Lipoxin A₄ blocks embryo implantation by controlling estrogen receptor α activity

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Abstract

Embryo implantation involves a complex regulatory network of steroid hormones, inflammatory cytokines, and immune cells. Lipoxin A₄ (LXA₄), a biologically active eicosanoid with specific anti-inflammatory and pro-resolving properties, was recently found to be a novel modulator of estrogen receptor α (ER α). In this study, we investigated the potential role of LXA₄ in implantation. We found that LXA₄ blocked embryo implantation in mice and significantly reduced the expression of inflammatory mediators associated with uterine receptivity and embryo implantation, including corticotropin-releasing factor (CRF), cyclooxygenase 2-derived prostaglandin I₂ and prostaglandin E₂, leukemia inhibitory factor, and interleukin 6, but this effect was independent of LXA₄ receptor. Subsequent investigation revealed enhanced ER α activity in the uteri of LXA₄-treated mice during the peri-implantation period. ER α and phosphorylated ER α were significantly increased following LXA₄ treatment. Finally, it was demonstrated that the inhibitory effect of LXA₄ on embryo implantation was mediated through ER α . In the presence of the ER α antagonist ICI 182 780, LXA₄ failed to block embryo implantation. LXA₄ also failed to inhibit CRF expression. These results suggested that LXA₄ blocks embryo implantation by controlling ER α activity, and this effect appeared to be related to the suppression of the inflammatory microenvironment necessary for implantation. *Reproduction* (2013) **145** 411–420

Introduction

Embryo implantation is a crucial step in mammalian reproduction. It can only take place during a very specific time, commonly known as the window of implantation. During this period the uterus undergoes a sequence of cellular and molecular events in the endometrium and differentiates to provide a specialized environment for the incoming embryo (Paria *et al.* 2002). Hormones play central roles in the regulation of uterine environment. The principal hormones that specify uterine receptivity are ovarian steroids estrogen and progesterone (Wang & Dey 2006). Estrogen is essential for an integrated uterine response, and estrogen signaling is primarily mediated through estrogen receptor (ER), ER α and ER β . ER α is dominantly expressed within the uterus, and studies on knockout mice models have revealed that ER α , but not ER β , is necessary for normal fertility (Paria *et al.* 2002, Wang & Dey 2006). However, estrogen activity must be tightly controlled during the peri-implantation period. Estrogen at a low level extends the window of uterine receptivity for implantation, but excessive high estrogen levels can prematurely close this window, transforming the uterus into a refractory state (Ma *et al.* 2003).

Although decidual development is governed by the ovarian steroid hormones, there is increasing evidence for the local immune regulation. Inflammatory micro-environment is closely related to endometrial remodeling during implantation. The fetal–maternal interface is rich in inflammatory cytokines (Jones *et al.* 2004) and dynamically populated with a wide variety of innate immune cells (Hanna *et al.* 2006, Plaks *et al.* 2008). One of the mediators that play important roles in the aseptic inflammatory process of implantation is corticotropin-releasing factor (CRF). CRF, a hypothalamic neuropeptide that modulates the activity of the hypothalamus–pituitary–adrenal axis, is also secreted in peripheral tissues and possesses potent pro-inflammatory properties (Theoharides *et al.* 1998, Venihaki *et al.* 2001, Cao *et al.* 2005). CRF is produced in several organs of the female reproductive system, including endometrial glands, decidualized stroma, and trophoblast (Mastorakos *et al.* 1996, Zoumakis *et al.* 2000). Locally produced CRF might stimulate the expression of FasL, thereby potentiating their ability to induce apoptosis of surrounding activated T lymphocytes (Makrigiannakis *et al.* 2001). Cyclooxygenase 2 (COX2)-derived prostaglandins (PGs) are also important

inflammatory mediators in implantation (Lim *et al.* 1999, Pakrasi & Jain 2008). PGs contribute to successful implantation via their effects on decidual angiogenesis, and *Ptgs2*-deficient mice show multiple reproductive failures, including defective decidualization and impaired implantation (Lim *et al.* 1997). Pro-inflammatory cytokines, such as leukemia inhibitory factor (LIF), interleukin 6 (IL6), IL8, and monocyte chemotactic protein 1 (MCP1), are also involved in the inflammatory response in implantation (Dominguez *et al.* 2005, van Mourik *et al.* 2009). Among the cytokine family, LIF is most pertinent to implantation. Gene targeting studies have showed the essential role of LIF in uterine preparation for implantation (Stewart *et al.* 1992). Implantation involves a complex regulatory network of steroid hormones, inflammatory cytokines, and immune cells as described earlier. However, how they interact and how they are coordinated are not clearly understood.

Lipoxins (LXs), which are derived from arachidonic acid in the context of inflammation, are the first recognized pro-resolving lipid mediators and are thought to be the 'stop signal' for inflammation. LXs have been extensively studied for their anti-inflammatory or pro-resolving effects (Serhan & Savill 2005, Serhan *et al.* 2008). Recently, our group revealed some new properties of LXs, including the regulatory mechanism in tumor immunoediting (Hao *et al.* 2011, Li *et al.* 2011). As discussed earlier, there is a close relationship between implantation and inflammation. So we are interested in the potential role of LXs in embryo implantation (Xiong *et al.* 2011). Moreover, a recent study described lipoxin A₄ (LXA₄) with estrogenic activity (Russell *et al.* 2011). This raises the question of whether estrogen signaling is involved in the effect of LXs on embryo implantation?

In this study, we investigated the effect of LXA₄ on embryo implantation, focusing on its influence on estrogen signaling and inflammatory mediators associated with uterine receptivity and embryo implantation. Our results showed that LXA₄ blocks embryo implantation by controlling ER α activity, and this effect appeared to be related to the suppression of inflammatory microenvironment necessary for implantation.

Results

LXA₄ blocks embryo implantation

LXA₄ was administered as described earlier. All control mice displayed implantation sites (mean $\pm$ S.E.M., 12.8 ± 1.7). However, none of the ten mice that received LXA₄ showed any implantation sites (Fig. 1). The pregnancy status of the mice without implantation sites was determined by flushing their reproductive tract for the presence of embryos. All mice treated with LXA₄ had no detectable implantation sites but embryos flushed from the uterus. Therefore, this study focused on the effect of LXA₄ on the uterus, specifically uterine receptivity for embryo implantation.

Figure 1 LXA₄ blocks embryo implantation. Representative images of uteri at day 5 of pregnancy showing the effect of LXA₄ treatment on embryo implantation. (A) A representative uterus from the control group showing implantation sites. Arrows indicate implantation sites. (B) A representative uterus from LXA₄ group showing no visible implantation sites. Scale bars: 1 cm.

LXA₄ adversely affects uterine receptivity for embryo implantation

An embryo transfer study was further performed to evaluate the effect of LXA₄ on uterine receptivity. All control mice with successful embryo transfer had implantation sites. There were 107 visible implantation sites, resulting from 200 embryos transferred (53.5%). However, none of the LXA₄-treated mice with successful embryo transfer had any detectable implantation sites (Fig. 2A, and B). Furthermore, histological examination of these transferred blastocysts and consequent implantation sites was performed to determine whether embryos properly attach to the uterine lumen. Successful attachment and invasion into uterine stroma of the embryos was observed in all controls. Moreover, the stromal bed surrounding these embryos underwent a decidual response. However, in LXA₄-treated mice, embryos failed to attach to the uterine luminal

Figure 2 LXA₄ adversely affects uterine receptivity for embryo implantation. Embryo transfer study was performed to confirm the effect of LXA₄ on uterine receptivity. (A) A representative uterus at day 5 from the control group showing implantation sites. Arrows indicate implantation sites. (B) A representative uterus at day 5 from LXA₄ group showing no visible implantation sites. Scale bars: 1 cm. (C) A representative photograph of hematoxylin and eosin (H&E)-stained histology in embryo implantation sites from the control group at day 5. The embryo is closely attached to the uterine luminal epithelium and the surrounding stroma is permeated as an initial decidual response. The arrow indicates the implanting blastocyst. (D) A representative photograph of H&E staining of uterine sections from LXA₄ group. The embryo remains unattached in the uterine lumen. The arrow indicates the location of unattached blastocyst. Scale bars: 200 μ m.

epithelium. An interesting observation was that the uteri showed an initial response to decidualization. However, the stromal bed showed poor vascular permeability resulting from reduced edema (Fig. 2C and D). Therefore, LXA₄ adversely affects uterine receptivity for embryo implantation.

LXA₄ influences uterine development during implantation

The above observations led us to examine uterine histology in LXA₄-treated mice. During implantation, the uterus undergoes a sequence of cellular and molecular events and differentiates into a state of receptivity. Cellular changes include the development of secretory glands, the emergence of large apical protrusions on the luminal epithelium (pinopodes), as well as the transformation of fibroblast-like stromal cells

into large and rounded decidual cells (decidualization) (Paria *et al.* 2002). Histological examination of the uteri of the control mice showed normal morphological appearance. Secretion from luminal epithelium or endometrial glands and stromal decidualization were observed. However, in LXA₄-treated mice, these changes did not occur. Increased endometrial vascular permeability at the implantation sites is also a key event in implantation (Wang & Dey 2006). However, the uterine stroma of LXA₄-treated mice showed poor vascular permeability with reduced extent of edema (Fig. 3).

LXA₄ reduces the expression of inflammatory mediators associated with uterine receptivity and embryo implantation

Inflammatory microenvironment is closely related to endometrial remodeling during implantation; we

Figure 3 LXA₄ influences uterine development during implantation. H&E staining was conducted for morphological analysis of uteri at day 5 of pregnancy, corresponding to the window of implantation. Secretion from luminal epithelium or endometrial glands (A and B) and stromal edema (C) were observed in control group, whereas in LXA₄ group, these changes were not detected (D, E and F). Scale bars: 200 μ m.

Figure 4 LXA₄ reduces the expression of inflammatory mediators associated with uterine receptivity and embryo implantation. (A and B) Immunohistochemical analysis of CRF in endometrium at day 5. The control group showed intense positive staining in glandular epithelium and also in stromal cells (A), whereas only weak staining was observed in LXA₄ group (B). Scale bars: 50 μ m. (C) Quantitative RT-PCR analysis of uterine CRF. (D) CRF analyzed by ELISA. There was a significant reduction in the CRF level in LXA₄-treated mice compared with the control mice. (E) COX2 expression measured by quantitative RT-PCR. The uterine expression of COX2 was significantly decreased in LXA₄-treated mice compared with control mice. (F) The levels of PGI₂ and PGE₂ in control mice and LXA₄-treated mice on day 5 of pregnancy as measured by ELISA. The levels of PGI₂ and PGE₂ were significantly lower in LXA₄-treated mice than in the control mice. (G) LIF expression measured by quantitative RT-PCR. LXA₄ treatment significantly suppressed the expression of LIF. (H) The levels of IL6, IL8, and MCP1 in control mice and LXA₄-treated mice on day 5 of pregnancy as measured by ELISA. LXA₄ treatment significantly suppressed the expression of IL6. Similar trends were also observed for IL8 and MCP1, although the difference did not reach statistical significance. Results represent means $\pm$ s.e.m.; $n=10$ mice/group. * $P<0.05$.

therefore examined the expression levels of a series of inflammatory mediators associated with uterine receptivity and subsequent embryo implantation in LXA₄-treated mice on day 5 of pregnancy. CRF is a key mediator in the

aseptic inflammatory process of implantation (Makrigianakis *et al.* 2001). Immunohistochemistry showed a marked reduction of CRF expression in LXA₄-treated mice (Fig. 4A, and B). To quantify this molecular change,

Figure 5 ALX is dispensable for the inhibitory effect of LXA₄ on embryo implantation. (A, B, C and D) Mice were treated with vehicle, LXA₄, and Boc2 alone or in combination, and the presence of implantation sites was assessed at day 5. (A) A representative uterus from the control group showing implantation sites. (B) A representative uterus from LXA₄ group showing no visible implantation sites. (C) A representative uterus from Boc2 group showing implantation sites. (D) A representative uterus from LXA₄ group pretreated with Boc2 showing no visible implantation sites. Scale bars: 1 cm. (E) The average number of implantation sites was recorded for each group. Both the control mice treated with vehicle or Boc2 showed no statistical difference (mean number of implantation sites 12.6 ± 2.0 vs 11.7 ± 2.3 respectively), suggesting that the amount of Boc2 used in this experiment has no effect on implantation. In mice pretreated with Boc2, administration of LXA₄ blocked implantation. Boc2 did not reverse the inhibitory effect of LXA₄ on embryo implantation. (F) Quantitative RT-PCR analysis of uterine CRF at day 5. Boc2 did not alter the inhibition of LXA₄ on CRF expression. Results represent means $\pm$ s.e.m.; $n=10$ mice/group. * $P<0.05$.

Figure 6 Enhanced ER α activity in the uteri of LXA₄-treated mice. (A) Serum levels of E₂ analyzed by RIA. LXA₄ does not affect the serum E₂ levels. Control mice and LXA₄-treated mice had similar serum levels of E₂ on day 5 of pregnancy. (B) Similar serum levels of P₄ in control mice and LXA₄-treated mice on day 5 of pregnancy as measured by RIA. (C) Quantitative RT-PCR analysis of uterine ER α . (D) Quantitative RT-PCR analysis of uterine PR. (E) Western blot analysis of ER α , phosphorylated ER α (p122), and PR. (F, G, H, I, J, and K) Immunohistochemical analysis of ER α , phosphorylated ER α (p122), and PR in the uteri of control mice and LXA₄-treated mice on day 5 of pregnancy. (F) Immunostaining of phosphorylated ER α (p122) in the control group. (G) Immunostaining of phosphorylated ER α (p122) in the LXA₄ group. (H) Immunostaining of ER α in the control group. (I) Immunostaining of ER α in the LXA₄ group. (J) Immunostaining of PR in the control group. (K) Immunostaining of PR in the LXA₄ group. ER α , phosphorylated ER α (p122), and PR were detectable in all endometrial cellular compartments, showing strong nuclear immunoreactivity. ER α and phosphorylated ER α (p122) (by assessing the relative proportion of immunopositive cells to total cells counted) were significantly higher in LXA₄-treated mice compared with controls. PR expression patterns (localization and intensity) were similar in the two groups. Scale bars: 50 μ m. Results represent means $\pm$ S.E.M.; $n=10$ mice/group. * $P<0.05$.

we performed quantitative real-time RT-PCR analysis and ELISA assay. These data confirmed the immunohistochemistry result. CRF expression was significantly reduced at both mRNA and protein levels in LXA₄-treated mice compared with controls (Fig. 4C, and D).

As COX2-derived PGs are also important inflammatory mediators in implantation (Lim *et al.* 1999, Pakrasi & Jain 2008), we further examined whether LXA₄ treatment could reduce COX2 expression and consequently inhibit the production of PGs during implantation. We found that the uterine expression of COX2 was significantly decreased in LXA₄-treated mice compared with the control mice (Fig. 4E). The levels of PGI₂ and PGE₂, the most abundant PGs at implantation sites, were also significantly lower in LXA₄-treated mice than in the control mice (Fig. 4F).

Pro-inflammatory cytokines, such as LIF, IL6, IL8, and MCP1, are also involved in the inflammatory response in implantation (Dominguez *et al.* 2005, van Mourik *et al.* 2009). To further confirm the inhibitory effect of LXA₄ on inflammatory microenvironment required for implantation and ascertain the possible inflammatory pathways involved, we examined whether LXA₄ treatment could also reduce the levels of these pro-inflammatory cytokines during implantation. It was also found that LXA₄ treatment significantly suppressed the expression of LIF and IL6. Similar trends were also

observed for IL8 and MCP1, although the difference did not reach statistical significance (Fig. 4G and H).

LXA₄ receptor is dispensable for the inhibitory effect of LXA₄ on embryo implantation

Biological actions of LXA₄ are mediated through several mechanisms. These include activation of LX-specific receptor (ALX), interaction with a subclass of cysteinyl-leukotriene receptors (CysLTs), as well as the nuclear receptor aryl hydrocarbon receptor (AhR; Chiang *et al.* 2006, Maderna & Godson 2009). LXA₄ exerts anti-inflammatory and pro-resolving effects specifically via ALX (Chiang *et al.* 2006, Ye *et al.* 2009). ALX has been identified in diverse cells and tissues, including endometrium (Motohashi *et al.* 2005, Macdonald *et al.* 2011). To determine the role of ALX in the observed inhibitory effect of LXA₄ on embryo implantation, additional experiment was performed in which the mice were pretreated with the formyl peptide receptor 2 (FPR2)/ALX antagonist N-*t*-butoxycarbonyl-Phe-Leu-Phe-Leu-Phe (Boc2). Interestingly, Boc2 did not reverse the inhibitory effect of LXA₄ on embryo implantation (Fig. 5A, B, C, D, and E). Furthermore, Boc2 did not alter the inhibitory effect of LXA₄ on CRF expression (Fig. 5F).

Figure 7 ER α is involved in the inhibitory mechanism of LXA₄ in embryo implantation. (A, B, C and D) Mice were treated with vehicle, LXA₄, and ICI alone or in combination, and the presence of implantation sites was assessed at day 5. (A) A representative uterus from the control group showing implantation sites. (B) A representative uterus from the LXA₄ group showing no visible implantation sites. (C) A representative uterus from the ICI group showing implantation sites. (D) A representative uterus from the LXA₄ group pretreated with ICI showing implantation sites. Scale bars: 1 cm. (E) The average number of implantation sites was recorded for each group. No difference in implantation sites was observed between the two groups treated with vehicle or ICI (mean number of implantation sites 12.7 ± 1.9 vs 13.2 ± 2.1 , respectively), suggesting that ICI, at the dose given here, has little effect on implantation. In the presence of ICI, LXA₄ failed to block embryo implantation. Mice from this group showed implantation sites (8.9 ± 2.8), although the number is slightly smaller than that for the controls. (F) Quantitative RT-PCR analysis of uterine CRF at day 5. In the presence of ICI, LXA₄ failed to inhibit CRF expression. Results represent means $\pm$ S.E.M.; $n = 10$ mice/group. * $P < 0.05$.

ER α activity is enhanced in the uteri of LXA₄-treated mice

Uterine functions are primarily directed by estrogen and progesterone, and their actions are largely mediated through ER α and progesterone receptor (PR) respectively. We then investigated the influence of LXA₄ on estrogen and progesterone signaling during implantation. We observed that serum levels of E₂ and P₄ in LXA₄-treated mice on day 5 were similar to those in the control mice (Fig. 6A, and B) and that LXA₄-treated mice had a normal expression pattern of PR (Fig. 6D, E, J, and K). However, uterine ER α expression was altered. As shown in Fig. 6C, ER α expression was significantly increased in LXA₄-treated mice compared with controls by qRT-PCR. This result was further confirmed by western blotting and immunohistochemical staining (Fig. 6E, H, and I).

It is well known that ER α activation involves ER α phosphorylation at specific sites. A major site of phosphorylation is located at serine 122 in mouse, corresponding to serine 118 of the human protein (Kato *et al.* 1995, Nilsson *et al.* 2001). To further determine whether these receptors are activated or not, we examined the phosphorylation status of ER α . Accordingly, the phosphorylation of ER α at serine 122 was significantly increased following LXA₄ treatment (Fig. 6E, F, and G). Collectively, these data showed a considerable increase in ER α expression and activation in the uteri of LXA₄-treated mice, implicating

involvement of ER α in the inhibitory mechanism of LXA₄ in embryo implantation.

ER α is involved in the inhibitory mechanism of LXA₄ in embryo implantation

Mice treated with LXA₄ exhibited implantation failure and enhanced ER α activity, suggesting that ER α might be involved in the inhibitory mechanism of LXA₄ in embryo implantation. To determine whether enhanced ER α activity is the prime cause of defective implantation, we assessed whether inhibition of ER α activity could rescue LXA₄-induced implantation defect. ICI 182 780 (ICI) a pure ER α antagonist, was used to inhibit ER α activity during the receptive period. ICI, at the dose given here, had little effect on implantation. As expected, LXA₄-treated mice failed to display embryo implantation, consistent with our previous observation. In contrast, in the presence of ICI, LXA₄ failed to block embryo implantation (Fig. 7A, B, C, D, and E). LXA₄ also failed to inhibit CRF expression (Fig. 7F). These results demonstrate that LXA₄ blocks embryo implantation through controlling ER α activity.

Discussion

Synchronizing uterine differentiation with embryonic development is crucial to successful implantation. Estrogen is essential for preparation of the progesterone-primed

uterus to the receptive state and has been shown to be a critical determinant that specifies the duration of the window of uterine receptivity for implantation. Estrogen at a low level extends the window of uterine receptivity, but excessively high estrogen levels can prematurely close this window, transforming the uterus into a refractory state. The uterine refractoriness at high estrogen levels is accompanied by aberrant expression of implantation-related genes (Ma *et al.* 2003). Thus, estrogen activity must be tightly controlled during the peri-implantation period. Disrupting estrogen activity is the basis of the estrogen-containing 'morning after pill' (Grimes & Raymond 2002). LXA₄ has been implicated in uterine physiology. The enzymes necessary for LXs biosynthesis and metabolism have previously been described in the endometrium (Lousse *et al.* 2010). Significantly higher expression of ALX is also detected in the endometrium (Motohashi *et al.* 2005). More importantly, the anti-inflammatory action of LXA₄ observed in the endometrium (Macdonald *et al.* 2011) suggests a potential role of LXA₄ signaling in the regulation of inflammatory events associated with endometrial remodeling during embryo implantation. Recently, LXA₄ is confirmed to be a novel ER modulator. Russell *et al.* (2011) showed that LXA₄ competes with E₂ for ER binding and possesses a dual capacity to alter estrogen response element transcriptional activity, and alkaline phosphatase activity, as well as expression of estrogen-regulated genes in human endometrial cells. Therefore, it will be of considerable interest to study the potential role of LXA₄ in embryo implantation. Here, we demonstrated that the estrogenic activity of LXA₄ is involved in contraceptive events. Our data showed that LXA₄ blocks embryo implantation by interference with the local inflammatory microenvironment necessary for implantation, and that this inhibitory effect of LXA₄ is mediated through ER α .

Although the embryonic effect of LXA₄ is not directly tested, we demonstrated a potent effect of LXA₄ on uterine receptivity. We observed that preimplantation treatment of LXA₄ resulted in implantation failure. None of the ten mice that received LXA₄ showed any implantation sites. First, as ovulation and fertilization happen during the dark cycle before 0500 h on gestation day 1 (Nagy *et al.* 2003), the LXA₄ treatment regimen in this study, which started at 0900–1000 h of gestation day 1, should not affect the ovulation and fertilization processes. Secondly, all mice treated with LXA₄ had no detectable implantation sites but embryos flushed from the uterus only, suggesting no effect of LXA₄ on embryo transport in the reproductive tract. Therefore, failed implantation following LXA₄ treatment might result from compromised uterine receptivity, which was subsequently confirmed by the embryo transfer study. To exclude any possible influence of LXA₄ on the embryo, we transferred embryos to LXA₄-treated pseudopregnant mice. These embryo-transferred mice also showed implantation failure. None

of LXA₄-treated mice with successful embryo transfer had any detectable implantation sites. These results suggested that LXA₄ blocks embryo implantation by altering uterine receptivity.

Inflammatory microenvironment is closely related to endometrial remodeling during embryo implantation. We therefore asked whether the inhibitory effect of LXA₄ on embryo implantation and uterine receptivity is associated with the change in the local inflammatory microenvironment in the endometrium. CRF, COX2-derived PGs, and pro-inflammatory cytokines including LIF, IL6, IL8, and MCP1 are important inflammatory mediators in implantation (Lim *et al.* 1999, Makrigianakis *et al.* 2001, Dominguez *et al.* 2005, Pakrasi & Jain 2008, van Mourik *et al.* 2009). So these inflammatory mediators were chosen as our primary research targets. We observed that LXA₄ treatment significantly reduced the expression levels of CRF, COX2, PGI₂, PGE₂, LIF, and IL6. Similar trends were also observed with respect to IL8 and MCP1 expression, although the difference did not reach statistical significance. These results confirmed that LXA₄ might hinder the local inflammatory microenvironment during implantation.

Biological actions of LXA₄ are mediated through various receptors, including ALX, and CysLTs, as well as AhR (Chiang *et al.* 2006, Maderna & Godson 2009). ALX is the first receptor cloned and identified as a G-protein-coupled receptor for lipoxygenase-derived eicosanoids with demonstrated cell-type-specific signaling pathways (Chiang *et al.* 2006). LXA₄ exhibits high-affinity binding with ALX, and the LXA₄–ALX axis is responsible for the anti-inflammatory and pro-resolving signals (Chiang *et al.* 2006, Ye *et al.* 2009). ALX is expressed in diverse cells and tissues, including endometrium (Motohashi *et al.* 2005, Macdonald *et al.* 2011). However, we observed that ALX is dispensable for the inhibitory effect of LXA₄ on embryo implantation. Boc2 did not reverse the inhibitory effect of LXA₄ on embryo implantation and CRF expression. Subsequent observation suggested that ER α is involved in the inhibitory mechanism of LXA₄ in embryo implantation. In the presence of ICI, LXA₄ failed to block embryo implantation. LXA₄ also failed to inhibit CRF expression. Therefore, the role of LXA₄ in implantation is not simply through anti-inflammatory and pro-resolving effects but also via the regulatory mechanisms involved in the endocrine–immune cross talk.

It is worth noting that the mice with LXA₄ treatment exhibited enhanced ER α activity in endometrium during embryo implantation. This is in contrast to the antiestrogenic action of LXA₄ observed in human endometrial epithelial cells *in vitro* (Russell *et al.* 2011). One reason for the different findings may be the relative concentrations of LXA₄ and E₂. The overall response is governed by complex interactions between LXA₄, E₂, and ER. Another possible reason is the *in vivo* cellular context. Stromal cells play a significant role in implantation (Franco *et al.* 2011, Shuya *et al.* 2011,

Das *et al.* 2012) and may respond differently to LXA₄ compared with epithelial cells.

We have also observed increased ER α expression in LXA₄-treated mice. The uterine response to estrogen is primarily mediated through ER, whose expression in turn is regulated by estrogen. Previous studies revealed that the expression of ER α varies during the estrous cycle and peaks during proestrous in accordance with the circulating E₂ levels, indicating that uterine ER α is specifically induced by endogenous estrogen (Winuthayanon *et al.* 2010). Therefore, this finding further confirmed the estrogenic activity of LXA₄ *in vivo*.

Additionally, to exclude the possible interference of endogenous estrogen, we also analyzed the serum levels of E₂. LXA₄ does not affect the serum E₂ levels. Thus, it can be speculated that LXA₄ does not disturb ovarian secretion.

In conclusion, this study first proposes the LXA₄/ER α axis existed in the endometrium during implantation and indicates that dysregulation of ER α activity by LXA₄ could be a promising female contraceptive. LXA₄ does not disturb ovarian secretion. What is more, LXA₄ is rapidly converted to biologically inactive lipid *in vivo* (Gilroy *et al.* 2004), so it is easy to restore reproductive function. Our results help to enrich functional properties of LXA₄ and provide novel insights into the endocrine-immune cross talk mechanisms during implantation.

Materials and Methods

Reagents and antibodies

LXA₄ (5S, 6R, 15S-trihydroxy-7E, 9E, 11Z, 13E-eicosatetraenoic acid) was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Boc2 (N-t-butyloxycarbonyl-Phe-Leu-Phe-Leu-Phe), an antagonist of FPR2/ALX, was purchased from GenScript (Piscataway, NJ, USA). ICI 182 780, a pure ER antagonist, was purchased from Sigma-Aldrich. Reagents were dissolved in ethanol or DMSO and further diluted in PBS on the day of administration. Equivalent concentrations of ethanol or DMSO were used as vehicle controls. Affinity-purified goat polyclonal antibody CRF (C-20) (sc-1759), rabbit polyclonal antibody ER α (MC-20) (sc-542), rabbit polyclonal antibody phosphorylated ER α (Ser 122) (sc-101675), and rabbit polyclonal antibody PR (C-19) (sc-538) were from Santa Cruz Biotechnology.

Animal study protocol

The study was approved by the Animal Care and Use Committee of Huazhong University of Science and Technology (S230). Experiments involving mice (Kunming White outbred strain) used animals obtained from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology. The mice were maintained in a controlled environment with a 14 h light:10 h darkness cycle. All animals appeared healthy throughout the experiments.

Female mice (8 weeks old) were naturally mated with fertile males of the same strain. The presence of a vaginal plug after

mating was designated as day 1 of pregnancy. Pregnant mice were randomly allocated to two groups, with ten animals per group. Group A received placebo vehicle and group B received LXA₄ (10 μ g/kg i.p.). Injections were done on days 1–3. The implantation sites on day 5 were identified by i.v. injection of 0.1 ml 1% Chicago blue (Sigma-Aldrich), and the number of implantation sites was recorded. If no implantation sites were detected, the uterine horns were flushed with PBS to determine the presence of embryos. The uteri were collected and placed in 4% buffered formalin overnight for morphological study or stored at –80 °C until ready for gene expression analysis.

The embryo transfer technique has been previously described (Nagy *et al.* 2003). Briefly, 8-week-old female mice were superovulated with i.p. injections of 5 IU equine chorionic gonadotropin (Sigma-Aldrich) and 48 h later with 5 IU human chorionic gonadotropin (Sigma-Aldrich) and subsequently mated with fertile males. Meanwhile, pseudopregnant females were prepared by mating with vasectomized males. The following day was designated as gestation day 1 when a vaginal plug was identified. The pseudopregnant females were given daily injections of placebo vehicle or 10 μ g/kg LXA₄ (10/group) on days 1–3. On day 4, blastocysts were harvested from superovulated females and transferred into the uteri of pseudopregnant females (10/horn). Resultant implantation sites were detected by blue dye injection on day 5. Histological examination of these transferred blastocysts and consequent implantation sites was performed to determine whether embryos properly attach to the uterine lumen.

To assess whether the effect of LXA₄ on embryo implantation is mediated through ALX or ER α , additional experiments were performed in which the mice were pretreated with the FPR2/ALX antagonist Boc2 (10 μ g/kg) or the ER α antagonist ICI (250 ng/kg) respectively. All these agents have been used at doses previously shown to produce the desired pharmacological effects (von der Weid *et al.* 2004, Lee *et al.* 2010). Thirty minutes after administration of one of these drugs, LXA₄ was given as stated earlier. Embryo implantation was assessed on day 5 of pregnancy, and uterine tissues were processed for further analysis.

Morphological analysis

Embryo implantation at day 5 of pregnancy was assessed macroscopically by i.v. injection of Chicago blue dye, and the implantation sites were visualized as distinct blue bands. For histological assessment, the isolated uterine samples were fixed overnight in 4% buffered formalin and processed for routine H&E staining. Immunohistochemical staining was performed by EnVision method. The expression of CRF, ER α , phosphorylated ER α , and PR were detected by anti-CRF (1:200), anti-ER α (1:200), anti-pER α (1:200), or anti-PR (1:200) primary antibody respectively. Control samples were run accordingly. As a negative control, non-immune serum was substituted for the primary antibody.

Quantitative real-time PCR

Total RNAs from uterine tissues were isolated using Trizol reagent (Invitrogen) and reverse transcribed into cDNA with

M-MLV (Invitrogen). qPCR analysis was performed with SYBR Green qPCR kit (Roche) on Stratagene Mx3000 Quantitative PCR System (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The sets of primer sequences used in this study were as follows: CRF forward 5'-ATGCTGCTGGTGGCTCTGT-3' and reverse 5'-GCTCCGGCTGCAAGAA-3'; COX2 forward 5'-CACCTCTGCGATGCTCTTCC-3' and reverse 5'-CTGGTCAAATCCTGTGCTCATAC-3'; LIF forward 5'-TTCCCATCACCCCTGTAAATG-3' and reverse 5'-CACTGTGCCATACGCCACCCA-3'; ER α forward 5'-GATGAAGGCGGCATACGG-3' and reverse 5'-CAGGGCTATTCTTCTTAGTGTGC-3'; PR forward 5'-CCAGATTCAGAAGCCA-GAA-3' and reverse 5'-CTCCACAGGTAAGCAC- GC-3'. The expression of target genes was normalized to that of GAPDH.

Western blotting

Proteins were extracted from uteri in the lysis buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.2 mM sodium vanadate, 1% phenylmethylsulfonyl fluoride, and 0.2% aprotinin). Protein concentration was measured by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The protein extracts were resolved by SDS-PAGE and subsequently transferred to nitrocellulose membranes. Immunodetection was performed by standard procedures. After being blocked in 5% nonfat milk in TBST for 1 h, the membranes were incubated with primary rabbit anti-ER α (1:200), anti-pER α (1:200), or anti-PR (1:200) overnight at 4 °C. The membranes were then washed three times in TBST and incubated with HRP-conjugated anti-rabbit secondary antibody (1:1000), followed by washing in TBST. The signals were developed with an ECL Kit (Millipore, Bedford, MA, USA). β -Actin served as an internal control.

ELISA

Levels of CRF, PGI₂, PGE₂, IL6, IL8, and MCP1 in the uterine extracts were quantified using respective ELISA kits as instructed by the manufacturer (R&D Systems, Minneapolis, MN, USA).

RIA

Serum was separated and serum levels of E₂ and P₄ were measured by RIA.

Statistical analysis

All data were presented as mean $\pm$ S.E.M. Relative gene expression data were analyzed using the 2^{- $\Delta\Delta$ CT} method (Livak & Schmittgen 2001). Statistical significance for differences between groups was determined by Student's *t*-test or by one-way ANOVA. The significance level was set at *P* < 0.05.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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