

RESEARCH PAPER

Lipoxin A₄ suppresses the development of endometriosis in an ALX receptor-dependent manner via the p38 MAPK pathway

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BACKGROUND AND PURPOSE

Lipoxins can function as endogenous 'breaking signals' in inflammation and play important roles in the progression of endometriosis. In this study, we further investigated the molecular mechanism by which lipoxin A₄ (LXA₄) suppresses the development of endometriosis.

EXPERIMENTAL APPROACH

Primary endometriotic stromal cells (ESCs) were treated with IL-1 β , or pre-incubated with LXA₄ before incubation with IL-1 β . The LXA₄ receptor (ALX receptor) antagonist Boc-2 and gene-silencing approaches were used to study the involvement of the ALX receptor in anti-inflammatory signalling responses in ESCs. An animal model of endometriosis was induced in BALB/c mice by i.p. injection of an endometrium-rich fragment.

KEY RESULTS

Decreased levels of LXA₄ and 15-LOX-2 expression but increased expression of ALX receptors were observed in endometriotic tissues. LXA₄ inhibited the release of inflammatory factors and phosphorylation of p38 MAPK in IL-1 β -induced ESCs, an effect mediated by ALX receptors. LXA₄ inhibited the proliferation of ESCs, as indicated by reduced DNA replication, caused G₀/G₁ phase cell cycle arrest and down-regulated the expression of proliferating cell nuclear antigen in ESCs. LXA₄ also attenuated the invasive activity of ESCs mainly by suppressing the expression and activity of MMP-9. *In vivo*, we further confirmed that LXA₄ could inhibit the progression of endometriosis by acting as an anti-inflammatory.

CONCLUSIONS AND IMPLICATIONS

LXA₄ exerted anti-inflammatory, anti-proliferative and anti-invasive effects on endometriosis through a mechanism that involved down-regulating the activities of p38 MAPK, which was mediated by ALX receptors.

Abbreviations

15-LOX-2, 15S-lipoxygenase-2; ALX receptor, lipoxin A₄ receptor; EM, endometriosis; ESCs, endometriotic stromal cells; LXA₄, lipoxin A₄

Table of Links

TARGETS	LIGANDS
ALX receptor	LXA ₄
15-LOX-2	IL-1 β
15-LOX-1	IL-6
p38 MAPK	MCP-1
MMP-9	TNF- α
	VEGF
	SB203580

This Table lists key protein targets and ligands in this document, which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a,b).

Introduction

Endometriosis (EM), characterized by the presence of uterine endometrial tissue outside of the normal location, is a common gynaecological disorder with a complex, multifactorial aetiology that caused chronic pelvic pain, dysmenorrhoea, and even infertility (Giudice and Kao, 2004). The underlying pathophysiological mechanism responsible for EM remains unknown. Growing evidence suggests that aberrant immune responses and inflammatory reactions play an important role in the pathogenesis of EM. Many of pro-inflammatory cytokines derived from endometriotic cells, activated macrophages and other immune cells in the abdominal cavities of women with EM have been thought to play a crucial role in the development of the disorder (Podgaec *et al.*, 2007).

The resolution of inflammation is now understood to be an active process that involves the action of anti-inflammatory and pro-resolution modulators, the lipoxins, resolvins and protectins, which activate specific biochemical pathways necessary for the resolution of acute inflammatory responses (Serhan *et al.*, 2008b). Lipoxins (LXs), a class of arachidonic acid (AA) metabolites, are typically formed by transcellular metabolism through distinct biosynthetic pathways depending on the cellular context, include the naturally occurring lipoxin A₄ (LXA₄) and B₄ (LXB₄) and the aspirin-triggered lipoxins (LXA₄) 15-epimeric lipoxin A₄ (15-epi-LXA₄) and 15-epimeric lipoxin B₄ (15-epi-LXB₄; Maderna and Godson, 2009). Three human lipoxygenases (LOXs), included 5-LOX, 12-LOX and 15S-lipoxygenase-2 (15-LOX-2), have been confirmed to involve the formation of LXs. The expression of these three LOXs was aberrant in many diseases including adenomatous polyps, human epidermoid carcinoma and EM (Melstrom *et al.*, 2008; Agarwal *et al.*, 2009; Borghese *et al.*, 2009). LXs mediate a number of physiologic and pathologic processes, including regression of proinflammatory cytokine production, inhibition of cell proliferation, modulation of cytokine-stimulated metalloproteinase activity and stimulation of macrophage clearance of apoptotic polymorphonuclear neutrophils (McMahon *et al.*, 2001; Bonnans *et al.*, 2006). These diverse actions are mainly medi-

ated by various membrane receptors including the LXA₄ receptor (ALX receptor), cysteinyl leukotriene receptor (CysLT1), GPCR 32 (GPR32), oestrogen receptor α (ER α , also known as NR3A1) and unidentified high-affinity surface-binding receptors (Maderna and Godson, 2009; Krishnamoorthy *et al.*, 2010; Russell *et al.*, 2011). However, because the A-type LX has been shown to bind with high affinity to the ALX receptor, more and more studies have focused on how LXA₄ exerts actions via it. The ALX receptor was known as formyl peptide receptor-like 1 (FPRL1) and formyl peptide receptor 2 (FPR2), a distinct GPCR of the formyl peptide receptor superfamily. LXA₄ is a dual acting mediator and activates specific cellular pathways via ALX receptor to elicit both anti-inflammatory and pro-resolution effects (Serhan *et al.*, 2008b). Studies also revealed that LXA₄ could attenuate NF- κ B activation and inhibit phosphorylation of p38 MAPK, ERK1/2, and Akt (Cezar-de-Mello *et al.*, 2008; Baker *et al.*, 2009), which suggested that LXA₄ might play its role in regulating the activation of these signalling pathways.

It is noteworthy that many of the factors that are attenuated by the LX-ALX receptor interaction are involved in the development of EM. Endometrium in experimental models of EM in rats and in women with EM showed higher expression of LXA₄ receptor compared with the normal tissues (Motohashi *et al.*, 2005), which suggested a possible role of LXA₄ and the receptor under pathophysiological condition of EM. We have previously reported that LXA₄ suppressed the growth of endometriotic lesion in an experimental mouse model of EM possible by inhibiting the expression and activities of MMP (Chen *et al.*, 2010a). Xu also found that LXA₄ could inhibit the progression of EM in mice by playing its roles of anti-inflammation and anti-angiogenesis (Xu *et al.*, 2012).

Recently, increasing evidence indicated that p38 MAPK, an intracellular signal-transducing molecule, might be involved in the pathogenesis of EM (Seval *et al.*, 2006; Yoshino *et al.*, 2006). Our previous studies found that SB203580, a p38 MAPK inhibitor, could suppress the development of EM in a murine model by inhibiting the expression of proinflammatory cytokines and proteolytic factors

(Zhou *et al.*, 2010). Collectively, we suppose that LXA₄ may play its anti-inflammatory and anti-invasive roles in EM via p38 MAPK signalling pathway.

In this study, we first investigated the levels of LXA₄, expressions of 15-LOX-2 and ALX receptor in human endometriotic tissues. Then, the effects of LXA₄ on proinflammatory cytokine production *in vivo* or *in vitro* were investigated. We also analysed the impacts of LXA₄ on cell proliferative and invasive activity in endometriotic stromal cells (ESCs). Furthermore, the effects of LXA₄ and its receptor ALX receptor on p38 MAPK signalling pathway were studied, which may explain the mechanism of LXA₄ inhibiting the progression of EM.

Methods

Patients and samples

Endometrial tissue samples (Supporting Information Table S1) were obtained from 53 patients who underwent surgery from September 2011 through to August 2012 for diagnosis or treatment of EM or for other benign gynaecological disorders (controls). To prevent the effect of tissue quality on PCR results, six cases were excluded from the study due to incompetent tissue quality as determined by the gynaecological pathologist. Finally, 47 patients were enrolled in this study. Among the patients, 20 patients with laparoscopically confirmed absence of EM, and 27 patients with both laparoscopically and histologically confirmed EM. All the EM patients had ovarian endometriotic cysts. The women's menstrual cycle phase were established according to their menstrual history and confirmed by endometrial histology using the criteria as described previously (Noyes *et al.*, 1975). Therefore, the division of the menstrual cycle was as follows: proliferative phase, days 1–14; secretory phase, days 15–29. The use of these tissues was approved by Ethics Committee of the First Affiliated Hospital of Xiamen University, and informed consent was obtained from each patient. These patients had not received hormonal treatment or any anti-inflammatory treatment for at least 6 months before surgery.

Isolation and culture of human ESCs

Primary ESC culture was all from ovarian endometriotic cyst tissues, based on previously published procedure with minor modifications (Lin *et al.*, 2012). Briefly, after endometriotic tissues were minced into small pieces and incubated in type IV collagenase, endometrial epithelial cells and stromal cells were separated from the cell suspensions by nylon cell strainers. Isolated ESCs were cultured in complete medium at 37°C in 5% CO₂. When the cells became 90% confluent, they were used for experiments. The purity of the endometrial stromal cells were assessed by staining with vimentin and CD 10, and the purity of the endometrial epithelial cells were determined by immunocytochemical staining with cytokeratin 19. As shown in Supporting Information Fig. S1, for ESCs in monolayer culture after the second passage, the percentages of vimentin- and CD 10-positive cells were approximately 96 and 89%, respectively, and remained consistent with passage. ESCs were used between passages 2 and 6.

Quantitative real-time PCR analysis

Total RNA was extracted from ESCs by using RNAiso Plus (Takara, Kyoto, Japan) following the manufacturer's protocol. Intact total RNA was confirmed by determining appropriate sharp 28 S and 18 S rRNA bands by agarose gel electrophoresis; 1 µg of total RNA was reverse transcribed in a system of 20 µL volume using PrimeScript RT reagent Kit (Takara). Real-time quantitative PCR assays were carried out by employing SYBR PrimeScript RT-PCR kit (Takara) using Rotor-Gene 6000 (Corbett Research, Sydney, Australia) as previously described (Zhou *et al.*, 2010). The primers used for RT-PCR and qRT-PCR were provided in Supporting Information Table S2. The 2^{-ΔΔCT} method was used to calculate the relative mRNA level of each gene (Schmittgen and Livak, 2008).

Western blotting analysis

Western blot analysis was performed as described previously (Zhou *et al.*, 2010). Briefly, protein was isolated from ESCs or endometriotic tissues using RIPA lysis buffer (Applygen Technologies Inc., Beijing, China) containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS and protease inhibitor cocktail (Roche, Basel, Switzerland). Then protein concentration was determined by using a BCA assay (Applygen Technologies Inc.). Protein lysates (cell for 30 µg per lane, tissue for 45 µg per lane) were separated on 10% SDS-PAGE under denatured conditions. The separated samples were transferred to PVDF membranes and exposed to primary antibodies included anti-15-LOX-2 (1:100), anti-ALX receptor (1:200), anti-p38 MAPK (1:1000), anti-phospho-p38MAPK (1:1000), anti-β-actin (1:1000) and β-tubulin (1:1000). The membranes were subsequently incubated with a HRP-conjugated secondary antibody (1:10000; Pierce, Rockford, IL, USA) for 1 h at room temperature and visualized using enhanced chemiluminescence (Pierce) and X-ray film.

Matrigel invasion assay

Effect of LXA₄ on the invasion ability of ESCs was determined using Matrigel invasion chambers (pore size: 8 mm, 24-well; BD Biosciences, Franklin Lakes, NJ, USA) following the manufacturer's protocol. The cells were trypsinized after serum-starvation for 24 h, and 1 × 10⁵ cells plated on transwell chambers pre-coated with 20 µg Matrigel. 500 µL medium-containing 10% FBS in the lower chamber served as chemotactant. After 24 h of co-incubation with LXA₄ (100 nM) or ethanol as a vehicle, cells that have invaded through the Matrigel and the 8.0 µm pore size membrane were fixed with 95% ethanol, stained by using crystal violet and counted.

ALX receptor and MMP-9 gene silencing in ESCs

The cells were transiently transfected with small interfering RNA (siRNA) against human ALX receptor or MMP-9 using TurboFect™ siRNA Transfection Reagent (Fermentas, Glen Burnie, MD, USA) according to the manufacturer's instructions. ALX receptor siRNA were 19-nucleotide long duplexes designed and synthesized by Shanghai GenePharma (Shanghai, China). The ALX receptor siRNA sequence is 5'-GAGGG AUUAUCCGGUUUGU-3' (forward) and 5'-ACAAACCGG AUAAUCCUC-3' (reverse). The MMP-9 siRNA sequence is 5'-UCACCUUCACUCGCGUGUA-3' (forward) and 5'-UACA

CGCGAGUGAAGGUGA-3' (reverse). As a negative control, a non-specific siRNA duplex with a random nucleotide sequence (Shanghai GenePharma) was used.

Statistical analysis

Statistical analysis was performed using SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). Results are reported as the mean ± SEM. After testing the data for normal distribution and equal variance, differences between two groups were analysed by Student's unpaired *t*-test, and differences between multiple groups were analysed by one-way ANOVA. Each assay was repeated in triplicate. *P* < 0.05 was considered statistically significant.

Results

Decreased LXA₄ levels and 15-LOX-2 expression in human endometriotic tissues

The levels of LXA₄ in normal endometrial (control) and endometriotic tissues (EM) were detected by ELISA. As shown in Figure 1A, both in proliferative phase and secretory phase, the levels of LXA₄ in EM group (*n* = 16) were significantly decreased compared with control group (*n* = 10; *P* < 0.01), but no difference was found in serum sample (data not shown). In proliferative phase, 15-LOX-2 mRNA expression was shown to be significantly reduced in EM group (*n* = 9) compared with control group (*n* = 10; *P* < 0.01; Figure 1B), but no

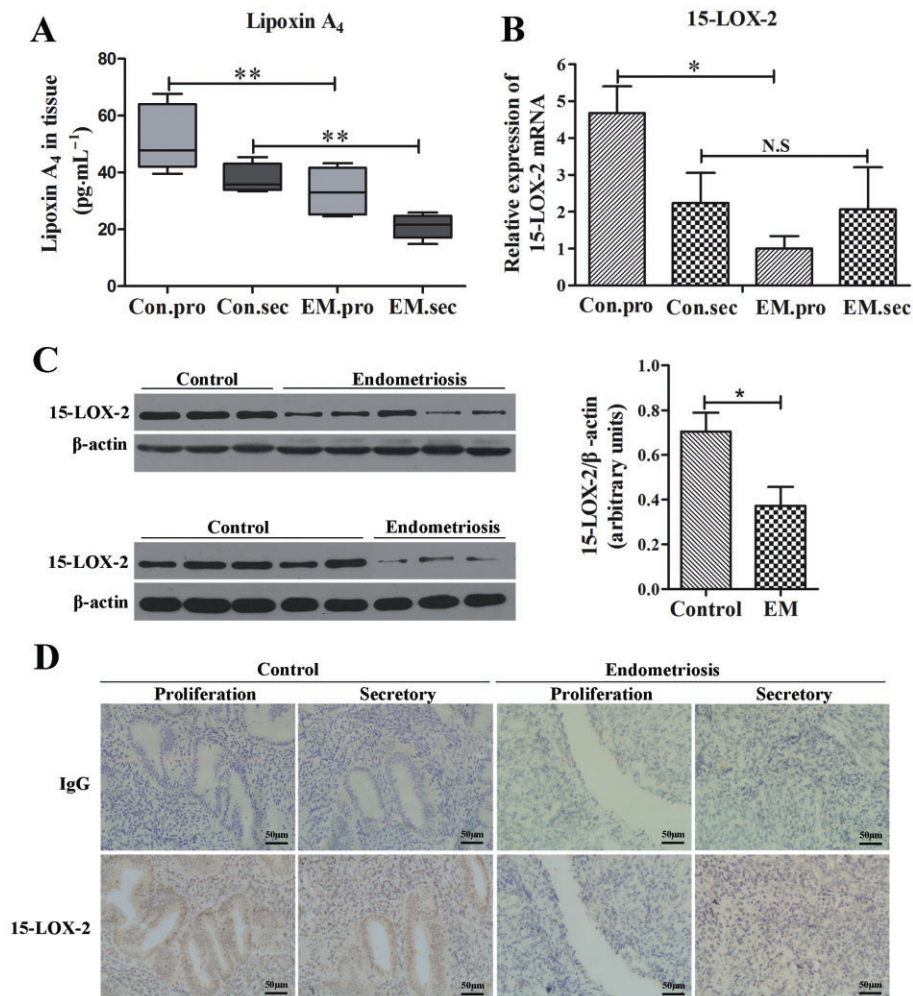


Figure 1

The expression of LXA₄ and 15-LOX-2 is down-regulated during the menstrual cycle in human endometriotic tissues. (A) LXA₄ levels in normal endometrial (control, *n* = 10) and endometriotic tissues (EM, *n* = 16) were evaluated by ELISA. (B) Gene expression of 15-LOX-2 was detected by real-time RT-PCR and normalized to GAPDH expression. 15-LOX-2 mRNA expression was significantly lower in the EM group (*n* = 8) versus control group (*n* = 9) in the proliferative phase, but no difference was found in the secretory phase. (C) Western blot analysis of 15-LOX-2 protein expression between normal endometrial (control, *n* = 8) and endometriotic tissues (EM, *n* = 8). Ratios of 15-LOX-2 to β-actin were determined following densitometry measurements of the specific protein bands. (D) Immunohistochemical staining for 15-LOX-2 in the human endometriotic tissues (EM, *n* = 12) and normal endometrium (control, *n* = 10) both in the proliferative phase and secretory phase (×400), scale bar = 50 μm. Sections were immunostained with 15-LOX-2 or IgG. Data are presented as means ± SEM; **P* < 0.05, ***P* < 0.01.

difference was found in secretory phase. We also analysed the expression and distribution of 15-LOX-2 by Western blot and immunohistochemistry. As shown in Figure 1C, 15-LOX-2 expression was shown to be significantly decreased in EM group ($n = 8$) compared with control group ($n = 8$; $P < 0.01$), and 15-LOX-2 was found in nucleus of glandular and stromal cells (Figure 1D). These data suggest that the low levels of LXA₄ may relate to abnormal expression of 15-LOX-2 in endometriotic tissues.

Up-regulation of ALX receptors in human ectopic endometrium

As shown in Figure 2A, the mRNA expression of ALX receptors in endometrium with EM was significantly higher than in control endometrium by RT-PCR ($P < 0.05$; Figure 2A). Real-time RT-PCR results further showed that, in proliferative phase, there was a 2.4-fold increase in mRNA expression of ALX receptor in EM group ($n = 9$) compared with that expressed in control group ($n = 8$; $P < 0.05$; Figure 2B). Immunolocalization of ALX receptors showed positive cytoplasmic staining in glandular epithelial and stromal cells in endometrium (Figure 2C). The results demonstrated a significant increase of ALX receptor protein expression in ectopic endometrial tissues compared with in the control endometrium. The expression of ALX receptor in ESCs was also examined. RT-PCR analysis, immunocytochemical staining and immunofluorescence assay demonstrated that the ALX receptor was expressed in ESCs (Figure 2D–F). Western blot analysis showed that ALX receptor protein expression in ESCs was significantly higher than in control endometrial stromal cells (NESCs; $P < 0.05$; Figure 2G). These results suggest that higher expression of ALX receptors in the ESCs derived from women with EM may be associated with their unique biological characteristics.

LXA₄ inhibits IL-1 β -induced cytokines release of ESCs via ALX receptors

In previous studies, we have demonstrated that the expression of ALX receptors in ESCs was higher than in NESCs. Here, we found that IL-1 β could up-regulate ALX receptor mRNA and protein expressions in ESCs (Figure 3A). To investigate whether the ALX receptor is involved in LXA₄ effect on cytokines release induced by IL-1 β in ESCs, the Boc-2 peptide, an effective antagonist of the ALX receptor, was used to block ALX receptor function. Pre-exposure of ESCs with Boc-2 (100 $\mu\text{mol}\cdot\text{L}^{-1}$, 15 min) abolished LXA₄ effect on IL-1 β -induced gene and protein expressions of IL-6, IL-8, MCP-1, TNF- α and VEGF (Figure 3B–D), showing the involvement of ALX receptors in LXA₄ anti-inflammatory actions on human ESCs.

LXA₄ suppresses the proliferative activity and cell cycle progression of ESCs

To investigate the role of LXA₄ in proliferative activity of ESCs, the EdU incorporation assay, cell cycle analysis and proliferating cell nuclear antigen (PCNA) staining were conducted after ESCs were treated with LXA₄ (100 $\text{nmol}\cdot\text{L}^{-1}$; Figure 4). Following pretreatment with LXA₄, the percentage of EdU-positive cells was reduced in ESCs compared with the controls (Figure 4A and B). Moreover, the proportion of cells

corresponding to the proportion of cells in the S and G₂/M phase was lower in LXA₄-treated ESCs (40.19 vs. 5.51%) than that in control cells (48.61 vs. 13.31%; Figure 4C). The G₀/G₁ phase was increased in LXA₄-treated ESCs (54.30%) compared with control cells (38.09%). It seems that treatment with LXA₄ resulted in cells that were blocked in G₀/G₁ phase and could not enter into S phase for DNA synthesis. Further analysis revealed that LXA₄ significantly decreased the proliferation index of ESCs ($P < 0.05$) (Figure 4D). PCNA is retained one of the most important index to estimate cell proliferation. The positive nuclear PCNA staining in LXA₄-treated ESCs was fewer than in control cells (Figure 4E). Counts of PCNA-positive cells were significantly decreased in LXA₄-treated ESCs compared with control cells ($P < 0.05$; Figure 4F).

LXA₄ attenuates the migration and invasion of ESCs

We first assessed the effect of LXA₄ on the migration in ESCs by wound healing assay. As shown in Supporting Information Fig. S2A and B, pretreatment with LXA₄ (100 nM) for 48 h resulted in a remarkable decrease in cell migration ability relative to control group ($P < 0.05$). We further investigated the effect of LXA₄ on the invasion of ESCs. As shown in Figure 5A and B, pretreatment with LXA₄ (10 and 100 nM) significantly reduced IL-1 β -stimulated (1 $\text{ng}\cdot\text{mL}^{-1}$) the mRNA and protein expressions of MMP-2 and MMP-9 in ESCs. To examine the effect of LXA₄ on IL-1 β -induced activities of MMP-2 and MMP-9 of ESCs, gelatin zymography assay was used. Only MMP-9 activity was detected in cell culture supernatants, which could be induced by IL-1 β in a dose-dependent manner. Treatment with LXA₄ (10 nM) significantly inhibited MMP-9 activity in ESCs stimulated by IL-1 β (1 $\text{ng}\cdot\text{mL}^{-1}$; $P < 0.05$) (Figure 5C). Cell Matrigel invasion assays were performed to evaluate the change in invasive ability of ESCs after LXA₄ treatment (100 nM), and the results showed that LXA₄ significantly decreased the invasive activity of ESCs ($P < 0.05$; Figure 5D). These results imply that LXA₄ could attenuate the invasive activity of ESCs by inhibiting the activity of MMP-9.

LXA₄ inhibits IL-1 β -induced phosphorylation of p38 MAPK of ESCs in an ALX receptor-dependent manner

We first examined the phosphorylated p38 MAPK immunoreactivity in both normal and ectopic endometrial tissues (Figure 6A). As was shown, phospho-p38 MAPK expressions were mainly detected in ESCs of ectopic endometrial tissues, which were barely detected in normal endometrial tissues. Phosphorylated/total p38 MAPK ratio was significantly higher in peritoneal cells of EM mice than in the control mice ($P < 0.01$; Figure 6B). Similarly, we also found that the phosphorylation level of p38 MAPK in ESCs was significantly higher than in NESCs ($P < 0.05$; data not shown).

In vitro, IL-1 β (1 $\text{ng}\cdot\text{mL}^{-1}$) significantly stimulated the phosphorylation of p38 MAPK in ESCs ($P < 0.01$; Figure 6C). To further clarify the effect of LXA₄ on the phosphorylation of p38 MAPK, ESCs were pretreated with LXA₄ or vehicle (0.01% ethanol) for 30 min before a further stimulation with IL-1 β for 15 min. Pretreatment with LXA₄ (10 nM) significantly reduced IL-1 β -stimulated phosphorylation of p38

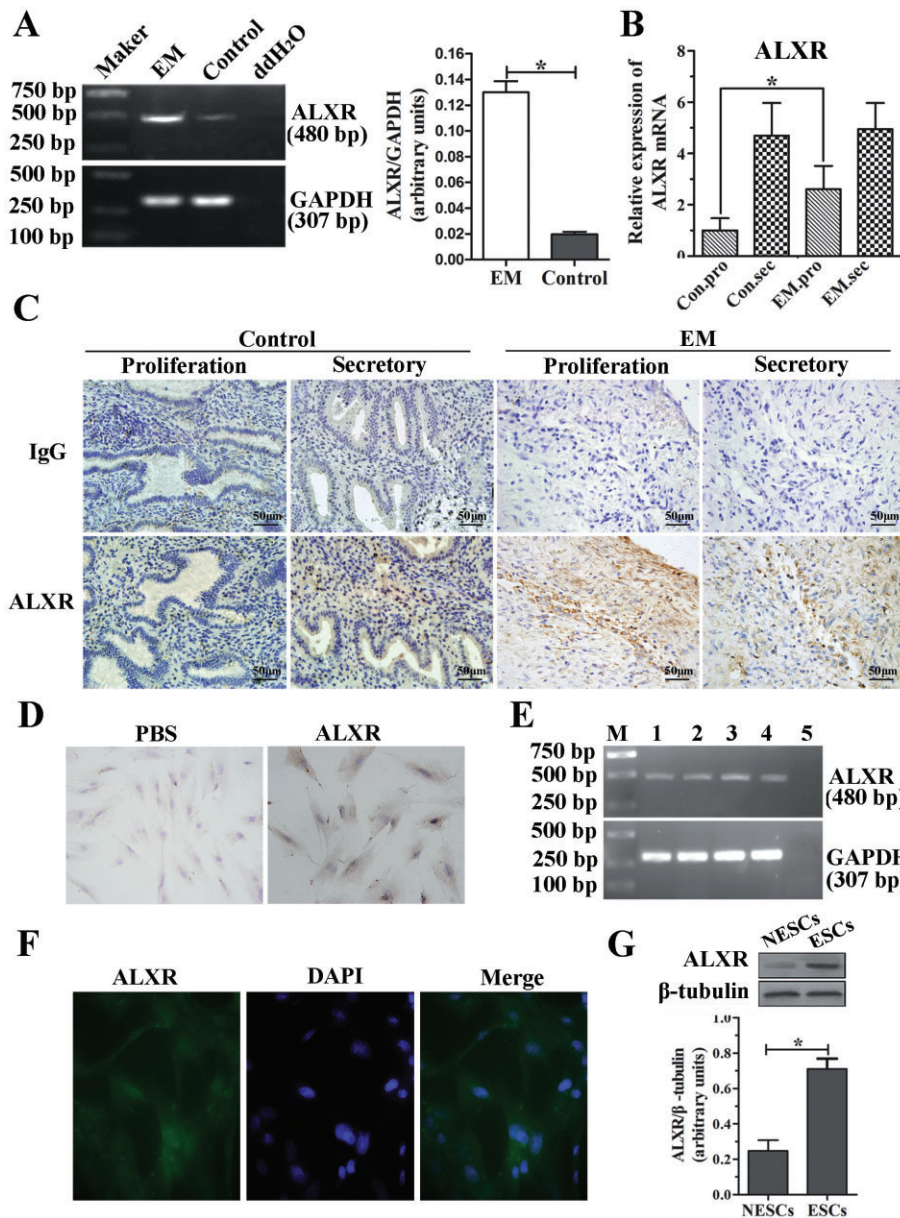


Figure 2

ALX receptor mRNA and protein are increased in endometriotic tissues and ESCs. (A) RT-PCR amplification of a 480 bp fragment from ectopic endometrial tissues and normal endometrium. Densitometric analysis of results are shown in column graph. (B) The ALX receptor mRNA expression was verified further in both control ($n = 20$) and ectopic endometrial tissues ($n = 27$) by real-time RT-PCR. The mRNA expression of ALX receptors was significantly greater in the EM group ($n = 8$) versus control group ($n = 9$) in the proliferative phase, but no difference was found in the secretory phase, $*P < 0.05$. (C) Immunohistochemical staining for ALX receptor in the human endometriotic tissues (EM, $n = 12$) and normal endometrium (control, $n = 10$). Sections were immunostained with anti-human ALX receptor antibody (ALXR) and IgG, scale bar = 50 μm . (D and E) Expression of ALX receptors in ESCs detected by immunocytochemical staining (D, $\times 400$), RT-PCR (E), and immunofluorescence assay (F, $\times 400$). (G) Western blot analysis of ALX receptor protein expression between normal (NESC) and ectopic (ESC) cells. A ratio of ALX receptor to β -tubulin was determined following densitometric measurements of the specific protein bands. Values are the mean \pm SEM of the combined data from three independent experiments; $*P < 0.05$.

MAPK ($P < 0.01$; Figure 6C). SB203580, a p38 MAPK specific inhibitor, significantly inhibited the phosphorylation of p38 MAPK induced by IL-1 β ($P < 0.001$). Interestingly, the inhibitory effect of SB203580 was also found in the peritoneal cells of EM mice (Figure 6B). To determine whether ALX receptor mediates LXA₄ attenuation of p38 MAPK phosphorylation in

ESCs induced by IL-1 β , we used ALX receptor-specific siRNA to silence the expression of ALX receptor by RNA interference. Transfection of ESCs with ALX receptor siRNA for 48 h resulted in strong reduction of ALX receptor mRNA and protein levels relative to mock-transfected cells (mock) or cells transfected with scrambled siRNA (control siRNA);

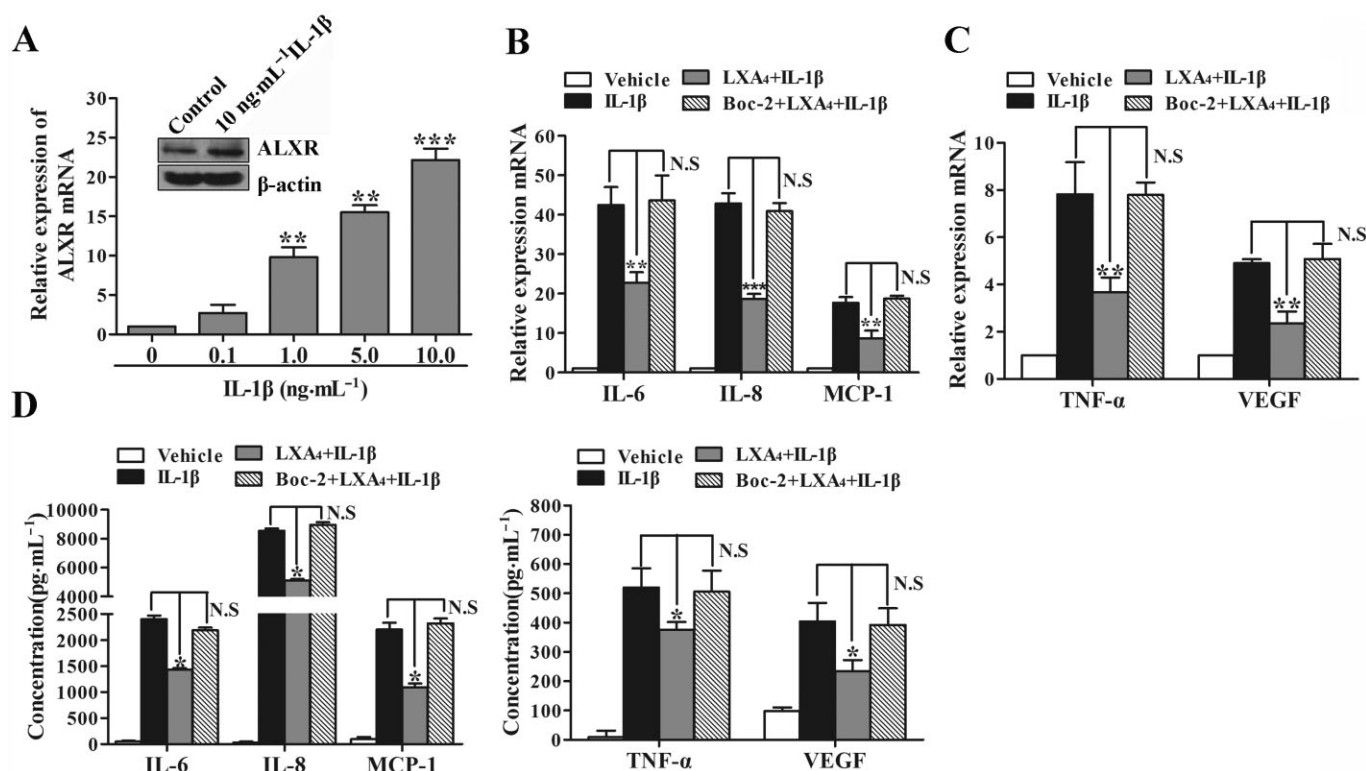


Figure 3

LXA₄ inhibition of IL-1β-induced cytokine release in ESCs is mediated by the ALX receptor. (A) ESCs were stimulated with IL-1β (0.1 to 10 ng·mL⁻¹) or vehicle (medium) for 24 h, and mRNA expression of the ALX receptor was determined by real-time PCR. Inset is a representative Western blot result showing that expression of ALX receptor protein is induced by IL-1β (10 ng·mL⁻¹). (B–D) ESCs were incubated with Boc-2 (100 μmol·L⁻¹, 15 min) or vehicle, then exposed to LXA₄ (10 nM, 30 min) and stimulated with IL-1β (1 ng·mL⁻¹, 24 h). Total RNA extracts were generated and converted into cDNA. The mRNA expressions of IL-6, IL-8, MCP-1, TNF-α and VEGF were detected by real-time PCR (B and C). Concentrations of IL-6, IL-8, MCP-1, TNF-α and VEGF were measured in supernatants by ELISA (D). Results were expressed as mean ± SEM of at least three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. N.S., no significant difference.

Figure 6D). LXA₄ had no effect on IL-1β-induced phosphorylation of p38 MAPK in ESCs transfected with ALX receptor siRNA (Figure 6D).

LXA₄ suppresses the growth of endometriotic lesion and the expressions of inflammatory factors in EM mouse model

To investigate the effect of LXA₄ on the progression of EM, we measured the weight and size changes of endometriotic lesions in EM mouse model after being treated by LXA₄ for 21 days. The results showed that LXA₄ inhibited the growth of endometriotic lesions in EM mice (Figure 7A–C). The weight of endometriotic lesions in the LXA₄ group (22.62 ± 12.66 mg) was significantly less than in EM group (67.62 ± 13.44 mg; *P* < 0.05; Figure 7B). LXA₄ also decreased the size of endometriotic lesions from 8.76 ± 1.88 mm³ to 3.38 ± 1.23 mm³ (*P* < 0.05) (Figure 7C).

Furthermore, we investigated the effects of LXA₄ on synthesis of IL-6, MCP-1 and VEGF in EM mice. The results showed that high levels of IL-6, MCP-1 and VEGF were observed in EM mice compared with control mice. LXA₄ significantly decreased the mRNA levels of IL-6, MCP-1 and VEGF in both endometriotic lesions and peritoneal cells of

EM mice (Figure 7D and E). The concentrations of IL-6, MCP-1 and VEGF in the peritoneal fluid were significantly lower in LXA₄ group than in EM group (*P* < 0.05). LXA₄ decreased the concentrations of IL-6 from 386.82 ± 49.33 pg·mL⁻¹ to 262.25 ± 29.12 pg·mL⁻¹, MCP-1 from 319.10 ± 19.26 pg·mL⁻¹ to 205.43 ± 26.36 pg·mL⁻¹ and VEGF from 250.09 ± 25.92 pg·mL⁻¹ to 105.51 ± 15.60 pg·mL⁻¹ (Figure 7F).

Discussion

15-LOX is an intracellular enzyme which has two distinct isoforms: 15-LOX-1 and 15-LOX-2. In terms of enzymatic characteristics, 15-LOX-1 preferentially metabolizes linoleic acid to 13-(S)-HODE, but also metabolizes AA to 15-hydroxyeicosatetraenoic acid (15-HETE). 15-LOX-2, on the other hand, converts AA exclusively to 15-HETE and plays a key role in the synthesis of LXA₄. The majority of earlier research on the role of 15-LOX focused on 15-LOX-1. For example, 13-(S)-HODE, the 15-LOX-1 metabolite, played an important role in activating the MAPK signalling pathway and subsequently decreasing PPAR expression in human colorectal carcinoma cells (Hsi *et al.*, 2002). However, growing

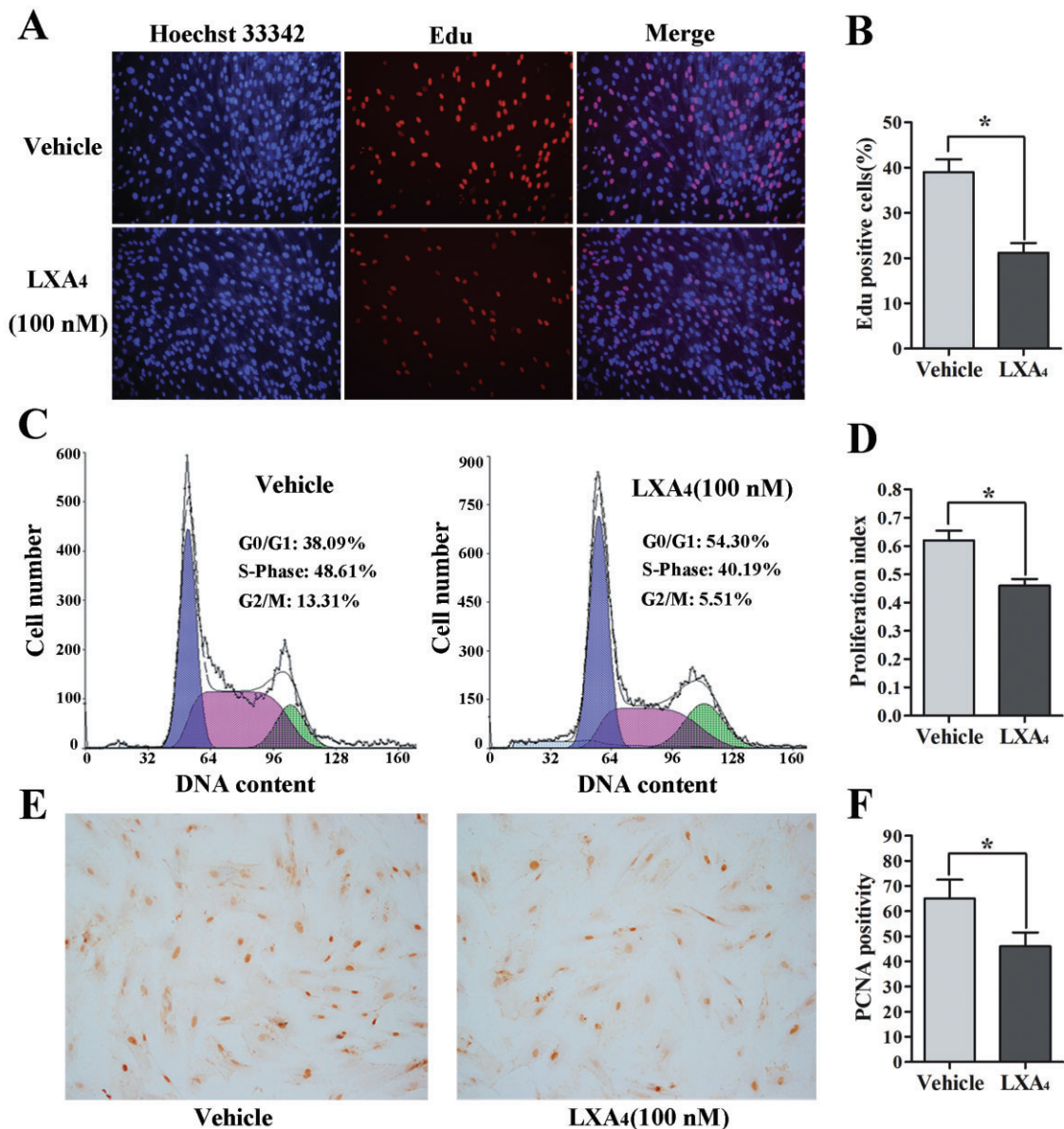


Figure 4

LXA₄ inhibits the proliferative activity of ESCs. (A) LXA₄ (100 nmol·L⁻¹) inhibited DNA replication in ESCs compared with controls as determined by the EdU incorporation assay, Original magnifications: ×200. EdU-positive cell counts were analysed by using software of the light microscope (B), *P < 0.05. (C) Cell cycle analysis indicated the increased population of cells in the G₀/G₁ phase and decreased S and G₂/M phase cells in ESCs treated with LXA₄ (100 nmol·L⁻¹). (D) Proliferation index = (S phase + G₂/M phase)/(G₀/G₁ phase + S phase + G₂/M phase). (E) LXA₄ decreased PCNA protein expression in ESCs detected by immunocytochemical staining. Original magnifications: ×100. PCNA-positive cell counts were analysed by using software of the light microscope (F). These experiments were done three times using different batches of cells and data are means ± SEM; *P < 0.05.

evidence showed that 15-LOX-2 and its metabolite (15-HETE) exerted an important role in some diseases. It was reported that the expression of 15-LOX-2 mRNA and protein declined in the prostate cancer tissue compared with the normal tissue (Tang *et al.*, 2009), suggesting 15-LOX-2 may play an important role in the development of prostate cancer, and 15-HETE could inhibit the MAPK signalling pathway and up-regulate a downstream target of MAPK signalling like PPAR (Hsi *et al.*, 2002). Interestingly, oestrogen negatively regulated the

intrinsic LXA₄ formation, especially ERβ-specific regulation of epithelial 15-LOX in the cornea, could provide novel mechanism that 15-LOX was down-regulated in some inflammatory disease (Wang *et al.*, 2012). The present study found that 15-LOX-2 existed in the endometriotic tissues, which was previously shown (Russell *et al.*, 2011), but the expression of 15-LOX-2 decreased comparing with control endometrial tissues in both proliferative and secretory phase. Collectively, the decreased levels of LXA₄ in endometriotic tissues suggest

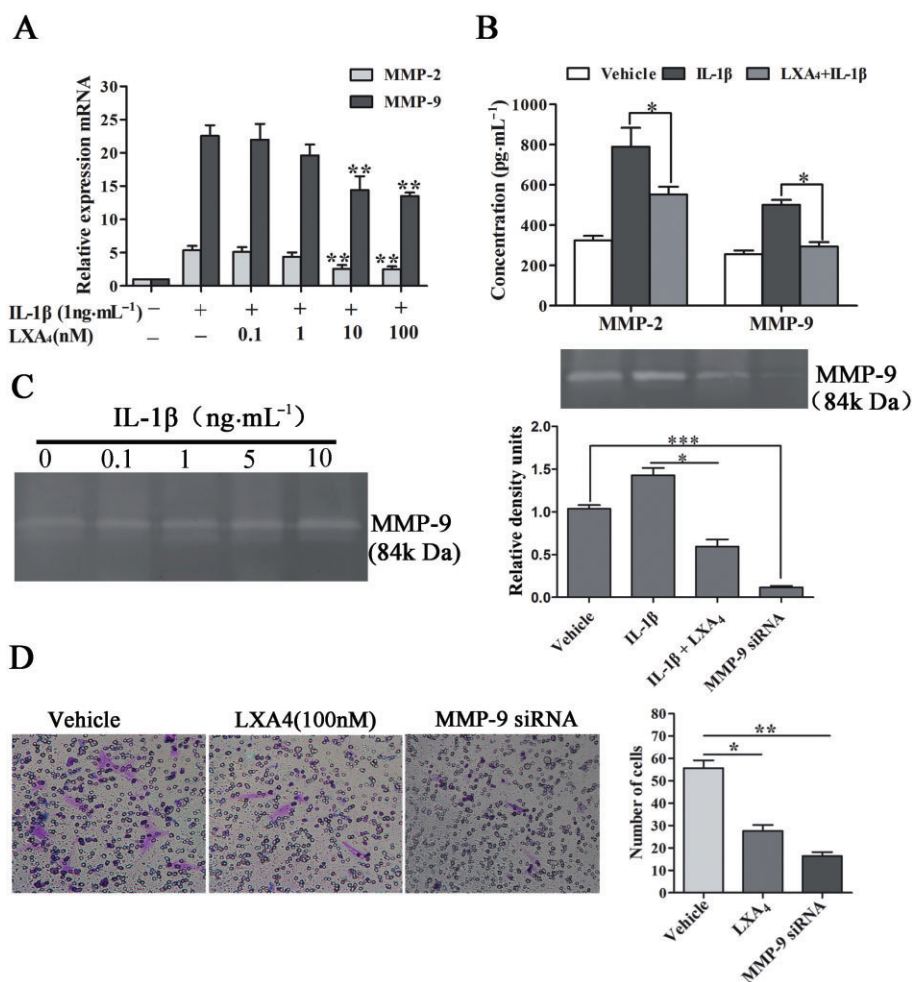


Figure 5

LXA₄ attenuates the invasion activity of ESCs by reducing the expression of MMP-9. (A and B) ESCs were stimulated with IL-1 β (1 ng·mL⁻¹) for 15 min or pre-incubated with LXA₄ (0.1 to 100 nM) for 30 min before IL-1 β treatment. The mRNA and protein expression of MMP-2 and MMP-9 were determined by real-time PCR and ELISA. (C) ESCs were stimulated with IL-1 β (0.1 to 10 ng·mL⁻¹) or vehicle (medium) for 24 h, and protein activities of MMP-9 was assessed by the Gelatin Zymography assay (left). The IL-1 β -increased MMP-9 was significantly inhibited by LXA₄ (right), (compared with IL-1 β alone). (D) Cell Matrigel invasion assays indicated the decreased number of ESCs, which invaded from upper chamber to lower chamber treated with LXA₄ (100 nM). The band intensities were analysed using Quantity One software. Data are presented as means \pm SEM; * P < 0.05, ** P < 0.01, *** P < 0.001.

that the endometrium is a source of LXA₄ production and decreased expression of 15-LOX-2 may lead to the reduction of LXA₄ in endometriotic tissues.

It has been demonstrated that ectopic endometrial cells need to undergo a three-step procedure (attachment-aggression-angiogenesis) to develop into EM (Robboy and Bean, 2010). Inflammatory reactions during this process might be associated with the development of EM. IL-1 β was well known to play a critical role in the pathogenesis of this disorder by inducing the growth, adhesion, invasiveness and angiogenesis of endometrial fragments outside the uterus. IL-6 was a multifunctional cytokine participating in immune response and had angiogenic effects, some of which were mediated via the induction of VEGF expression (Wu and Ho, 2003). Many studies have reported increased expression of IL-6 in EM (Umezawa *et al.*, 2008). IL-8, a CXC chemokine, was elevated in women with EM and the levels correlate with

the severity of the disease (Ulukus *et al.*, 2005). MCP-1 was a chemotactic cytokine that could influence both innate immunity through its effects on monocytes and adaptive immunity via T helper cell polarization (Gu *et al.*, 2000). Findings indicated that MCP-1 might result in the development of immunotolerance by increasing apoptosis of leukocytes and thereby supporting the survival of ectopic endometrial cells (Selam *et al.*, 2006). VEGF was an important vasoactive growth factor being involved in the development of peritoneal EM (Dziunycz *et al.*, 2009). Our previous studies have found an increase production of MMP2 and MMP-9 in EM mice, which might associate with the development of EM (Chen *et al.*, 2010b). In the present study, we also observed that IL-1 β stimulated the release of IL-6, IL-8, MCP-1, TNF- α and VEGF in ESCs (Supporting Information Fig. S3). Together, inflammation plays an important role in the pathophysiological processes of EM.

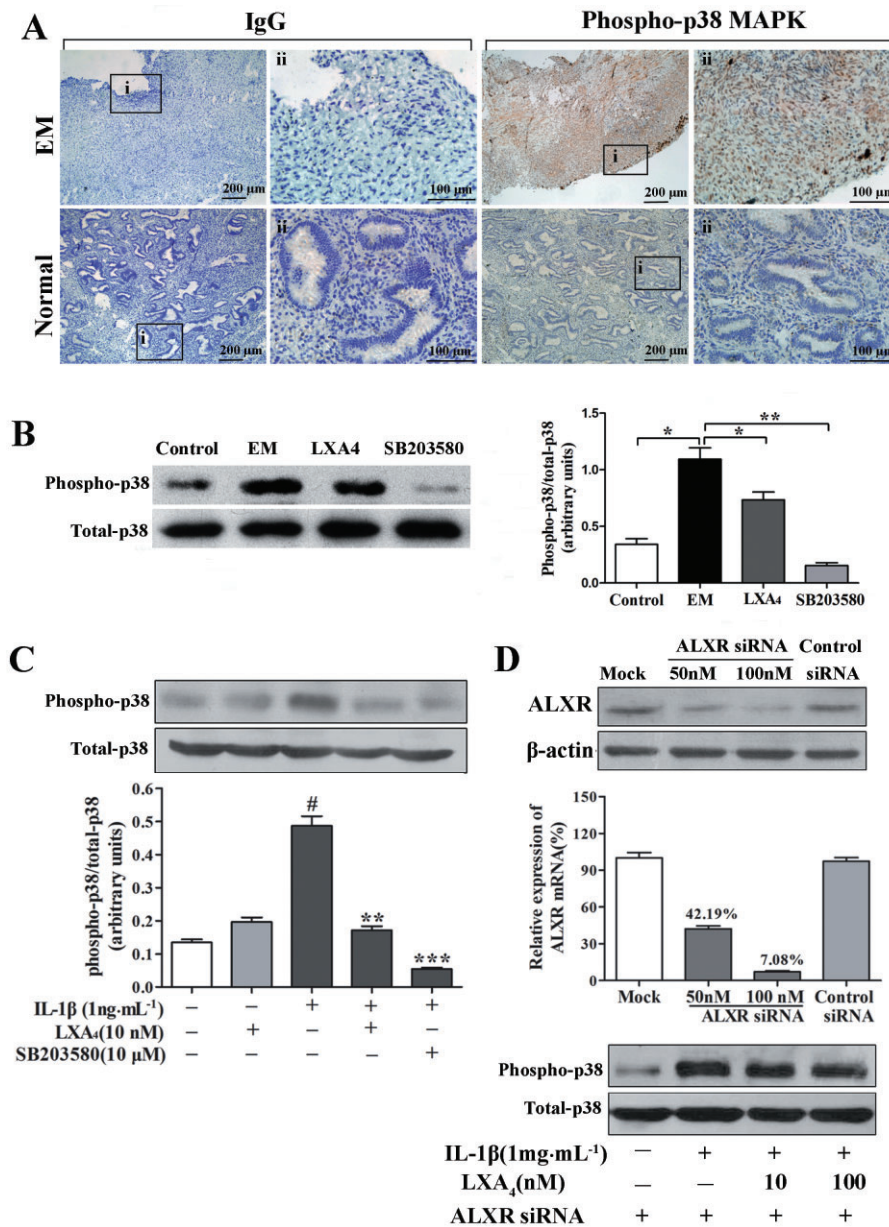


Figure 6

LXA₄ inhibition of IL-1 β -induced phosphorylation of p38 MAPK in ESCs is ALX receptor-dependent. (A) Immunohistochemical staining for phosphorylated p38 MAPK in ectopic endometrial tissues (EM, $n = 12$) and normal endometrial tissues (control, $n = 10$). Sections were immunostained with phosphorylated p38 MAPK (phospho-p38 MAPK) antibody and IgG. Original magnification: (i) $\times 100$ and (ii) $\times 400$. (B) A representative Western blot result demonstrated that expression of phospho-p38 MAPK was inhibited by LXA₄ in peritoneal cells of EM mice. $*P < 0.05$, $**P < 0.01$. (C) IL-1 β -induced p38 MAPK phosphorylation was inhibited by LXA₄. ESCs were pre-incubated with LXA₄ for 30 min before IL-1 β treatment for an additional 15 min. The band intensities were analysed using Quantity One software. $\#P < 0.001$ (compared with vehicle); $**P < 0.01$, $***P < 0.001$ (compared with IL-1 β alone). (D) ALX receptor knockdown with siRNA blocked the inhibitory effect of LXA₄ on IL-1 β -induced p38 MAPK phosphorylation in ESCs. Cells were transiently transfected with siRNA duplexes for the ALX receptor or scrambled siRNA (control) for 48 h. ALX receptor mRNA and protein levels significantly decreased in the presence of ALX receptor siRNA (D) Mock: the cells were transfected with transfection reagent alone. A representative Western blot result showed that LXA₄ had no effect on IL-1 β -induced p38 MAPK phosphorylation in ESCs transfected with ALX receptor siRNA. Experiments were repeated in ESCs from at least three different subjects.

Recently, LXA₄ was described a anti-inflammatory and pro-resolution lipid mediator, which elicits its actions via the GPCR ALX receptor (Serhan and Chiang, 2008a). LXA₄ and its receptor ALX receptor involved regulating inflammatory

events in the human endometrium and decidua of early pregnancy (Macdonald *et al.*, 2011). The ectopic endometrial tissues from women with EM showed an elevated expression of LXA₄ receptor compared with the normal tissues

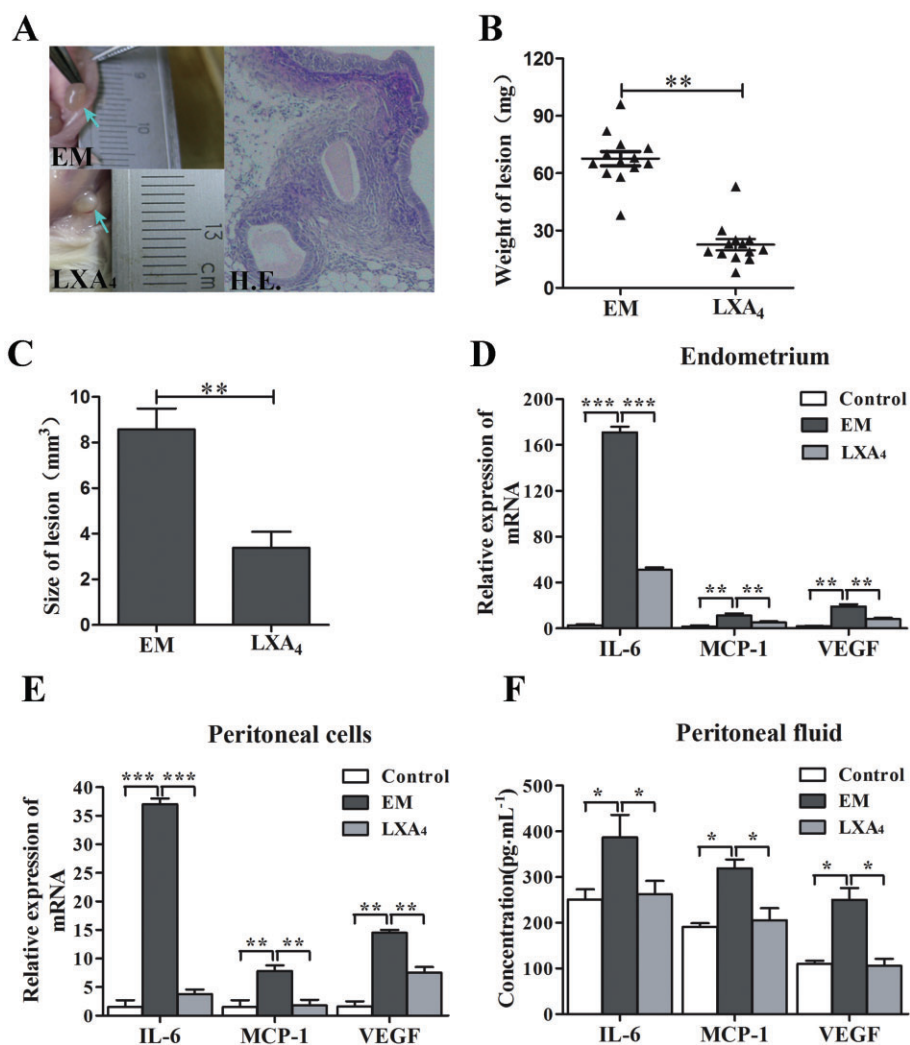


Figure 7

LXA₄ inhibits the proliferative activity of endometriotic lesions and the production of IL-6, MCP-1 and VEGF in EM mice. (A) LXA₄ suppressed the growth of the endometriotic lesions in EM mice. Three days before the EM induction, the LXA₄ group was i.p. injected LXA₄ at a dose of 5 $\mu\text{g}\cdot\text{kg}^{-1}$ on a daily basis for 24 days. Arrows indicate endometriotic lesions. The weight (B) and size (C) of endometriotic lesions were significantly reduced by LXA₄ treatment. Horizontal bars represent the mean weight of endometriotic lesions. * $P < 0.05$. (D and E) The mRNA expressions of IL-6, MCP-1 and VEGF in both endometrial tissues and peritoneal cavity cells were detected by real-time PCR. (F) Concentrations of IL-6, MCP-1 and VEGF in peritoneal fluids were measured by ELISA. Results are expressed as mean \pm SEM of at least three independent experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

(Motohashi *et al.*, 2005; Canny and Lessey, 2013). The present finding also demonstrated a significant increase of ALX receptor gene and protein expression in ectopic endometrial tissues compared with the control endometrium. ALX receptor protein expression in ESCs was significantly higher than in normal endometrial stromal cells (NESC). Therefore, we speculated that the high expression of ALX receptor in EM patients was responsible for responding to the inflammatory reactions, although the LXA₄ levels in ectopic endometrial tissues were significantly decreased. These findings suggest a possible role of LXA₄ and its receptor under pathological condition as EM. In another study, we found that LXA₄ could regulate ER in ESCs. Treatment with LXA₄ (100 nM) for 48 h, LXA₄ could attenuate ER α and augment ER β expression in ESCs, and inhibit oestrogen response

element activity (data not shown). The results suggest that LXA₄ may exert its biological function in EM via ALX receptor or ER β .

LXA₄ has been previously demonstrated to elicit anti-inflammatory, anti-proliferative, and anti-angiogenic actions in a variety of cell types, including epithelial cells, endothelial cells, fibroblasts, by reducing the expression of inflammatory factors and growth factors (Baker *et al.*, 2009; Janakiram *et al.*, 2011). In the present study, the elevated expressions of proinflammatory cytokines in IL-1 β -induced ESCs were significantly inhibited by pre-treatment with LXA₄. It has been shown that LXA₄ exerted its biological actions through its specific receptor named FPRL1 or ALX receptor. In this study, we found that Boc-2, an antagonist of ALX receptor, could abolish inhibition of LXA₄ on IL-1 β -induced expressions of

IL-6, IL-8, MCP-1, TNF- α and VEGF in ESCs. These data indicate the involvement of ALX receptor in LXA₄ anti-inflammatory actions on EM.

In *in vivo* experiment, LXA₄ could suppress the weight and size of endometriotic lesions in EM mouse model. In *in vitro* experiment, we further found that LXA₄ could inhibit the DNA replication and cause G₀/G₁ phase cell cycle arrest in ESCs, suggesting an anti-proliferative potential of LXA₄ with endometriotic lesion. LXA₄ could also down-regulate synthesis of PCNA protein in ESCs. PCNA is a cofactor of DNA polymerases that coordinates cell proliferation by recruiting crucial players to the DNA replication fork (Moldovan *et al.*, 2007). It was reported that the positive nuclear PCNA staining of human endometrium in women with EM showed a significant persistence compared with in fertile healthy women (Hapangama *et al.*, 2009). Therefore, PCNA may play an important role in the development of EM, and LXA₄ may decrease the proliferative activity of endometriotic lesion by inhibiting the synthesis of PCNA. Moreover, some evidence indicated that LXA₄ could play a critical role in regulating cell invasion and migration (Zhou *et al.*, 2009; Hao *et al.*, 2011), and few was reported about its role in EM. In the present study, we also observed that LXA₄ attenuated the invasive activity of ESCs possibly by inhibiting the activity of MMP-9, which was consistent with the previous reported *in vivo*. Wound healing assay also demonstrated that LXA₄ attenuated the ESCs migration ability. Therefore, these results imply that LXA₄ may be involved in suppressing the implantation of endometriotic cells in ectopic sites.

P38 MAPK, an intracellular signal-transducing molecule, has been demonstrated to involve in the regulation of many cellular processes including inflammatory reaction, cell differentiation, cell proliferation and cell death. Activation of p38 MAPK by extracellular stimuli, such as bacterial pathogens and cytokines, mediates signal transduction into the nucleus to turn on the responsive genes (Ono and Han, 2000). In the present study, we found that IL-1 β significantly stimulated the phosphorylation of p38 MAPK in endometriotic cells. High levels of phosphorylated p38 MAPK were also observed in human ectopic endometrial tissues. These findings suggest that p38 MAPK may serve as an essential signal transducer and thereby play a key role in the pathogenesis of EM. P38 MAPK signalling pathway was reported to involve inhibition of pro-inflammatory cytokine production in endothelial cells by LXA₄ (Wu *et al.*, 2008). In the present investigation, the attenuation of IL-1 β -induced p38 MAPK phosphorylation by LXA₄ in ESCs was observed. In addition, the RNA interference experiment demonstrated that LXA₄ had no effect on IL-1 β -induced phosphorylation of p38 MAPK in ESCs transfected with ALX receptor siRNA. These data suggest that LXA₄ may reduce the expression of pro-inflammatory cytokines in EM by inhibiting p38 MAPK activation, which was AXL receptor-dependent.

In summary, our findings have uncovered a novel role of LXA₄ in regulating the pathological processes of EM. These results demonstrated that LXA₄ markedly suppressed the development of EM by inhibiting activation of p38 MAPK through the ALX receptor, which led to the down-regulation of pro-inflammatory cytokines and proteolytic factors. Cross-talk between these factors presents a mechanism for the inhibitory effects of LXA₄ in EM. In addition, these studies

point to a novel therapeutic strategy for endogenous anti-inflammatory lipid mediators in EM.

Acknowledgements

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Author contributions

R. W. and W. Z. contributed equally to this work. They mainly achieved all the experimentation. S. C., Y. S., L. S. and M. Z. participated in clinical material collection, cell culture, ELISA test, immunohistochemistry. Q. C. and Q. C. contributed towards designing of this study, supervision of experiments, interpretation of data and composed the article.

Conflict of interest

None declared.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

<http://dx.doi.org/10.1111/bph.12816>

Figure S1 The immunocytochemistry identified of human endometrial glandular epithelial and stromal cells (40×), scale bar = 50 μm. (A) Endometrial glandular epithelial cells (left, negative control IgG; right, CK19); (B) Endometrial stromal cells (left, negative control IgG; right, vimentin 9); (C) Endometrial stromal cells (left, negative control IgG; right, CD10);

Figure S2 LXA₄ inhibited the migration of ESCs. (A) A wound healing assay was performed on ESCs (5 × 10⁵, 6 cm plate) incubated with either ethanol (control) or LXA₄ (100 nM). The 'wounded' areas were photographed by Nikon Eclipse 50i fluorescent microscope at various time points (0 h, 24 h or 48 h). One representative experiment was shown, scale bar = 50 μm. (B) Quantitative analysis of wound-induced migration assay from (A). The results were presented as mean ± SEM of three experiments done in duplicate and

differences with $P < 0.05$ on the Student's *t*-test were considered statistically significant (*).

Figure S3 LXA₄ inhibits IL-1β-induced expressions of IL-6 (A), IL-8 (B), MCP-1 (C), TNF-α (D), and VEGF (E) mRNA in a dose-dependent manner. ESCs were pretreated for 30 min with vehicle (0.01% ethanol) or with the indicated concentrations of LXA₄ before IL-1β (1 ng·mL⁻¹) treatment for an additional 24 h. Total RNA extracts were generated and converted into cDNA. Changes in the expressions of IL-6, IL-8, MCP-1, TNF-α and VEGF mRNA were detected by real-time PCR. To determine the dose effects of IL-1β on cytokines synthesis of ESCs, cells were stimulated with IL-1β (0.1 to 10 ng·mL⁻¹) or vehicle (medium) for 24 h, and mRNA expressions of IL-6 (A), IL-8 (B), MCP-1 (C), TNF-α (D) and VEGF (E) were also determined by real-time PCR. The results were presented mean ± SEM of three independent experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure S4 According to the manufacturer's instructions and previous reports, appropriate positive control was used with each antibody during the staining run. (A) Immunohistochemical staining for 15-LOX-2 in the human normal renal tissues ($n = 3$). Sections were immunostained with anti-human 15-LOX-2 antibody (15-LOX-2) and negative control (IgG). Scale bar = 50 μm; (B) Immunohistochemical staining for ALX receptor in the human gastric cancer tissues ($n = 3$). Sections were immunostained with anti-human ALX receptor antibody (ALXR) and negative control (IgG). Scale bar = 50 μm; (C) Immunohistochemical staining for Phospho-p38 MAPK in the human gastric cancer tissues ($n = 3$). Sections were immunostained with anti-human Phospho-p38 MAPK antibody and negative control (IgG). Scale bar = 50 μm.

Table S1 Clinical characteristics of patients and controls.

Table S2 Primer sequences for RT-PCR and quantitative real-time PCR.

Appendix S1 Supplemental materials and methods.