Expression of Endothelial Angiotensin II Receptor mRNA in Pregnancy-Induced Hypertension

Yuko Takeda-Matsubara, Keiichi Matsubara, Hiroshi Ochi, Masaharu Ito, Masaru Iwai, and Masatsugu Horiuchi

Objective: The normal suppression of vascular sensitivity to angiotensin II (Ang II) in pregnancy is lost in pregnancy-induced hypertension (PIH). To examine the mechanism, we investigated Ang II receptor subtype 1 (AT1R) and 2 (AT2R) expression in human umbilical vein endothelial cells (HUVEC) and vascular smooth muscle cells (VSMC).

Methods: The HUVEC and VSMC were incubated with serum from normal pregnant women and PIH patients for 0 to 12 h. The AT1R and AT2R mRNA were semi-quantified as the ratio to glyceraldehyde-3-phosphate dehydrogenase mRNA, using multiplex reverse transcription-polymerase chain reaction. The AT1R expression was also evaluated by immunocytochemistry.

Results: Serum from PIH patients significantly increased AT1R mRNA of HUVEC (1.48 ± 0.44) after a 12-h incubation compared with that from normal pregnant women (0.25 ± 0.14). On the other hand, AT2R mRNA of HUVEC incubated with serum from PIH patients (0.14 ± 0.02) was significantly decreased compared with HUVEC incubated with serum from normal pregnant women (0.31 ± 0.08). The AT1R mRNA of VSMC was significantly increased by serum from both PIH patients and normal pregnant women. The AT1R-to-AT2R mRNA ratio increased by serum from PIH patients was significantly reduced by anti-tumor necrosis factor-α (TNF-α) antibody (20 μg/mL). Valsartan (an AT1R antagonist, at 1 to 10 nmol/L) significantly increased AT2R mRNA of HUVEC. Also, immunocytochemistry demonstrated that endothelial AT1R expression was strongly increased by PIH sera and reduced by anti-TNF-α antibody.

Conclusions: Endothelial AT1R expression is increased and AT2R expression is decreased in PIH. The TNF-α is related to the pathogenesis of PIH by reduced AT2R mRNA through an increase of AT1R mRNA. Am J Hypertens 2003;16:993–999 © 2003 American Journal of Hypertension, Ltd.

Key Words: Pregnancy-induced hypertension, angiotensin II receptor, tumor necrosis factor-α, vascular smooth muscle cell, human umbilical vein endothelial cell.

Pregnancy-induced hypertension (PIH) is an important cause of maternal and perinatal mortality. Although the etiology of PIH has not yet been resolved, endothelial cell (EC) dysfunction is proposed as a pathophysiology typical of PIH. Dysfunctional endothelium in the uteroplacental circulation not only increases peripheral vascular resistance, but also influences generalized vasoconstriction through humoral factors released from the placenta because hypertension is rapidly improved after delivery. Angiotensin II (Ang II) is the most active hormone of the renin-angiotensin system, playing key roles in the regulation of blood pressure (BP), sodium homeostasis, and vasoconstriction. However, the maternal circulating renin-angiotensin system (RAS) is stable or reduced in PIH. Furthermore, the concentrations of angiotensin II receptors in normal and pre-eclamptic chorionic-placental tissues do not differ. On the other hand, vascular responsiveness to Ang II decreases early in pregnancy; however, pregnant women who subsequently develop pre-eclampsia are exquisitely sensitive to the pressor effects of infused Ang II.

Angiotensin II receptor (ATR) consist of subtype 1 (AT1R) and 2 (AT2R). The AT1R is predominantly located in the vascular system, liver, kidney, and adrenal cortex and mediates vasoconstriction, aldosterone secretion, and renal sodium resorption induced by Ang II.
Table 1. Characteristics of the study subjects

<table>
<thead>
<tr>
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<th>Normal Pregnancy (n = 13)</th>
<th>PIH (n = 10)</th>
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<tbody>
<tr>
<td>Maternal age (y)</td>
<td>31 ± 5</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>Nulliparity (n)</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Gestational age at sampling (wk)</td>
<td>37 ± 4</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.0 ± 3.3</td>
<td>28.5 ± 3.7</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>106 ± 11</td>
<td>167 ± 15*</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>66 ± 10</td>
<td>98 ± 6*</td>
</tr>
<tr>
<td>Gestational age at delivery (wk)</td>
<td>39 ± 1</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3246 ± 436</td>
<td>3019 ± 499</td>
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</table>

Body mass index = body weight (kg)/body height (m²).
* P < .01 v normal pregnancy.

Whereas AT2R is located in the uterus, brain, and adrenal medulla, and affects vasodilatation, antieell growth, and proapoptosis. A major cause of PIH is thought to be increased vascular sensitivity to Ang II. The balance of expression between the two subtypes of receptor is potentially important in the regulation of vascular tone in pregnancy. We examined the mechanism of vascular refractoriness to Ang II in normal pregnancy and its loss in PIH to understand the effects of ATR.

Methods

Subjects

Informed consent was obtained from all participants before enrollment in this study and the protocol was approved by the local ethics committee. Peripheral blood collected from the antecubital vein of 13 normal pregnant women and 10 PIH patients was immediately separated by centrifugation. Sera were stored at −80°C until measurement.

A diagnosis of PIH was confirmed when BP was 140/90 mm Hg or higher while sitting on two occasions at least 4 h apart during pregnancy with no hypertensive event before 20 gestational weeks with or without proteinuria and edema. We are not studying the clinical phenomenon of pre-eclampsia, the diagnosis of which requires additional criteria aside from just BP changes. All cases were uncomplicated singleton pregnancies. The characteristics of each group are detailed in Table 1.

Materials

Valsartan was donated by Novartis Pharma (Bern, Switzerland). Monoclonal antihuman tumor necrosis factor-α (TNF-α) antibody and collagenase type I were purchased from Sigma Chemical Co. (St. Louis, MO). All oligonucleotide primers were synthesized by Amersham Pharmacia Biotech (Uppsala, Sweden). Minimum essential medium (MEM) was purchased from GIBCO Life Technologies (Paisley, UK). Smooth muscle cell growth medium-2 (SmGM-2), microvascular endothelial cell growth additive agent (EGM-MV), and human vascular smooth muscle cells (VSMCs) were purchased from Cambrex Co. (East Rutherford, NJ). Ribonuclease inhibitor was purchased from Promega (Madison, WI). AMV reverse transcriptase XL was purchased from Takara Biochemicals (Tokyo, Japan). Taq polymerase was purchased from Perkin Elmer (Shelton, CT). ISOGEN was purchased from WAKO (Tokyo, Japan). Vistra Green was purchased from Amersham Life Science Ltd. (Buckinghamshire, UK). All other reagents were analytical grade.

Cell Culture

Human umbilical vein ECs (HUVECs) were obtained using the modified method of Jaffe from umbilical veins of normal pregnant women. The HUVECs were cultured in MEM with EGM-MV. The VSMCs were cultured in MEM with SmGM-2 at 37°C in an atmosphere of 5% CO₂ and 95% air. Subconfluent HUVECs (second passage) and VSMCs (fifth passage) were studied. Cells were cultured for 12 h in serum in serum-free medium before the experiment.

Experimental Protocol

The HUVECs and VSMCs were incubated with MEM containing 10% serum from normal pregnant women or PIH patients for 1, 2, 6, and 12 h. In some experiments, HUVECs were preincubated for 10 min with AT1R antagonist valsartan (1, 10 nmol/L) before incubation with sera from PIH patients. In some experiments, human anti-TNF-α monoclonal antibody (20 µg/mL) and sera from PIH patients with normal mouse IgG (20 µg/mL) were incubated for 1 h at 4°C before the experiment.

Isolation of Total RNA and Multiplex Reverse Transcription-Polymerase Chain Reaction

The HUVECs and VSMCs were lysed and total RNA was extracted using the ISOGEN reagent according to the manufacturer’s instructions, then resuspended in 15 µL of diethylpyrocarbonate water.

Total RNA was reverse transcribed to cDNA by incubating 1 µg of RNA with 50 ng of random primer in a total volume of 10.35 µL at 70°C for 10 min, followed by 4°C for 10 min. Tris-Cl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl₂, 10 mmol/L DTT, 0.5 mmol/L dNTPs, 5.5 U AMV reverse transcriptase XL, and 20 U of ribonuclease inhibitor were added to a final volume of 20 µL and incubated at 42°C for 1 h. The samples were then heated for 5 min at 99°C to terminate the reaction and stored at 4°C.

The AT1R, AT2R, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotide primers were constructed from published nucleotide sequence databases (Table 2). The level of GAPDH mRNA served as an
internal standard for normalization of ATR mRNA levels. The reverse transcription-polymerase chain reaction (RT-PCR) conditions were optimized to ensure that amplification proceeded within the linear portion of the reaction. The multiplex RT-PCR amplification profile consisted of denaturation at 95°C for 10 min, followed by 35 cycles of 94°C for 1 min, and 58°C (AT1R) or 55°C (AT2R) for 1 min, with an extension at 72°C for 7 min.

The following reagents were used in each multiplex RT-PCR: 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L MgCl₂, 0.2 mmol/L of each dGTP, dATP, dTTP, and dCTP, 0.2 µmol/L of sense and antisense oligonucleotides (AT1R or AT2R), 0.2 µmol/L of sense and antisense oligonucleotides (GAPDH), 0.1 µL Taq polymerase, and 0.8 µL cDNA, in a final volume of 20 µL. The AT1R-to-AT2R ratio was calculated in the same sample as the density of AT1R divided with that of AT2R, independent of the density of GAPDH.

The PCR products (5 µL) were electrophoresed on 2% agarose gels and stained with Vistra green for 30 min. Bands were visualized and digitally photographed using a luminescent image analyzer (FluoImager, Beckton Dickinson) and quantified using Image Quant (Beckton Dickinson).

**Immunocytochemistry for AT1R**

The HUVECs were cultured on eight-chamber slides (Nunc, Inc., Naperville, IL) coated with collagen type 1 (Sigma Chemical Co.). After rinsing in ice cold phosphate buffered saline (PBS; 10 mmol/L PBS and 0.14 mol/L NaCl at pH 7.4), the HUVECs were fixed in 4% paraformaldehyde. Immunolocalization was accomplished using rabbit anti-AT1R polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Localization of specific staining was visualized by indirect immunoperoxidase detection using aminoethyl cabazole as the chromogen (IHC kit, InnoGenex, San Ramon, CA). Controls consisted of replacing the primary antibody with antirabbit IgG (Santa Cruz Biotechnology) at the same concentration as the primary antibody.

**Statistical Analysis**

Values are presented as means ± SD. Statistical analysis was performed by Mann-Whitney U test or analysis of variance (ANOVA) followed by the post-hoc test (Bonferroni). A P value of < .05 was considered a significant difference.

**Results**

The expression of AT1R mRNA in VSMCs was significantly and similarly increased by stimulation with sera from normal pregnant women and PIH patients for 12 h (Fig. 1). The expression of AT1R mRNA in HUVECs was significantly increased to 1.48 ± 0.44 (n = 13) after 12 h of stimulation with sera from PIH patients (P < .05). Incubation with sera from normal pregnant women did not change AT1R mRNA expression (0.25 ± 0.14 after 12 h) (Fig. 2A).

Fig. 2B shows that the expression of AT2R mRNA in HUVECs was significantly increased to 0.31 ± 0.08 (n = 13) after 12 h of stimulation with sera from normal pregnant women (P < .005). However, the expression level of AT2R mRNA was not changed by sera from PIH patients (0.14 ± 0.02).

These results indicate that the relative ratio of AT1R mRNA-to-AT2R mRNA (AT1R/AT2R) in HUVECs was significantly decreased from 4.64 ± 1.61 (basal level) to 0.62 ± 0.14 (12 h incubation) by sera from normal pregnant women (P < .01). On the other hand, AT1R/AT2R was significantly increased to 10.62 ± 2.30 by 12 h incubation with sera from PIH patients (P < .01).

Anti-TNF-α monoclonal antibody significantly decreased the AT1R mRNA level to 0.35 ± 0.14 in 12 h incubation (n = 10, P < .005) (Fig. 3A) and significantly increased the AT2R mRNA level to 0.41 ± 0.08 in 12 h (n = 10, P < .01) (Fig. 3B). Consequently, AT1R/AT2R was significantly decreased to 0.65 ± 0.14 by TNF-α inhibition (P < .01). The AT1R-specific antagonist, valsartan (1 and 10 nmol/L), significantly increased the level of AT2R mRNA (0.30 ± 0.04 and 0.68 ± 0.10, respectively) induced by 12 h of incubation with sera from PIH patients (P < .05) (Fig. 3B).

Using immunocytochemistry we demonstrated the expression of AT1R in the HUVECs (Fig. 4). The AT1R expression was observed in HUVECs and the expression was increased with PIH sera compared with normal pregnant sera. The effect of PIH sera was abolished by anti-TNF-α antibody. No immunoreactivity was observed with control normal IgG run in parallel to AT1R antibody.

**Table 2. List of oligonucleotide primers used**

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<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Length of DNA Product</th>
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<tr>
<td>AT1R</td>
<td>5'-GTC ACC TGC ATC ATC ATT TGG C-3' (sense)</td>
<td>266 bp</td>
</tr>
<tr>
<td></td>
<td>5'-TCA TAA GCC TTC TTT AGG GCC TTC-3' (antisense)</td>
<td></td>
</tr>
<tr>
<td>AT2R</td>
<td>5'-TAT GGC TTT CCC ACC TGA GA-3' (sense)</td>
<td>216 bp</td>
</tr>
<tr>
<td></td>
<td>5'-AAC AGC AGC TGC CAT CTT CA-3' (antisense)</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-CCA CCC ATG GCA AAT TCC ATG GCA-3' (sense)</td>
<td>622 bp</td>
</tr>
<tr>
<td></td>
<td>5'-TCT AGA CGG GAG TCC ACC-3' (antisense)</td>
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Discussion

Vascular responsiveness to Ang II is increased in pre-eclampsia; however, the serum Ang II concentration is not affected. It is thought that the change of Ang II receptor expression plays an important role in the pathogenesis of PIH. The AT1R is expressed in vascular smooth muscle cells or kidney and it mediates vasoconstriction and renal sodium reabsorption induced by Ang II. We demonstrated that sera from either normal pregnant women or PIH patients stimulated AT1R expression in VSMCs. The responsiveness of vascular smooth muscle to Ang II may be stimulated during pregnancy.

The expression of AT1R in ECs was also stimulated by...
FIG. 3. Effect of anti-tumor necrosis factor-α (TNF-α) antibody or angiotensin II receptor subtype 1 (AT1R) antagonist on AT1R or angiotensin II receptor subtype 2 (AT2R) mRNA expression in human umbilical vein endothelial cells. TNF-α inhibition significantly decreased AT1R mRNA expression (A) but increased that of AT2R mRNA (B). AT2R mRNA expression was significantly increased by AT1R inhibition. Data are expressed as means ± SD. *P < .05 vs anti-TNF-α antibody or valsartan. **P < .01 vs anti-TNF-α antibody.

FIG. 4. Immunocytochemistry of endothelial angiotensin II receptor subtype 1 (AT1R) expression in control (medium alone) and after 12 h of incubation with sera from normal pregnant women, sera from patients with pregnancy-induced hypertension (PIH), and anti-tumor necrosis factor-α (TNF-α) antibody-treated sera from PIH patients. The AT1R expression was increased by sera from PIH patients and the effect was reduced by anti-TNF-α antibody.
sera from PIH patients. The AT1R stimulates endothelin production in ECs and amplifies vasoconstriction. In PIH, the increased vascular response to Ang II is thought to be mediated by both activated AT1R in smooth muscle and endothelium. On the other hand, the BP of normal pregnant women is reduced during the middle of pregnancy. The expression of AT1R in ECs that is reduced by sera from normal pregnant women may be partly related to the decreased BP.

The AT2R causes vasodilatation through the release of bradykinin, nitric oxide, and prostaglandin I2 (PGI2). The dilation is abolished by the dissection of endothelium, suggesting that the effect of AT2R is endothelium-dependent. The present study showed that AT2R in ECs was increased by sera from normal pregnant women. The AT2R is thought to be important for the reduced vascular responsiveness to Ang II and decreased BP during normal pregnancy. On the other hand, AT2R in ECs was decreased by sera from PIH patients. The AT1R-to-AT2R ratio in ECs is usually about 4:1. Vascular motion induced by Ang II may be affected by the ratio of AT1R and AT2R expressed in ECs, and increased AT1R/AT2R in ECs induced by sera from PIH patients is thought to be important for the loss of the vascular reactivity to Ang II in PIH patients. The Ang II can enhance sympathovagal transmission by acting on AT1R, which is located on sympathetic nerve terminals. Greenwood et al reported that central sympathetic output was increased in normal pregnancy and was even greater in hypertensive pregnancy. Increased AT1R/AT2R may cause hypertension through sympathovagal activation. Conversely, decreased AT1R/AT2R in ECs would reduce BP during normal pregnancy. The balance of AT1R and AT2R is therefore important for the regulation of BP during pregnancy.

The triggers in serum that change the AT1R/AT2R ratio in ECs remain unclear. Endothelial dysfunction is associated with the onset of PIH, which appears to be caused by circulating factors derived from the placenta because their concentration rapidly decreases after delivery. An important proinflammatory cytokine produced by macrophages or trophoblastic cells, TNF-α, is involved in ECs dysfunction and inflammation. The TNF-α inhibits endothelial nitric oxide synthase, contributes to the impaired endothelium-dependent relaxation, affects vasoconstriction leading to hypertension, and thrombosis through EC dysfunction. The TNF-α concentration in sera from PIH patients is so high from early in pregnancy and may be a key factor in the pathogenesis of PIH.

The TNF-α is reported to upregulate AT1R in cardiac fibroblasts and downregulate AT2R expression in the adrenal gland. Peng et al reported that TNF-α-induced AT1R upregulation enhanced Ang II-mediated cardiac fibroblast proline incorporation and protein production, which leads to fibrosis in myocardial infarction. The TNF-α may be related to the changes of the AT1R/AT2R ratio also in ECs. The present study found that anti-TNF-α antibody reduced the expression of AT1R in ECs and stimulated that of AT2R. Thus, TNF-α may regulate the vascular reactivity to Ang II in PIH by stimulating AT1R expression and reducing AT2R expression.

Valsartan is an efficient antihypertensive drug that blocks AT1R. The AT1R antagonists inhibit AT1R and stimulate AT2R, because increased angiotensins might act preferentially on AT2R. Angiotensin-converting enzyme inhibitors and AT1R inhibitors are a contraindication during pregnancy. However, AT1R inhibitor is an important medicine to elucidate the effect of AT1R and AT2R on the fetal and maternal circulation, as ATR is playing a key role in the pathogenesis through the vascular reactivity to Ang II and it could be related to fetal growth.

Sera from PIH patients stimulated the expression of AT1R mRNA in ECs; however, it did not stimulate the expression of AT2R mRNA. Instead, TNF-α inhibition decreased the increased expression of AT1R mRNA; in contrast, the expression of AT2R was increased. Therefore, the balance of expression between the two subtypes of Ang II receptor (AT1R and AT2R) is potentially important in the regulation of vascular tone during pregnancy. Increased AT1R/AT2R induced by TNF-α may explain the loss of vascular reactivity to Ang II in the pathogenesis of PIH. It is unknown the extent to which any of the PIH patients might also have had pre-eclampsia. We should evaluate not only the effect of BP but also the effect of proteinuria on AT1R/AT2R.

Acknowledgments
We thank Dr. Masakuni Fukuzumi for technical advice.

References