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Placental expression of retinol-binding protein 4 in patients with preeclampsia

E. Shim[1], J.Y. Park[2], H.J. Seol[2]

[1] Department of Medicine, Graduate School, Kyung Hee University, Seoul
[2] Department of Obstetrics & Gynecology, Kyung Hee University Hospital at Gangdong, Seoul (Republic of Korea)

Summary

Purpose of investigation: The aim of this study was to investigate retinol-binding protein 4 (RBP4) expression in the placenta of patients with preeclampsia and in normal pregnant women in the third trimester of pregnancy. *Materials and Methods*: Sixteen patients with preeclampsia and 11 normal healthy pregnant women were included. Placental RBP4 mRNA levels were quantified by real-time polymerase chain reaction (RT-PCR) and RBP4 protein expression in placental tissues was determined by western blot analysis and immunofluorescence. *Results*: Placental RBP4 mRNA expression was increased in patients with preeclampsia compared to normal controls. No association was observed between placental RBP4 mRNA expression and maternal body mass index or neonatal weight. *Conclusion*: Placental RBP4 expression was upregulated in patients with preeclampsia. This result suggested that placental RBP4 may play a role in exaggerated insulin resistance in preeclampsia.

Key words: Retinol-binding protein 4; Placenta; Preeclampsia.

Introduction

Preeclampsia is a leading cause of maternal and neonatal morbidity and mortality, affecting 2–8% of all pregnancies depending on geographical area [1, 2]. Preeclampsia is characterized by hypertension and proteinuria developed after 20 weeks of gestation; however, clinical manifestations vary, and the underlying pathophysiology remains to be determined [3]. One of the universal clinical features of preeclampsia is the systemic inflammatory response involving major organs such as the heart, kidney, liver, brain, and blood vessels [4]. Increased inflammatory stress also affects adipose tissue, leading to abnormalities in lipid metabolism in preeclampsia, which is often associated with exaggerated insulin resistance compared with that in normal pregnancy [5]. After recovery of acute preeclampsia for mothers and fetuses during pregnancy, preeclampsia is also associated with long-term morbidity in mothers, with increased risk of cardiovascular disease, and metabolic syndrome compared to women without preeclampsia [6, 7].

Retinol-binding protein 4 (RBP4), an adipokine associated with the obesity-induced insulin resistance in patients with diabetes [8], is also suggested to be associated with chronic inflammation in non-diabetic and non-obese adults [9]. Recent research has focused on the role of RBP4 in the cardiovascular disease [10, 11]. Additionally, several reports have described maternal circulating RBP4 levels in patients with preeclampsia; however, inconsistent results have been observed, with some investigators reporting increased circulating RBP4 levels in preeclampsia [12-14], whereas others found no differences in RBP4 levels [15, 16]. It is unclear whether RBP4 expression is altered in placenta of patients with preeclampsia. RBP4 is mainly synthesized in the liver but has also been detected in the placenta of pregnant women [17]. Thus, the present authors hypothesized that placental RBP4 expression may be increased in patients with preeclampsia. The aim of this study was to evaluate the placental expression of RBP4 in patients with preeclampsia and in normal pregnant women in the third trimester.

Materials and Methods

Sixteen patients with preeclampsia and 11 normal pregnant women who had singleton pregnancies in the third trimester were included in this study. Preeclampsia was defined as sustained hypertension with blood pressure of 140/90 mmHg or higher combined with significant proteinuria as 1+ protein on a dipstick of random urine or at least 300 mg protein per 24 hours. Women who had diabetes, chronic hypertension or preexisting hypertension and other maternal medical disease were excluded. The normal control group composed of healthy term pregnant women who gave birth without any complications during the study period. All placentae were collected immediately after delivery. Small pieces (1×1 cm) were obtained from the center of the placenta, after removal of the chorionic and basal plates, and the placentae were frozen in liquid nitrogen bath and stored at -80°C for further analysis. The study was approved by the Institutional Review Board of the authors' institution.

RNA extraction and purification were carried out using an RNeasy mini kit as described in the manufacturer's protocol. The

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concentration of RNA was measured using a spectrophotometer, and RNA quality was confirmed on agarose gels. cDNA was generated using total RNA samples (two μg/sample) with a synthesis system for real-time polymerase chain reaction (RT-PCR) in a 20-μL scale. RNA was reverse transcribed using the following conditions: 25 mM $MgCl_2$, 10 mM dNTP mix, 10× RT buffer, 0.1 M DTT, 200 U of SuperScript III, 40 U of RNaseOut, and 50 μM oligo d(T) primers in a final volume of 20 μL. The reaction was run at 65°C for five minutes and 50°C for 50 minutes, and then the enzyme was heat inactivated at 85°C for five minutes. Next, four μL of reaction product was used for RT-PCR reaction to quantify human RBP4 mRNA. The expression of RBP4 mRNA was normalized using GAPDH as an endogenous housekeeping gene. The primers and probes were designed for human RBP4 using Primer Express 2.0. RBP4 mRNA levels were quantified using TaqMan real-time PCR with an ABI system. Gene-specific probes and primer pairs for RBP4 (Assays-on-Demand, Hs00924047_m1) were used. For each probe/primer set, a standard curve was generated, with a linear relationship between the readout and increasing amounts of cDNA. The amplification conditions were two minutes at 50°C, ten minutes at 95°C, and 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Protein lysates were obtained with a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl2, one mM EDTA, 10% glycerol, 1% Triton X-100, and a mixture of protease inhibitors (aprotinin, phenylmethylsufonyl fluoride [PMSF], and sodium orthovanadate). Total tissues lysates were prepared by homogenization. The concentration of extracted protein was measured using Bradford method. Equal amounts of total protein were resolved on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes. After blocking (TBS, 0.1% Tween-20) at 4°C overnight, the membranes were incubated with primary anti-mouse RBP4 (dilution 1:1000) and anti-mouse GAPDH antibodies (dilution 1:2000) for 24 hours, followed by incubation with secondary antibodies linked to horseradish peroxidase (HRP). Immunoreactive proteins were visualized by chemiluminescence using SuperSignal West Dura Extended Duration substrate and signals were detected on an X-ray film.

Immunofluorescence was used to localize RBP4 and examine the distribution of RBP4. Tissue fragments were embedded in OCT. Tissue sections were stained with mouse anti-human RBP4 monoclonal antibodies, and DAPI was used for nuclear staining. Alexa-Fluor 488 were used as the secondary antibody. The images were obtained with an IX-71 laser scanning confocal microscope.

Demographic variables and RBP4 expression for least three independent experiments were shown as the mean ± standard error of the means (SEM). Data were analyzed using the Mann-Whitney U test. Differences with p values less than 0.05 were considered statistically significant.

Results

Table 1 shows the demographic variables of all participants. There were no significant differences in age, parity, pre-pregnancy body mass index (BMI), BMI, and placental weight. There were no significant differences in delivery mode and the percentage of women who had labored. Gestational age at delivery was lower in patients with preeclampsia than in normal pregnant women (34.3 ± 4.0 *vs*. 38.1 ± 2.1 weeks, respectively; $p = 0.011$), and accordingly, neonatal weights were significantly lower in patients with preeclampsia than in normal pregnant women (2,018 ± 621 *vs*. 3,055 ± 535 grams, respectively; $p = 0.001$). Placental RBP4 mRNA expression was significantly increased in patients with preeclampsia compared with that in normal pregnant women (6.0 ± 3.0 *vs*. 3.0 ± 0.6, $p = 0.007$). Western blot analysis revealed that protein levels of RBP4 were also increased in the placentae of patients with preeclampsia compared with normal pregnant women (151 ± 33.1 *vs*. 74.9 ± 26.5, respectively; $p < 0.001$; Figure 1). Figure 2 shows representative immunofluorescence images demonstrating increased RBP4 expression in patient with preeclampsia compared with those of normal pregnant women. RBP4 mRNA levels in the placenta were not correlated with pre-pregnancy BMI, BMI, placental weight, or neonatal weight (data not shown).

Table 1. — *Comparison of demographic variables between normal pregnant women and patients with preeclampsia.*

	Normal (n=11)	Preeclampsia (n=16)	*p*
Age (years)	31.4±4.9	32.5±4.3	NS
Parity	0.55	0.68	NS
Pre-BMI (kg/m^2)	21.2±2.4	23.3±3.8	NS
BMI (kg/m^2)	27.9±2.8	29.6±3.8	NS
Gestational age at delivery (weeks)	38.1±2.1	34.3±4.0	0.011
Blood pressure (mmHg)			
Systolic	118.7±9.8	166.8±10.7	<0.001
Diastolic	72.7±10.0	108.1±9.1	<0.001
C/S (%)	8 (72.7)	13 (81.3)	NS
Labored (%)	4 (36.3)	7 (43.7)	NS
Placenta weight (grams)	495.5±117.3	458.1±162.6	NS
Neonatal weight (grams)	3055±535	2018±621	<0.001

Discussion

RBP4 is a newly identified insulin resistance that has been extensively studied in metabolic and cardiovascular diseases [10, 18-21]. Preeclampsia can be caused by abnormal placentation, leading to placental hypoxia or ischemia, and it is often associated with increased insulin resistance [22, 23]. Several anti-angiogenic molecules are associated with preeclampsia and these molecules have been confirmed to be derived from the placenta and to decrease after delivery of placenta [23]. In this study, placental expression of RBP4 was increased in patients with preeclampsia compared with that in normal pregnant women, suggesting that the placenta may contribute to exacerbation of insulin resistance in patients with preeclampsia. Several studies have examined whether circulating RBP4 levels would predispose a patient to preeclampsia, although the results are inconclusive [13, 14, 24]. Vaisbuch *et al.* first reported that maternal circulating RBP4 levels are increased in patients with early-onset preeclampsia

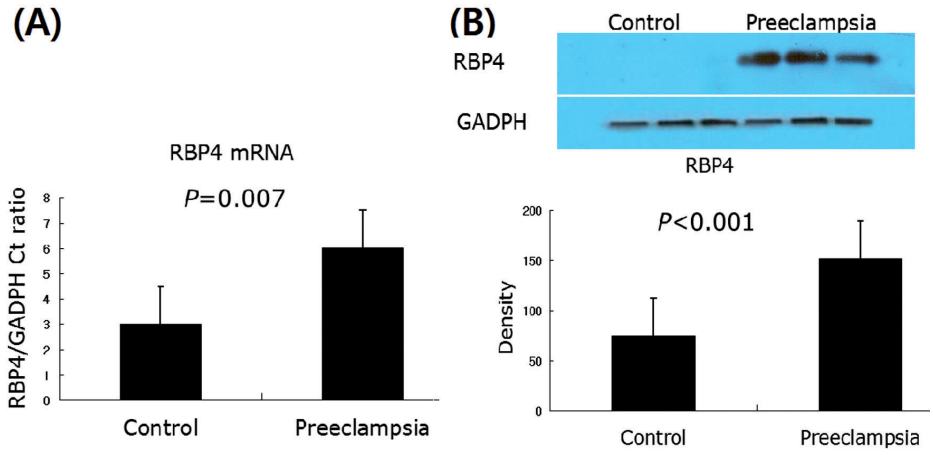

Figure 1. — Placental expression of RBP4 in normal pregnant women and patients with preeclampsia. (A) RT-PCR demonstrates that RBP4 mRNA is significantly increased in the placentae of the patients with preeclampsia as compared with that in normal placenta. (B) Western blot analysis of protein extracts from the placentae of all participants. Densitometric analysis of RBP4 immune complexes reveals that protein levels of RBP4 are significantly increased in the placentae of patients with preeclampsia. Data are means ± SEM.

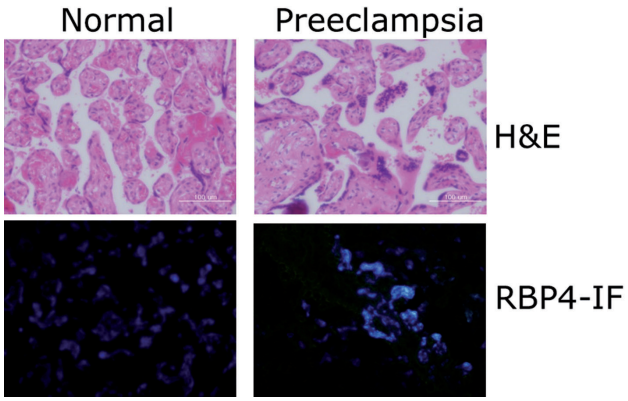

Figure 2. — H&E staining and immunofluorescence of RBP4 in representative placentae of normal pregnant women and patients with preeclampsia. (A) H&E staining shows increased syncytial knots in the placenta of a patient with preeclampsia (×200). (B) Immunofluorescence analysis shows increased expression of RBP4 in the placenta of a patient with preeclampsia.

[13]. Masuyama *et al.* reported that RBP4 levels and an index of insulin resistance were increased in overweight patients with late-onset preeclampsia compared with that in overweight normal pregnant women. These results suggested that RBP4 may have a role in the pathophysiology of preeclampsia via increased insulin resistance [14]. Very recently, Yliniemi *et al.* proposed that maternal circulating RBP levels during the first trimester could be a predictive marker for preeclampsia [24].

Studies of RBP4 and pregnancy have focused mainly on gestational diabetes mellitus (GDM), and increased RBP4 levels have been observed in Asian women with GDM in a meta-analysis [25, 26]. However, the origin of increased RBP4 in maternal blood in patients with GDM has yet to be determined. Kuzmicki *et al.* found that there was no significant difference in placental RBP4 mRNA expression between patients with GDM and normal pregnant women [27]. In contrast, Rong *et al.* demonstrated that RBP4 was significantly upregulated in the placenta of patients with GDM [28].

Recent studies of the preeclamptic placenta have suggested that early- and late-onset preeclampsia exhibit different pathologies [29, 30]. Thus, one limitation of the present study was that the preeclampsia group was a heterogeneous population consisting of patients at various gestational ages from 31 to 41 weeks, because the authors aimed to examine preeclampsia in the third trimester. Most enrolled patients (12/16) exhibited late-onset preeclampsia, whereas only four patients exhibited early-onset preeclampsia, making it difficult to determine whether placental RBP4 expression would be altered between early- and late-onset preeclampsia. Another limitation of this study was that the authors did not determine whether maternal or fetal circulating RBP4 levels were correlated with placental RBP4 expression. However, despite these limitations and the fact that the exact role of placental RPB4 in the pathophysiology of preeclampsia has not yet been elucidated, this study suggested that RBP4 may be associated with the pathophysiology of preeclampsia by overexpression in the placenta, similar to other placenta-derived molecules. These data may also provide important insights into preventive interventions for women with a history of preeclampsia because, although most patients with preeclampsia recover from disease with the end of pregnancy, these women have increased risk for developing cardiovascular disease and other long-term morbidities such as metabolic syndrome and insulin resistance, compared with normal pregnant women [3, 7, 24, 31].

Conclusion

Placental RBP4 expression was significantly increased in patients with preeclampsia compared with normal pregnant women. There were no significant correlations between placental RBP4 expression and maternal BMI,

neonatal weight, or placental weight.

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Coresponding Author:
H.J. SEOL, M.D., Ph.D.
Department of Obstetrics & Gynecology
Kyung Hee University Hospital at Gangdong
892, Dongnam-ro, Gangdong-gu
Seoul, 05278 (Republic of Korea)
e-mail: seolhj@khu.ac.kr