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<td>Author(s)</td>
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Kyoto University
Decreased Lectin-Like Oxidized LDL Receptor 1 (LOX-1) and Low Nrf2 Activation in Placenta Are Involved in Preeclampsia

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Context: Serum concentration of oxidized low-density lipoprotein (oxLDL) is higher in women with preeclampsia than in normal pregnant women. Lectin-like oxLDL receptor-1 (LOX-1) is one of the scavenger receptors for oxLDL and is abundantly expressed in placenta. It is well known that oxLDL activates nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of antioxidant and cytoprotective genes such as heme oxygenase-1 (HO-1), which play an important role in preeclampsia. However, it has yet to be elucidated whether LOX-1, along with Nrf2, participates in the pathology of preeclampsia.

Objective: The objective of the study was to assess LOX-1 expression and Nrf2 activation in pre-eclamptic placentas and to manifest their physiological roles in preeclampsia.

Methods: Expression and regulation of LOX-1, HO-1, and Nrf2 were evaluated by real-time quantitative RT-PCR and Western blotting. The functions of LOX-1 and Nrf2 were examined using an anti-LOX-1 antibody and Nrf2 activator in JAR, a choriocarcinoma cell line, and placental explants.

Results: Both LOX-1 expression and Nrf2 activation were significantly decreased in preeclamptic placentas compared with normal controls. A significant decrease in LOX-1 mRNA was found in placental explant cultures under hypoxic conditions. Activation of Nrf2 up-regulated HO-1 in both the JAR cells and placental explants. Furthermore, oxLDL increased HO-1 mRNA, whereas the blockade of LOX-1 inhibited the increase of HO-1 mRNA in JAR cells.

Conclusion: Decreasing LOX-1 expression in preeclamptic placenta may contribute to high oxLDL concentration, low Nrf2 activation, and low HO-1 expression. These findings provide novel insights into the crucial role of LOX-1 and Nrf2 in the pathogenesis of preeclampsia. (J Clin Endocrinol Metab 97: E1862–E1870, 2012)
A). LOX-1 is a 52-kDa, type 2, single-transmembrane receptor cloned by Sawamura in 1997 (9) and present primarily on endothelial cells and macrophages (10). Although the basal expression of LOX-1 is very low, it can be rapidly induced by various stimuli and several pathological conditions, such as oxLDL, diabetes (11), and hyperlipidemia (12). Intriguingly, placenta is the organ with the highest LOX-1 expression, even in a healthy state (9), suggesting that LOX-1 is crucial for maintaining pregnancy. However, the underlying precise mechanism by which LOX-1 elicits its effects in placenta and contributes to the maternal lipid metabolism during pregnancy remains to be elucidated. In particular, the function of LOX-1 in placenta in context of preeclampsia remains largely unknown.

Recently several studies have highlighted the importance of heme oxygenase-1 (HO-1) in pregnancy (13, 14). HO-1 is an inducible enzyme that catalyzes the degradation of heme, yielding biliverdin, iron, and carbon monoxide, a potent vasodilator (15). It has been implicated in several physiological functions including the control of vascular tone (16) and the regulation of the inflammation (17) as well as contributing to the antioxidant capabilities (18). It has been reported that the protein level of HO-1 is decreased in preeclamptic placentas (17). Overexpression of HO-1 in endothelial cells results in a significant decrease in production of soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (14). They are the most important antiangiogenic circulating factors that are tightly associated with preeclampsia (19, 20). Furthermore, induction of HO-1 attenuates hypertension in pregnancy hypertension model animals (13). However, the primordial cause for the decrease of HO-1 in preeclamptic placenta has yet to be elucidated. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key molecule that is activated in response to oxidative stress and regulates antioxidant responsive element mediated induction of cytoprotective genes (21). Although Nrf2 is widely acknowledged as the predominant upstream regulator of HO-1 and is activated by oxLDL (22), the role of Nrf2 in placenta and how LOX-1 involves in Nrf2 activation have not been clarified.

In the present study, we hypothesized that LOX-1 may have an influence in preeclamptic placenta, leading to maternal pathogenesis of preeclampsia through high serum level oxLDL and endothelial dysfunction. We analyzed the expression and regulation of LOX-1 and postulated the relationship between LOX-1 and HO-1. We have also focused on Nrf2 and investigated its relevance to LOX-1 in preeclamptic placenta. Based on these results, we will discuss the putative role of LOX-1 and Nrf-2 on the pathogenesis of preeclampsia.

### Materials and Methods

#### Patient characteristics and tissue collection

The clinical characteristics of patients enrolled in this study are shown in Table 1. Preeclampsia was defined as maternal systolic blood pressure of 140 mm Hg or greater and/or diastolic blood pressure of 90 mm Hg or greater in two consecutive measurements, with an interval of 6 h, and proteinuria of 300 mg or greater per 24 h after 20 wk of gestation. Placental villous tissues were collected from normal pregnant subjects (n = 16) and preeclamptic subjects (n = 16), immediately after cesarean section without labor at Kyoto University Hospital (Kyoto, Japan). The tissues were stored in RNAlater (Ambion, Austin, TX) after brief rinsing in saline. The local ethics committee of the Graduate School of Medicine, Kyoto University, approved the study protocol, and written informed consent was obtained from each patient enrolled in this study.

#### Real-time quantitative RT-PCR

Total RNA extraction from placental tissues and cells was performed using the RNaseq Mimi kit (QIAGEN, Germantown, MD) according to the manufacturer’s instructions. RNA quality and quantity was measured using a ND-1000 spectrophotometer (Nanodrop, Wilmington, NC). Reverse transcriptase of 1 μg RNA was performed using the Rever Tra Ace (TOYOBO, Osaka, Japan) according to the manufacturer’s instructions. The following primers were designed using GeneFisher 2 software (Bielefeld University Bioinformatics Service, Bielefeld, Germany): LOX-1 (OLR1) (GenBank accession no. NM_002543.3), sFlt-1 (U01134.1), CD36 (NM_001001548.2), MSR1 (NM_138716.2), CD63, C/EBPα (NM_001150.4), IL6, IL1B, TNF (NM_001475.3), IL10 (NM_001383.3), IL12B (NM_000268.3), and STAT3 (NM_006682.2).

### Table 1. Clinical characteristics of the normal and preeclamptic (PE) patient groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normal (n = 16)</th>
<th>PE (n = 16)</th>
<th>P value</th>
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<tr>
<td>Patient’s age at delivery (yr)</td>
<td>35.6 ± 5.2 (27-44)</td>
<td>33.8 ± 4.0 (27-41)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Primipara (n)</td>
<td>7/16</td>
<td>11/16</td>
<td></td>
</tr>
<tr>
<td>Gestational age at delivery (wk)</td>
<td>38 [37–38]</td>
<td>34 [32–37]</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Body mass index at delivery (kg/m²)</td>
<td>25.2 ± 2.1 (21.2–29.1)</td>
<td>25.0 ± 3.3 (21.0–33.4)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Systolic blood pressure at delivery (mm Hg)</td>
<td>106 ± 6 (90–116)</td>
<td>168 ± 16 (145–192)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diastolic blood pressure at delivery (mm Hg)</td>
<td>63 ± 8 (52–80)</td>
<td>101 ± 12 (80–124)</td>
<td>&lt;0.0001</td>
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<tr>
<td>Neonatal weight (g)</td>
<td>2969 [2756.5–3189.5]</td>
<td>1583.5 [1290.5–2440]</td>
<td>&lt;0.0001</td>
</tr>
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Values are the mean ± sd and (range) or median value with [interquartile range]. Dashes indicate the median value with interquartile range.
Cytosolic and nuclear extract preparation

Placental tissues were homogenized in radioimmunoprecipitation assay buffer (50 mmol/liter Tris-HCl, pH 8.0; 150 mmol/liter sodium chloride; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate; 1.0% Nonidet P-40 substitute) supplemented with cocktail protease inhibitor Complete Mini (Roche Diagnostics, Mannheim, Germany) based on the following run conditions: 50 μg total protein for initial denaturing heating from 60 to 97 C to ensure amplification specificity. The following run conditions: 95 C for 30 sec for initial denaturing followed by 95 C for 5 sec and 60 C for 30 sec for 40 cycles. For dissociation after PCR amplification, the protocol included slow heating from 60 to 97 C to ensure amplification specificity. The end result for gene expression was estimated using comparative crossing point method for relative quantification. All data were normalized and expressed relative to the GAPDH gene as an internal control. All samples were run in duplicates and quantitative detection was averaged.

Western blotting

Thirty micrograms of the cytosolic (for LOX-1 and HO-1 expression) or the nuclear (for Nrf2 expression) protein were separated on 10% (LOX-1), 12% (HO-1), or 7.5% (Nrf2) sodium dodecyl sulfate-polyacrylamide gels, respectively. The separated proteins were transferred onto nitrocellulose membranes, which were blocked with 5% fat-free milk overnight at 4 C. The cells were washed with ice-cold PBS, and cytosolic protein was extracted in the same manner. Nuclear proteins from tissues and cells were prepared using CellLyte NuCLEAR extraction kit (Sigma Aldrich, St. Louis, MO) according to the manufacturer’s instructions. Protein concentrations were determined with a bicinchoninic assay protein assay kit (Thermo Scientific, Rockford, IL).

Immunohistochemistry

Immunohistochemical staining was carried out using the streptavidin-biotin-peroxidase method. Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and antigen retrieval was performed in Tris-EDTA buffer (pH 9.0) at 120 C for 5 min. Endogenous peroxidase activity was blocked with 0.3% H2O2. The sections were incubated with rabbit polyclonal antibody against LOX-1 (1:50; ABGENT) or normal rabbit IgG overnight at 4 C, followed by incubation with biotinylated goat antirabbit secondary antibody (Nichirei, Tokyo, Japan). Then they were incubated with streptavidin-peroxidase complex solution for 30 min. Signals were generated by treatment with diaminobenzidine. Finally, the sections were counterstained with hematoxylin and observed under the microscope.

Tissue and cell culture

Placental villous tissues were obtained from normal-term pregnancies delivered by elective cesarean section in the absence of labor. Small fragments of placental villi (~10–20 mg) were dissected from the placenta and washed in ice-cold PBS. Two fragments were placed in six-well plates with 3 ml culture medium (RPMI 1640 containing 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin) per well. For hypoxia treatment, explants (n = 6) were cultured at 37 C, 5% CO2 in an atmosphere of 20 or 1% O2 for 24 h. The JAR (HTB-144) ch
riocarcinoma cell line was obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C, 20% O2, 5% CO2.

Diethylmaleate (DEM) treatment
The JAR cells grown in six-well plates and a 10-cm dish for RNA extraction and protein extraction, respectively, were treated with or without 100 μM DEM (Wako Pure Chemical Industries, Osaka, Japan), a typical Nrf2 activating electrophilic agent. After 3, 6, 9, and 24 h, Nrf2 protein and mRNA of HO-1 and LOX-1 were measured. The experiments were performed six times in duplicates (n = 6). Placental explants (n = 6) were cultured at 37 °C, 20% O2, 5% CO2 with or without 100 μM DEM. After 6 and 24 h, both protein and mRNA of HO-1 were measured.

oxLDL treatment
oxLDL and native LDL (nLDL) were purchased from Intracel (Frederick, MD). Purified normal human IgG was purchased from R&D Systems (Minneapolis, MN). TS92, an antihuman LOX-1 antibody, was a kind gift from Dr. T. Sawamura (Osaka, Japan). JAR cells grown in 48-well plates or a 6-cm dish were treated with or without oxLDL (100 μg/ml) for 3, 6, 9, and 24 h. The cells were also tested after being pretreated with TS92 (30 μg/ml) or normal human IgG (30 μg/ml) and later treated with oxLDL (100 μg/ml) or nLDL (100 μg/ml) for 9 h. After harvesting cells, mRNA and protein of HO-1 and Nrf2 was measured. The experiments were performed six times in triplicates (n = 6).

Statistical analysis
The results of normally distributed continuous variables are expressed as the mean ± SEM (range), whereas those with skewed distribution were expressed as the median value with (interquartile range). Statistical comparisons were performed with a Mann-Whitney U test and a two-way ANOVA followed by Bonferroni test, and a one-way ANOVA followed by a Tukey test as appropriate, using Prism 4.0 (GraphPad Software, La Jolla, CA). P < 0.05 was deemed statistically significant.

Results

Patient characteristics
The features of patients are shown in Table 1. Gestational age at delivery was earlier in the preeclampsia group than in the normal pregnancy group. Neonatal weight was lighter in the preeclampsia group than in the normal pregnancy group. Among 16 preeclamptic women, seven were early-onset (<34 wk gestation) preeclampsia and nine were late-onset (>34 wk gestation) preeclampsia. All patients are not habitual smokers and all preeclamptic women were diagnosed as severe preeclampsia.

Expression of LOX-1 in normal and preeclamptic placentas
Quantitative real-time PCR analysis showed that mRNA expression of LOX-1 was significantly decreased in preeclamptic placentas compared with...
normal placetas (n = 16 in each group) (Fig. 1A). The mRNA expressions of CD36 and SR-A, predominant receptors for oxLDL, were also significantly lower in preeclamptic placetas (Fig. 1, B and C). We confirmed the significantly higher mRNA of sFlt-1 and leptin in preeclamptic placetas compared with normal controls because they are well known to be up-regulated in preeclamptic placenta (Fig. 1, D and E). Western blot analysis of placental lysates demonstrated that protein level of LOX-1 was significantly reduced in preeclamptic placetas compared with the normal controls (n = 5 in each group) (Fig. 2, A and B). With immunohistochemistry, LOX-1 immunostaining was observed in villous trophoblasts in normal term placenta; on the other hand, this was not detected in vascular endothelial cells (Fig. 2, C and D).

The mRNA expression of LOX-1 in placental explants under hypoxic condition

We cultured placental explants under normal and hypoxic conditions (20 and 1% O2, respectively) (n = 6 in each group). After 24 h cultures, hypoxia resulted in significantly decreased LOX-1 mRNA expression (Fig. 2E).

Nrf2 activation and HO-1 mRNA are decreased in preeclamptic placetas

We analyzed Nrf2 activation by Western blot analysis using nuclear extracts from normal and preeclamptic placetas (n = 5 in each group). Nuclear accumulation of Nrf2 was significantly decreased in preeclampsia compared with normal controls (Fig. 3, A and B). Next, we assessed mRNA expression of HO-1, which is a main target gene of Nrf2, and found significant low expression of that in preeclamptic placetas by quantitative real-time PCR (n = 16 in each group) (Fig. 3C).

Regulation of HO-1 mRNA by Nrf2 activation

We cultured the JAR choriocarcinoma cell line with or without DEM (100 µM), a typical Nrf2-activating agent. DEM administration significantly up-regulated HO-1 mRNA at 6 h and then decreased (Fig. 4A). The DEM treatment also augmented nuclear accumulation of Nrf2 and HO-1 protein expression after 6–9 h (Fig. 4B), whereas it did not up-regulate LOX-1 mRNA (Fig. 4C). In cultured placental explants, DEM (100 µM) significantly increased HO-1 mRNA at 6 h and then decreased (Fig. 4D). The HO-1 protein expression was also increased by DEM treatment (Fig. 4E). The experiments were performed six times in duplicates (n = 6).

Role of LOX-1 on the regulation of HO-1 by oxLDL

In JAR cells, HO-1 mRNA was up-regulated by oxLDL (100 µg/ml) and increased in a time-dependent manner up to 9 h and then decreased (Fig. 5A). Both nuclear accumulation of Nrf2 and HO-1 protein expression were increased by oxLDL treatment (Fig. 5B). In the same culture model, we inhibited the LOX-1 mediated signal by TS92, an antihuman LOX-1 antibody, and this pretreatment significantly alleviated the HO-1 up-regulation induced by oxLDL for 9 h, whereas nLDL did not affect HO-1 mRNA expression (Fig. 5C). The experiments were performed six times in duplicates (n = 6).

Discussion

In the present study, we first accurately demonstrated that LOX-1 expression in preeclamptic placetas is decreased...
at both the mRNA and the protein level. Although LOX-1 is the most abundant in placenta, there have been few studies on LOX-1 in placenta. Contrasting to our results, Lee et al. (23) reported the elevated LOX-1 expression in the placentas of women with preeclampsia by Western blotting and immunohistochemistry. It is unclear what causes this discrepancy. The differences in phenotype of preeclamptic patients enrolled in these studies may be one of the reasons. We tried to collect placental tissues from the homogenous group with regard to mode of delivery, age, body mass index, and other factors. In addition, to confirm the quality of the samples, we also evaluated the sFlt-1 and leptin, in which the up-regulation in preeclampsia are well established (19, 24), and we found both genes were significantly increased in our preeclamptic placentas. We therefore believed the samples were appropriately obtained from preeclamptic placentas and concluded that LOX-1 expression is significantly decreased in preeclamptic placentas. Ethier-Chiasson et al. (25) demonstrated that placental LOX-1 expression is higher in women with hyperlipidemia or gestational diabetes mellitus. Satoh et al. (26) have shown higher expression of LOX-1 mRNA in the first-trimester placenta than in the term placenta and suggested a connection with increasing oxidative stress at the end of the first trimester in placenta. It is reasonable that hyperlipidemia, gestational diabetes mellitus, or oxidative stress may cause the increase of LOX-1; however, our results suggested that LOX-1 is not increased in preeclamptic placenta despite oxidative stress, although the mechanism has not been elucidated.

In Western blot analysis, we detected three bands for LOX-1. Similar results were reported previously and suggested that two of the three bands were precursor forms, and the other one was the mature form (27). LOX-1 is synthesized as a precursor form and processed into mature form by glycosylation (28). Xie et al. (29) found that the extracellular C-terminal lectin-like domain is sufficient for the binding to oxLDL, and this domain is not glycosylated, which suggests the glycosylation of LOX-1 is not a prerequisite for the binding of ligand. Accordingly, we regarded these three signals as functional LOX-1 and quantified. Immunohistochemistry revealed that LOX-1 immunostaining was observed in villous trophoblasts but not in vascular endothelial cells. This is similar to the previous report by Satoh et al. (26), and LOX-1 is expressed mainly in trophoblasts.

We also demonstrated the significantly decreased LOX-1 mRNA in cultured placental explants under hypoxic condition, indicating that hypoxia can down-regulate LOX-1 expression in placenta. Although several stimuli or pathological conditions have been studied to

FIG. 4. Effect of DEM on JAR cells and placental explant culture. Time course of HO-1 (A) and LOX-1 (C) mRNA expression in JAR cells treated with DEM (100 μM). Values were normalized to those of GAPDH. A, A representative Western blotting image for the time courses of Nrf2 nuclear accumulation and HO-1 expression in cytosolic protein in JAR cells treated with DEM (100 μM). B, A representative Western blotting image for the time course of HO-1 expression in cytosolic protein of placental explant treated with DEM (100 μM). Values were normalized to those of GAPDH. E, A representative Western blotting image for the time course of HO-1 expression in cytosolic protein of placental explant treated with DEM (100 μM). Data are presented as the mean ± SEM. *, P < 0.05. The experiments were performed six times in duplicates (n = 6).
enhance LOX-1 gene expression (11, 12), little is known so far with regards to factors reducing LOX-1 expression. Here, for the first time, we revealed the pathological condition that down-regulates LOX-1 expression in placenta. Hypoxia is one of the factors involved in the onset of preeclampsia. At the early stage of pregnancy, impaired invasion and adaptation of extravillous trophoblast to maternal spiral artery leads to reduced uteroplacental perfusion and placental hypoxia (2). It has been implicated in the pivotal pathogenesis of preeclampsia. So the decrease in LOX-1 expression may be derived from the early pathological alteration in placenta.

LOX-1 expression in placenta is the most abundant among the organs (9), and it can be the key molecule to regulate the serum oxLDL level. Ishigaki et al. (30) reported an actual example demonstrating the involvement of LOX-1 in systemic lipid metabolism regulation. Hepatic LOX-1 overexpression enhanced oxLDL uptake in apolipoprotein E-deficient mice, and the plasma oxLDL level was markedly decreased. In women with preeclampsia, serum lipid levels are higher (4), and elevated oxLDL can be a risk factor of preeclampsia (31), suggesting that aberrant lipid metabolism may have a role in the pathogenesis of preeclampsia. In addition to LOX-1, CD36 and SR-A are considered predominant receptors for oxLDL (22), and mRNA expressions of both were revealed to be lower in preeclamptic placenta in the present study. These results suggest that the scavenger function against oxLDL is weakened in preeclamptic placentas. Therefore, it can be speculated that hypoxia at an early stage lead to decreased oxLDL receptors, and consequently, it may cause increasing serum oxLDL, which can give rise to further maternal endothelial dysfunction.

Next, we focused on Nrf2, a transcriptional factor that regulates antioxidant responsive element mediated induction of cytoprotective genes in response to oxidative stress (21). Under basal conditions, Nrf2 is sequestered in cytoplasm by binding to the Kelch-like ECH-associated protein 1 and is degraded by a proteasome pathway (32). However, upon exposure to oxidative or electrophilic stress, Nrf2 is dissociated from Kelch-like ECH-associated protein 1, accumulates in the nucleus, and induces the antioxidant genes.

Nrf2 is widely acknowledged as the predominant upstream regulator of HO-1. Despite its importance, there has been only one study that dealt with Nrf2 activation in placentas so far, providing nuclear accumulation of Nrf2 in cytotrophoblast by immunohistochemistry (33). We showed here not only significantly lower Nrf2 activation but also decreased HO-1 mRNA in preeclamptic placentas. To investigate the role of Nrf2 in placenta, we examined the effect of DEM, an Nrf2 activator, in the JAR cell line and placental explant culture. We found that DEM certainly up-regulated HO-1 expression in both JAR cells and placental explants.

Although it is reported that oxLDL activates Nrf2 in murine macrophages (22), Nrf2 was less activated in pre-
eclamptic placenta despite a high serum level of oxLDL and increasing oxidative stress in women with preeclampsia. To address this query, we assessed the role of LOX-1 in JAR cells using TS92, a blocking anti-LOX-1 antibody. We confirmed in advance that only LOX-1, but neither CD36 nor SR-A, was expressed in JAR and considered that blockade of LOX-1 was enough to prevent JAR cells from oxLDL uptake. We revealed that oxLDL activated Nrf2 and up-regulated HO-1 expression in JAR cells and that LOX-1 blockade resulted in the alleviation of increasing HO-1 mRNA induced by oxLDL. These results suggest that oxLDL might be less internalized due to decreased LOX-1 in preeclamptic placenta than in the healthy state, leading to lower Nrf2 activation. When Nrf2 is not activated appropriately in trophoblasts, they fail to increase antioxidative genes, and both the mother and fetus may be affected against oxidative stress. In this study, LOX-1 mRNA was unchanged by the Nrf2 activator. It is currently unclear how the LOX-1 expression was decreased in preeclamptic placenta, and that is now under investigation.

In conclusion, to the best of our knowledge, this is the first study describing decreased LOX-1 expression and reduced Nrf2 activation in preeclamptic placenta. In addition, we have also shown the relevance between LOX-1 and Nrf2 through the assessment of HO-1 expression induced by oxLDL. The decrease in LOX-1 expression may contribute not only to maternal high serum oxLDL in woman with preeclampsia, although this needs further investigation, but also to lower Nrf2 activation in placenta. Our findings provided novel insights into the crucial role of LOX-1 and Nrf2 in placenta and paved the way for the precise comprehension of the pathogenesis of preeclampsia.

Acknowledgments

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Disclosure Summary: The authors have nothing to disclose, and there is no conflict of interest among the authors.

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