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<th>Reduced ABCA1 expression and low Nrf2 activation due to decreased lectin-like oxidized LDL receptor 1 (LOX-1) in the placenta are involved in preeclampsia</th>
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<td>Chigusa, Yoshitsugu</td>
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<tr>
<td>Citation</td>
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</tr>
<tr>
<td>Issue Date</td>
<td>2014-03-24</td>
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<tr>
<td>URL</td>
<td><a href="https://dx.doi.org/10.14989/doctor.k18125">https://dx.doi.org/10.14989/doctor.k18125</a></td>
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ATP-binding Cassette Transporter A1 Expression is Decreased in Preeclamptic Placentas

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Grants: This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports, Japan (No. 21592096, 22591822 and 23791833) and by a grant from the Smoking Research Foundation.

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

Preeclampsia is a pregnancy-specific multisystem disorder characterized by hypertension and proteinuria. Accentuated maternal hyperlipidemia, especially high serum levels of oxidized LDL (oxLDL), is one of the features of preeclampsia. We previously reported that lectine-like oxidized LDL receptor 1 (LOX-1) expression was decreased in preeclamptic placentas. Here we show that decreased LOX-1 expression is associated with low expression of ATP-binding cassette transporter A1 (ABCA1) in the placenta. ABCA1 mediates cellular efflux of cholesterol, and Liver X receptors (LXRs) are its predominant transcriptional regulators. Both ABCA1 and LXR expressions were significantly lower in preeclamptic placentas than those in normal controls. Oxidized LDL upregulated ABCA1 expression, while LOX-1 blockade resulted in the alleviation of increasing ABCA1 mRNA in JAR cells. These results suggest that low LOX-1 expression may lead to insufficient oxLDL uptake, thereby contributing to reduced LXR activation and decreased ABCA1 expression in preeclamptic placentas.

Keywords: preeclampsia, oxidized LDL, LOX-1, ABCA1, LXR

Introduction

Preeclampsia is a pregnancy-specific disorder clinically characterized by de-novo development of hypertension and proteinuria.¹ Complicating 2-8 % of pregnancies, preeclampsia is a major cause of maternal and neonatal morbidity and mortality.² Although the etiology of preeclampsia remains
enigmatic, it is generally agreed that the placenta plays a critical role in the pathogenesis of this
disorder.

One of the striking changes that occurs in lipid metabolism during normal pregnancy is maternal
hyperlipidemia. This feature is observed more noticeably in women with preeclampsia. Indeed, serum
lipid levels such as triglycerides, low-density lipoproteins (LDLs), and small dense LDLs, which are
susceptible to oxidation, are higher in women with preeclampsia than those in normal pregnant
women.\(^3\,^4\) In addition, oxidized LDL (oxLDL) is also increased in the serum of preeclamptic
women.\(^5\,^6\)

ATP-binding cassette transporter A1 (ABCA1) is a membrane transporter that mediates cellular efflux
of cholesterol and phospholipids to lipid-poor apolipoprotein A1, the precursor of high density
lipoprotein (HDL). ABCA1 is highly expressed in the human placenta,\(^7\,^8\) and is thought to play a
central role in cholesterol metabolism. Importantly, placental malformation and intrauterine growth
restriction were observed in ABCA1 null mice.\(^9\) The predominant transcriptional regulator of ABCA1
is the Liver X receptor (LXR). LXRs are nuclear receptors that modulate the expression of genes
involved in cholesterol and lipid metabolism in response to changes in cellular cholesterol status.\(^10\)

Two isotypes have been identified: LXR\(\alpha\) is mainly expressed in the liver, adipose tissue, and
macrophages, while LXR\(\beta\) is ubiquitously expressed in human tissues. Both LXRs are activated by
oxysterols, oxidized derivatives of cholesterol.\(^10\)
We previously reported that lectine-like oxidized LDL receptor 1 (LOX-1), a major oxLDL scavenger receptor was decreased in preeclamptic placentas. LOX-1 is responsible for the binding and internalization of oxLDL. However, it is presently unclear whether reduced LOX-1 affects ABCA1 expression through LXR in preeclamptic placentas. We hypothesized that LOX-1 and oxLDL may be involved in the regulation of ABCA1 expression in the placenta. The aim of this study was to clarify the relationship between LOX-1 and ABCA1 in the placenta, particularly in the context of preeclampsia.

**Materials and Methods**

**Patients**

Twenty women with singleton pregnancy were enrolled in this study (Table 1). Ten women had normal pregnancies, while ten women were complicated with preeclampsia. Women with chronic hypertension and renal disease or other pregnancy complications were excluded from this study. Preeclampsia was defined as maternal systolic blood pressure $\geq 140$ mmHg and/or diastolic blood pressure $\geq 90$ mmHg in 2 consecutive measurements at least 6 h apart, and proteinuria $\geq 300$ mg per 24 h after 20 weeks of gestation. We calculated the number of standard deviations (SD) depending on the appropriate normal mean for gestational age in Japanese singleton pregnancies, and this was expressed as delta neonatal weight (SD).
Placental tissues

Placental villous tissues were obtained from normal pregnancies (n = 10) and preeclamptic pregnancies (n = 10), immediately after Cesarean section in the absence of labor, at Kyoto University Hospital, Japan. Villous tissues were collected from the central part of the placenta and were macroscopically free of infarction or calcification. After brief rinsing in saline, these tissues were stored in RNALater (Ambion, Austin, TX) at −80 °C until RNA extraction. 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.

Real-time quantitative RT-PCR

Total RNA extraction from placental tissues and JAR cells was performed using the RNeasy Mini kit (QIAGEN, Germantown, MD) according to the manufacturer’s instructions. RNA quality and quantity was measured using an ND-1000 spectrophotometer (Nanodrop, Wilmington, NC). Reverse transcription of 1 µg RNA was performed using the Rever Tra Ace (TOYOBO, Osaka, Japan) according to the manufacturer’s instructions. Primers for the genes examined (Table 2) were designed using GeneFisher 2 software (Bielefeld University Bioinformatics Service, Bielefeld, Germany). Real-time quantitative RT-PCR was performed using SYBR premix Ex TaqII (Takara Bio, Otsu, Japan) on the LightCycler 480 Real-Time PCR system (Roche Diagnostics, Mannheim, Germany) with the following run conditions: 95 °C for 30 s for initial denaturing, followed by 95 °C for 5 s and
60 °C for 30 s (40 cycles). For dissociation after PCR amplification, the protocol included slow heating from 60 to 97 °C to ensure amplification specificity. Gene expression was estimated using the comparative crossing point (Cp) method for relative quantification. All data were normalized using GAPDH as an internal control and expressed relative to controls. All samples were run in duplicate and quantitative detection was averaged.

**Western blot**

Placental tissues were homogenized in RIPA buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L Sodium Chloride, 0.5 % sodium Deoxycholate, 0.1 % Sodium Dodecyl Sulfate, 1.0 % NP-40 substitute) supplemented with cocktail protease inhibitor Complete Mini (Roche Diagnostics). Homogenized tissues were centrifuged at 10,000 g for 20 min at 4 °C, and the supernatant was saved as cytosolic extract from placental tissues. Cells were washed with ice-cold phosphate buffered saline, and cytosolic protein was extracted in the same manner. Protein concentrations were determined with BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Twenty µg of proteins was separated on 7.5 % SDS-polyacrylamide gels. Separated proteins were transferred onto nitrocellulose membranes, which were blocked with 5 % fat free milk overnight at 4 °C. We confirmed an equal amount of protein loading by Ponceau S staining. Membranes were probed with mouse monoclonal antibody against ABCA1 (ab18180) (1:1000; Abcam Cambridge UK). Rabbit polyclonal antibody against β-actin (1:5000; Abcam) was used as a loading control. Blots were subsequently incubated with an
appropriate secondary antibody (1:10000; Santa Cruz Biotechnology). Signals were detected with Western Blotting Substrate Plus (Thermo Scientific) and visualized by the ChemiDoc system (BioRad, Hercules, CA).

Cell culture

The JAR (HTB-144) choriocarcinoma cell line was purchased from the American Type Culture Collection (Manassas, VA) and cultured in RPMI medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. OxLDL and native LDL (nLDL) were purchased from Intracel (Frederick, MD). JAR cells grown in 48 well plates were treated with 100 µg/ml of oxLDL or nLDL for 3, 6, 9, and 24 h. Next, after pretreating with 30 µg/ml of TS92, anti-human LOX-1 antibody, or normal human IgG purchased from R&D Systems (Minneapolis, MN), JAR cells were treated with oxLDL (100 µg/ml) for 9 h. TS92 was a kind gift from Dr. T. Sawamura, Osaka, Japan. Cells were harvested and mRNA expressions of ABCA1, LXRα, and LXRβ were measured by qPCR. Protein expression of ABCA1 was analyzed by Western blotting. Six experiments were performed in triplicate (n = 6).

Statistical analysis

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

Results

Patient characteristics

The clinical characteristics of patients enrolled in this study are shown in Table 1. No patients were habitual smokers. Gestational age at delivery was earlier in the preeclampsia group than that in the normal pregnancy group. Neonatal weight was also lighter in the preeclampsia group than that in the normal pregnancy group. Although delta neonatal weight was larger in the preeclampsia group, it was not statistically significant. Meanwhile, no differences between groups were observed concerning the age and body mass index of patients at delivery. Among 10 preeclamptic women, 4 were early-onset (≤ 34 weeks gestation) preeclampsia and 6 were late-onset (> 34 weeks gestation) preeclampsia. All preeclamptic women were diagnosed with severe preeclampsia according to American College of Obstetricians and Gynecologists criteria.

Expression of ABCA1 in normal and preeclamptic placentas

First, we assessed mRNA expressions of ABCA1, LXRα, and LXRβ in normal and preeclamptic placentas. Both LXRα and LXRβ are predominant upstream regulators of ABCA1. Quantitative real-time PCR analysis showed that mRNA expressions of these genes were significantly lower in
preeclamptic placentas than those in normal placentas (Figure 1A and B), indicating that LXR activation was reduced in preeclamptic placentas. Western blot analysis of placental lysates demonstrated that protein levels of ABCA1 were also significantly reduced in preeclamptic placentas compared with those of the normal controls (Figure 2A and B).

ABCA1 upregulation by oxLDL in JAR cells

In preeclamptic women, serum levels of oxLDL are higher than in normal pregnant women.\textsuperscript{5,6} To investigate the efficacy of oxLDL to ABCA1 gene expression in trophoblast cells, we treated JAR cells with 100\,µg/ml of oxLDL or nLDL, as a control, for 3, 6, 9 and 24 h. OxLDL treatment significantly increased the expression of ABCA1 mRNA at 6 to 24 h (Figure 3A left), while nLDL did not alter ABCA1 mRNA expression (Figure 3A right). In Western blot analysis, we found increased ABCA1 protein levels in JAR cells treated with oxLDL at 9 h (Figure 3B). Moreover, oxLDL upregulated LXR\(\alpha\) mRNA significantly in a time dependent manner (Figure 3C left). Though LXR\(\beta\) mRNA tended to increase with oxLDL at 9 to 24 h, it was not statistically significant (Figure 3C right).

Attenuation of increasing ABCA1 mRNA by LOX-1 blockade

In order to determine the possible involvement of reduced LOX-1 expression in decreased ABCA1 expression in preeclamptic placentas, JAR cells were pretreated with TS92 (30\,µg/ml), an anti-human
LOX-1 antibody, and then stimulated with oxLDL (100µg/ml) for 9 h. In this culture model, TS92 significantly inhibited ABCA1 upregulation induced by oxLDL (Figure 4). Normal human IgG did not affect increased ABCA1 mRNA expression and nLDL did not upregulate ABCA1 mRNA.

**Discussion**

In placental tissue, ABCA1 is localized in villous cytotrophoblast cells, the surface of the syncytiotrophoblast membrane, and placental endothelial cells. Based on this evidence, ABCA1 is believed to be engaged in not only cholesterol homeostasis in the placenta during pregnancy, but also feta-placental cholesterol transport. Indeed, studies of ABCA1 knockout mice revealed aberrant placental development and fetal growth restriction. However, whether or not altered ABCA1 expression in the human placenta is associated with pathological pregnancies, including preeclampsia, has not been explored thoroughly. In the present study, we first found that ABCA1 expression was significantly lower in preeclamptic placentas than those in normal placentas in both mRNA and protein levels. Intriguingly, our results were inconsistent with previous studies. Plosch et al revealed upregulation of ABCA1 in early-onset preeclamptic placentas, and Albrecht et al reported unchanged expression of ABCA1 in placentas from women with preeclampsia, while they found decreased ABCA1 expression in those with antiphospholipid syndrome (APS). Although the causes of these disparities remain unclear, they might derive from the phenotype of preeclampsia, especially the timing of onset or disease duration of preeclampsia. In addition, we must take into account that
our sample number was limited and the gestational age in preeclamptic group ranged from 26 to 40 weeks. Meanwhile, as a pilot study, we confirmed that our preeclamptic placentas were appropriate samples by revealing high expressions of soluble fms-like tyrosine kinase 1 (sFlt-1) and leptin mRNA (1.89 fold, \( P < .05 \); 33.7 fold, \( P < .001 \), respectively. Data not shown), since it is widely acknowledged that these mRNAs were increased in preeclamptic placentas.\(^{17,18}\) Remarkably, Lindegarrd et al treated C57Bl/6 mice with an LXR agonist, and demonstrated significant upregulation of placental ABCA1 mRNA expression and increased maternal-fetal cholesterol transfer, which is beneficial for some congenital fetal diseases.\(^{19}\) On the other hand, the patients with ABCA1 mutations, known as Tangier disease, present with low levels of HDL and develop premature atherosclerosis.\(^{20}\) Thus, suppression of ABCA1 function in the placenta may lead to maternal aberrant lipid metabolism in preeclampsia or may cause fetal growth restriction.

LXR is a predominant upstream regulator of ABCA1, and the LXR pathway regulates lipid metabolism and inflammation. We showed that mRNA expressions of LXRs were significantly downregulated in preeclamptic placentas, which is consistent with the report by Weedon-Fekjaer et al.\(^{21}\) Taken together, these results suggest that LXR activation is reduced and can be one of the causes of decreases in ABCA1 expression in preeclamptic placentas. Moreover, LXR has a powerful anti-inflammatory effect that may contribute to antiatherosclerotic potency,\(^{22}\) and many studies have established that an LXR agonist results in the attenuation of atherosclerosis in vivo.\(^{23-25}\) On the other hand, a combined deficiency of LXR\(\alpha\) and LXR\(\beta\) in mice was associated with increased LDL levels.
and foamy macrophage accumulation in the arterial wall.\textsuperscript{26} Interestingly, the spiral arteries of preeclamptic placental decidua often exhibit lipid deposition and the involvement of foamy macrophages.\textsuperscript{27} This phenomenon is called acute atherosis and resembles the early stages of atherosclerotic development. Given these considerations, it is possible that decreasing ABCA1 expression due to disruption of LXR signaling in term placentas is intimately related with the pathophysiology of preeclampsia.

Endogenous LXR ligands are oxysterols (oxidized cholesterol derivatives). Increasing intracellular concentrations of oxysterols subsequently activate LXR, and upregulate ABCA1 expression. We demonstrated that ABCA1 expression was increased by oxLDL, not by nLDL in JAR cells. OxLDL also increased only LXR\textalpha{} mRNA expression. Whitney et al showed that both natural and synthetic LXR agonists upregulated LXR\textalpha{}, but not LXR\textbeta{} gene expression in human macrophages, adipocytes, and hepatocytes.\textsuperscript{28} Moreover, Arai et al found that oxLDL activates LXR in macrophages, and hence, our results suggest that oxLDL acts as an LXR agonist also in trophoblasts.

However, the reason why ABCA1 expression was decreased in preeclamptic placentas in spite of high maternal serum levels of oxLDL remained unexplained. To address this query, we focused on LOX-1, a predominant oxLDL scavenger receptor. In our previous study, we revealed the expressions of scavenger receptors for oxLDL, including LOX-1, were decreased in preeclamptic placentas.\textsuperscript{29} In the present study, LOX-1 blockade resulted in the attenuation of increasing ABCA1 mRNA induced by oxLDL. These results robustly suggest that oxLDL mediated ABCA1 regulation partially depends on
LOX-1 expression in the placenta. In other words, insufficient oxLDL uptake due to decreased LOX-1 expression may lead to low ABCA1 expression in preeclamptic placentas (Figure 5). However, we are currently ignorant of precise mechanism by which LOX-1 was decreased in preeclamptic placentas, and thus, further investigation is required.

Conclusion
In conclusion, to the best of our knowledge, this is the first study to reveal decreased ABCA1 expression in both mRNA and protein levels as well as mRNA expression of LXRs in preeclamptic placentas. Moreover, we demonstrated that oxLDL upregulated ABCA1 expression, while LOX-1 blockade resulted in the alleviation of increasing ABCA1 mRNA in JAR cells. These results strongly suggest that low LOX-1 expression may lead to insufficient oxLDL uptake, thereby contributing to reduced LXR activation and decreases in ABCA1 expression in preeclamptic placentas. Our findings provided new insight into the pathophysiology of preeclampsia particularly in context of lipid metabolism.

Acknowledgements
This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports, Japan (No. 21592096, 22591822 and 23791833) and by a grant from the Smoking Research Foundation. We are greatly indebted to Dr. Tatsuya Sawamura for
his kind gift of anti-LOX-1 antibody (TS92) and informative guidance. We also thank Ms. Akiko Abe for her secretarial and technical assistance.

Legends

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

Table 2. Primer sequences used in real-time quantitative PCR

Figure 1. Messenger RNA expressions of (A) ABCA1, and (B) LXRα and LXRβ in normal and preeclamptic placentas (n = 10 in each group). Values were normalized to those of GAPDH. Data are presented as the median value with interquartile range. *P < .05, **P < .01.

Figure 2. Expression of ABCA1 protein in normal and preeclamptic placentas (n = 10 in each group). (A) Western blot for ABCA1 in placentas. (B) Densitometric analysis of ABCA1 protein expression normalized to β-actin. Data are presented as the median value with interquartile range. *P < .05.

Figure 3. ABCA1 upregulation by oxLDL in JAR cells. (A) Time courses of ABCA1 mRNA expression in JAR cells treated with oxLDL (100 μg/ml) or nLDL (100 μg/ml) (B) A representative
Western blot image for ABCA1 in JAR cells treated with or without oxLDL (100 µg/ml) for 9 h. (C)

Time courses of LXRα and LXRβ mRNA expression in JAR cells treated with oxLDL (100 µg/ml).

Values were normalized to those of GAPDH. Data are presented as the mean ± SEM. * P < .05, ** P < .01. Six experiments were performed in triplicate (n = 6).

Figure 4. Attenuation of increasing ABCA1 mRNA by LOX-1 blockade. ABCA1 mRNA expression in JAR cells treated with oxLDL (100 µg/ml) in the presence or absence of TS92 (30 µg/ml) or normal human IgG (30 µg/ml), and that in JAR cells treated with nLDL (100 µg/ml) for 9 h. Values were normalized to those of GAPDH. Data are presented as the mean ± SEM. * P < .05, *** P < .001. Six experiments were performed in triplicate (n = 6).

Figure 5. Scheme of oxLDL mediated signaling pathways in trophoblasts. The enhancement in ABCA1 expression following increased oxLDL is suppressed in preeclamptic placentas.

References


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<td>10 / 10</td>
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<td>Gestational age at delivery (weeks)</td>
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<td>35 ± 4.6 (26 - 40)</td>
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<td>Body mass index at delivery (kg/m²)</td>
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<td>Systolic blood pressure at delivery (mmHg)</td>
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<td>Neonatal weight (g)</td>
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<td>Delta neonatal weight (SD)</td>
<td>-0.12 ± 0.46 (-0.97 - +0.57)</td>
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Figure 1

A

![](image)

B

![](image)
Figure 2

A

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B

Box plot showing the ABCA1/β-actin ratio for Normal and PE groups. There is a significant difference indicated by an asterisk (*).
Figure 3

A

[Bar graph showing expression levels of ABCA1/GAPDH mRNA over time with oxLDL 100μg/ml treatment.]

B

[Western blot images showing ABCA1 and β-actin at 254 kDa and 42 kDa respectively, with oxLDL treatment indicated as (-) and (+).]

C

[Bar graph showing expression levels of LXRα/GAPDH mRNA over time with oxLDL 100μg/ml treatment.]