by neutrophils resolved after delivery in preeclampsia, whereas activation persisted postpartum in women with essential hypertension. The different transition of neutrophil superoxide production in response to pregnancy appears to be that preeclampsia is characterized by the presence of serum factors that enhance neutrophil superoxide production. This suggests that serum factors bear a more essential role producing superoxide than a behavior of neutrophils in preeclampsia.

Perspectives

In this study, we assess the difference in the neutrophil superoxide production between preeclampsia and essential hypertension in pregnancy. In preeclampsia, increased superoxide production by neutrophils resolved after delivery, whereas activation persisted postpartum in women with essential hypertension. This difference in the transition of neutrophil superoxide production in response to pregnancy results from the enhancement of neutrophil superoxide production in the preeclamptic pregnancy. Further study is necessary to identify the serum factor(s) in order to understand, treat, and eventually prevent preeclampsia.

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Disclosures

None.

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Difference in Neutrophil Superoxide Generation During Pregnancy Between Preeclampsia and Essential Hypertension

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