Treatment of Lipoxin A₄ and its analogue on low-dose endotoxin induced preeclampsia in rat and possible mechanisms

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ABSTRACT

Preeclampsia (PE) is known to represent an exaggerated maternal inflammatory response to pregnancy. Lipoxin A₄ (LXA₄), considered as an endogenous stop signal in inflammation, has been extensively studied pre clinically for its inflammatory pro-resolving effects. Thus, in the current study, we tested the effect of BML-111 (synthetic analogue of LXA₄) on experimental PE rats induced by low-dose endotoxin (LPS) and of LXA₄ on human extravillous trophoblast cell line (TEV-1). In vivo experiment results showed that systolic blood pressure, 24-h urinary albumin excretion, serum TNF-α and IL-8 levels and morphologic damage of placenta and kidney caused by LPS were all effectively alleviated by BML-111. LXA₄ also inhibited LPS-triggered apoptosis, activation of NF-κB, TNF-α and IL-8 mRNA and protein expression in TEV-1 cells. At the same time, BML-111 protected the cells from LPS-reduced proliferation. The current study demonstrated for the first time that LXA₄ could alleviate the symptoms of PE in endotoxin exposed rats.

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1. Introduction

Preeclampsia (PE) is a pregnancy-specific disorder that affects 3–5% of pregnant women worldwide and is one of the most frequently encountered medical complication of pregnancy [1]. It is characterized by de novo hypertension, proteinuria and edema occurring after the 20th week of gestation. During early placental development, extravillous trophoblasts (EVTs) of fetal origin invade the uterine spiral arteries of the decidua and myometrium. These invasive cytotrophoblasts replace the endothelial layer of the maternal spiral arteries, and transform them from small, high-resistance vessels into large-caliber capacitance vessels which can provide adequate placental perfusion to nourish the fetus [1]. Although the pathophysiology of PE is not completely understood, failure of EVTs to adequately invade and remodel spiral arteries is widely recognized as one of the predisposing factor [2].

A series of intriguing findings have led to the suggestion that pregnancy is a condition of controlled mild maternal systemic inflammation [3], whereas severe PE is associated with an exaggerated inflammation response. The pro-inflammatory cytokines produced by ischemia or hypoxia–reoxygenation injured placenta may be released into the maternal circulation where they interact with maternal endothelium and innate immunocytes, causing the maternal symptoms of PE.

Lipoxins (LXs), including Lipoxin A₄ (LXA₄), Lipoxin B₄ (LXB₄) and aspirin-triggered LXs, are trihydroxytetraene-containing bioactive eicosanoids generated by transcellular lipoxygenation of arachidonic acid. As the most potent endogenous stop signals for inflammation, LXs have been extensively studied preclinically for their inflammatory pro-resolving effects [4,5]. Our group provided the first evidence for the anti-angiogenic role of LXA₄ on hypoxic human umbilical vein endothelial cells (HUVECs) and we also proved that LXA₄ could prevent the EC hyperpermeability induced by LPS in HUVECs [6,7]. Given that placenta hypoxia and vascular endothelial dysfunction are typical features of patients with PE [8,9], our findings indicated that LXA₄ might be a promising candidate for treatment of PE. However, little is known about the direct effect of LXs on placental trophoblasts.

LXs are rapidly converted to inactive lipids with lower potencies as anti-inflammatory agents. To circumvent such degradation,
a panel of synthetic, stable LXA₄ analogues has been designed [10]. These longer acting analogues serve as useful tools to evaluate the potential of pharmacological manipulation of LXs which are not suitable for long term in vivo experiments. Among the LXs analogues studied, 5(S), 6(R)-7-trihydroxymethyl heptanoate (BML-111) is a C-7 truncated analogue that was originally proven to be equipotent with LXA₄ in the inhibition of leukotriene B₄-induced neutrophil chemotaxis [11]. Our group previously demonstrated that, similar to LXA₄, BML-111 protected mice against carbon tetrachloride-induced liver injury [12], regulated the cytosolic calcium concentration [13] and inhibited hepatocarcinoma [14]. Thus, in the following in vitro experiments involving human EVT, LXA₄ was applied, while in in vivo experiments involving experimental PE rat, BML-111 was used to mimic the effect of LXA₄.

The current study demonstrated for the first time that BML-111 could alleviate the symptoms of PE in low dose endotoxin exposed pregnant rats. We also found that LXA₄ promoted the proliferation but inhibited the apoptosis and pro-inflammatory cytokines secretion in TEV-1 cells. These findings might explain the therapeutic effect of exogenous LXAs on PE rats.

2. Materials and Methods

2.1. Materials and reagents

LXA₄, from Cayman Chemical Company (USA), was stored at –80 °C until being diluted in serum-free culture medium immediately before use. BML-111, purchased from Biomol (USA), was stored in methanol at –20 °C at a concentration of 1 mM. On the day of administration, it was diluted in saline. The daily working stocks were routinely discarded after use. Endotoxin (lipopolysaccharide, or LPS) from Escherichia coli O127:B8, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were from Sigma Aldrich (USA). Bacterial or fungal serum (FBS) were from Gibco (Australia). Annexin V-FITC and PI apoptosis detection kits were from Biolegend (USA). ProteoJET mammalian cell lysis reagent, NE-PE nuclear and cytoplasmic extraction reagents were from Fermentas (USA). FITC-conjugated goat anti-mouse and BCA protein assay kit were purchased from Pierce (USA). ELISA kits were from BD (USA). Trizol was from Invitrogen (USA).

2.2. In vitro experiment

2.2.1. Animals

Female Sprague-Dawley rats, 10–12-weeks old, weighing 220–250 g were purchased from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology. Animals were housed individually in plastic cages with wood chips as bedding under pathogen-free conditions, in a controlled environment of temperature at 20–25 °C and 12 h cycles of light and dark. Rats were fed a standard laboratory diet and water ad libitum. The female rats were mated overnight with fertile male rats. A positive vaginal smear for sperm defined day 1 of pregnancy (duration of gestation is 21 days). All animal work was conducted according to relevant national and international guidelines. All the usage and procedures were approved by the committee for experimental animals of Tongji Medical College.

2.2.2. Experimental protocol

Rats were randomly divided into control group (n = 8), LPS group (n = 8), LPS + BML-111 group (n = 6) and LPS + methanol group (n = 6). Experimental PE was induced by infusion of LPS (1 μg/kg body weight) in 2 ml of sterile saline through an infusion pump into the tail vein (infusion rate, 2 ml/h) on day 14 of pregnancy [15]. Normal pregnant control rats were infused with 2 ml of saline alone. 1 mg/kg BML-111 or methanol in 2 ml saline was administrated intraperitoneally 30 min prior to LPS on day 14 and once daily through day 19 of pregnancy. Since commercially obtained BML-111 was dissolved in methanol solution, 0.5% methanol in saline was applied as vehicle control.

2.2.3. Measurement of systolic blood pressure

The systolic blood pressures (SBPs) were measured in conscious, restrained pregnant rats once every 2 days in the morning from day 8 through 20 of pregnancy. An automated system with a photoelectric sensor linked to a dual channel recorder (BP-98A, Softron, Japan), tail cuff and sphygmomanometer was used to obtain indirect blood pressure measurements [16].

2.2.4. Determination of urinary albumin excretion

For 24-h urine collection, on day 12 and 19 of pregnancy, the rats were placed in metabolic cages. To avoid contaminating the collected urine, rats were restricted from food; however, they were allowed free access to water. To avoid the adverse effects of fasting, rats were fed in other cages for 30 min every 6 h. Urine samples were centrifuged at 2000 rpm for 15 min at room temperature, and the top layer was stored at –80 °C for later analysis of protein. Urine protein concentrations were determined with a BCA protein assay kit using bovine serum albumin as standard.

2.2.5. Specimen collection

On day 14 of pregnancy before saline or LPS administration, about 0.5 ml blood specimen was drawn from tail vein. On day 20 of pregnancy, after the rats were anesthetized, about 1 ml blood specimen was drawn by heart punctio. Serum was stored at –80 °C for further assessment. Fetal pups, placenta and kidneys were removed and weighed. Three placentae and one kidney were randomly selected from each rat and fixed with 10% neutral-buffered formalin for histological evaluation. Three placentae from each rat were stored at –80 °C for RT-PCR assessment.

2.2.6. Histology assay

Placentae and kidneys specimens in 4-μm paraffin sections were stained with hematoxylin and eosin for conventional morphological evaluation under light microscope (Nikon eclipse 90i, Tokyo, Japan).

2.3. In vitro experiment

2.3.1. Cell culture

The human first-trimester EVT cell line TEV-1, was a gift from Dr. Tsao S.W. (University of Hong Kong, China) [17]. Cells were plated in 6-well plates for RT-PCR, Western blotting and ELISA assay, or in 96-well plates for MTT assay and in 24-well plates for FACS analysis. Cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂ in DMEM/F-12 medium supplemented with 10% heat-inactivated FBS, 25 mM/italic L-HEPES, 100 U/ml penicillin and 100 U/ml streptomycin for 3 days. The serum-free DMEM/F-12 then was used to replace the culture medium. After 12 h of starvation, cells were divided into 4 groups according to different treatment for 24 h: (1) control cells, since LXA₄ and Boc-2 were dissolved in methanol solution, an appropriately diluted methanol was treated as vehicle control; (2) LPS cells, treated with 1 μg/ml LPS; (3) LPS + LXA₄, 30 min before LPS treatment pretreated with 200 nM LXA₄; (4) LPS + LXA₄ + Boc-2 cells, 30 min before LPS and LXA₄ treatment, pretreated with 10 μM Boc-2. Boc-2 is an effective antagonist of LXA₄ receptor, and was used at a concentration (10 μM) known to block LXA₄ effects [18]. Each experiment was repeated three times, and each treatment was performed in three duplicates.

2.3.2. Cell proliferation assay

Cell proliferation was measured by MTT assay as previously reported [19].

2.3.3. FACS analysis of apoptosis

Annexin V-FITC and PI apoptosis detection kits were used according to manufacturer’s instructions to measure cell apoptosis. Cellular apoptotic rate was analyzed with a FACSscan flow cytometer (BD, Biosciences).

2.3.4. Enzyme-linked immunosorbent assay

TNF-α and IL-8 protein levels in serum or TEV-1 cells were measured with ELISA kits according to the manufacturer’s instructions.

2.3.5. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA (4 μg) was isolated from rat placental specimens or TEV-1 cells by Trizol reagent according to manufacturer’s instructions. PCR amplification was performed with Taq DNA polymerase. The sequences of primers were as follows: rat TNF-α, forward 5′-GCT CCC AAC AAG GAC GAG AAG-3′ and reverse 5′-CAC CCT CAC CCT AAC AC-3′; human TNF-α, forward 5′-AGC TAC ATG GCC TGA ACC G-3′ and reverse 5′-CAG GAT CAT CCC AAA GTA-3′; rat IL-8, forward 5′-GAA CAT CCA GAG TTG GAA-3′ and reverse 5′-GGT ACA GAA CGG ATG AAC AC-3′; human IL-8, forward 5′-ATG ACT GCC CTT GCC GTG CCT-3′ and reverse 5′-TCT CAG CCT TCC TAA ACT TCT-3′; rat GAPDH, forward 5′-GGT CAA AAG GCC TCT AAT CGT-3′ and reverse 5′-TCT CAG CCA GGG CAG TAA TC-3′; human GAPDH, forward 5′-ACC AGC CCC AAG ACC ACA AG-3′, reverse 5′-TTC AAG GGT TCA TCC TCA TCT-3′. Amplified cDNA was separated by electrophoresis on 1.6% agarose gel and identified with Gold View.

2.3.6. Immunofluorescence assay

Regular immunofluorescent assay was performed to detect NF-κB p65 expression. Anti-NF-κB p65 (1:50) and FITC-conjugated goat anti-mouse (1:100) antibodies were applied. Cell nuclei were counter stained with Hoechst. Fluorescent images were processed using LSM710 and Confocor3 Microscope Systems (Carl Zeiss, Germany).

2.3.7. Western blotting assay

Total, nuclear and cytoplasmic proteins were extracted using ProteoJET™ mammalian cell lysis, NE-PE nuclear and cytoplasmic extraction reagents, respectively.
Protein concentrations were determined using a BCA protein assay kit. Equal amounts (40 μg) of protein were then subjected to 12% SDS-PAGE electrophoresis and transferred to nitrocellulose membranes. Anti-hiB-α (1:500) and NF-κB p65 (1:500) were applied. β-actin (1:500) served as an internal control. The bound antibody was detected by enhanced chemiluminescence on an X-ray film.

2.4. Statistics

All statistical analyses were done using the SPSS 13.0 software. Numerical results were expressed as mean ± SEM of multiple experiments. The means of the different groups were compared by a 1-way ANOVA followed by a Student–Newman–Keuls test. A values of p < 0.05 was considered significant.

3. Results

3.1. Effects of BML-111 on experimental PE rats

Infusion of low dose endotoxin into pregnant rats, causing hypertension, proteinuria, and DIC [15], had been applied in the study of inflammatory aspects of PE for several years. In current study, none of the rats that were exposed to LPS infusion developed any sign of lethargy, behavioral changes or loss of appetite. No fetal anomaly or demise was observed in any group. There were no statistically significant differences in maternal body weight gain over treatment period between all groups (p > 0.05, Fig. 1A). There were no statistically significant differences in fetus or placenta weight between all groups (p > 0.05, Fig. 1B).

Fig. 1C showed that in control group, SBP did not change obviously during 20 days of pregnancy, while it significantly elevated after infusion with 1 μg/kg LPS. The SBP at 16, 18 and 20 days of pregnancy were 112 ± 9, 127 ± 7 and 128 ± 9 mmHg, respectively (p < 0.05 versus control group at the corresponding time point). Treatment of LPS-infused rats with BML-111 at 1 mg/kg-d for a consecutive 6 days significantly alleviated SBP to 106 ± 5 and 114 ± 9 mmHg on day 18 and 20 of pregnancy, respectively (p < 0.05 versus LPS group), whereas, methanol decreased SBP on day 18 and 20 of pregnancy but not as much as BML-111.

Before infusion of LPS or saline solution, mean urinary albumin excretion of rats showed no difference between control group and LPS group (0.35 ± 0.08 versus 0.44 ± 0.09 mg/24 h on day 12 of pregnancy, p > 0.05, Fig. 1D), but on day 19, this level was significantly higher in LPS group (0.86 ± 0.09 versus 0.44 ± 0.08 mg/24 h, p < 0.05). Treatment of BML-111 also caused a marked reduction in urinary albumin from 0.86 ± 0.09 to 0.52 ± 0.09 mg/24 h (p < 0.05) compared to LPS-treated rats.

It was shown that LPS infusion caused the morphological changes in both placenta and kidney characterized by thickening of the media of placental vessel walls and glomerular endotheliosis with accumulation of inflammatory leukocytes, respectively (seen in Fig. 2). These findings were remarkably improved by treatment with BML-111, but not by methanol alone.

3.2. BML-111 inhibited TNF-α and IL-8 generation in experimental PE rats

As potent pro-inflammatory cytokines, TNF-α and IL-8 were also involved in the pathogenesis of PE [20–22]. In current study, ELISA assay showed that in the control group, both cytokines in
Fig. 2. The effects of BML-111 on placenta and kidney morphological features in experimental PE rats. Placenta and kidney specimens were obtained on day 20 of pregnancy and examined by hematoxylin and eosin staining. (A) Representative images of placenta. The magnifications were 100× (a1–a4), and 400× (a5–a8), respectively, bar = 20μm. (B) Representative images of kidney. The magnification was 200×. Black arrows indicated leukocytes. Blue arrows indicated glomerular endotheliosis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

serum were slightly elevated from day 14 to day 20 of pregnancy but without statistical differences (p > 0.05). Both TNF-α and IL-8 were significantly increased on day 20 in LPS-treated animals, relative to controls (p < 0.05, Fig. 3A and B). Treatment with BML-111 (1 mg/kg-d) for 6 days reduced the upregulation of TNF-α and IL-8 in LPS-treated animals (p < 0.05).

TNF-α and IL-8 expressions in placenta were also detected by RT-PCR on day 20 of pregnancy. A much stronger signal for both

Fig. 3. The effects of BML-111 on pro-inflammatory cytokines generation in experimental PE rats. Blood samples were obtained on day 14 or day 20 of pregnancy. Placenta was obtained on the day 20 of pregnancy. Serum TNF-α (A) and IL-8 (B) protein levels were analyzed by ELISA. (C) Placenta TNF-α and IL-8 mRNA levels were analyzed by RT-PCR. The representative photographs for PCR were from one of three independent experiments that yielded similar results. (D) Densitometric analysis of the visualized bands was performed using Gel-Pro analyzer 4 software to normalize TNF-α and IL-8 mRNA expression with the corresponding GAPDH levels, and expressed as a percentage of control group. All data were expressed as mean ± SEM. *p < 0.05 versus control group on day 20 of pregnancy; †p < 0.05 versus data from the corresponding group on day 14 of pregnancy; ‡p < 0.05 versus LPS group on the corresponding day of pregnancy.
TNF-α and IL-8 were observed after LPS infusion. BML-111, but not methanol, inhibited this up-regulation by LPS (Fig. 3C and D).

3.3. LXA₄ promoted the proliferation of LPS-stimulated TEV-1 cells

We first assessed the effect of LPS on the viability of TEV-1 cells by MTT assay. Cells showed decreased viability in a dose-dependent manner when stimulated with indicated concentration of LPS for 24 h (p < 0.05 versus control, Fig. 4A). In the following in vitro experiment, LPS with middle concentration (1 µg/ml) was applied to establish the inflammatory circumstance.

The effect of 50–800 nM LXA₄ on the viability of LPS-stimulated TEV-1 cells was further studied. LXA₄ increased the viability in a dose-dependent manner, and reached a maximal effect at 200 nM (p < 0.05 versus LPS group, Fig. 4B), where viability was similar to untreated controls. Based on the results, 200 nM LXA₄ was chosen for further in vitro study.

3.4. LXA₄ inhibited LPS-stimulated apoptosis in TEV-1 cells

Numerous articles have been published addressing the association between altered apoptosis and PE. Excessive apoptotic activity of trophoblast in the placental bed of PE women inhibits its invasion into the spiral artery [23]. We further investigated the effect of LXA₄ on LPS-induced apoptosis in TEV-1 cells with the combination of Annexin V and PI double staining through FACS assay.

As shown in Fig. 4C and D, after TEV-1 cells were challenged with LPS for 24 h, cell apoptotic rate was enhanced from 4.6 ± 1.0 to 19.1 ± 2.9% (p < 0.05 versus control group). Pretreatment of the cells with 200 nM LXA₄ significantly reduced cell apoptotic rate to 8.9 ± 1.6% (p < 0.05 versus LPS cells). The inhibitory effect of LXA₄ could be partly reversed by 10 µM Boc-2, antagonist to LXA₄ receptor.

Given that Bcl-2 family plays a key role in the regulation of cell apoptosis, we then explored whether it was also involved in the anti-apoptosis effect of LXA₄. As presented in the western blotting photograph, both Bcl-2 and Bax protein could be detected in control cells (Fig. 4E and F) LPS down-regulated Bcl-2 to 59.9 ± 16.5% of control (p < 0.05), while up-regulated Bax to 301 ± 33.8% of control (p < 0.05). Both effects of LPS on these two proteins expression could be inhibited by LXA₄. At the same time, Boc-2 partially blocked the inhibition by LXA₄.

3.5. LXA₄ inhibited TNF-α and IL-8 secretion in LPS-stimulated TEV-1 cells

TNF-α and IL-8 mRNA in TEV-1 cells were measured by RT-PCR. Exposure of the cells to 1 µg/ml LPS for 24 h lead to higher mRNA expression of both genes which were increased by 200 nM LXA₄ by 53.2% and 63.4%. At the same time, Boc-2 reduced the inhibitory effect of LXA₄ on TNF-α and IL-8 gene expression (Fig. 5A and B).

Production of TNF-α and IL-8 by TEV-1 cells was also studied by ELISA. As seen in Fig. 5C and D, LPS resulted in increased secretion of TNF-α and IL-8 (p < 0.05 versus control group). Pretreatment of the cells with 200 nM LXA₄ significantly reduced the secretion of both pro-inflammatory cytokines induced by LPS (p < 0.05). This effect was blocked by the presence of 10 µM Boc-2 (p < 0.05 versus LXA₄ group).

3.6. LXA₄ inhibited the LPS stimulated activation of NF-κB in TEV-1 cells

Since NF-κB activation was known to be essential for LPS-stimulated pro-inflammatory cytokines secretion and apoptosis [24], we analyzed how it was changed when LXA₄ was present.

With immunofluorescence assay, it was shown in Fig. 6A that NF-κB p65 was mainly located in cytoplasm of the control cells while translocated into nucleus after stimulation with LPS for 24 h. However, if the cells were cotreated with LXA₄, nuclear translocation could be clearly inhibited and this inhibition was blocked by the presence of Boc-2.

Next, we examined the effects of LXA₄ on IκB-α, cytoplasmic NF-κB p65 and nuclear NF-κB p65 protein level by Western blotting analysis. The results showed that stimulation of the cells with LPS induced an increment in nuclear NF-κB p65 expression, while decrease in cytoplasmic NF-κB p65 and IκB-α. Pretreatment of the cells with LXA₄ prior to LPS exposure blocked these effects of LPS (Fig. 6B and C).

4. Discussion

Although the exact etiology and pathogenesis of PE still remain obscure, there is now consensus that inflammation caused by placental dysfunction is a common underlying reason of PE [25]. In current study, endotoxin was chosen to mimic inflammatory circumstances in rats. Similar with previous reports [15,26,27], low doses of endotoxin exposure led to some typical clinical PE manifestations such as hypertension, proteinuria and thickening in the media of placental vessel walls. At the same time, glomerular endotheliosis with accumulation of leukocytes revealing the typical changes in maternal endothelium in PE was presented in the kidney. Elevated levels of TNF-α and IL-8 in serum and placentae were also observed in pregnant rats under LPS stimulation, seen clinically in women with PE [28–30]. Severe PE in humans is usually associated with fetal growth restriction, however, in our study, low dose LPS infusion at 1 µg/kg on day 14 of pregnancy had no effect on fetal or placental weight. A similar study was reported by Sakawi [27], but at a higher dose of LPS (100 µg/kg/day for 7 days in pregnant rats) significantly reduced the size of fetuses and increased fetal demise. LPS at this high concentration obviously has some toxic effects [31].

There are some reports in the literature that non-steroidal anti-inflammatory drugs, targeted at blocking pro-inflammatory pathways may prevent the incidence or reduce the severity of PE in women [32,33]. However, formal clinical trials have shown that low doses of these medications had no beneficial effect [34,35]. The possible maternal and fetal side effects of chronic treatment with these agents (e.g., constriction of fetal ductus arteriosus and reduced renal blood flow) must also be considered when using these drugs [36].

Ever since inflammation resolution phase has been identified as an active process following the pro-inflammatory activities, these new mechanisms and pathways have challenged the existing paradigm and opened new avenues for therapeutics that are controlling excessive inflammation without apparent immunosuppression [37]. The current study showed for the first time that BML-111, a synthetic analogue of LXA₄, efficiently alleviated the PE manifestations in rats exposed to low dose endotoxin without obvious influence on fetal or placental weight. This indicated the therapeutic effect of LXA₄ on PE. Moreover, as an endogenous autostability molecule and novel immune regulator in vivo, LXA₄ may have a potential preventive effect on PE. Further studies are required to evaluate this effect. 5-lipoxygenase (5-LO), an indispensable enzyme to synthesize LXs, has been reported to localize in human placental syncytiotrophoblasts, decidua and amniotic membrane [38]. Furthermore, we found that LXA₄ receptor, formylpeptide receptor like-1 (FPRL-1), was highly expressed in human placentae (seen in Supplementary data). All these point out the importance of endogenous LXs in maintaining the normal pregnancy and indicated that LXs might be a promising target for PE treatment.
Fig. 4. The effects of LXA4 on LPS-stimulated TEV-1 cells proliferation and apoptosis. (A) and (B) TEV-1 cells were stimulated with indicated concentration of LPS for 24 h with or without indicated concentration of LXA4 treatment 30 min in advance. Cell proliferation was measured by MTT assay. A representative of three independent experiments with the similar results was shown. (C) The cells were treated with 1 μg/ml LPS for 24 h. 200 nM LXA4 with or without 10 μM Boc-2 were added 30 min prior to LPS. Representative photograph showed cell apoptosis being analyzed with the combination of Annexin V and PI double staining through FACS assay. The percentage of cells at each different apoptotic stage is presented. The lower-left field represents viable cells, the lower-right field apoptotic cells, the upper-left field necrotic cells, and upper-right field late-apoptotic cells. (D) Bar graph showed the apoptosis rates in different groups. (E) Western blotting analysis of Bcl-2 and Bax protein expression in TEV-1 cells. β-Actin served as an internal control. The representative photographs were from one of three independent experiments that yielded similar results. (F) Densitometric analysis of the visualized bands was performed using Gel-Pro analyzer 4 software to normalize Bcl-2 and Bax protein expression with the corresponding β-Actin levels, and expressed as a percentage of control cells. All data were expressed as mean ± SEM. *p < 0.05 versus control cells. *p < 0.05 versus LPS cells. *p < 0.05 versus LPS + LXA4 cells.
Reduced placental perfusion has been postulated to be the initial event in PE which leads to widespread dysfunction of the maternal vascular endothelium [39]. Most studies have suggested that abnormal cytotrophoblast invasion of spiral arterioles is a crucial factor leading to abnormal placental perfusion. Interestingly, compared to later stages, expression of 5-LO in the chorion-decidua was significantly higher in the first trimester in which EVTs invade the maternal decidua and contribute to generate successful pregnancy [40]. Thus, to explore whether EVTs were involved in the protective effect of LXs on PE, TEV-1 cells, of which the phenotypic and characteristic were similar to human EVTs [17], were used in the in vitro experiment.

In accord with the results from the in vitro experiment, the inhibitory effect of LXA4 on LPS-stimulated pro-inflammatory cytokines production was presented on the cellular level. And, both protein expression and localization assays showed that LPS-triggered activation of NF-κB could be inhibited by LXA4. As we know, in the unstimulated cells, NF-κB normally resides in the cytoplasm as a heterodimer composed of p65 and p50 subunits linked to IκB-α. Activation of NF-κB results in phosphorylation and degradation of IκB-α, causing the release of p65 and p50 which translocate to the nucleus, where it participates in transcription of genes involved in the regulation of inflammatory responses [41]. Our results show that the down-regulation of TNF-α and IL-8 by LXA4 might occur through NF-κB, which has also been reported to be elevated in women with PE [42]. Although the biological effects of LXA4 were mainly elicited via FPRL-1, other receptors such as cysteinyI-leukotriene receptors, growth factor receptors and Ah receptor were reported to mediate LXA4 action in vivo [43]. Our results show that Boc-2 partially blocked the effects of LXA4; this suggested that there were other receptors involved.

We further analyzed the change of cell apoptosis which is crucial to the development and homeostasis of human tissues, including placenta. Apoptosis has been confirmed in placentas throughout gestation by many techniques, including light microscopy, electron microscopy, and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling staining. It could be detected in trophoblast cells of normal placental tissue especially with advancing gestational age suggesting that apoptosis is a part of normal placental aging process [44]. Both cytotrophoblasts and syncytiotrophoblasts are in a steady state under normal pregnancy, while it is likely that the placental insults would alter this condition by modulating the trophoblast cell turnover [19]. Studies have also demonstrated that increased apoptosis in trophoblast cells contributes to development of PE [44–46]. Previously, LXA4 was shown to augment apoptosis in LPS-treated neutrophils or renal interstitial fibroblasts [47]. On the other hand, it was found to inhibit apoptosis of human monocyte and macrophages induced by LPS [48,49]. As far as we know, the current study showed for the first time that LXA4 inhibited LPS-stimulated TEV-1 cells apoptosis.

Although highly conserved, apoptosis pathways are now increasingly recognized as cell type- and stimulus-specific. The list of pro- and anti-apoptotic signaling molecules is ever-expanding. Among these, we selected the Bcl-2 family of genes for analysis in our studies. The Bcl-2 gene family, isolated from a B-cell lymphoma, includes apoptosis-promoting (Bax) and apoptosis-inhibiting (Bcl-2 and Bcl-x) members. Bcl-2 has been immunolocalized to the syncytiotrophoblast of the chorionic villi, persisting from the first
to the third trimester of pregnancy but decreases as gestation progresses [50,51]. According to our results, LXA4 might exert its anti-apoptotic effect in vitro by up-regulating anti-apoptotic Bcl-2 and down-regulating pro-apoptotic Bax.

In conclusion, this is the first description of a potential therapeutic effect of BML-111 in rats with LPS-induced PE, which appears to be mediated through inhibition of inflammatory processes in the placenta. Till now, despite research advances in understanding the pathogenesis of PE there remains no treatment except delivery of the placenta [1]. Although further studies need be carried out to evaluate the effects of LXS on other models of PE and further explore the mechanisms, our findings may open a new way for development of anti-inflammatory therapeutic strategies on PE or other inflammatory complications of pregnancy.

**Conflict of interest**

There are no conflicts of interest.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2012.09.009.

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