Spontaneous Miscarriages Are Explained by the Stress/Glucocorticoid/Lipoxin A4 Axis

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Spontaneous Miscarriages Are Explained by the Stress/Glucocorticoid/Lipoxin A4 Axis

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Despite various suspected causes, ranging from endocrine and genetic to infectious and immunological aspects, the molecular mechanisms of miscarriage still remain enigmatic. This work provides evidence that downregulation of 11β-hydroxysteroid dehydrogenase (HSD) type 2, the key enzyme inactivating glucocorticoid activities, insults the pregnant inflammatory milieu by inhibiting the biosynthesis of lipoxin A4 (LXA4), a metabolite of arachidonic acid, leading to an early loss of the pregnancy. Both LXA4 and its biosynthetic enzymes were found to be decreased in women with spontaneous miscarriages and in the murine miscarriage model. Replenishing LXA4 reversed LPS-induced miscarriages in mouse models, whereas blocking LXA4 signaling resulted in miscarriages in the pregnant mice. The protective effect of LXA4 might be explained by LXA4’s role in regulating uterine and placental inflammatory factors and mast cells. The underlying molecular mechanism involved miscarriage-inducing infections or stresses that downregulate the expression of 11β-HSD2, but not 11β-HSD1, resulting in increases in glucocorticoid activity and decreases in LXA4. Together, these findings suggest that the stress/glucocorticoid/LXA4 axis might be a common pathway through which miscarriages occur. The Journal of Immunology, 2013, 190: 6051–6058.
remains unclear whether 11β-HSD1/11β-HSD2 plays a role in regulating pregnancy. Interestingly, glucocorticoids have been reported to suppress LXA4 in severe asthma (18) and shown to down-regulate 15-LO and 12-LO, two enzymes involved in LXA4 biosynthesis (19, 20), implying that the regulation of LXA4 by glucocorticoids might also have a role in pregnancy. This study provides evidence that spontaneous miscarriages may be triggered through stress/glucocorticoid/LXA4 pathways.

Materials and Methods

Patient samples

Sixteen patients with spontaneous abortions and 16 women with normal pregnancies were recruited for this study. The diagnosis of a spontaneous abortion as separate from a normal pregnancy was based on color Doppler ultrasonography and serum human chorionic gonadotropin inspection. We used color Doppler ultrasound to detect gestational sac, fetal pole, and fetal heart motion of women after 8 wk gestation. If the gestational sac, fetal pole, and fetal heart motion can be detected, we define it as normal pregnancy; if we only detect gestational sac and fetal pole, but failure to detect fetal heart motion, in company with the decline of human chorionic gonadotropin and progesterone, we define it as spontaneous abortion.

The patients were chosen according to the following criteria: 1) patients were 12-35 y old and known to use cigarettes or illicit drugs, or were not suffering from any medical conditions; 2) patients had normal liver and kidney functions and did not suffer from high blood pressure or endocrine illness; 3) patients did not have in vitro fertilization-embryo transfer and infertility history; 4) none of the patients with spontaneous abortion had undergone any therapy prior to sampling; and 5) patients with abnormal karyotype were eliminated. Blood and placental villi samplings were obtained from diagnosed patients after informed consent, and the protocol was approved by the Ethical Committee of the Medical Faculty of Tongji Medical College in accordance with the Declaration of Helsinki.

Mice and pregnancy model

BALB/c mice (8 wk old and 20–25 g in weight) were purchased from Center for Medical Experimental Animals of Hubei Province (Wuhan, China) for studies approved by the Animal Care and Use Committee of Tongji Medical College. Female mice were checked for estrus and were mated with fertile males for mating. The females with positive vaginal plugs present the next morning were considered to be on day 0.5 of pregnancy.

ELISA

For LXA4 detection, the human or mouse serum and human chorionic villus tissues were assessed by ELISA kits (R&D Systems, Minneapolis, MN). For 12-HETE and 15-HETE detection, the human serum was assessed by ELISA kits (Abnova, Walnut, CA). For estradiol and progesterone detection, the human or mouse serum was assessed by ELISA kits (Westang, Shanghai, China). For CCL2, TGF-β, IFN-γ, IL-10, and IL-6 detection, the mouse uterus tissues were assessed by ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions. For cortisol detection, the human serum was assessed by ELISA kits (R&D Systems). Human chorionic villus tissues and the mouse uterus tissues were homogenized in PBS (0.5 ml) containing 100 μM PMSF (Sigma-Aldrich, St. Louis, MO), 1% (v/v) aprotinin (Sigma-Aldrich), 2 μg/ml leupeptin (Sigma-Aldrich), and 1 μg/ml pepstatin (Sigma-Aldrich). After centrifugation, the supernatant was assessed by ELISA kits.

In addition, peripheral blood extracted from pregnant women was allowed to sit for 20 min at room temperature. The serum supernatants were transferred to a 1.5-ml Eppendorf tube and mixed quickly. After centrifugation, serum was collected and assessed by ELISA kits.

RT-PCR and real-time RT-PCR

Blood and tissues were lysed or homogenized with TRIzol reagent (Invitrogen, Carlsbad, CA), and the total RNA was extracted according to the manufacturer’s instructions. A RT-PCR procedure was used to determine the expression level of mRNA (One-Step RT-PCR kit; Qiagen, Valencia, CA). The primer sequences were presented in Supplemental Table 1.

For real-time RT-PCR assays, the cDNA sequences of all detected genes were retrieved from the National Center for Biotechnology Information database. The primers were designed with the Oligo Primer Analysis 4.0 software (Molecular Biology Insights, Cascade, CO), and the sequences were subjected to BLAST analysis. The total RNA (100 ng) was used for reverse transcription using Superscript II RNase H reverse transcriptase (Invitrogen) in a volume of 25 μl. Next, 2 μl cDNA was amplified with SYBR Green Universal PCR Mastermix (Bio-Rad, Richmond, CA) in duplicate. The resulting data were analyzed with the comparative cycle threshold method for relative gene expression quantification against GAPDH.

Histology and immunohistochemistry

Mouse placenta and uterus tissues and human chorionic villus were fixed in a 10% neutral buffered formaldehyde solution. After dehydration procedures, the samples were blocked in paraffin, and 4-μm sections were cut with a microtome and collected for H&E and immunohistochemical staining. The serial sections of the chorionic villus, placenta, and uterus tissues were immunohistochemically stained for 5-LO, 12-LO, 15-LO, vascular endothelial growth factor (VEGF), 11β-HSD1, and 11β-HSD2. The tissue sections were incubated overnight with primary Abs at 4°C, followed by secondary Abs and peroxidase-labeled avidin for 30 min. Rabbit anti-5-LO, 12-LO, 15-LO, and VEGF Abs were purchased from Santa Cruz (Santa Cruz, CA), and anti-11β-HSD1 and anti-11β-HSD2 Abs were purchased from Biosynthesis Biotechnology (Beijing, China). The sections were subsequently incubated with 3,3-diaminobenzidine and counterstained with methyl green. The negative controls without primary Abs were included. The mounted slides were examined under a light microscope. The pathologist assessing the treatment effects was blind to the treatment groups.

Positive staining was judged if the cell plasma, cell membranes, or the nuclei had turned brown. The analysis of immunohistochemical staining was performed by Image-Pro Plus (IPP 6.0; Media Cybernetics, Silver Spring, MD), according to the measurement parameter mean density. The mean density was calculated by the formula, mean density = integrate OD/area sum. Intensity was averaged from 10 fields of view. The original magnification for the immunohistochemistry study was ×400.

Pontamine blue reaction

A pontamine blue reaction test was carried out to assess the status of implantation in pregnant animals. The animals were etherized, and 0.3 ml 1% pontamine blue (Sigma-Aldrich) in sterile normal saline was injected into each animal through a tail vein. To monitor the pregnancy outcomes, the pontamine blue positive/negative sites in the uterine horns were evaluated by performing laparotomy 3 min after tail i.v. injection.

Statistics

Results were expressed as mean values plus or minus SD and interpreted by repeated-measure ANOVA. Differences were considered to be statistically significant if the p value was <0.05.

Results

Levels of LXA4 are decreased in women with spontaneous miscarriages

This study first sought to determine whether LXA4 is correlated with spontaneous miscarriages. In this regard, serum and chorionic villus tissue were collected from both women with normal pregnancies and those with spontaneous miscarriages. As shown in Fig. 1A, the serum LXA4 levels of women with spontaneous miscarriages were 3-fold decreased, compared with women with normal pregnancies. In line with this, the levels of LXA4 in chorionic villus tissue of spontaneous abortion women were found to be significantly lower than those in women with normal pregnancies (Fig. 1B). In addition, the decrease of LXA4 levels was not due to the chromosomal abnormalities in the miscarriage patients, because the clinical genetic detection did not show abnormality. These clinical data suggest that low levels of LXA4 are correlated to women with spontaneous miscarriages.

Downregulation of enzymes catalyzing LXA4 biosynthesis in women with spontaneous miscarriages

LXA4 is the metabolite of arachidonic acid. Three enzymes are involved in its biosynthesis through one of two pathways, as follows (6, 7): arachidonic acid catalyzed to 5-hydroxyeicosatetraenoic acid by 5-LO, which, in turn, is converted to leukotriene A4, and then catalyzed to LXA4 by 12-LO; arachidonic acid catalyzed to 15(S)-hydroxyeicosatetraenoic acid by 15-LO


and further catalyzed to LXA4 by 5-LO. To validate the above data, the expressions of 5-LO, 12-LO, and 15-LO in periphery and chorionic tissues were studied. As shown by RT-PCR and real-time RT-PCR in Fig. 2A and 2B, although 5-LO mRNA expression remained at similar levels in all the samples, the mRNA expressions of 12-LO and 15-LO were strikingly downregulated in women with spontaneous miscarriages. Similar changes of 12-LO and 15-LO at the protein levels were also confirmed by immunohistochemical staining (Fig. 2C, 2D). In addition, the activities of 12-LO and 15-LO were determined by measuring the serum levels of 12-HETE and 15-HETE, the substrates of 12-LO and 15-LO. Consistently, the activities of 12-LO and 15-LO were also lower in spontaneous miscarriages. Similar changes of 12-LO and 15-LO at the protein levels were also confirmed by immunohistochemical staining of uterus and placenta, respectively (Fig. 3G). Together, these data further supported the idea that the LPS-induced miscarriages are mediated by the decrease of LXA4 in pregnant mice.

Blocking LXA4 signaling induces miscarriages in pregnant mice

But was LXA4 really significant for the healthy progress of early pregnancies? A comparable approach was used to further elucidate the role of LXA4 in miscarriages. The peptide N-Boc-Phe-Leu-Phe-Leu-Phe, an antagonist of LXA4 receptor (21), was used to block LXA4 signaling pathway in pregnant mice. Administering the antagonist from day 5.5 after fertilization and at the rate of 0.15 μg/g LPS to female mice on day 8.5 of the pregnancy. Symptomatically, the treated mice expressed vaginal bleeding and weight loss and produced no pups. When, however, the pregnant mice were given i.p. injections of LXA4 (5 μg/kg) 2 h prior to and after the administration of LPS, the LXA4 treatment appeared to inhibit the LPS-induced fetal loss (Fig. 3A), increasing the number of viable pregnancies (Fig. 3B) and maintaining weight gain (Fig. 3C). Again, it was clear that LXA4 treatment, in comparison with the LPS group, significantly reduced the rate of fetal reabsorption (Fig. 3D), suggesting that LXA4 effectively prevents LPS-induced miscarriage in pregnant mice.

The above data prompted further confirmatory investigations into the reduced LXA4 levels in LPS-induced miscarriages. Serum levels of LXA4 and mRNA expressions of 5-LO, 12-LO, and 15-LO were carefully determined after LPS administration. As expected, the LPS injections resulted in the decrease of both mRNA expressions of lipoxygenases and LXA4 levels (Fig. 3E, 3F). Consistently, similar changes of 5-LO, 12-LO, and 15-LO at the protein levels were also confirmed by immunohistochemical staining of uterus and placenta, respectively (Fig. 3G). Together, these data further supported the idea that the LPS-induced miscarriages are mediated by the decrease of LXA4 in pregnant mice.

FIGURE 1. Low levels of LXA4 in women with spontaneous miscarriage. (A) Comparison of serum LXA4 levels between abortion and normal pregnancy (310.4 ± 83.6 pg/ml vs 1054.1 ± 411.6 pg/ml, n = 16, p < 0.001). (B) Comparison of chorionic villus tissue LXA4 levels between abortion and normal pregnancy (336.9 ± 85.6 pg/ml vs 693.1 ± 155.2 pg/ml, n = 16, p < 0.001).

FIGURE 2. LXA4-synthesizing enzymes are downregulated in women with spontaneous miscarriage. (A) Blood samples and chorionic villus were collected from women with normal pregnancy at ∼50-d gestation or spontaneous miscarriage, and the total RNAs were isolated from PBMCs and chorionic tissues, respectively. The expressions of 5-LO, 12-LO, and 15-LO were analyzed by RT-PCR. The results shown were representative from eight samples. (B) Sixteen blood samples were collected (n = 8, each group above), and the expression of 5-LO, 12-LO, and 15-LO was analyzed by real-time RT-PCR (p < 0.001). (C) The immunohistochemical staining of 5-LO, 12-LO, and 15-LO in chorionic tissues (original magnification ×400). (D) The density mean was calculated, as described in Materials and Methods (p < 0.005). (E) Comparison of the activities of 12-LO and 15-LO between normal pregnancies and miscarriages. Serum samples (n = 8, each group) from patients were used to determine the activities of 12-LO and 15-LO by measuring the productions of 12-HETE and 15-HETE by the ELISA kits (p < 0.05).
pregnancy and its blocking may result in early loss of the pregnancy.

**LXA4 antagonizes the proinflammatory effects of LPS in early pregnancies**

To address the question of how LXA4 prevented LPS-induced miscarriages, focus was turned to a set of inflammation-associated factors arising from the proinflammatory properties of LPS. These inflammation-associated factors included, but were not limited to inducible NO synthase (iNOS), CCL2, TGF-β, IFN-γ, IL-6, and IL-10 in the placenta 24 h after LXA4 treatment were detected by RT-PCR and real-time RT-PCR. As shown in Fig. 5A and 5B, the expressions of iNOS, CCL2, TGF-β, IFN-γ, and IL-6 were downregulated, whereas IL-10 mRNA was upregulated after LXA4 treatment. ELISA results showed decreases in CCL2, IFN-γ, and TGF-β and increases in IL-10 (Fig. 5C). Moreover, when the murine uterus and placenta tissues were analyzed by H&E staining, it was clear, even though the inflammatory alteration was not obviously manifested in the uterus, that...
the LPS-induced pathological changes had been reversed by LXA4 treatment (Fig. 5D). LXA4 has been implicated in the regulation of VEGF (22, 23). Given the importance of angiogenesis to the maintenance and development of pregnancy, we additionally determined the influence of LXA4 on the expression of VEGF. The RT-PCR result showed that LPS upregulated the expression of VEGF, which, however, was reversed by the administration of LXA4 (Fig. 5A, 5B). The similar result was further confirmed by the immunohistochemical staining (Fig. 5E). Once again, the data suggest that LXA4 must have a central role in keeping the homeostatic balance in the inflammatory microenvironment of early pregnancy through regulating inflammation-associated factors.

Glucocorticoids are involved in the downregulation of LXA4
Glucocorticoids play an integral part in the response to stress, which, in turn, is known to be a critical factor in spontaneous miscarriages. Are decreases in LXA4 levels due to the effect of glucocorticoids during early pregnancy? To address this question, dexamethasone, in dosages of 0.5, 2.5, 5, or 10 mg/kg, were i.v. administered from day 5.5 of mice pregnancies, once per day for 5 d thereafter. Although 0.5 and 2.5 mg/kg dosages appeared not to affect the murine pregnancies or normal labor, higher dosages of either 5 or 10 mg/kg induced vaginal bleeding, indicating the occurrence of a miscarriage. In line with this result, the levels of LXA4 were significantly decreased after the administration of 5 or 10 mg/kg dexamethasone (Fig. 6A). Similar decreases in 5-LO, 12-LO, and 15-LO were also observed in those mice (Fig. 6B, 6C). Besides dexamethasone, we also tested corticosterone, an endogenous active glucocorticoid in mice. A consistent result was obtained (Supplemental Fig. 1). These data suggested that glucocorticoid hormones might be important to the regulation of the levels of LXA4 in miscarriages.

**FIGURE 5.** LXA4 ameliorates the inflammatory microenvironment in early pregnancy. LXA4 antagonized the proinflammatory effects of LPS. Mice on day 8.5 of pregnancy were treated with LPS in the presence or absence of LXA4 for 24 h. (A) The inflammation-associated factors in the placenta were analyzed by RT-PCR. (B) The analysis of inflammation-associated factors by real-time RT-PCR (*p < 0.01). (C) The protein levels of CCL2, TGF-β, IFN-γ, and IL-10 in the placenta were analyzed by ELISA (*p < 0.01). (D) LXA4 ameliorated the inflammation of uterine and placenta in LPS-induced mouse abortion model. The uterus and placenta were examined by histological staining (H&E, original magnification ×200). (E) The immunohistochemical staining was conducted for VEGF proteins in placenta (original magnification ×400). The density mean was calculated, as described in Materials and Methods (*p < 0.05).

**FIGURE 6.** Levels of LXA4 are regulated by glucocorticoid. (A) Glucocorticoid downregulated LXA4 levels in pregnant mice. Dexamethasone (0.5, 2.5, 5, or 10 mg/kg) was injected i.v. into mice on day 5.5 of pregnancy (n = 8) once per day for 5 d. The levels of LXA4 were measured at different time points. (B) Dexamethasone downregulated the mRNA expression of 5-LO, 12-LO, and 15-LO. Dexamethasone (0.5, 2.5, 5, or 10 mg/kg) was injected i.v. into mice on day 5.5 of pregnancy. Five hours later, the mRNA expression of 5-LO, 12-LO, and 15-LO was detected by RT-PCR. (C) The immunohistochemical examinations of 5-LO, 12-LO, and 15-LO in the uterus (original magnification ×400). The density mean was calculated, as described in Materials and Methods (*p < 0.001).
Glucocorticoid activity is upregulated through the 11β-HSD1/11β-HSD2 pathway in stress-triggered miscarriages

The above data prompted further investigation as to whether infections and noninfectious stresses induce miscarriages through the glucocorticoid/LXA4 axis. Analyzing blood samples of clinical patients with miscarriages showed none of the otherwise expected corresponding increases in the levels of cortisol (Fig. 7A). Given that 11β-hydroxylation is a prerequisite for glucocorticoid functions and is regulated by 11β-HSD1 and 11β-HSD2 (16), the expressions of these two enzymes were, therefore, determined in patients with spontaneous miscarriages. Surprisingly, when compared with normal pregnancies, the expression of 11β-HSD1 was not changed, whereas 11β-HSD2 was strikingly decreased in chorionic villus of miscarriages at both mRNA and protein levels, evaluated by conventional RT-PCR, real-time RT-PCR, and immunohistochemical staining, respectively (Fig. 7B, 7C). Therefore, in women with spontaneous miscarriages, reductions in 11β-HSD2 might result in increases in the villus or placenta local activity, but not blood cortisol levels.

Infection- and noninfectious stress-induced miscarriage mouse models were used to validate the above human data. Administration of LPS represented bacterial infection or inflammation-induced stress in the mouse miscarriage model. As expected, 11β-HSD1 and 11β-HSD2 were expressed in normal pregnancy. However, the expression of 11β-HSD2 was significantly downregulated in the placenta after the injection of LPS to day 8.5 of the pregnancies (Fig. 7C, 7E). Significantly, the administration of LXA4 reversed the decrease of 11β-HSD2 (Fig. 7D, 7E).

In addition to LPS, vesicular stomatitis virus VSV was also used to represent a virus infection-related miscarriage model, whereas CoCl2 was used to represent a hypoxia-related miscarriage model (24). Vaginal bleeding was induced by i.v. injection of VSV once per day for 3 d or alternatively by s.c. injection of 20 μg/kg CoCl2 once on day 5.5 of the pregnancies. The mice were found with vaginal bleeding, indicating successful induction of miscarriage under both conditions. A biopsy was then made of the placenta tissue to determine the above two enzymes. Similarly, the hypoxic stress by CoCl2 or virus infection by VSV resulted in the decrease of 11β-HSD2 levels, but had no effect on 11β-HSD1 expression (Fig. 7F, 7G).

The above data suggest that infections or stresses may induce the downregulation of HSD2, leading to increased glucocorticoid activities in early pregnancies. However, previous studies had shown that progesterone downregulated HSD2 expression, and both progesterone and estrogen were inhibitors of 11β-HSD2.

**FIGURE 7.** Infections or stresses regulate glucocorticoid activity through 11β-HSD1/11β-HSD2 pathway. (A) Levels of glucocorticoid were not increased in patients with miscarriage. Serum levels of glucocorticoid were measured in patients with normal pregnancy and miscarriage (n = 16). (B) The mRNA expression of 11β-HSD2 was downregulated in the chorionic villus of miscarriage patients. The human chorionic villus tissue was used to isolate the total RNA, and the expression of 11β-HSD1 and 11β-HSD2 was analyzed by RT-PCR (top) and real-time RT-PCR (bottom) (n = 6, *p < 0.001). (C) The immunohistochemical examinations of 11β-HSD1 and 11β-HSD2 in human chorionic villus (original magnification ×400). The density mean was calculated, as described in Materials and Methods (*p < 0.001). (D) The HSD2 expression was decreased in LPS-induced miscarriage. Mice on day 8.5 of pregnancy were injected i.p. with 0.15 μg/g LPS. Some mice were additionally i.p. injected with LXA4 (5 μg/kg) 2 h prior to and after LPS. Five hours later, placenta expressions of 11β-HSD1 and 11β-HSD2 were detected by RT-PCR (top) and real-time RT-PCR (bottom). (E) The immunohistochemical examinations of 11β-HSD1 and 11β-HSD2 in the placenta of LPS- or LPS/LXA4-treated mice above (original magnification ×400). The density mean was calculated, as described in Materials and Methods (*p < 0.001). (F) The HSD2 expression was decreased in VSV- or CoCl2-induced miscarriage. Mice on day 8.5 of pregnancy were s.c. injected with CoCl2 (20 μg/kg; Sigma-Aldrich) or injected i.v. with VSV (provided by C. Zheng, Wuhan Institute of Virology). Forty-eight hours later, the expression of 11β-HSD1 and 11β-HSD2 in placenta was detected by RT-PCR (top) and real-time RT-PCR (bottom). (G) The immunohistochemical examinations of 11β-HSD1 and 11β-HSD2 in the placenta of CoCl2- or VSV-treated mice above (original magnification ×400). The density mean was calculated, as described in Materials and Methods (*p < 0.001).
activity in human placenta (25). To discern whether the differential HSD2 levels are attributable to differential levels of progesterone and estrogen, we i.p. injected estradiol or progesterone to mice with 8.5 d pregnancy to determine the expression of 11β-HSD2 in placenta tissues. Neither estradiol nor progesterone was found to have effect on HSD2 expression, evaluated by RT-PCR, real-time RT-PCR, and immunohistochemical staining (Supplemental Fig. 2A–C), suggesting that, in our system, the differences in HSD2 levels might not be attributable to the differential sex steroid hormone expression. In addition, the levels of estradiol and progesterone were also measured. The blood levels of estradiol and progesterone in spontaneous miscarriage women were found to be significantly lower than those in normal pregnancies (Supplemental Fig. 2D). Consistently, the blood levels of estradiol and progesterone in LPS-treated pregnant mice were significantly lower than those in the pregnant mice treated with LPS plus LXA4 (Supplemental Fig. 2E). Such striking decreases in estradiol and progesterone might be ascribed to the loss of the baby (Fig. 8).

**Discussion**

To date, the molecular mechanisms underlying miscarriages remain elusive. The present study provides evidence that stresses might enhance the activity of glucocorticoids, leading to a miscarriage via the lipid mediator LXA4 pathway. Despite the fact that inflammation plays an important role in the early stages of pregnancies and late stages preceding labor (26, 27), mounting evidence suggests that spontaneous miscarriages are associated with a bias toward a proinflammatory cytokine profile with respect to normal pregnancies (28). Thus, the body has to employ an elaborate mechanism to optimally and advantageously regulate inflammation in pregnancy. LXA4, a local endogenous eicosanoid, might play such a role. In counter-regulating the evolvement of inflammation, LXA4 may exert its function by inhibiting neutrophil and eosinophil recruitment and down-regulating NF-κB activation (29). Furthermore, LXA4 blocks dendritic cell IL-12 production by increasing suppressor of cytokine signaling-2 expression (30) and stimulates phagocytosis of apoptotic cells by reprogramming macrophages from M1 to M2 types (31). In addition, LXA4 may reduce angiogenesis, cell proliferation, and fibrosis through antagonizing leukotrienes, growth factor receptors (such as VEGF and platelet-derived growth factor), and others (23).

In other words, LXA4 may promote inflammatory resolution at different levels. However, whether LXA4 regulates pregnant inflammation remains unclear. This study has shown that LXA4 effectively inhibits LPS-induced proinflammatory factors, including iNOS, CCL2, IFN-γ, and IL-6, while increasing IL-10 expression in the placenta. Perhaps more interestingly, our observations showed that the administration of LPS to pregnant mice increased mast cell density in mouse uterus and placenta, whereas the administration of LXA4 counteracted such effects, suggesting that LXA4 might regulate the migration of mast cells to the maternal–fetal interface (J. Zhao and B. Huang, unpublished observations). As a central player of inflammation, mast cells release a variety of inflammatory mediators, including histamine, proteases, and cytokines, such as IL-6, IFN-γ, etc., thereby mediating inflammatory development (32, 33). Our unpublished observations probably implicate an important role of mast cells in miscarriages. Taken together, we propose in this work that endogenous LXA4 is a key factor in keeping normal pregnancies by regulating inflammation-associated factors, mast cells, and probably others.

A key issue that remained unresolved was how LXA4 decreased in a miscarriage. It is commonly known that LXA4 is biosynthesized by lipoxigenases 5-LO/12-LO or 15-LO/5-LO. Therefore, factors that induce miscarriages might regulate activities of those enzymes. Although not all the etiologies nor mechanisms of a miscarriage are well defined, all the causes might be linked to endogenous glucocorticoids, key steroid hormones, involved in stress responses and essential for the maturation of fetal organs, growth, and preparation of the fetus for birth (34, 35). This study found that dexamethasone negatively regulated lipoxigenases in LPS-induced murine miscarriages, a finding consistent and in line with previous reports (18–20). Thus, glucocorticoids can be identified in this study as the molecular basis underlying decreases in LXA4 levels in a miscarriage. The exact molecular regulation pathway, however, is beyond the current study and is worthy of investigation in the future.

What has been the important finding of this study lies in finding that whereas systemic cortisols are not changed, their utilization in chorionic villus or placenta is significantly enhanced in a miscarriage. As the key part of homeostatic control mechanisms, glucocorticoids affect perhaps ~5% of the genome. Therefore, multiple mechanisms are evolved to strictly regulate glucocorticoid signaling. Besides the regulation of its receptor, the enzymes 11β-HSD1 and 11β-HSD2 are critical for the regulation. Glucocorticoid must be hydroxylated to acquire its functional activity. The 11β-HSD1 uses NADPH to generate active glucocorticoid. In contrast, 11β-HSD2 uses NAD+ to produce a dehydroxy form of glucocorticoid. In the placenta, 11β-HSD2 represents the active component of the functional placental barrier that controls the trans-placental passage of germinal centers protecting the fetus against excessive exposure (36, 37). The reduced amount and/or activity of 11β-HSD2 in placentas, and the accompanying increase in exposure of the placenta and embryo/fetus to active germinal centers, could be a consequence of impaired placental perfusion and hypoxia (38, 39). This study, respectively, used LPS, VSV, and CoCl2 to construct a mouse model of miscarriage. Regardless of the cause of miscarriage, the placenta is characterized by 11β-HSD2 decreases with unchanged 11β-HSD1. This local increase of the utility of glucocorticoid is very important, because it leads to the avoidance of the systemic side effect of high concentration of glucocorticoid. However, how infections or stresses reduce the expression of 11β-HSD2 in miscarriage is not addressed in this study, and the further elucidation of the underlying mechanism is worthy of investigation.

**FIGURE 8.** A scheme of spontaneous miscarriages induced by the stress/glucocorticoid/LXA4 axis.
Based on the above findings, this study proposes a common mechanism underlying the occurrence of miscarriage (Fig. 8), providing new insights into early loss of pregnancies. This study also suggests that the administration of LXA4 might be a potential strategy to prevent miscarriages in early pregnancies.

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Disclosures
The authors have no financial conflicts of interest.

References

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Supplemental Figure 1. Lipoxygenases are regulated by corticosterone. 0, 0.5, 2.5, 5 and 10mg/kg corticosterone was i.v. injected to mice on Day 5.5 of pregnancy (n=8) once per day for 5 days. The expressions of 5-LO, 12-LO and 15-LO in the uterus were determined by immunohistochemical staining. The results were 400× magnification. The density mean was calculated as described in Materials and methods. * P <0.05, compared to normal control.
Supplemental Figure 2. HSD2 is not regulated by estradiol or progesterone. (A) Estradiol (25μg/kg) or progesterone (1mg/mouse) were i.p. injected to mice with 8.5 d pregnancy. The expression of 11β-HSD2 in placenta tissues 6h and 12h after injection were determined by immunohistochemical staining (magnification×400), the mean density was calculated as described in Materials and methods (*, \(P>0.05\)). (B and C) The expression of 11β-HSD2 was determined by RT-PCR (B) and real time RT-PCR (C). (D) The blood levels of estradiol and progesterone in normal human pregnancies and miscarriages (n=8 per group, *, \(P<0.001\)). (E) The blood levels of estradiol and progesterone in pregnant mice treated with LPS or LPS+LXA4 (n=7 per group, *, \(P<0.01\)).
### Supplemental Table 1

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<th>Target mRNA</th>
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<td>5'-CTGGACCTGATGGTGGAC-3'</td>
</tr>
<tr>
<td><strong>Mouse 5-LO</strong></td>
<td>Forward</td>
<td>5'-CATCGAGTTCACATGTACC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CCTCTCTGACTCTCGTCT-3'</td>
</tr>
<tr>
<td><strong>Mouse 12-LO</strong></td>
<td>Forward</td>
<td>5'-CAACACAGCAGCACTTGG-3'</td>
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<tr>
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<td>Reverse</td>
<td>5'-GTAGGCAAAGAAGTGCATC-3'</td>
</tr>
<tr>
<td><strong>Mouse 15-LO</strong></td>
<td>Forward</td>
<td>5'-TGAGAAGAAGAGAGAATCTG-3'</td>
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<tr>
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<td>Reverse</td>
<td>5'-ATCCGCTTCAACAGAGTCG-3'</td>
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<tr>
<td><strong>Mouse 11-βHSD1</strong></td>
<td>Forward</td>
<td>5'-AACCACATCAGCAAGATCTC-3'</td>
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<td>5'-ACCCATAACAGCAAACTTG-3'</td>
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<tr>
<td><strong>Mouse 11-βHSD2</strong></td>
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<td>5'-GAAGCTCACTCTTGAC-3'</td>
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<tr>
<td><strong>Human GAPDH</strong></td>
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<td>5'-CCCTCATTGACCTCACAATC-3'</td>
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<td>Reverse</td>
<td>5'-GGTGAAGGTATCCATTTG-3'</td>
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<tr>
<td><strong>Human 5-LO</strong></td>
<td>Forward</td>
<td>5'-CATCTCAAGCAACACCGAC-3'</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-TCAGGAGACTGTTGGACCAAC-3'</td>
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<tr>
<td><strong>Human 12-LO</strong></td>
<td>Forward</td>
<td>5'-GACCGTAAAGGATGATC-3'</td>
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<tr>
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<td>Reverse</td>
<td>5'-CTGCAACAGGGAACATTC-3'</td>
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<tr>
<td><strong>Human 15-LO</strong></td>
<td>Forward</td>
<td>5'-GGAAGAGAGAAGGAGGATG-3'</td>
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<td>Reverse</td>
<td>5'-GCTACAGAGAATGACCTTG-3'</td>
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<tr>
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