Preeclamptic Serum Enhances Endothelin-Converting Enzyme Expression in Cultured Endothelial Cells

Satoshi Nishikawa, Atsushi Miyamoto, Hiroyuki Yamamoto, Hideyo Ohshika, and Ryuichi Kudo

Increased vascular sensitivity to vasoconstrictors, such as angiotensin II and epinephrine, is observed in preeclampsia (PE). Recently, it was suggested that abnormal endothelial function might contribute to the pathophysiologic changes in PE. We investigated vasoconstrictor (angiotensin II and epinephrine)-induced endothelin-1 (ET-1) release from human umbilical vein endothelial cells incubated with sera from women with PE compared with normotensive pregnant and nonpregnant women. Moreover, inositol 1,4,5-trisphosphate production and endothelin-converting enzyme (ECE) expression in human umbilical vein endothelial cells were also evaluated. There were no significant differences in ET-1 release without vasoconstrictors among the three groups (nonpregnant, normotensive pregnant, and PE). No significant differences in basal inositol 1,4,5-trisphosphate production and ECE expression without vasoconstrictors were detected among the three groups. No significant differences in vasoconstrictor-induced inositol 1,4,5-trisphosphate production among the three groups. However, ECE expression after incubation with vasoconstrictor was significantly increased by PE sera. Our results suggest that ET-1 release from endothelial cells may contribute to the increased vascular sensitivity to vasoconstrictors observed in PE, and that vasoconstrictor-induced ET-1 release may be related to enhanced ECE expression. Am J Hypertens 2001;14:77–83 © 2001 American Journal of Hypertension, Ltd.

Key Words: Preeclampsia, endothelin-1, endothelin-converting enzyme, human umbilical vein endothelial cells.

Preeclampsia (PE) is a pregnancy-specific disease that causes fetal growth retardation, infant morbidity and mortality, premature birth, and maternal death. This disease is considered to be one of the most significant health problems in human pregnancy, complicating about 6% to 8% of all gestations of more than 20 weeks, but the cause is not clearly understood. Increased vascular sensitivity to vasoconstrictors such as angiotensin II (Ang II) and epinephrine (Epi) is observed in PE.1,2 Recently, it was suggested that endothelial dysfunction may contribute to the pathophysiologic changes observed in PE.3,4

Endothelial cells play an important role in the regulation of vascular smooth muscle tone by releasing several active substances. Endothelin-1 (ET-1) is a potent vasoconstrictor peptide produced by vascular endothelial cells. ET-1 release is augmented by intracellular free-calcium ions caused by inositol 1,4,5-trisphosphate (IP3) production in endothelial cells.5 ET-1 appears to play a role in the pathogenesis of some forms of hypertension,6,7 but the role of ET-1 in hypertension is still unclear. ET-1 has been thought to play an important role in the maintenance of blood pressure (BP) and some role in the response to injury of endothelial cells.8 In PE plasma, an increased concentration of ET-1 has been reported.9–12 Therefore, it is important to clarify the regulatory mechanism of ET-1 production by endothelial cells in PE. In this report, we evaluated ET-1 production by human umbilical vein endothelial cells (HUVEC) incubated with PE sera. We also examined the mechanism of Ang II- and Epi-induced ET-1 release from HUVEC incubated with PE sera.
Methods

Subjects

We obtained serum samples from 15 healthy nonpregnant (NP) women, 21 normotensive pregnant (NTP) women, and 21 women with PE after receiving informed consent. Subjects were recruited with a protocol approved by the Hospital Ethics Committee. All samples were collected before delivery, and samples from PE women were collected at the time of the first diagnosis of PE. None of the women with PE had received any drugs such as magnesium sulfate or antihypertensive drugs. The serum was stored at −80°C until experiments. BP was recorded just before blood collection. PE criteria are hypertension, proteinuria, and reversal of hypertension and proteinuria after pregnancy. Hypertension was defined as an absolute BP ≥140/90 mm Hg. Proteinuria was defined as ≥2+ on dipstick or =0.3 g/24 h. The NP, NTP, and PE groups were matched for mean age. The NTP and PE groups were matched for the mean gestational age at blood sampling. Patients with fever or a prior history of chronic disease (such as chronic hypertension, diabetes, renal disease, or cardiovascular disease) were excluded.

Endothelial Cell Culture

Isolation and culture of HUVEC were performed by a modification of the method of Jaffe, as described previously. Briefly, endothelial cells were isolated with 0.05% trypsin and grown in RPMI 1640 medium containing 10% fetal bovine serum, 30 μg/mL endothelial cell growth supplement, 100 U/mL penicillin, 100 μg/mL streptomycin, and 3 μg/mL L-glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The cells were identified as endothelial cells by contact-inhibited monolayer growth with a cobblestone appearance and the presence of Factor VIII antigen by immunocytochemical study. The primary cultures were dispersed and passaged with 0.01% trypsin, plated in 16-mm (24-well plates) or 30-mm collagen (type I)-coated plastic dishes (Iwaki Glass, Funabashi, Japan), and grown as confluent monolayers. Second-passage cells were used for all experiments.

Study Design

For measurement of ET-1 release, HUVEC in culture medium without fetal bovine serum were incubated with 10% serum (NP, NTP, and PE) for 6 h with or without Ang II (50 μmol/L) and Epi (50 μmol/L). For measurement of ET-1 release, HUVEC were incubated in the centers of 24-well plates using RPMI 1640 medium. For measurement of IP₃ production and endothelin-converting enzyme (ECE) expression, HUVEC in RPMI 1640 medium without fetal bovine serum were incubated with 10% serum (NP, NTP, and PE) for 6 h in 30-mm dishes, and cells were collected after 30-sec stimulation by Ang II (50 μmol/L) and Epi (50 μmol/L).

Determination of ET-1

The ET-1 concentrations in the culture medium and serum samples were determined using the ET-1 ELISA system (Amersham, Tokyo, Japan) and RIA system (Amersham, Tokyo, Japan), respectively.

Determination of IP₃

Incubation of HUVEC was terminated by the addition of ice-cold 30% perchloric acid, and the cells were placed on ice for 30 min. The perchloric acid extract was neutralized and IP₃ production in HUVEC was determined using an IP₃ [³H] assay system (Amersham) as described previously.

Determination of Endothelin-Converting Enzyme Expression

ECE expression was determined by Western immunoblotting. Gel electrophoresis and immunoblotting were performed using the procedures previously described. Culture dishes were rinsed twice with cold phosphate-buffered saline solution (136.9 mmol/L NaCl, 2.7 mmol/L KCl, 0.2 mmol/L KH₂PO₄, 7.8 mmol/L Na₂HPO₄, and 1 mL of ice-cold solution A (50 mmol/L Tris-HCl, pH 7.4), containing 10 μg/mL soybean trypsin inhibitor, 5 μg/mL leupepsin, 200 μg/mL bacitracin, 2 mmol/L EDTA, and 100 μg/mL phenylmethylsulfonfonyl fluoride added to prevent proteolysis of protein. HUVEC were then scraped from culture dishes on ice with a rubber policeman. Collected cells were centrifuged at 1000 g for 15 min at 4°C. Sedimented cells were resuspended in solution A and homogenized by a Kinematica Polytron (10 sec at setting 6) on ice and stored at −80°C. On the day of the experiment, sample buffer containing 62.5 mmol/L Tris/HCl (pH 6.8), 2% SDS, 10% glycerol, 0.025% bromophenol blue, and 4% 2-mercaptoethanol was added to cell membrane suspensions and the mixture was boiled for 5 min. Aliquots (15 μg/protein/lane) were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis with 4/20% polyacrylamide gel, and proteins were transferred electrophoretically (30 V, 90 min) to nitrocellulose membranes. Blots were washed with 0.01 mol/L Tris buffer (pH 7.4) containing 0.9% NaCl, blocked by incubation (42°C) with 0.01 mol/L Tris/0.9% NaCl buffer containing 3% bovine serum albumin. After subsequent incubations with the primary ECE monoclonal antibody, and antimouse IgG labeled with horseradish peroxidase, immunoreactivity was detected with the enhanced chemiluminescence Western Blot detection system followed by exposure to Hyperfilm-ECL. After suitable exposure periods, immunolabeled ECE bands were analyzed densitometrically using a Molecular Dynamics laser densitometer (PDSI, Molecular Dynamics, Sunnyvale, CA). Protein was determined by the method of Lowry et al using bovine serum albumin as the standard.
Table 1. Clinical characteristics

<table>
<thead>
<tr>
<th></th>
<th>Preeclampsia (n = 21)</th>
<th>Normotensive Pregnant (n = 21)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (yr)</td>
<td>30.7 ± 1.51</td>
<td>30.8 ± 1.36</td>
<td>NS</td>
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<tr>
<td>Parity</td>
<td>1.35 ± 0.43</td>
<td>0.95 ± 0.19</td>
<td>NS</td>
</tr>
<tr>
<td>Gestational age at blood sampling (wk)</td>
<td>33.8 ± 0.83</td>
<td>33.5 ± 0.70</td>
<td>NS</td>
</tr>
<tr>
<td>Blood pressure (mm Hg)</td>
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<tr>
<td>Systolic</td>
<td>167.3 ± 4.60</td>
<td>108.8 ± 2.48</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Diastolic</td>
<td>98.4 ± 3.74</td>
<td>61.7 ± 1.49</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Urinary protein (g/day)</td>
<td>3.99 ± 1.28</td>
<td>0</td>
<td>&lt;.005</td>
</tr>
<tr>
<td>Plasma uric acid concentration (mg/dL)</td>
<td>6.72 ± 0.45</td>
<td>61 ± 17</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Gestational age at delivery (wk)</td>
<td>34.8 ± 0.79</td>
<td>39.0 ± 0.22</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Infant birth weight (kg)</td>
<td>1.91 ± 0.21</td>
<td>3.03 ± 0.09</td>
<td>&lt;.0005</td>
</tr>
</tbody>
</table>

NS = not significant.
Values are mean ± SE.

Reagents

RPMI 1640 and L-glutamine were obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum was obtained from Dainihon Seiyaku (Osaka, Japan) and JRH Biosciences Co. (Lenexa, KS), respectively. Trypsin, penicillin, and streptomycin were from GIBCO BRL (Grand Island, NY). Endothelial cell growth supplement, Ang II, and Epi were from Sigma Chemical Co. (St. Louis, MO). CV-11974 was obtained from Takeda Chemical Industries LTD. (Osaka, Japan). ECE monoclonal antibody (AEC 32-236) was a kind gift from Dr. Kazuhiko Tanzawa, Sankyo Co. (Tokyo, Japan). All other reagents were either of reagent grade or molecular biology grade, and were purchased from commercial sources.

Data Analysis

Data are expressed as mean ± SE. Statistical analysis was accomplished by Student’s t test and ANOVA. A P value < .05 was considered to be significant.

Results

Characteristics of Patients

The clinical characteristics of the study groups are shown in Table 1. Between the NTP and PE groups, there were significant differences in the serum ET-1 concentration (2.91 ± 0.17 pg/mL v 4.60 ± 0.44 pg/mL, P < .005), mean gestational age at delivery (39.0 ± 0.22 weeks v 34.8 ± 0.79 weeks, P < .0001), and mean infant birth weight (3.03 ± 0.09 kg v 1.91 ± 0.21 kg, P < .0005). There were no differences in maternal age, parity, or gestational age at blood sampling between the two groups. The mean age of the NP group was 30.5 ± 1.46 years, and was similar to those of the NTP and PE groups. Serum ET-1 concentration in NP groups were 2.68 ± 0.18 pg/mL. No significant difference in serum ET-1 concentration was detected between the NP and NTP groups. Serum ET-1 concentration in the PE groups were significantly higher than those in the NP groups (P < .005).

ET-1 Release From HUVEC

Although ET-1 release from HUVEC into the medium with or without 10% human serum (NP, NTP, and PE) increased time-dependently during 24-h culture, actual ET-1 release from HUVEC for the first 6 h was the highest, as described previously. ET-1 release from HUVEC incubated without human serum was 86.0 ± 5.5 fmol/10^5 cells. Levels of ET-1 release from HUVEC incubated with 10% serum from NP, NTP, and PE women were 99.7 ± 9.6, 99.1 ± 4.4, and 101.1 ± 6.8 fmol/10^5 cells, respectively. ET-1 release from HUVEC incubated with each 10% serum was significantly higher than without human serum (P < .05). However, ET-1 release from HUVEC for 6 h did not differ among the three groups (Fig. 1, top).

Ang II- and Epi-Induced ET-1 Release From HUVEC

Levels of Ang II (50 μmol/L)- and Epi (50 μmol/L)-induced ET-1 release from HUVEC incubated without human serum for 6 h were significantly higher than those without stimulants (P < .05; data not shown). Ang II-induced ET-1 release from HUVEC treated with PE serum increased significantly more than without stimulants (5.90 ± 4.20 fmol/10^5 cells, P < .05), but that from those treated with NP and NTP sera did not increase (3.75 ± 2.34 and 1.89 ± 0.44 fmol/10^5 cells, respectively). Epi-induced ET-1 release from HUVEC treated with PE serum also increased significantly more than that from those without stimulants (10.95 ± 5.38 fmol/10^5 cells, P < .05), but that from those treated with NP and NTP sera did not increase (1.42 ± 3.63 and 1.25 ± 4.14 fmol/10^5 cells, respectively) (Fig. 1, bottom). Levels of Ang II- and Epi-induced ET-1 release from HUVEC for 6 h were inhibited by 100 nmol/L CV-11974, used as an Ang II type 1 receptor antagonist.
(AT₁) receptor antagonist, and by 10 μmol/L prazosin, used as an α-adrenoceptor antagonist (data not shown).

**IP₃ Production in HUVEC**

IP₃ production in HUVEC treated without human serum was the highest 30 sec after stimulation by Ang II (50 μmol/L) and Epi (50 μmol/L). IP₃ production in HUVEC treated with 10% serum (NP, NTP, and PE) was also the highest 30 sec after stimulation (data not shown). The IP₃ level in HUVEC incubated without human serum was 0.85 ± 0.08 pmol/10⁵ cells. Basal IP₃ levels in HUVEC incubated with 10% serum from the NP, NTP, and PE groups were 1.34 ± 0.09, 1.32 ± 0.25, and 1.38 ± 0.22 pmol/10⁵ cells, respectively.

HUVEC incubated with 10% sera had significantly higher basal IP₃ levels than without serum (P < .05). However, IP₃ production did not differ among the three groups (NP, NTP, and PE). Changes of IP₃ production in HUVEC treated with 10% serum (NP, NTP, and PE) for 30 sec after stimulation by Ang II and Epi (bottom). Ang II (50 μmol/L) and Epi (50 μmol/L)-induced IP₃ production in HUVEC treated with each serum increased. However, IP₃ production with stimulants did not differ among the three groups. Values are means ± SE of 10 to 15 different experiments.

*Significantly different from ET-1 release without stimulants.
showed significantly higher expression of ECE than that with control medium ($P < .05$). However, ECE expression in HUVEC did not differ among the three groups (NP, NTP, and PE) (Fig. 3, top). On the other hand, densitometric levels of Ang II (50 μmol/L)-stimulated ECE expression in HUVEC incubated with 10% NP, NTP, and PE sera were 120.4% ± 8.1%, 132.2% ± 11.8%, and 146.3% ± 13.1% of the control level, respectively. Those of Epi (50 μmol/L)-stimulated ECE expression in HUVEC incubated with 10% NP, NTP, and PE sera were 119.4% ± 12.7%, 133.6% ± 10.0%, and 153.3% ± 13.9% of the control level, respectively. Ang II- and Epi-stimulated ECE expression in HUVEC incubated with PE serum were significantly higher than those without stimulants ($P < .05$) (Fig. 3, bottom). However, Ang II- and Epi-stimulated ECE expression in HUVEC incubated with NP and NTP sera were not significantly higher than those without stimulants.

**Discussion**

The influence of PE serum or plasma on ET-1 release from cultured endothelial cells is controversial. Some investigators reported suppression of ET-1 release from cultured endothelial cells by PE serum.\(^{18,19}\) On the other hand, we previously reported that ET-1 release from HUVEC did not differ among serum groups (NP, NTP, and PE).\(^{14}\) Our data supported the findings that ET-1 release from endothelial cells was not increased by serum from the PE group compared with serum from the NTP group, as the other researchers reported.\(^{20–22}\) In this study, we carried out further experiments with increased serum sample numbers and obtained the same result that ET-1 release from HUVEC did not differ among the three groups. As in our results, Yamamoto et al\(^{21}\) reported that sera from women with PE did not increase ET-1 release from HUVEC. However, they reported that antiendothelial cell antibody positive sera from women with PE significantly increased ET-1 release from HUVEC.

Concerning ET-1 release from endothelial cells incubated with PE serum, discrepancies between our study and those of other investigators may have resulted from differences such as selection of the study population, the concentration of serum used in experiments, the cell type used, and incubation time with serum. The severity of PE and other factors such as gestational age at delivery and infant birth weight, medication using magnesium sulfate or antihypertensive drugs, as well as sample collection (such as gestational age at sampling) differ in various reports. Medication with magnesium sulfate or antihypertensive drugs may have some influence on ET-1 production by endothelial cells, therefore we excluded patients taking any medication. We used second passage HUVEC in these experiments. We used 10% sera in this study because ET-1 release from HUVEC were at the same levels with 10% and 20% sera (data not shown). ET-1 release from HUVEC into the medium with or without

**ECE Expression in HUVEC**

Fig. 3 shows Western blot analysis of ECE expression in HUVEC incubated with control medium or medium containing 10% sera from NP, NTP, and PE groups for 6 h (top). Densitometric levels of expression of ECE in HUVEC incubated with medium containing NP, NTP, and PE sera were 115.0% ± 7.0%, 118.0% ± 8.5%, and 119.5% ± 9.4% of the control level, respectively. HUVEC incubated with 10% sera

100 nmol/L CV-11974, used as an AT\(_1\) receptor antagonist, and by 10 μmol/L prazosin, used an α-adrenergic receptor antagonist (data not shown).
human serum (NP, NTP, and PE) increased time dependently during 24 h culture. However, actual ET-1 release from HUVEC for the first 6 h was the highest. Therefore, we chose 6 h for the incubation time with sera.

Ang II- and Epi-induced ET-1 release from HUVEC treated with PE serum increased significantly more than without stimulants. However, Ang II- and Epi-induced ET-1 release from HUVEC treated with NP and NTP sera did not increase compared to without stimulants. These results suggested that the pronounced vascular sensitivity to vasoconstrictors, such as Ang II and Epi, observed in PE in vivo might be mediated by AT₃ receptors and α-adrenoceptors. ET-1 release is augmented by intracellular free-calcium ions caused by IP₃ production in endothelial cells.⁵

Previously, we reported that HUVEC incubated with 10% sera had significantly higher basal IP₃ levels than without serum, but IP₃ production did not differ among the three groups. Ang II- and Epi-induced ET-1 release from HUVEC was mediated by IP₃ production stimulated by AT₃ receptors and α-adrenoceptors, respectively. Ang II- and Epi-induced ET-1 release was significantly increased by PE serum treatment. Ang II- and Epi-induced IP₃ production in HUVEC incubated with PE serum was increased, but not significantly compared with other sera. Considering these findings, it is suggested that Ang II- and Epi-induced ET-1 release from HUVEC incubated with PE serum may be mediated by a mechanism other than increased IP₃ production.

Another important factor that regulates the local concentration of ET-1 in endothelial cells is ECE. Endothelin-1 is synthesized from a proform of ET-1 (pro ET-1) through an intermediate form, big ET-1.²³ Big ET-1 is converted into the mature peptide ET-1 by ECE. The endothelial ECE is a glycosylated, membrane-bound metalloproteinase.²⁴–²⁶

The vasoconstrictive activity of big ET-1 is much less than that of ET-1.²⁷ ECE plays an important role in vascular tone in response to ET-1. Therefore, we evaluated ECE expression in HUVEC incubated with NP, NTP, and PE sera. HUVEC incubated with 10% serum from each group for 6 h had significantly higher expression of ECE than with control medium. However, ECE expression in HUVEC did not differ among the three groups, as ET-1 release did not. Ang II- and Epi-stimulated ECE expression in HUVEC incubated with PE serum were significantly higher than those without stimulants. However, Ang II- and Epi-stimulated ECE expression in HUVEC incubated with NP and NTP sera was not significantly higher than without stimulants. Our results suggest that the increased vascular sensitivity to vasoconstrictors observed in PE may be mediated by ET-1 release from endothelial cells caused by enhanced ECE expression.

Taken together, our results suggest that ET-1 release from endothelial cells may contribute to the increased vascular sensitivity to vasoconstrictors observed in PE, and that vasoconstrictor-induced ET-1 release may be related to enhanced ECE expression.

References