ATP-binding Cassette Transporter A1 Expression is Decreased in Preeclamptic Placentas

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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: LXRα is mainly expressed in the liver, adipose tissue, and macrophages, while LXRβ 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.[11] 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 ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg in 2 consecutive measurements at least 6 h apart, and proteinuria ≥ 300 mg per 24 h after 20 weeks of gestation. We calculated the number of standard deviations (SD) depending on the appropriate nomal 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 RNA*later* (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 % CO_2. OxLDL and native LDL (nLDL) were purchased from Intracel (Frederick, MD). JAR cells grown in 48 well plates were treated with 100μg/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 (100μg/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 $P < .05$ 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.[5,6] To investigate the efficacy of oxLDL to ABCA1 gene expression in trophoblast cells, we treated JAR cells with 100μg/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 LXRα mRNA significantly in a time dependent manner (Figure 3C left). Though LXRβ 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,[12] the surface of the syncytiotrophoblast membrane,[13] and placental endothelial cells.[14] 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.[9] 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,[15] 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).[16] 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 LXRα and LXRβ in mice was associated with increased LDL levels

243 and foamy macrophage accumulation in the arterial wall.[26] Interestingly, the spiral arteries of
244 preeclamptic placental decidua often exhibit lipid deposition and the involvement of foamy
245 macrophages.[27] This phenomenon is called acute atherosis and resembles the early stages of
246 atherosclerotic development. Given these considerations, it is possible that decreasing ABCA1
247 expression due to disruption of LXR signaling in term placentas is intimately related with the
248 pathophysiology of preeclampsia.
249 Endogenous LXR ligands are oxysterols (oxidized cholesterol derivatives). Increasing intracellular
250 concentrations of oxysterols subsequently activate LXR, and upregulate ABCA1 expression. We
251 demonstrated that ABCA1 expression was increased by oxLDL, not by nLDL in JAR cells. OxLDL
252 also increased only LXRα mRNA expression. Whitney *et al* showed that both natural and synthetic
253 LXR agonists upregulated LXRα, but not LXRβ gene expression in human macrophages, adipocytes,
254 and hepatocytes.[28] Moreover, Arai *et al* found that oxLDL activates LXR in macrophages, and hence,
255 our results suggest that oxLDL acts as an LXR agonist also in trophoblasts.
256 However, the reason why ABCA1 expression was decreased in preeclamptic placentas in spite of high
257 maternal serum levels of oxLDL remained unexplained. To address this query, we focused on LOX-1,
258 a predominant oxLDL scavenger receptor. In our previous study, we revealed the expressions of
259 scavenger receptors for oxLDL, including LOX-1, were decreased in preeclamptic placentas.[29] In the
260 present study, LOX-1 blockade resulted in the attenuation of increasing ABCA1 mRNA induced by
261 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 pathophisiology of preeclampsia particularly in context of lipid metabolism.

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Legends

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

Values are the mean ± SD and (range).

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.

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

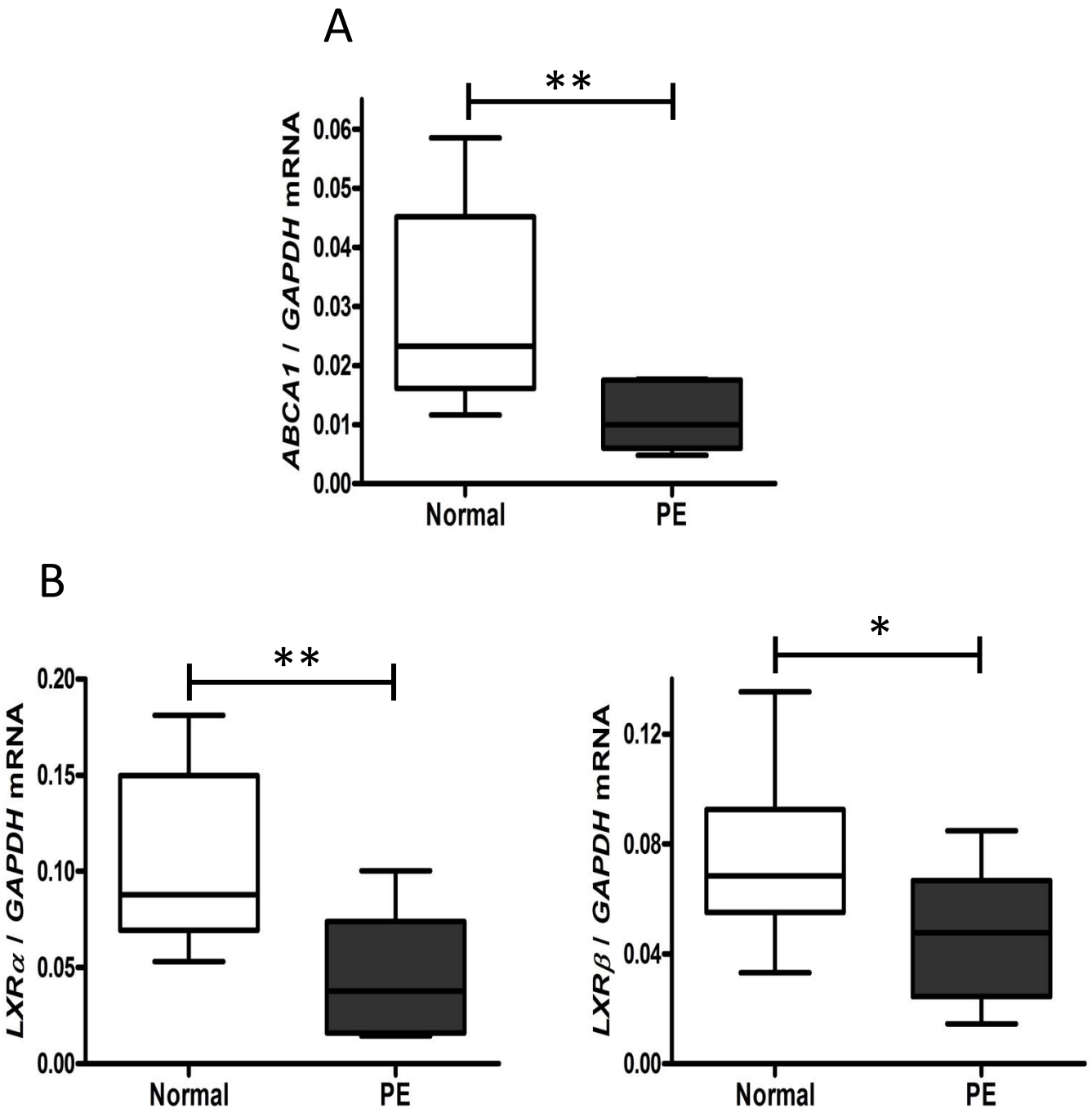
	Normal (n = 10)	**PE (n = 10)**	***P* value**
Patient's age at delivery (years)	36.8 ± 5.4 (27 - 44)	34.1 ± 3.3 (29 - 39)	n.s.
Primipara (n)	3 / 10	8 / 10	-
Cesarean section (n)	10 / 10	10 / 10	-
Gestational age at delivery (weeks)	38 ± 0.9 (37 - 39)	35 ± 4.6 (26 - 40)	< .05
Body mass index at delivery (kg/m^2)	25.4 ± 2.6 (21.2 - 29.1)	25.4 ± 3.8 (21.0 - 33.4)	n.s.
Systolic blood pressure at delivery (mmHg)	106 ± 7 (90 - 116)	173 ± 17 (145 - 192)	< .0001
Diastolic blood pressure at delivery (mmHg)	65 ± 9 (52 - 80)	105 ± 11 (90 - 124)	< .0001
Neonatal weight (g)	2902 ± 245 (2494 - 3296)	2085 ± 943 (576 - 3460)	< .05
Delta neonatal weight (SD)	-0.12 ± 0.46 (-0.97 - +0.57)	-0.84 ± 0.96 (-2.0 - +0.71)	n.s

Values are the mean ± SD and (range).

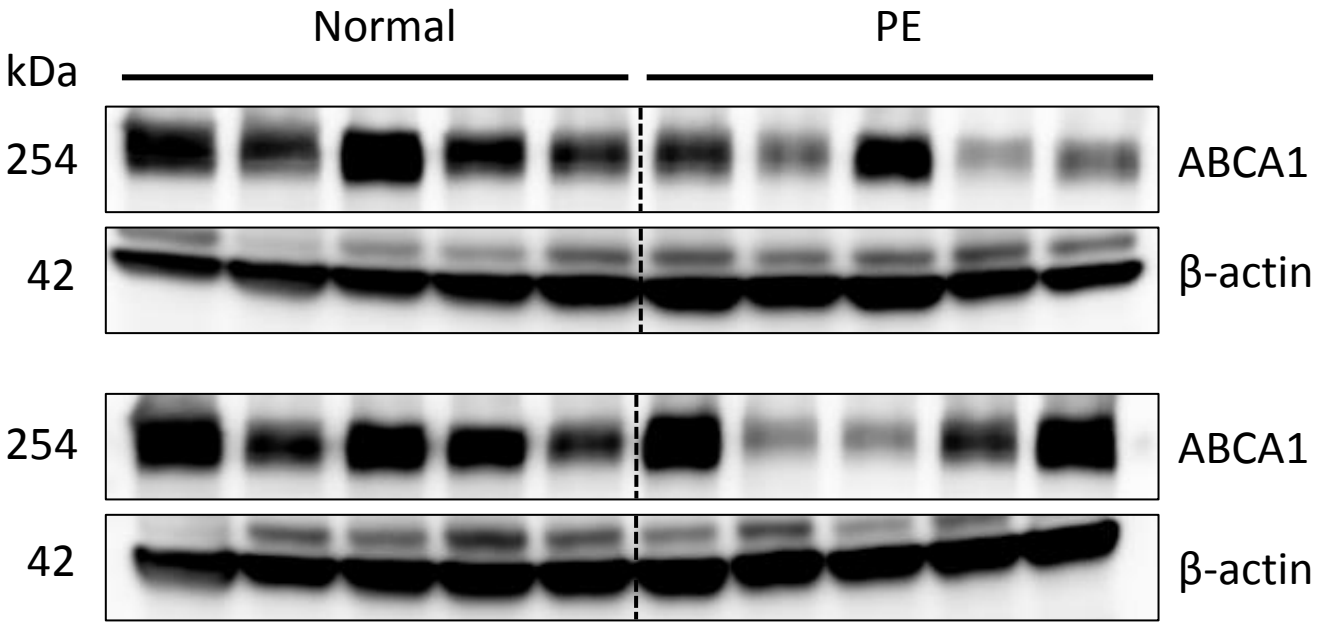
Table 2. Primer sequences used in real-time quantitative PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Entrez Gene ID
ABCA1	GGAACAGGCTACTACCTGACCTTGG	ATCGATGGTCAGCGTGTCACTCTC	19
LXRα	GATCGAGGTGATGCTTCTGG	ACTCGAAGATGGGGTTGATG	10062
LXRβ	GATCGTGGACTTCGCTAAGCAAGTG	GTCCTTGCTGTAGGTGAAGTCCTTC	7376
GAPDH	GAGTCAACGGATTTGGTCGTATTGG	GCCATGGGTGGAATCATATTGGAAC	2597

Figure. 1


A
**
ABCA1 / GAPDH mRNA
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Normal
PE
B
**
LXRα / GAPDH mRNA
0.20
0.15
0.10
0.05
0.00
Normal
PE
*
LXRβ / GAPDH mRNA
0.12
0.08
0.04
0.00
Normal
PE

A Figure. 2


Normal
PE
kDa
254
ABCA1
42
β-actin
254
ABCA1
42
β-actin

B

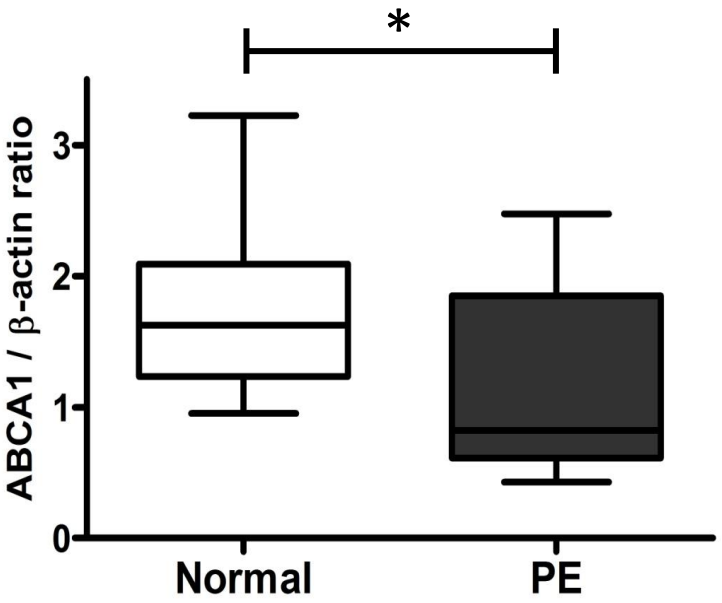

*
ABCA1 / β-actin ratio
3
2
1
0
Normal
PE

Figure. 3

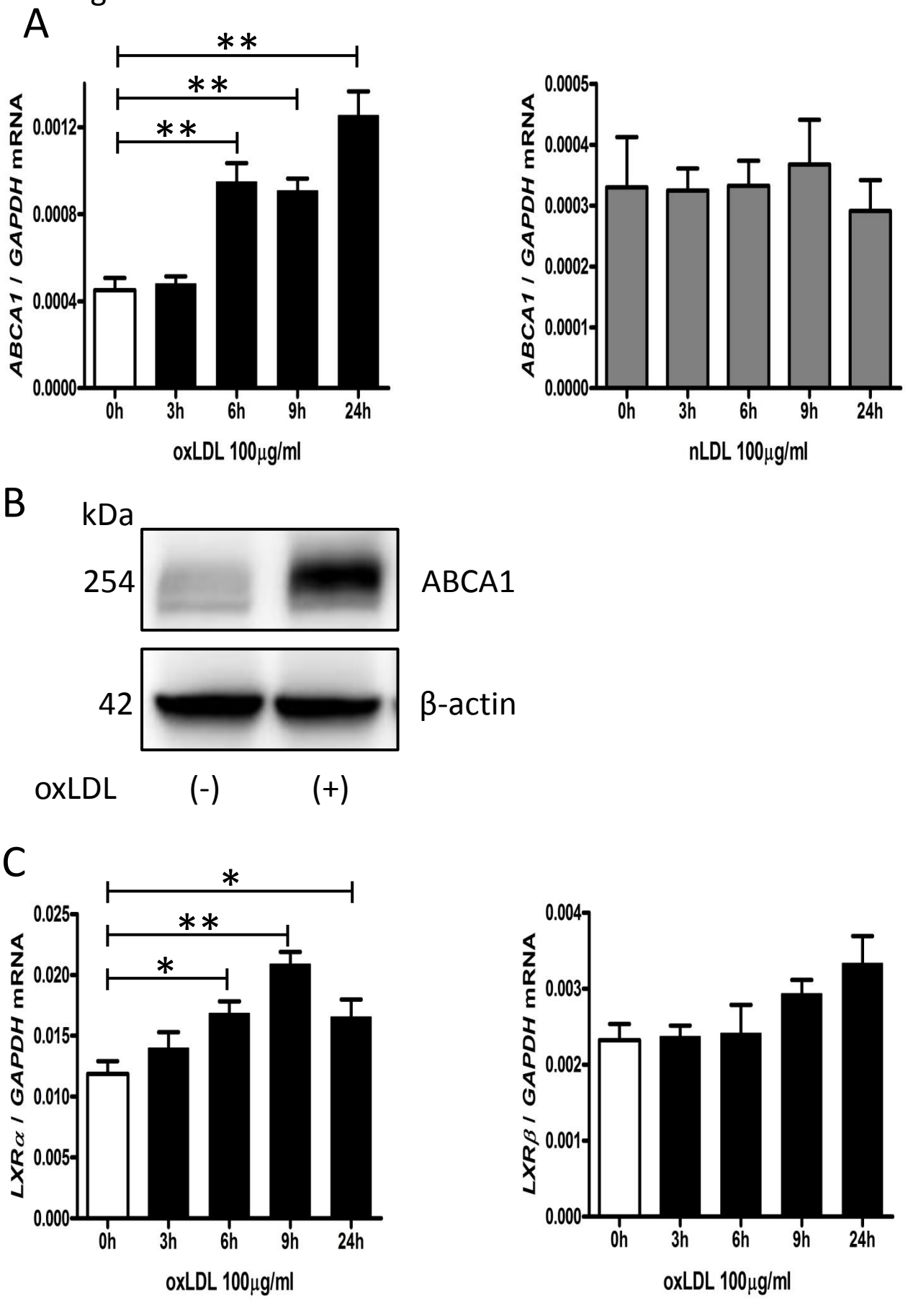

A
**
**
**
ABCA1 / GAPDH mRNA
0.0012
0.0008
0.0004
0.0000
0h
3h
6h
9h
24h
oxLDL 100μg/ml
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
nLDL 100μg/ml
B
kDa
254
ABCA1
42
β-actin
oxLDL
(-)
(+)
C
*
**
*
LXRα / GAPDH mRNA
0.025
0.020
0.015
0.010
0.005
0.000
oxLDL 100μg/ml
LXRβ / GAPDH mRNA
0.004
0.003
0.002
0.001
0.000

Figure. 4

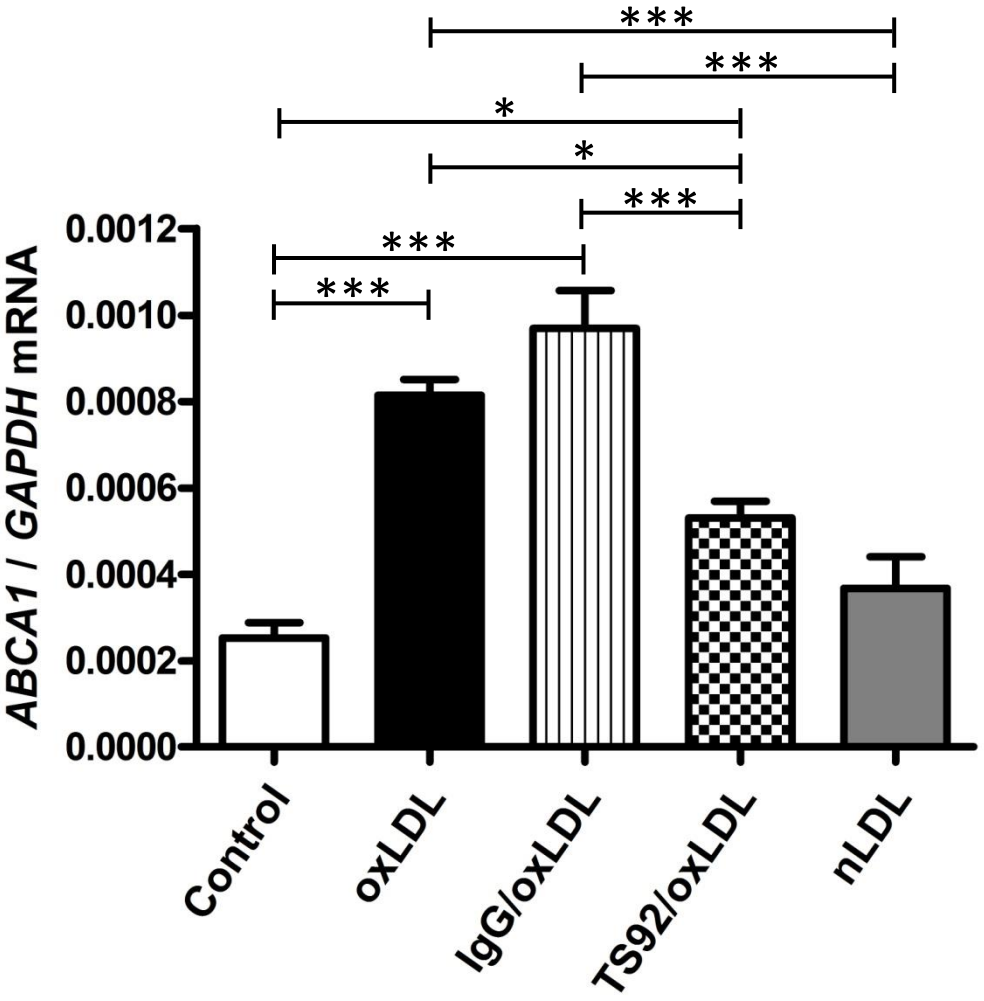
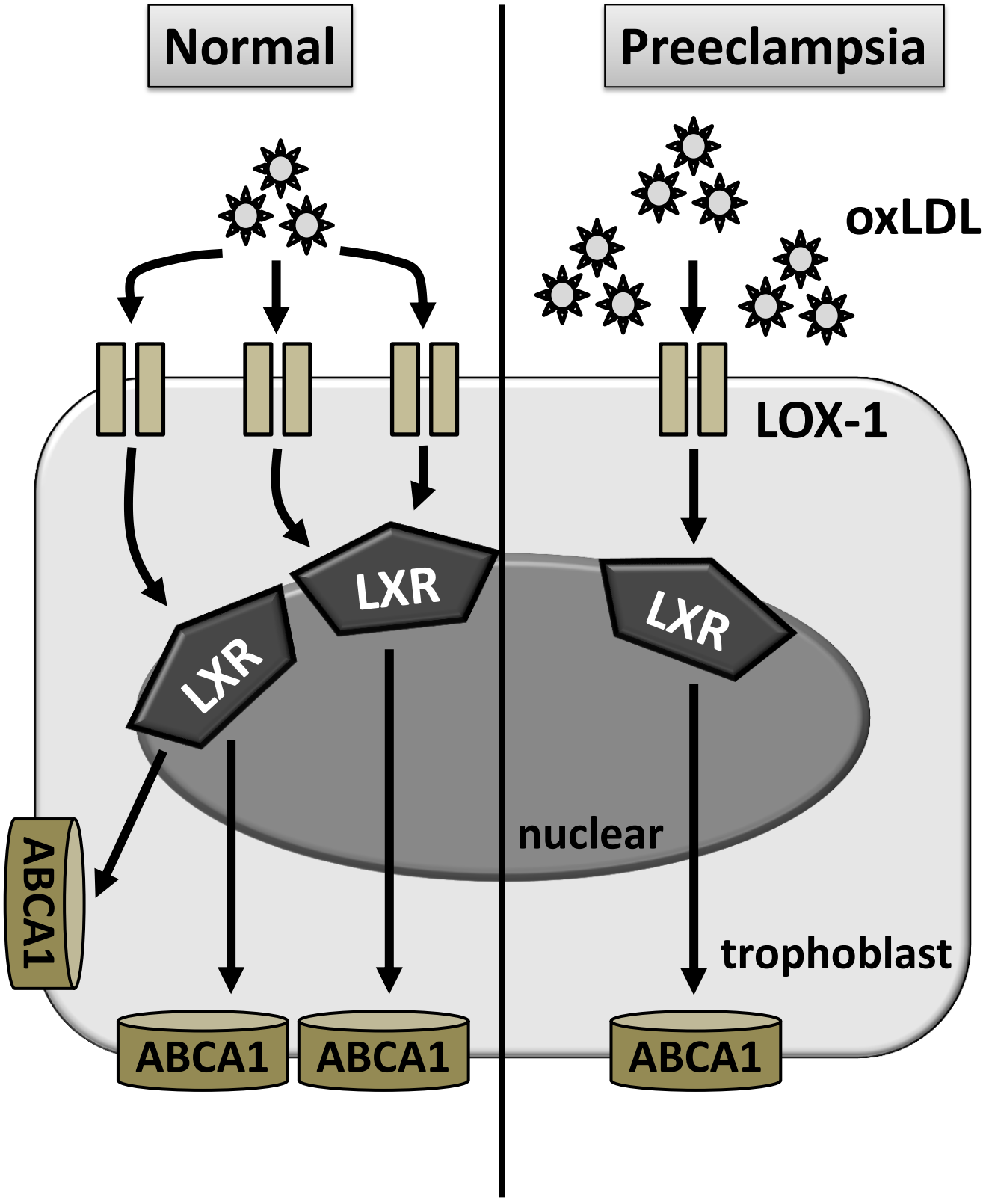

***
***
*
*
***
***
***
0.0012
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
ABCA1 / GAPDH mRNA
Control
oxLDL
IgG/oxLDL
TS92/oxLDL
nLDL

Figure. 5


Normal
Preeclampsia
oxLDL
LOX-1
LXR
LXR
LXR
nuclear
trophoblast
ABCA1
ABCA1
ABCA1
ABCA1