Leptin, leptin receptors and hypoxia-induced factor-1α expression in the placental bed of patients with and without preeclampsia during pregnancy

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Abstract. The mechanism underlying the pathogenesis of preeclampsia (PE) has been previously investigated but remains to be elucidated. Among numerous biomarkers that are associated with the pathogenesis of PE, leptin is most frequently investigated. Although studies concerning the association between PE and the expression of leptin in the serum and placenta have been conducted, the results are conflicting and inconsistent. Furthermore, the expression of leptin and its receptors in the placental bed and their association with PE, to the best of our knowledge, has not been previously reported. Therefore, to determine the association between the expression of leptin and its receptor, and pathogenesis and onset period of PE, placental bed tissues were obtained from cesarean section deliveries. The mRNA and protein expression levels of leptin and its receptor were investigated in normal pregnancies (n=18), pregnancies complicated with early-onset PE (n=9) and late-onset PE (n=9) by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. The results demonstrated that the mRNA and protein expression of leptin in the placental bed was significantly increased in the PE groups compared with normal controls and was associated with the onset period of PE. Furthermore, as evidenced by immunostaining, leptin was upregulated in endothelial cells of the placental bed in the PE groups, with a particularly strong upregulation in activated endothelial cells from patients with early-onset PE. The results of the present study indicate that altered expression of leptin in the placental bed may contribute to the pathogenesis of PE.

Introduction

Preeclampsia (PE) is a pregnancy-specific syndrome that is characterized by hypertension and proteinuria following 20 weeks of gestation (1). Although it is a major contributor to maternal and perinatal morbidities, mechanisms underly ing the pathogenesis of PE have not been elucidated. Various potential etiologies associated with development of PE have been investigated, including angiogenesis imbalance, coagulation abnormalities, immunological maladaptation and exaggerated inflammatory response (2-4). Among the etiologies of PE, incomplete spiral artery remodeling, leading to dysregulated uteroplacental perfusion and placental oxidative stress, is considered to markedly impact the development of PE (2,3). Remodeling of spiral arteries during human pregnancy is precisely regulated by angiogenic factors and their associated receptors (5-7). One potential mechanism underlying PE may be associated with faulty vascular transformation of the uteroplacental unit due to an imbalance in levels of angiogenic factors and hypoxia-induced oxidative stress (5,7).

Leptin, a 16-kDa protein encoded by the ob gene, is secreted by adipose tissues and functions in the regulation food intake and energy expenditure (8). During pregnancy, leptin is also produced in syncytiotrophoblasts and endothelial cells of the placenta (9,10). Furthermore, in a previous study, leptin secretion in BeWo cells (villus trophoblast tumor-derived cells) increased under hypoxic conditions, and these observations indicate that placental production of leptin may be upregulated in severe PE (11).

The pathophysiological role of leptin in the placental bed of patients with PE is currently unclear. However, it has been hypothesized that leptin may serve a role in the pathogenesis of PE by regulating angiogenesis and vascular smooth muscle cell development in the placenta and placental bed (12-15). Among previous reports concerning the angiogenic properties of leptin, Sierra-Honigmann et al (12) indicated that it may...
demonstrate angiogenic activity, and leptin has also been hypothesized to promote angiogenesis through activation of vascular endothelial growth factor (VEGF) receptor 2 (13). However, Islami et al (14) reported that leptin reduces the release of VEGF in cytotoxoplasms in a dose dependent manner, while Bohlen et al (15) demonstrated that leptin inhibits growth and decreases the number of human vascular smooth muscle cells by downregulating the short isoform of leptin receptor.

Another potential mechanism of leptin activity is regulation of inflammatory mediators in the placenta (16,17). Normal pregnancy promotes a mild systemic inflammation, as evidenced by the activation of leukocytes in the blood (18). PE exacerbates this maternal response in the presence of an embryo and placenta (18). Furthermore, the development of PE is associated with activation of the coagulation-hemostasis system (19). Activated platelets stimulate leukocyte activity by secreting cytokines (20).

Leptin may serve a role in the placenta by stimulating maternal energy resources for fetal use and development, via leptin receptors, which are present in several isoforms. The long isoform of the leptin receptor activates the mitogen-activated protein kinase cascade in human placental tissue, while the mechanism and action of the short isoform is less understood (21,22). Regulation of leptin and its receptors in the uteroplacental unit remains to be elucidated. One study reported that the expression of leptin receptors is induced under hypoxic conditions in vitro (23); however, the use of human placenta from patients with PE demonstrates conflicting results (22). To date, to the best of our knowledge, there have no previous reports concerning the expression of leptin receptor isoforms in the placental bed tissue, therefore, the present study aimed to elucidate the expression pattern of leptin receptor mRNA and protein in patients with PE.

In previous studies, hypoxia inducible factor-1 (HIF-1), a major regulator of the hypoxic response, was indicated to function as a mediator of leptin expression (24,25). HIF-1 is composed of a heterodimeric HIF-1α/HIF-1β complex and hypoxia leads to enhanced leptin expression via HIF-1α (24). Previous reports have also demonstrated an association between hypoxia and leptin in PE (26,27).

The present study aimed to determine alterations in the expression of these factors in placental bed tissue. Despite the perception that pathophysiological alterations of placental bed tissue are implicated in pathogenesis of PE, research into further understanding the placental bed has not been conducted due to difficulty in obtaining a sample containing utero-placental arteries, whose primary function is the maintenance of a sufficient blood supply to the intervillous space of the placenta (28,29). It was previously reported that the density of extravillous trophoblasts and depth of invasion of utero-placental arteries are increased in the central region of the placental bed (30).

Previous studies on expression of leptin, leptin receptor and HIF-1α during pregnancy are limited in the placental tissue and serum and to the best of the authors knowledge, no studies have been conducted using placental bed (10,11,31-35). Currently, to the best of our knowledge, no previous studies have investigated the expression of these factors in the placental bed of women with PE. Therefore, the purpose of the present study was to investigate the association between the expression of leptin, isoforms of leptin receptor and HIF-1α in the placental bed of pregnant females with and without PE.

**Materials and methods**

**Study participants.** A total of 36 pregnant women (32±4.8 years), 18 with normal pregnancies, 9 with early-onset PE (EOPE) and 9 with late-onset PE (LOPE) were included in the present study. The Institutional Review Board of Pusan National University Hospital approved the research protocol of the present study (approval no. 1302-005-015) and all participants signed written informed consent forms prior to recruitment. PE was diagnosed based on increased blood pressure (≥140/90 mmHg) that occurred in pregnant women following 20 weeks of amenorrhea accompanied by proteinuria (≥0.3 g/24 h or 1+ dipstick value of protein concentration in urine), using criteria defined by the report of the American College of Obstetricians and Gynecologists Task Force on Hypertension in Pregnancy (1). EOPE and LOPE were defined as those diagnosed at <34 and ≥34 weeks gestation, respectively (36).

**Placental bed collection.** Placental bed tissues were collected from 18 patients with PE (9 with EOPE and 9 with LOPE) and 18 gestational age-matched normotensive controls of third trimester pregnancies at Pusan National University Hospital (Busan, Korea) between May 2014 and December 2015. To avoid the effect of labor on the results, only individuals that delivered by cesarean section were included. Placental bed tissues were sampled using punch biopsy forceps by a single operator as previously described by Dixon and Robertson (28). Tissue samples were washed with 0.9% NaCl and placed in sterile tubes. These were stored at -70°C until subsequent extraction of total RNA and proteins or fixed in 4% paraformaldehyde for 16-24 h at room temperature prior to histological analysis. All placental bed tissue was verified by immunostaining with antibodies against cytokeratin (1:50; cat. no. ab7753; Abcam, Cambridge, MA, USA) to detect trophoblast and against desmin (1:100; cat. no. ab8592; Abcam) to detect muscle, as previously described (37). Subsequently, confirmed placental bed tissues were used for evaluation of the expression of leptin, isoforms of leptin receptor and HIF-1α.

**RNA preparation, reverse transcription-polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR).** Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. cDNA was synthesized from 1 µg total RNA using Avian Myeloblastosis Virus Reverse Transcriptase (Promega Corporation, Madison, WI, USA) using random hexamers (Takara Bio, Inc., Otsu, Japan), 0.2 mM deoxynucleotide triphosphate (dNTP) mixture (Cosmo Genetech, Co., Ltd., Seoul, Korea) and 1X buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, and 10 mM DTT] at 42°C for 1 h.
followed by inactivation of the enzyme at 95°C for 5 min. Gene expression was assessed using RT-PCR. Each cDNA was subjected to PCR using 2.5 U of Taq polymerase (Cosmo Genetech, Co., Ltd.), 10X Taq buffer, 0.2 mM of dNTP mixture and 100 pmol of each gene-specific primer (Table I). The following thermocycling conditions were used for the PCR: Initial denaturation at 95°C for 5 min, 35-45 cycles of denaturation at 95°C for 30 sec, primer-specific annealing temperature for 30 sec and extension at 72°C for 30 sec; and final extension at 72°C for 10 min. RT-PCR products were visualized on 2% agarose gels by ethidium bromide staining and UV illumination. Data are representative of at least three independent experiments. The relative density of PCR bands was quantified and normalized to the control GAPDH bands using ImageJ software (version 1.35d; National Institutes of Health, Bethesda, MD, USA). The analytical performance of qPCR and the optimal number of cycles were determined prior to performing RT-PCR. qPCR was performed using SYBR-Green Premix Reagent (Takara Bio, Inc.), as previously described (38). Each experiment was conducted in duplicate and repeated 3 times. The relative expression levels of mRNA in each sample were calculated using the \( \Delta \Delta C_q \) method.

Western blot analysis. Proteins were extracted by mechanical homogenization of placental bed tissues in the presence of 200 µl ice-cold lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA] containing protease inhibitor and extracted protein concentrations were determined using a Bradford assay. A total of 60 µg protein/lane was separated by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The transfer was performed at a constant voltage of 15 V for 90 min. For western blotting, the membrane was incubated with anti-leptin polyclonal (1:100; cat. no. sc-842; Santa Cruz Biotechnology, Inc.) and anti-rabbit IgG (1:5,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) horseradish peroxidase conjugated secondary antibodies for 30 min at room temperature. Following washing with TBST, protein bands were visualized using an enhanced chemiluminescence detection system (Amersham ECL Advance Western Blotting Detection kit) according to the manufacturer's protocol (GE Healthcare, Chicago, IL, USA). Protein bands were quantified and normalized to the control bands with ImageJ software.

Immunohistochemistry. Serial sections (4 µm thick) of formalin-fixed, paraffin-embedded placental beds were spread on coated-slides using a microtome and transferred to a 37°C water bath with distilled water for 1 h. Slides were deparaffinized in xylene to solubilize and rehydrated in a graded ethanol series (100% ethanol twice, 95% ethanol and 85% ethanol, 1 min each) at room temperature. The deparaffinized sections were washed with PBS, and microwaved in 10 mM citrate buffer, pH 6.0, for 15 min in a conventional microwave (98°C). Subsequently, endogenous peroxidase was quenched by immersion in 0.3% H₂O₂ in methanol for 5 min as previously described (38). The sections were blocked with 10% normal rabbit serum (cat. no. ab166640; Abcam) for 30 min at room temperature to prevent non-specific binding and subsequently incubated with a primary antibody against leptin (cat. no. sc-842; Santa Cruz Biotechnology, Inc.) at a dilution of 1:300 in PBS and 3% bovine serum albumin (Sigma-Aldrich; Merck KGaA) overnight at 4°C. Following four washes with PBS for 15 min each, samples were incubated with a biotinylated secondary antibody (diluted 1:200 in PBS; SuperPicture™ kit; cat. no. 879263; Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at room temperature and washed three times with PBS. The samples were subsequently incubated with streptavidin-peroxidase conjugate (Zymed; Thermo Fisher Scientific, Inc.; 1:500 in PBS; cat. no. S-911; Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at room temperature followed by an incubation with 3, 3'diaminobenzidine chromogen (Sigma-Aldrich; Merck KGaA). Sections were then rinsed in distilled water, and counterstained for 5 min in Mayer's hematoxylin (Sigma-Aldrich; Merck KGaA) at room temperature and mounted using Histomount solution (Invitrogen; Thermo Fisher Scientific, Inc.). Results were assessed by two blinded pathologists under a light microscope.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Annealing Tm, °C</th>
<th>Cycles</th>
<th>Product size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td>GATGACACCAAAAAACCTCACT</td>
<td>GGCCACCACTCTGTGGAGT</td>
<td>59</td>
<td>45</td>
<td>354</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>CAGCTATTGCGTGTAGAGAAA</td>
<td>ACCAAGCAGGTCATAGGTGTT</td>
<td>57</td>
<td>35</td>
<td>471</td>
</tr>
<tr>
<td>Leptin-RL</td>
<td>CTAAGAGAACCTTGCCGACT</td>
<td>GAAGATGTTGCCAACCCTCAAGA</td>
<td>60</td>
<td>42</td>
<td>428</td>
</tr>
<tr>
<td>Leptin-RS</td>
<td>GGAAGTGGCACAATGGGTC</td>
<td>CCATTGGAAGTACCATCTTG</td>
<td>60</td>
<td>42</td>
<td>330</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GTGGTTCCTCCTACTGCTCAAC</td>
<td>TCTCCTCTCTGTGCTTGG</td>
<td>57</td>
<td>35</td>
<td>212</td>
</tr>
</tbody>
</table>

Tm, temperature; HIF-1α, hypoxia-induced factor 1α; leptin-RL, long leptin receptor isoform; leptin-RS, short leptin receptor isoform.

Table I. Primers sequences used for quantitative polymerase chain reaction amplification and conditions.
Clinical characteristics of participants. The clinical characteristics of participants of the present study are summarized in Table II. There were no statistically significant differences in maternal age, parity, gravidity and body mass index among the normotensive, LOPE and EOPE groups. Systolic and diastolic blood pressures were significantly increased in the PE groups compared with the normotensive group (P<0.01). Gestational age at the time of delivery in the EOPE group was significantly shorter compared with the LOPE and normotensive groups (P<0.05). Birth weights of newborns were significantly lower in LOPE and EOPE groups compared with the normotensive group; furthermore, the EOPE group demonstrated significantly lower birth weights compared with the LOPE group (P<0.01).

mRNA and protein expression of leptin and HIF-1α in the placental bed of normotensive, EOPE and LOPE groups. The results of RT-PCR demonstrated that the expression of placental bed leptin mRNA was significantly increased in PE groups compared with the normotensive group. In addition, the leptin mRNA expression level in the EOPE group was significantly increased compared with the LOPE group as analyzed by RT-PCR (Fig. 1A and B). Expression levels of HIF-1α mRNA in the EOPE group demonstrated a pattern similar to the results for leptin expression (Fig. 1A and B). Furthermore, qPCR analysis demonstrated that mRNA expression levels of leptin and HIF-1α were increased ~2-fold (248 and 196%, respectively) in the EOPE group compared with the normotensive control group (100%: both P<0.01; Fig. 1C and D). However, based on RT-PCR results, the expression of leptin receptor isoforms was not significantly different between the PE and the normotensive groups (Fig. 2). Therefore, qPCR was not performed.

Similar to the results for mRNA expression, western blot analysis revealed that the protein expression of leptin and HIF-1α in the placental bed was significantly increased in the PE groups compared with the normotensive group (P<0.01; Fig. 3). In addition, leptin and HIF-1α protein expression were significantly increased in the EOPE group compared with the LOPE group (P<0.01; Fig. 3).

Table II. Characteristics of study participants.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normotensive (n=18)</th>
<th>LOPE (n=9)</th>
<th>EOPE (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>30.9±2.3</td>
<td>33.5±3.6</td>
<td>32.9±0.37</td>
</tr>
<tr>
<td>Gestational age, weeks</td>
<td>35.8±2.4</td>
<td>35.0±2.0</td>
<td>32.9±2.1c</td>
</tr>
<tr>
<td>Parity</td>
<td>0.9±0.8</td>
<td>1.0±1.3</td>
<td>0.6±0.7</td>
</tr>
<tr>
<td>Gravidity</td>
<td>2.1±1.1</td>
<td>2.5±1.8</td>
<td>2.2±1.1</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.4±5.3</td>
<td>25.9±3.1</td>
<td>26.2±3.6</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>109.3±8.0</td>
<td>143.7±13.7</td>
<td>158.0±13.2b</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>68.7±7.4</td>
<td>86.7±5.2b</td>
<td>111.3±5.0b</td>
</tr>
<tr>
<td>Neonatal birth weight, g</td>
<td>2,800.7±570.4</td>
<td>2,208.3±504.0</td>
<td>1,455.6±357.3d</td>
</tr>
</tbody>
</table>

*P<0.05 and **P<0.01 vs. the normotensive control; †P<0.05 and ‡P<0.01 vs. the LOPE group. Data are presented as the mean ± standard deviation. LOPE, late-onset preeclampsia; EOPE, early-onset preeclampsia; BMI, body mass index; BP, blood pressure.
Immunohistochemical analysis of leptin expression in the placental bed of normotensive, EOPE and LOPE groups. Immunohistochemistry was performed to investigate the localization of leptin protein in the placental bed. Leptin was positively stained in the endothelial cells of all groups. The endothelial cells of the intima in the EOPE group exhibited increased activation compared with the normotensive group, based on a cuboidal morphology compared with the flattened morphology of the normotensive group, indicating damage of endothelial cells in the EOPE groups (Fig. 4). Endothelial expression of leptin in the EOPE group was increased compared with the LOPE and normotensive control groups, as indicated by more intense staining. These observations indicate that increased leptin expression may be associated with endothelial cell activation in the placental bed.

Discussion

A number of studies have investigated leptin levels in maternal serum, cord blood and placental tissue in patients with or without PE (10,11,31-35). The majority of studies have reported increased serum leptin levels and increased placental leptin expression in pregnancies with PE compared with normotensive pregnancies (10,11,34). However, conflicting results have also been reported (31,32,40). The present study demonstrated that the expression of placental bed leptin and HIF-1α were significantly elevated in pregnancies with PE compared with the normotensive control group. To the best of our knowledge, the present study is the first to report on the expression of leptin, leptin receptor isoforms and HIF-1α in the third trimester placental bed from patients with PE and normotensive controls, and indicated an association between the expression of these factors in the placental bed and the pathogenesis of PE.
Dysregulation of leptin during pregnancy has been associated with the pathogenesis of various maternal complications, including PE, fetal growth restriction and gestational diabetes (9). However, mechanisms underlying pathogenesis of PE, as well as the effects of leptin on pregnancy, are diverse (11). Therefore, the association between the elevated expression of leptin and HIF-1α in the placental bed and PE remains to be elucidated, but several mechanisms have been postulated.

One potential mechanism involves the angiogenic property of leptin. Placental hypoperfusion-ischemia has been hypothesized to be the major pathogenic mechanism underlying PE, resulting in hypoxia (11). Hypoxia was reported to increase VEGF production and the expression of placental leptin (11,41). The association between leptin and angiogenesis is complex. According to in vitro studies on cytotrophoblasts and human vascular endothelial cells, leptin inhibited angiogenesis (14,15). In one study, the level of leptin mRNA expression was associated with the expression levels of placental HIF-1α mRNA (10). The present study demonstrated that placental bed leptin and HIF-1α expression was markedly increased in patients with PE compared with normotensive controls. Therefore, it may be hypothesized that reduced placental perfusion and hypoxia caused by PE results in an increased production of leptin, which may further inhibit angiogenesis in endothelial cells.

Another plausible mechanism to be considered is the association of leptin with inflammation (16,17). The expression and release of leptin is affected by inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1α and IL-6 (42). Previous studies have hypothesized that increased production of systemic inflammatory cytokines may result in PE due to impaired trophoblast invasion with vascular damage during spiral artery remodeling (43-45). The endothelial cells observed by immunostaining in the present study exhibited hypertrophic cuboidal morphology with ovoid nuclei which indicated possible cell damage (46). The present study demonstrated that PE groups manifested damaged endothelial cells of the intima, while the normotensive group exhibited normal endothelial cells with a flattened morphology. Therefore, it may be hypothesized that elevated leptin expression in the placental bed may be a consequence of endothelial cell damage induced by increased inflammation. However, further studies investigating the expression of inflammatory cytokines and leptin in the placental bed are required to verify this hypothesis.

Previous studies have investigated the expression of the leptin receptor in PE. Klaffenbach et al (23) demonstrated increased expression of leptin receptor in placental cells under hypoxic conditions, while other studies revealed no difference in leptin receptor expression between normotensive controls, and mild and severe PE groups (10,12). The present study determined the expression of leptin receptor isoforms in the placental bed and revealed no differences between the normotensive control and PE groups. Therefore, the results of the present study indicate that the degree of leptin expression may have been below the effective level to trigger upregulation of the leptin receptor, or that the development of PE may be influenced by leptin rather than the amount of leptin receptor under hypoxic conditions.

In conclusion, the expression of leptin, its receptor and HIF-1α in the third trimester placental bed of pregnancies with PE was investigated in the present study and significant alterations of leptin expression associated with onset period were detected. The results indicated that leptin and HIF-1α expression level in the placental bed may be associated with the development and onset period of PE. Although the results of the present study demonstrate differential expression of leptin and HIF-1α between the normotensive control and the PE groups, certain limitations are present. Experimental samples were obtained from third trimester placental beds and it cannot be established whether the alterations are a cause or a consequence of established PE. Although the pathological alterations of PE were examined after the gestation period was complete, samples (blood or placenta) in early or mid-trimesters were not examined in the current study. Further studies based on a larger number of diverse samples may further confirm the association between leptin and the pathogenesis of PE, and elucidate the precise molecular mechanisms underlying this association.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

MJP and SCK conceived the idea and designed the experiments. MJP, BSJ and YJL did experiments and analysis of results. DHL contributed to the idea generation. JKJ, SCK and KSL provided resources. MJP, BSJ and SCK wrote the manuscript.

Ethics approval and consent to participate

The Institutional Review Board of Pusan National University Hospital approved the research protocol of the present study (approval no. 1302-005-015) and all participants signed written informed consent forms prior to recruitment.

Consent for publication

Written informed consent was obtained from all participants.

Competing interests

The authors declare that they have no competing interests.
Possible involvement of placental hypoxia


