Examination of the Expression of Cyclooxygenase-2 in Placenta Villi from Sufferers of Pregnancy Induced Hypertension

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Objectives: The purpose of this paper is to elucidate the roles of phospholipase A2 (PLA2), cyclooxygenase-2 (COX-2), and prostaglandin I2 (PGI2) synthase in pregnancy induced hypertension (PIH). Methods: In placentas from normal pregnant women and pregnant women with severe PIH, the enzyme expression of PLA2, COX-2, and PGI2 synthase was measured using real time reverse transcription-polymerase chain reaction (RT-PCR). Results: The expression of each enzyme was compared between normal (n=12) and PIH (n=12) groups. The expression levels of COX-2 and PGI2 synthase during PIH pregnancy were significantly decreased to about 51% and 68%, respectively, of their values in normal pregnancy. However, the expression of PLA2 was hardly changed by PIH. Conclusions: The decreases in COX-2 and PGI2 synthase expression in severe PIH placentas may be causal factors in the disruption of the PGI2–thromboxane A2 (TXA2) balance in favor of TXA2. The decrease in COX-2 was more marked than that of PGI2 synthase.

Key words pregnancy induced hypertension; cyclooxygenase; placenta; prostaglandin I2; phospholipase A2

Pregnancy induced hypertension (PIH) causes hypertensive cerebrophathy, deep vein thrombosis, pulmonary embolism lung edema, intrauterine growth retardation, and premature delivery. It is therefore considered to be a serious disease that affects both mother and fetus. However, the mechanism through which PIH induces its clinical symptoms is unknown. At first, damage to vascular endothelial cells induces vasoconstriction and hemocoagulation, resulting in fetoplacental circulation dysfunction. Then, clinical symptoms such as hypertension and protein urea are induced.

As described above, the damage caused to vascular endothelial cells is the main pathogenic factor in PIH and results in decreased concentrations of vasodilatory factors. Therefore, it is assumed that circulation homeostasis is disrupted. The prostaglandin I2 (PGI2)–thromboxane A2 (TXA2) adjustment system is present in many circulation control systems. Prostaglandin I2 is synthesized in the endovascular system and acts as a vasodilator in addition to its inhibitory effect on platelet aggregation. In contrast, TXA2 is synthesized by platelets, acts as a vasoconstrictor, and induces platelet aggregation. Furthermore, PGI2 and TXA2 are synthesized from the same precursor, arachidonic acid. These prostaglandins, which have opposite effects, are synthesized in close proximity to each other, such as in blood vessels and platelets, and maintain circulation homeostasis. Therefore, the PGI2–TXA2 balance is considered to be a complex mechanism for circulation control. Additionally, PGI2 and TXA2 are synthesized in the placenta, umbilical vein, uterine blood vessel, amnion, chorionic villi, and decidua. The PGI2–TXA2 balance is considered to be very important in the fetoplacental circulatory system; therefore, an imbalance in this system may play an important part in PIH pathogenesis.1) In another report, production of PGI2 was decreased and TXA2 was increased in the blood, urine, and tissues of PIH maternal and fetal tissues, indicating that the PGI2–TXA2 equilibrium in these tissues is pushed towards TXA2. Similar results have also been reported in umbilical and placental tissue.2)

These differences may depend on (1) the activity of prostaglandin production enzymes, (2) the content of these enzymes, and (3) the activity of inhibitors for these enzymes.

Satoh et al. reported that the activity of enzymes such as cyclooxygenase-2 (COX-2), which takes part in the synthesis of PGI2, is decreased in the endothelium of the umbilical vein in patients with severe PIH, resulting in an imbalance in the PGI2–TXA2 system that leads to TXA2 predominance.3) However, in their report, no distinction between COX-1 and COX-2 was made because the isozymes of COX had not yet been discovered. In addition, Keirse et al. determined the contents of COX and PGI2 synthetase using an immunoradiometric assay with monoclonal antibody.4,5) In their report, COX was induced and increased with increasing gestational age, but there was no difference in the level of COX between patients suffering from PIH and normal pregnant women. However, they did not distinguish between COX-1 and COX-2. It is now known that COX isozymes exist and that the inducible type enzyme, COX-2, is a key enzyme in various illnesses. Therefore, it is necessary to reexamine the activity and content of these enzymes.

In the present study, we determined the expression of phospholipase A2 (PLA2), COX-2, and PGI2 synthase, which play important roles in PGI2 production by real time reverse transcription-polymerase chain reaction (RT-PCR). We compared the expression of these enzymes between PIH sufferers and normal pregnant women.

MATERIALS AND METHODS

Materials For immunohistology, anti COX-2 polyclonal antibody and IgG rabbit negative controls were obtained from NEO MARKERS (California, U.S.A.). A Histfine SAB-PO(R) kit was obtained from NICHIREI Bio Science (Tokyo, Japan). Hematoxylin and NuSieve® 3 : 1 Agarose were purchased from Bio Genex (San Ramon, U.S.A.) and Cambrex (Wokingham, U.K.), respectively. For electrophore-
sis, DNA Ladder (20 bp) and TBE (Tris–Borate–EDTA) powder were obtained from TaKaRa (Shiga, Japan). For RT-PCR, SYBER® Premix Ex Taq™ and a SYBER® RT-PCR Kit were obtained from TaKaRa (Shiga, Japan). ISOGEN® was purchased from NIPPON GENE Co., Ltd. (Toyama, Japan). Preparation of specific primers for PLA2, COX-2, PGI2 synthase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was done by Invitrogen (Tokyo, Japan).

**Patients** Placental tissue was obtained from patients who had undergone a caesarean section at Saitama Medical Center from September 2004 to August 2005. The control group (n=12) had suffered no complications except for the caesarean section. The severe PIH group (n=12) included eight preeclampsia and four superimposed preeclampsia cases.

Women who maintained a diastolic/systolic blood pressure of >160/110 mmHg with or without protein urea were defined as having severe PIH according to the definition of the Japan Society of Obstetrics and Gynecology. The research protocol was approved by the ethics committee of Saitama Medical Center, which established the procedures for obtaining informed consent. All pregnant women in this study were informed of the purpose and design of this study and gave their consent. The mean gestational age at delivery in the normal and severe PIH pregnant groups was 37.2±0.4 weeks (37–38 weeks) and 31.2±4.3 weeks (24–37 weeks), respectively. The mean gestational age was significantly lower in the PIH pregnant group than in normal pregnant group (p<0.01).

**Immunohistology** Immunohistochemical staining of placental tissues was performed using the streptavidin labeled biotinylation method. Placental sections were fixed in paraffin. After deparaffinization, the antigen was activated by boiling the sections in citrate buffer solution (pH 6.0) mixed with autoclave (121 °C for 1 min), and endogenous peroxidase was then eliminated with 3% hydrogen peroxide in methanol. The sections were then placed on slides and treated with 10% goat normal serum, before being incubated with primary polyclonal antibody against COX-2 antigen (dilution 1:100) for 60 min at room temperature. Staining was completed using a SAB-PO(R) kit. Normal rabbit serum was used as the negative control.

**Real Time Reverse Transcription-PCR (RT-PCR)** Placental tissue samples were obtained from the middle of the placentas, which were removed immediately after expulsion. The amnion and decidua were removed from the tissue samples. The tissue samples were then immediately washed with phosphate buffered saline to remove the excess blood and stored at −80 °C until further processing. The tissue (0.5 g) was homogenized in 5 ml ice cold ISOGEN® using a Polytron homogenizer for 2 min. The resulting homogenate was centrifuged at 5000×g for 15 min at 4 °C, and total RNA was then extracted from 1 ml supernatant. cDNA was synthesized from the total RNA using reverse transcriptase. Specific primer pairs for PLA2, COX-2, PGI2 synthase, and GAPDH (internal standard) were synthesized (Table 1). The reaction mixture contained the above primer, SYBR® premix Ex Taq™, ROX Reference Dye, DNA extract, and distilled water. After denaturation for 10s at 95 °C, a cycle of reaction of 5 s at 95 °C and 30 s at 60 °C was repeated until the product yield reached the threshold. The amplification products were treated with 6% agarose gel electrophoresis and ethidium bromide staining. The intensity of the ethidium bromide staining was detected at 320 nm.

**Statistical Analysis** Statistical analysis was performed by the Student t-test. The results of the RT-PCR are shown as the mean±standard deviation (S.D.) and p values <0.05 were accepted as statistically significant.

**RESULTS**

**Immunohistology** COX-2 immunoreactivity was found in the amnion and decidua. There was also immunoreactivity in the chorionic villi, which allow material exchange among mother and fetus, as well as in the placental vascular smooth muscle (Fig. 1). Similar results were reported for PLA2 and PGI2 synthase immunoreactivity.6,7 No positive staining could be found for negative control (data not shown). As a result, we confirmed the existence of COX-2 in placental villous tissue before the real time PCR experiment. Significant differences were not seen between normal and preeclampsia placental tissues (data not shown).

**RT-PCR** In order to identify the specificity of each primer, agarose gel electrophoresis was performed. Bands of PLA2, COX-2, and PGI2 synthetase were detected at 66 bp, 80 bp, and 67 bp, respectively (data not shown). The COX-2 expression in the PIH pregnant group was significantly decreased to approximately 51% of the value in the control group. Furthermore, the PGI2 synthase expression in the PIH pregnant group was significantly decreased to about 68% of the control value. However, no significant differences in PLA2 expression were detected between the PIH and normal pregnant groups (Table 2). Figure 2 shows the distribution of (A) PLA2/GAPDH ratios in the normal (0.014—0.008) and PIH (0.017—0.004) groups, (B) COX-2/GAPDH ratios in the normal (0.095—0.051) and PIH (0.053—0.009) groups, and (C) PGI2 synthetase/GAPDH ratios in the normal (0.043—0.023) and PIH (0.038—0.005) groups. Significant differences were not seen between preeclampsia and superimposed preeclampsia.

**DISCUSSION**

In many reports, difference of normal and PIH pregnant women in COX-1 and COX-2 content in placental tissues was discussed. Wetzkla et al. reported that there were no significant differences in COX-1 and COX-2 expression between precritical and postcritical PIH sufferers.8 On the other hand, Borekci et al. reported that the COX-1 and COX-2 activity in severe and mild PIH patients was significantly lower than that in a normal pregnant group.9 No differences in PGI2 synthase or TXA2 synthase were reported between normal and PIH pregnant groups.10 In villi and deciduas, no

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**Table 1. Primer Sequences**

<table>
<thead>
<tr>
<th>Gene</th>
<th>PLA2</th>
<th>Reverse</th>
<th>COX-2</th>
<th>Reverse</th>
<th>PGI2</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5'-GAC TGG AGA GCA ACC CTG AAG</td>
<td>5'-GCC AGG GCC ACC TTC TTT CA</td>
<td>5'-CAG ATG TTC ACC GTA AAT ATG ATT TAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GCG AGG GCC ACC GAA GCA</td>
<td>5'-GGT ACC CAT GAA AGC</td>
<td>5'-TGG CGA AAG GTG TGG AAG A</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
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differences in COX-1 and COX-2 expression were found between normal and PIH pregnant groups, but the TXA2 synthase content was increased in the PIH pregnant group.11)

In the present study, the existence of COX-2 in normal and PIH placental tissues became clear as a result of immunohistochemical staining. However, there was no difference for COX-2 staining. Therefore, the mRNA levels of tissue samples were evaluated by RT-PCR which is sensitive method.

Result for RT-PCR, COX-2 and PGI2 synthase expressions were significantly decreased in severe PIH. Furthermore, the rate of decrease of COX-2 was more marked than that of PGI2 synthase (Table 2). Generally, COX is regarded as a rate-limiting enzyme in prostaglandin production. Therefore, our data that indicate the importance of COX-2 in PIH may be valid. The decrease in COX-2 and PGI2 synthase expression in severe PIH indicates a reduction of PGI2 synthesis, resulting in the PGI2–TXA2 balance leaning towards TXA2.

In this study, placental COX-2 expression was significantly decreased in PIH pregnancy compared with normal pregnancy. It is reported that the amount of COX increases with increasing gestational age.5) In our study, the gestational weeks were significantly smaller in PIH pregnancy than in normal pregnancy. Therefore, we cannot rule out the effect of gestational weeks on COX-2 expression. In order to confirm the effect of the duration, gestational age-matched controls are needed. However, severe PIH leads to early onset, mean-

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Table 2. Expression of PGI2 Related Enzymes

<table>
<thead>
<tr>
<th></th>
<th>Normal pregnant women (n=12)</th>
<th>Severe PIH women (n=12)</th>
<th>PIH/Normal ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA2</td>
<td>0.011 ±0.002</td>
<td>0.010 ±0.005</td>
<td>0.909</td>
</tr>
<tr>
<td>COX-2</td>
<td>0.067±0.014</td>
<td>0.034±0.014</td>
<td>0.507**</td>
</tr>
<tr>
<td>PGI2 synthase</td>
<td>0.031±0.006</td>
<td>0.021±0.009</td>
<td>0.677*</td>
</tr>
</tbody>
</table>

Values are given as mean±S.D. of enzyme/GAPDH ratio. **p<0.01 compared with normal pregnant women. *p<0.05 compared with normal pregnant women.
ing that premature labor placentas can be used as matched controls. As COX-2 activity is reported to be lower in premature labor than in full term in placental tissue, using early onset placentas as the normal control may be problematic. Therefore, we cannot rule out possibility that differences in gestational age affect COX-2 content. However, it is difficult to prepare a matched control for severe PIH. We did not investigate the existence of inhibitory factors of COX-2 induction in this study. It has been reported that a serum component found in PIH patients causes damage to villous trophoblasts. Therefore, it may be necessary to investigate COX-2 content and inhibitory factors. PIH is not regarded as a disease with a single cause, but rather, it is a syndrome. Thus, various symptoms may be found in PIH. This may be the reason why no established opinion concerning COX-1 and COX-2 in PIH has been reached.

In this study, we revealed that COX-2 expression is significantly reduced in placental tissues from patients with severe PIH. These results suggest that a decrease in COX-2 expression is at least partially responsible for the pushing of the PGI₂–TXA₂ equilibrium towards TXA₂.

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REFERENCES