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Obeticholic Acid Protects against Lipopolysaccharide-Induced Fetal Death and Intrauterine Growth Restriction through Its Anti-Inflammatory Activity

Yuan-Hua Chen,*,†,‡,[1] Xiao-Guang Hu,*,†,[1] Yan Zhou,*,† Zhen Yu,*,† Lin Fu,* Gui-Bin Zhang,* Qing-Li Bo,*,† Hua Wang,*,† Cheng Zhang,* and De-Xiang Xu*,†

Farnesoid X receptor (FXR) is expressed in human and rodent placentas. Nevertheless, its function remains obscure. This study investigated the effects of obeticholic acid (OCA), a novel synthetic FXR agonist, on LPS-induced fetal death and intrauterine growth restriction. All pregnant mice except controls were i.p. injected with LPS (100 μg/kg) daily from gestational day (GD) 15 to GD17. Some pregnant mice were orally administered with OCA (5 mg/kg) daily from GD13 to GD17. As expected, placental FXR signaling was activated by OCA. OCA pretreatment protected against LPS-induced fetal death. In addition, OCA pretreatment alleviated LPS-induced reduction of fetal weight and crown-rump length. Additional experiments showed that OCA inhibited LPS-evoked TNF-α in maternal serum and amniotic fluid. Moreover, OCA significantly attenuated LPS-induced upregulation of placental proinflammatory genes including *Tnf-α*, *Il-1β*, *IL-6*, *Il-12*, *Mip-2*, *Kc*, and *Mcp-1*. By contrast, OCA elevated anti-inflammatory cytokine IL-10 in maternal serum, amniotic fluid, and placenta. Further analysis showed that OCA blocked nuclear translocation of NF-κB p65 and p50 subunits in trophoblast giant cells of the labyrinth zone. These results provide a mechanistic explanation for placental FXR-mediated anti-inflammatory activity. Overall, this study provides evidence for roles of FXR as an important regulator of placental inflammation. ***The Journal of Immunology*, 2016, 197: 000–000.**

Lipopolysaccharide is a toxic component of cell walls and the outer cell membranes in gram-negative bacteria, and it is widely present in the digestive tracts of humans and animals (1). Gastrointestinal inflammatory diseases and excess alcohol intake are known to increase permeability of LPS from the gastrointestinal tract into blood (2). LPS has been associated with adverse developmental outcomes. According to several earlier reports, maternal LPS exposure at early gestational stage induced early embryo loss and fetal demise in rodent animals (3, 4). Moreover, maternal LPS exposure at middle gestational stage caused fetal death and abortion (5). Recently, several reports from our laboratory demonstrated that maternal LPS exposure during organogenesis induced neural tube defects (NTDs) in mice (6–9). Others and we found that maternal LPS exposure at late gestational stage caused preterm delivery, fetal growth restriction, and skeletal development retardation (10–12). Moreover, prenatal LPS exposure caused age- and sex-dependent impairments of neurobehavioral development (13). In addition, prenatal LPS exposure permanently impaired steroidogenesis and spermatogenesis in male mice (14).

Several epidemiological studies demonstrate that proinflammatory cytokines, such as TNF-α, and chemokines, such as IL-8, are associated with adverse pregnant outcomes including preterm delivery and fetal growth restriction (15, 16). Numerous animal experiments indicate that proinflammatory cytokines, such as TNF-α, contribute to LPS-induced fetal demise, NTDs, and intrauterine growth restriction (IUGR) (7, 8, 11, 17). Indeed, maternal LPS exposure during pregnancy elevated proinflammatory cytokines and chemokines in maternal serum and amniotic fluid (18). Several studies found that some chemicals alleviated LPS-induced abortion, preterm birth, fetal demise, NTDs, and fetal growth restriction through their anti-inflammatory activities (7–9, 12, 19–21).

The farnesoid X receptor (FXR) is a ligand-activated transcription factor that plays an important role in positively regulating genes involved in bile acid homeostasis (22). Increasing evidence demonstrates that FXR is also an immune regulator with anti-inflammatory activities (23). Obeticholic acid (OCA), a novel synthetic FXR agonist, is currently in phase II clinical trials for nonalcoholic steatohepatitis (24). Recently, an experimental report showed that OCA inhibited inflammation and preserved intestinal barrier in inflammatory bowel disease (25). Moreover, OCA alleviated experimental autoimmune encephalomyelitis (26). Indeed, FXR is also expressed in human and rodent placentas (27–29). Nevertheless, its function remains obscure.

The aim of this study was to investigate the effects of pretreatment with OCA on LPS-induced fetal death and IUGR in mice. We showed that pretreatment with OCA activated placental FXR signaling. We demonstrate that pretreatment with OCA protects against LPS-induced fetal death and IUGR through its anti-inflammatory activity. Our results provide evidence for roles of FXR as an important regulator of placental inflammation.

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Abbreviations used in this article: *Cox-2*, *cyclooxygenase-2*; FXR, farnesoid X receptor; GD, gestational day; IUGR, intrauterine growth restriction; NTD, neural tube defect; OCA, obeticholic acid; TGC, trophoblast giant cell; VDR, vitamin D receptor.

Materials and Methods

Chemicals and reagents

LPS (*Escherichia coli* LPS, serotype 0127:B8) and OCA were purchased from Sigma Chemical (St. Louis, MO). NF-κB p65, FXR, β-actin, and Lamin A/C Abs were from Santa Cruz Biotechnologies (Santa Cruz, CA). NF-κB p50 Ab was from Cell Signaling Technology (Beverley, MA). p-IκB and IκB Abs were purchased from Abcam (Cambridge, MA). Chemiluminescence (ECL) detection kit was from Pierce Biotechnology (Rockford, IL). TRI reagent was from Molecular Research Center (Cincinnati, OH). RNase-free DNase was from Promega (Madison, WI). All the other reagents were from Sigma or as indicated in the specified methods.

Animals and treatments

The ICR mice (10- to 12-wk-old; male mice: 30–34 g; female mice: 28–30 g) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories The animals were allowed free access to food and water at all times and were maintained on a 12-h light-dark cycle in a controlled temperature (20–25°C) and humidity (50 ± 5%) environment for a period of 1 wk before use. For mating purposes, four females were housed overnight with two males starting at 9:00 PM. Females were checked by 7:00 AM the next morning, and the presence of a vaginal plug was designated as gestational day (GD) 0. To investigate the effects of pretreatment with OCA on LPS-induced fetal death and growth restriction, 48 pregnant mice were divided into four groups randomly. In LPS alone and OCA+LPS groups, pregnant mice were i.p. injected with three doses of LPS (100 μg/kg) daily from GD15 to GD17. The dose of LPS used in this study referred to our previous study (30). In OCA alone and OCA+LPS groups, pregnant mice were orally administered with OCA (5 mg/kg) daily from GD13 to GD17. The dose of OCA used in this study referred to others (25, 26). All dams were sacrificed on GD18 and gravid uterine weights were recorded. For each litter, the number of live fetuses, dead fetuses, and resorption sites were counted. Live fetuses were weighed and crown-rump length was measured. In this study, the definition of IUGR used referred to others (17). We defined IUGR as a fetal weight falling below the 10th percentile for gestational age. The threshold of IUGR was determined through evaluating the distribution of all fetal weights from the saline-treated control cohort. Fetuses with weights <10th percentile were designated as IUGR. To investigate the effects of OCA on LPS-induced placental inflammation, 48 pregnant mice were divided into eight groups randomly. In LPS alone and OCA+LPS groups, pregnant mice were i.p. injected with LPS (100 μg/kg) on GD15. In OCA alone and LPS+OCA groups, pregnant mice were orally administered with OCA (5 mg/kg) daily from GD13 to GD15. All dams were sacrificed either 1 or 6 h after LPS injection. Maternal serum was collected for measurement of TNF-α and IL-10. Placentas, including decidual zone, spongiotrophoblast zone, and labyrinth zone, were collected for real-time RT-PCR and immunoblots. Some placentas were collected for immunohistochemistry. All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

Isolation of total RNA and real-time RT-PCR

Total RNA from placentas was extracted using TRI reagent. RNase-free DNase-treated total RNA (1.0 μg) was reverse-transcribed with avian myeloid leukemia virus reverse transcriptase (Promega). Real-time RT-PCR was performed with a LightCycler 480 SYBR Green I kit (Roche Diagnostics).

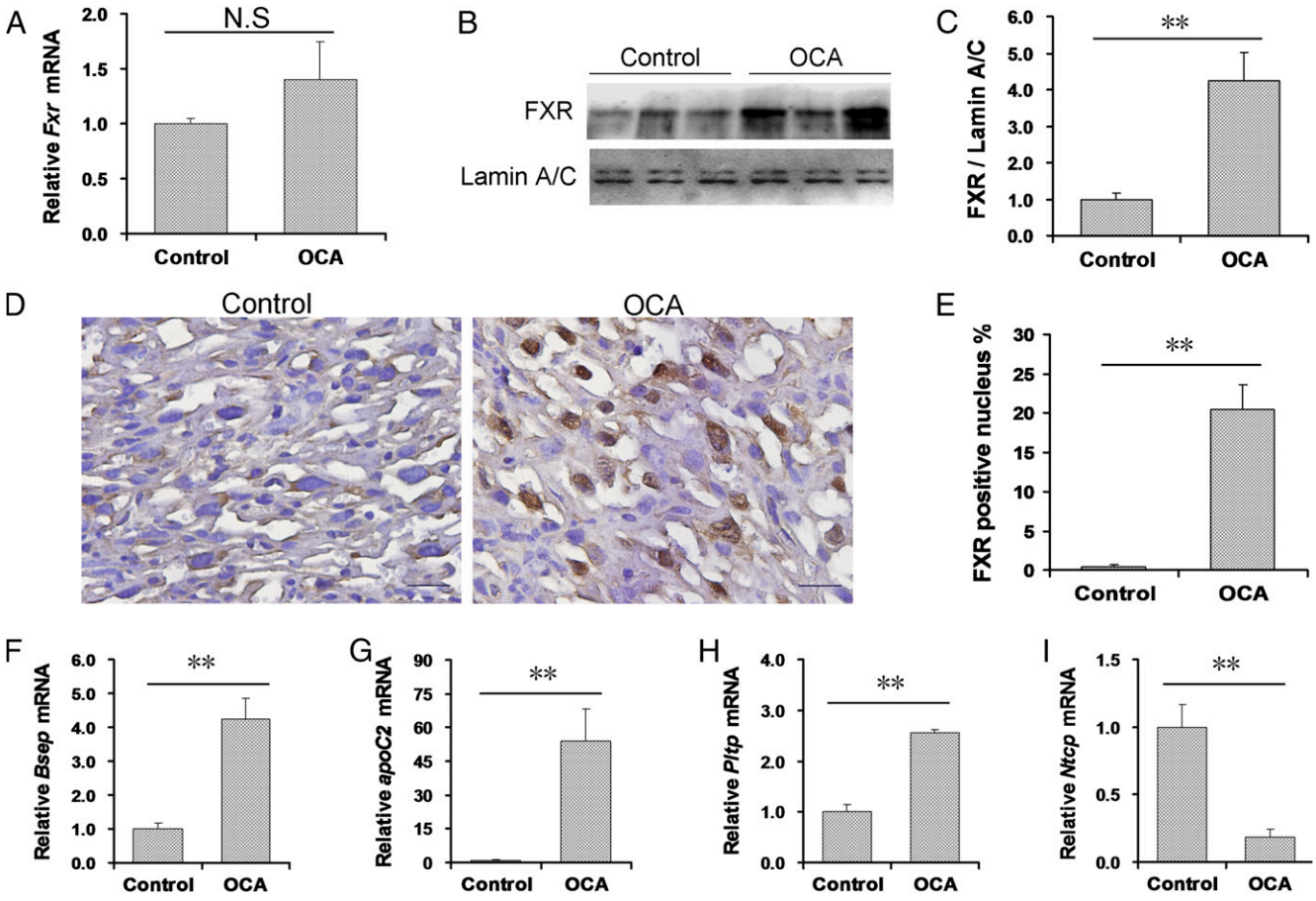

FIGURE 1. OCA pretreatment activates placental FXR signaling. Pregnant mice were administered with OCA (5 mg/kg) by gavage daily from GD13 to GD15. Mouse placentas were collected on GD15. (**A**) Placental *Fxr* mRNA was measured using real-time RT-PCR. All data were expressed as means ± SE of six samples from six different pregnant mice. (**B** and **C**) Nuclear FXR was measured using immunoblot. (B) A representative gel for FXR (upper panel) and Lamin A/C (lower panel) was shown. (C) All data were expressed as means ± SE of six samples from six different pregnant mice. (**D** and **E**) Nuclear translocation of FXR was analyzed using IHC. (D) Representative photomicrographs of placental histology from mice treated with saline (as control) and OCA alone are shown. Original magnification ×200. Nuclear translocation of FXR was observed in mononuclear sinusoidal TGCs of the labyrinth zone (arrows). (E) FXR^+ cells were analyzed. All data were expressed as means ± SE of six samples from six different pregnant mice. (**F–I**) Placental *Bsep*, *apoC2*, *Pltp*, and *Ntcp* mRNAs were measured using real-time RT-PCR. All data were expressed as means ± SE of six samples from six different pregnant mice. $^{**}p < 0.01$.

Table I. Oligonucleotide sequences and sizes of primers

Genes	Sequences	Sizes (bp)
18S	Forward: 5′-GTAACCCGTTGAACCCCATT-3′	151
	Reverse: 5′-CCATCCAATCGGTAGTAGCG-3′	
Bsep	Forward: 5′-GGTCGGAGGCCCAGACTTAT-3′	165
Pltp	Reverse: 5′-GGCGACCAAGCTTAGTAGTGT-3′	117
apoC2	Forward: 5′-TCCGGCTTGATGAGAACAGG-3′	193
	Reverse: 5′-CAGTACTCCAGTGGGTTGGC-3′	
Ntcp	Forward: 5′-TCTTTTATTTGCCCACAGCTACA-3′	134
	Reverse: 5′-CATCCTCCGACCCAGTGAAC-3′	
Fxr	Forward: 5′-GCATTACCAAGAACGCCGTG-3′	161
	Reverse: 5′-ACACTGGATTTCAGTTAACAAACCT-3′	
Tnf-α	Forward: 5′-CCCTCCTGGCCAACGGCATG-3′	109
	Reverse: 5′-TCGGGGCAGCCTTGTCCCTT-3′	
Il-1β	Forward: 5′-GCCTCGTGCTGTCGGACCCATAT-3′	143
	Reverse: 5′-TCCTTTGAGGCCCAAGGCCACA-3′	
Il-6	Forward: 5′-AGACAAAGCCAGAGTCCTTCAGAGA-3′	146
	Reverse: 5′-GCCACTCCTTCTGTGACTCCAGC-3′	
Il-12	Forward: 5′-ACCTGCCACAAAGGAGGCGAG-3′	155
	Reverse: 5′-GAGCACGTGAACCGTCCGGAG-3′	
Mip-2	Forward: 5′-TTGCCTTGACCCTGAAGCCCCC-3′	175
	Reverse: 5′-GGCACATCAGGTACGATCCAGGC-3′	
Kc	Forward: 5′-ACTCAAGAATGGTCGCGAGG-3′	123
	Reverse: 5′-GTGCCATCAGAGCAGTCTGT-3′	
Mcp-1	Forward: 5′-GGCTGGAGAGCTACAAGAGG-3′	93
	Reverse: 5′-GGTCAGCACAGACCTCTCTC-3′	
Il-10	Forward: 5′-TTCCCAGTCGGCCAGAGCCA-3′	198
	Reverse: 5′-TGCCTGGGGCATCACTTCTACCA-3′	
Inos	Forward: 5′-GCTCGCTTTGCCACGGACGA-3′	146
	Reverse: 5′-AAGGCAGCGGGCACATGCAA-3′	
Cox-2	Forward: 5′-GGGCTCAGCCAGGCAGCAAAT-3′	187
	Reverse: 5′-GCACTGTGTTTGGGGTGGGCT-3′	

The amplification reactions were carried out on a LightCycler 480 Instrument (Roche Diagnostics) with an initial hold step (95˚C for 5 min) and 50 cycles of a three-step PCR (95˚C for 15 s, 60˚C for 15 s, 72˚C for 30 s).

Immunoblots

For nuclear extraction, total lysate from mouse placentas was suspended in hypotonic buffer and then kept on ice for 15 min. The suspension was then mixed with detergent and centrifuged for 30 s at 14,000 × *g*. The nuclear pellet obtained was resuspended in complete lysis buffer in the presence of the protease inhibitor mixture, incubated for 30 min on ice, and centrifuged for 10 min at 14,000 × *g*. Protein concentrations were determined with the bicinchoninic acid protein assay reagents (Pierce Biotechnology) according to the manufacturer's instructions. For immunoblots, the same amount of protein (40–80 μg) was separated electrophoretically by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membranes were incubated for 2 h with the following Abs: FXR, p-IκB, IκB, NF-κB p65, and p50. For nuclear protein, lamin A/C was used as a loading control. For total proteins, β-actin was used as a loading control. After washes in Dulbecco's PBS containing 0.05% Tween 20 four times for 10 min each, the membranes were incubated with goat anti-rabbit IgG or goat anti-mouse Ab for 2 h. The membranes were then washed four times in Dulbecco's PBS containing 0.05% Tween 20 for 10 min each, followed by signal development using an ECL detection kit.

Immunohistochemistry

Placenta was fixed in 4% formalin and embedded in paraffin according to the standard procedure. Paraffin-embedded tissues were cut 5 μm thick. For immunohistochemistry, paraffin-embedded placental sections were deparaffinized and rehydrated in a graded ethanol series. After Ag retrieval and quenching of endogenous peroxidase, sections were incubated with anti-p65, anti-p50, or anti-FXR mAbs (1:200) at 4˚C overnight. The color reaction was developed with the HRP-linked polymer detection system and counterstaining with hematoxylin. The identification of positive cell in this study referred to others (31–33). Mononuclear sinusoidal trophoblast giant cells (TGCs) have large nuclei, line maternal blood sinusoids in the labyrinth zone, and are readily identified based on position and morphology.

ELISA

Commercial ELISA (R&D Systems, Abingdon, Oxon, U.K.) kits were used to determine levels of TNF-α and IL-10 according to the manufacturer's protocol.

Statistical analysis

The litter was considered the unit for statistical comparison among different groups. Fetal mortality was calculated per litter and then averaged per group. For fetal weight and crown-rump length, the means were calculated

Table II. Fetal outcomes among different groups

Parameters	Control	OCA	LPS	OCA+LPS
Pregnant mice (*n*)	12	12	12	12
Abortions (*n*)	0	0	0	0
Resorptions per litter (*n*)	0.3 ± 0.20	0.5 ± 0.17	0.2 ± 0.15	0.3 ± 0.12
Dead fetuses per litter (*n*)	0.2 ± 0.12	0.3 ± 0.12	3.3 ± 0.82[b]	0.4 ± 0.17[d]
Live fetuses per litter (*n*)	14.2 ± 0.93	13.8 ± 0.56	11.8 ± 0.72[a]	14.0 ± 0.44c
Fetal weight (g)	1.37 ± 0.023	1.37 ± 0.015	1.15 ± 0.020[b]	1.26 ± 0.019[d]
Crown-rump length (cm)	2.41 ± 0.257	2.42 ± 0.135	2.24 ± 0.212[b]	2.30 ± 0.211[c]
Average placental weight (g)	0.097 ± 0.003	0.095 ± 0.001	0.083 ± 0.002[b]	0.091 ± 0.00[c]
Placental diameter (mm)	8.062 ± 0.086	7.993 ± 0.057	7.511 ± 0.047[b]	7.739 ± 0.07[c]

[a]$p < 0.05$, [b]$p < 0.01$ as compared with control group; [c]$p < 0.05$, [d]$p < 0.01$ as compared with LPS group.

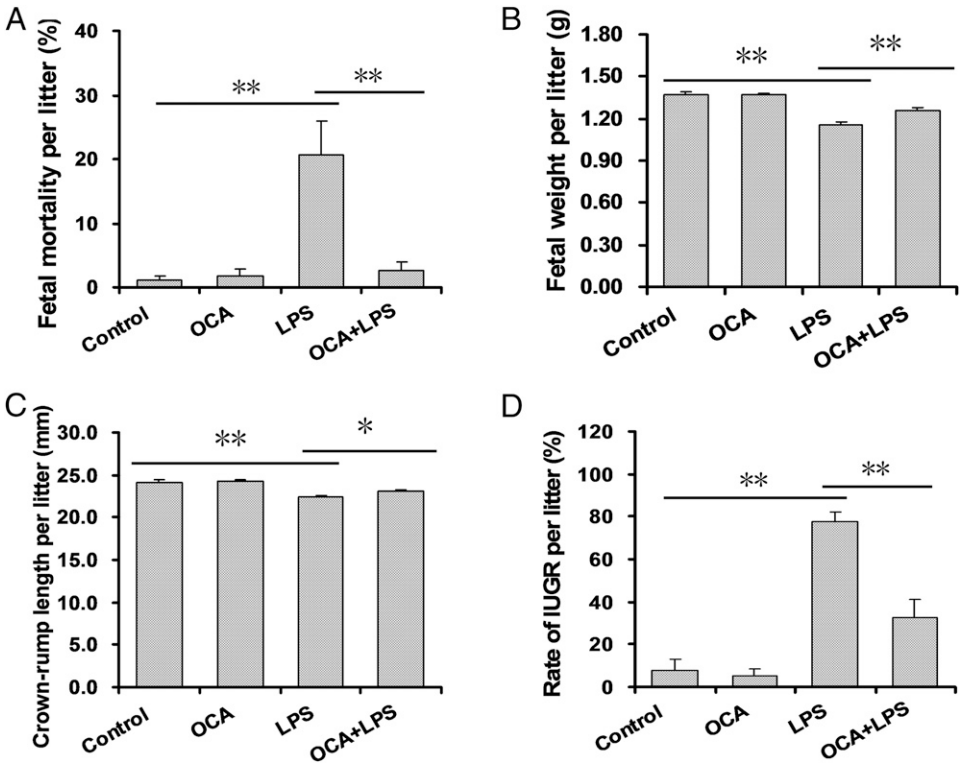

FIGURE 2. OCA pretreatment alleviates LPS-induced fetal death and growth restriction. In LPS alone and OCA+LPS groups, pregnant mice were i.p. injected with LPS (100 μg/kg) daily from GD15 to GD17. In OCA alone and OCA+LPS groups, pregnant mice were administered with OCA (5 mg/kg) by gavage daily from GD13 to GD17. All dams were sacrificed on GD18. The numbers of live fetuses and dead fetuses were counted. (**A**) Fetal mortality. (**B**) Fetal weight. (**C**) Fetal crown-rump length. (**D**) Rate of IUGR fetuses. All data were expressed as means ± SE (n = 12). $^{**}p < 0.01$.

per litter and then averaged per group. Quantified data were expressed as means ± SE at each point; $p < 0.05$ was considered statistically significant. ANOVA and the Student–Newman–Keuls post hoc test were used to determine differences between the treated animals and the control and statistical significance.

Results

OCA pretreatment activates placental FXR signaling

The effects of OCA on placental FXR signaling are presented in Fig. 1. Although OCA pretreatment had little effect on placental *Fxr* mRNA (Fig. 1A), the level of placental nuclear FXR was elevated in OCA-pretreated mice (Fig. 1B, 1C). Immunohistochemistry showed that nuclear translocation of FXR was observed in mononuclear sinusoidal TGCs of the labyrinth zone (Fig. 1D, 1E). There are at least four subtypes of unique TGCs in mouse placenta: spiral artery-associated TGC, parietal TGC, canal TGC, and sinusoidal TGC (32). This study had not observed nuclear FXR translocation in spiral artery-associated TGC, parietal TGC, and canal TGC (data not shown). Finally, we analyzed the effects of OCA on several target genes of FXR in mouse placenta. Gene-specific primers are presented in Table I. As expected, placental *Bsep*, *apoC2*, and *Pltp*, three target genes of FXR, were upregulated by OCA pretreatment (Fig. 1F–H). By contrast, placental *Ntcp*, a suppressive target gene of FXR, was downregulated in OCA-pretreated mice (Fig. 1I).

OCA pretreatment alleviates LPS-induced fetal death

No dams died throughout the pregnancy. In addition, all pregnant mice completed pregnancy. The rate of litters with dead fetuses and the number of dead fetuses per litter were analyzed. As expected, OCA alone did not increase fetal mortality. Intraperitoneal injection with LPS daily from GD15 to GD17 resulted in 100% (12/12) of pregnant mice with dead fetuses. The number of dead fetuses per litter was 3.3 in the LPS group (Table II). Further analysis showed that maternal LPS injection resulted in 21.9% dead fetuses in dams that completed the pregnancy (Fig. 2A). Interestingly, the number of dead fetuses per litter was reduced to 0.4 in OCA-pretreated mice (Table II). Moreover, OCA pretreatment significantly reduced LPS-evoked fetal mortality (Fig. 2A).

OCA pretreatment alleviates LPS-induced fetal growth restriction

The effects of OCA on fodder consumption were observed. As expected, OCA had no effect on fodder consumption of pregnant mice (data not shown). Fetal weight and crown-rump length were then analyzed. As expected, fetal weight and crown-rump length was significantly reduced in the LPS group (Fig. 2B, 2C). Interestingly, OCA pretreatment significantly alleviated LPS-induced reduction of fetal weight and crown-rump length (Fig. 2B, 2C). To determine the threshold of IUGR, we evaluated distribution of all fetal weights from saline-treated controls (12 dams and 170 live fetuses). Fetuses with weights <1.2307 g (<10th percentile) were designated as IUGR. The rate of IUGR per litter was ~80% in the LPS group (Fig. 2D). Interestingly, the rate of IUGR per litter was reduced to <40% in OCA+LPS group (Fig. 2D).

OCA pretreatment inhibits LPS-induced proinflammatory cytokines and chemokines

The effects of pretreatment with OCA on LPS-induced placental proinflammatory cytokines and chemokines were analyzed. Gene-specific primers are presented in Table I. As expected, the level of TNF-α in maternal serum and amniotic fluid was significantly elevated 1 h after LPS injection (Fig. 3A, 3B). Correspondingly, placental *Tnf-α*, *Il-1β*, *Il-6*, and *Il-12* mRNAs, four proinflammatory genes, were upregulated 1 h after LPS and remained elevated 6 h after LPS injection (Fig. 3C–F). Moreover, placental *Mip-2*, *Kc*, and *Mcp-1* mRNAs, three inflammatory chemokine genes, were significantly upregulated 1 h after LPS and remained elevated 6 h after LPS injection (Fig. 3G–I). Interestingly, pretreatment with OCA repressed LPS-induced release of TNF-α in maternal serum and amniotic fluid (Fig. 3A, 3B). Moreover, pretreatment with OCA repressed LPS-induced upregulation of placental

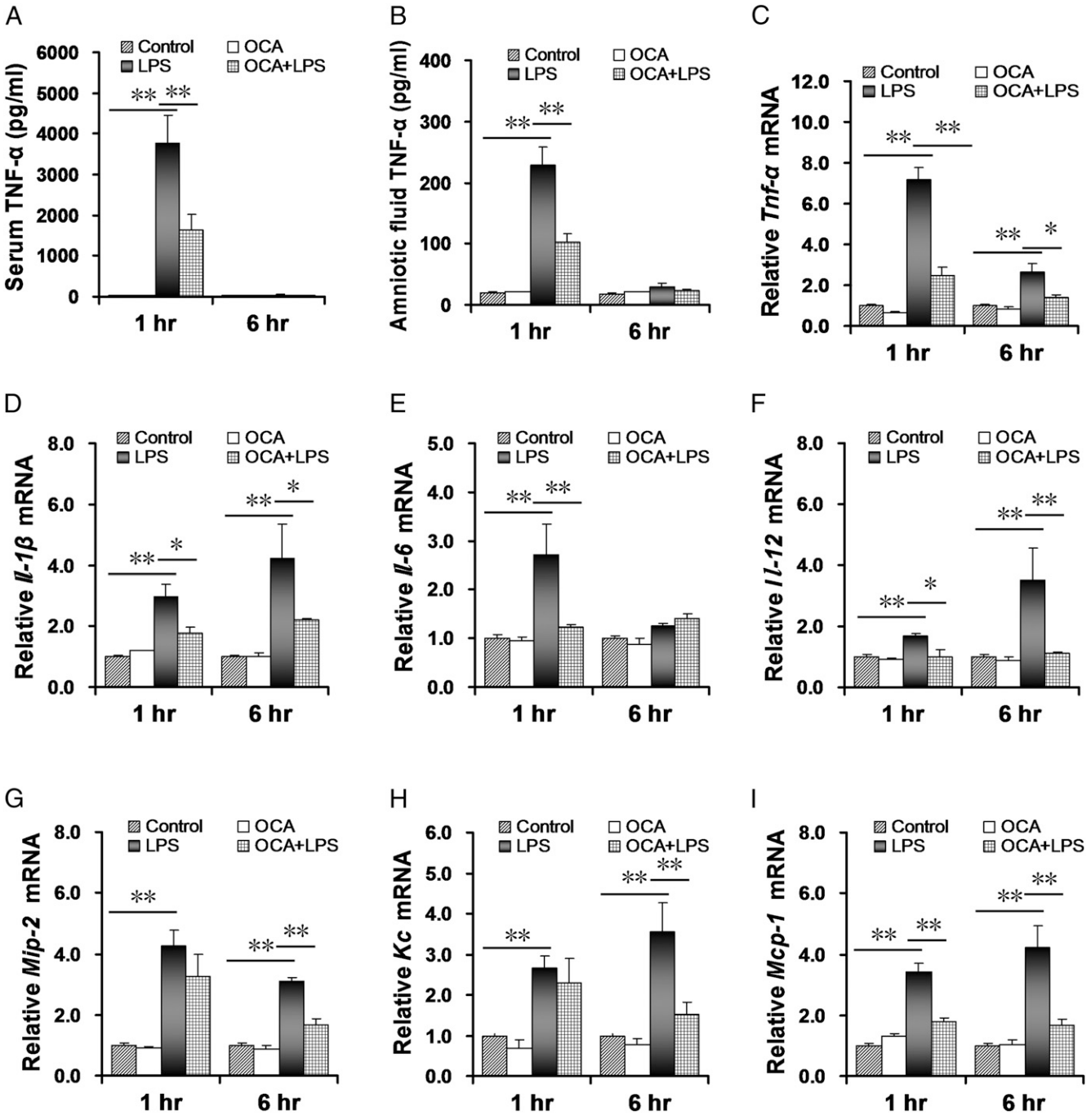

FIGURE 3. OCA pretreatment inhibits LPS-induced proinflammatory cytokines and chemokines. In LPS alone and OCA+LPS groups, pregnant mice were i.p. injected with a single dose of LPS (100 μg/kg) at GD15. In OCA alone and OCA+LPS groups, pregnant mice were administered with OCA (5 mg/kg) by gavage daily from GD13 to GD15. (**A**) Maternal serum was collected 1 and 6 h after LPS injection. TNF-α was measured using ELISA. (**B**) Amniotic fluid was collected 1 and 6 h after LPS injection. TNF-α was measured using ELISA. (**C–I**) Mouse placentas were collected 1 and 6 h after LPS injection. Placental *Tnf-α*, *Il-1β*, *Il-6*, *Il-12*, *Mip-2*, *Kc*, and *Mcp-1* mRNAs were measured using real-time RT-PCR. (C) *Tnf-α*. (D) *Il-1β*. (E) *Il-6*. (F) *Il-12*. (G) *Mip-2*. (H) *Kc*. (I) *Mcp-1*. All data were expressed as means ± SE of six samples from six different pregnant mice. $^*p < 0.05$, $^{**}p < 0.01$.

proinflammatory genes (Fig. 3C–F). In addition, pretreatment with OCA repressed LPS-induced upregulation of placental chemokine genes (Fig. 3G–I).

OCA pretreatment induced further elevation of IL-10 in LPS-treated pregnant mice

The levels of IL-10 in maternal serum and amniotic fluid were analyzed. As shown in Fig. 4A and 4B, the level of IL-10, an anti-inflammatory cytokine in maternal serum and amniotic fluid, was elevated at 1 h after LPS and remained elevated 6 h after LPS injection. Correspondingly, placental *Il-10* mRNA was significantly upregulated 1 h after LPS and remained elevated 6 h after LPS injection (Fig. 4C). The effects of OCA on LPS-induced IL-10 were then analyzed. As shown in Fig. 4A and 4C, OCA alone had little effect on IL-10 in maternal serum and *Il-10* mRNA in placenta. Moreover, OCA alone only slightly elevated the level of IL-10 in amniotic fluid (Fig. 4B). Interestingly, pretreatment with OCA significantly elevated LPS-induced release of IL-10 in maternal serum and amniotic fluid (Fig. 4A, 4B). Pretreatment with OCA significantly elevated placental *Il-10* mRNA in LPS-treated pregnant mice (Fig. 4C).

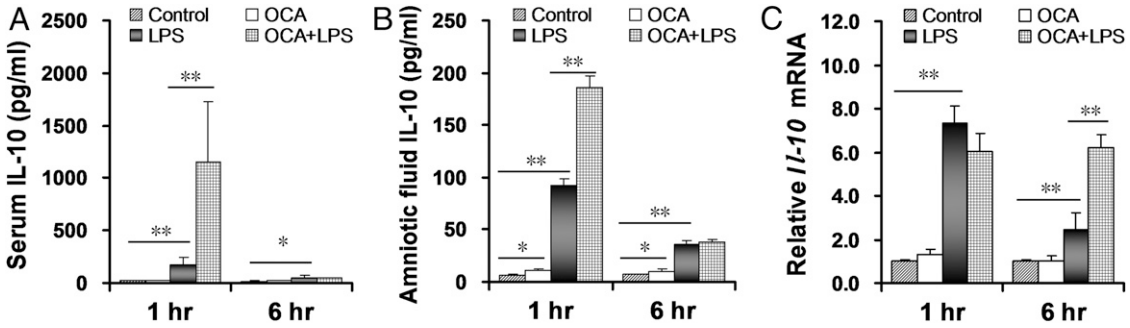

FIGURE 4. OCA pretreatment elevates IL-10 in maternal serum and placenta. In LPS alone and OCA+LPS groups, pregnant mice were i.p. injected with a single dose of LPS (100 μg/kg) on GD15. In OCA alone and OCA+LPS groups, pregnant mice were administered with OCA (5 mg/kg) by gavage daily from GD13 to GD15. (**A**) Maternal serum was collected 1 and 6 h after LPS injection. IL-10 was measured using ELISA. (**B**) Amniotic fluid was collected 1 and 6 h after LPS injection. IL-10 was measured using ELISA. (**C**) Mouse placentas were collected 1 and 6 h after LPS injection. Placental *Il-10* mRNA was measured using real-time RT-PCR. All data were expressed as means ± SE of six samples from six different pregnant mice. $*p < 0.05$, $**p < 0.01$.

OCA pretreatment has no effect on LPS-induced placental Inos *and* Cyclooxygenase-2 *expression*

The effects of pretreatment with OCA on LPS-induced placental *Inos* and *cyclooxygenase-2* (*Cox-2*) were analyzed. Gene-specific primers are presented in Table I. As shown in Fig. 5A, the level of placental *Inos* mRNA was elevated 6 h after LPS injection. In addition, placental *Cox-2* mRNAs were significantly upregulated 1 h after LPS and remained elevated 6 h after LPS injection (Fig. 5B). Unexpectedly, pretreatment with OCA did not inhibit LPS-induced placental *Inos* and *Cox-2* expression (Fig. 5).

OCA pretreatment inhibits LPS-induced placental NF-κB activation

To investigate the effects of pretreatment with OCA on LPS-induced placental NF-κB activation, we measured placental p-IκB and IκB. As expected, placental p-IκB elevated by about 4-fold 1 h after LPS and remained elevated 6 h after LPS injection (Fig. 6A, 6B). On the contrary, placental IκB was significantly reduced 1 h after LPS injection (Fig. 6A, 6B). Interestingly, pretreatment with OCA significantly attenuated LPS-induced placental IκB phosphorylation (Fig. 6A, 6B). Placental nuclear NF-κB p65 and p50 subunits were then measured. As expected, nuclear NF-κB p65 subunit was significantly increased 1 h after LPS injection (Fig. 6C, 6D). In addition, nuclear NF-κB p50 subunit was significantly increased 1 h after LPS and remained elevated 6 h after LPS injection (Fig. 6C, 6D). Immunohistochemistry showed that nuclear translocation of NF-κB p65 and p50 was mainly observed in mononuclear sinusoidal TGCs of the labyrinth zone (Fig. 6E, 6G). Interestingly, pretreatment with OCA significantly attenuated LPS-induced elevation of placental nuclear NF-κB p65 and p50 subunits (Fig. 6C, 6D). Immunohistochemistry showed that pretreatment with OCA significantly attenuated LPS-evoked nuclear translocation of placental NF-κB p65 and p50 in mononuclear sinusoidal TGCs of the labyrinth zone (Fig. 6F, 6H).

Discussion

In this study, we investigated the effect of pretreatment with OCA, a novel FXR agonist, on LPS-induced fetal death and IUGR in mice. Although OCA alone had no effect on pregnant outcomes, pretreatment with OCA markedly reduced LPS-induced fetal mortality. In addition, pretreatment with OCA significantly attenuated LPS-induced reduction of fetal weight and crown-rump length. These results suggest that pretreatment with OCA protects against LPS-induced fetal death and IUGR.

Numerous reports showed that maternal LPS exposure during pregnancy elevated the levels of proinflammatory cytokines in maternal serum and amniotic fluid (18, 34), of which TNF-α was the major mediator leading to fetal death and IUGR (11, 17, 35). Indeed, OCA has an anti-inflammatory activity (25, 26). Recently, two experimental reports demonstrated that OCA reduced bacterial translocation and inhibited intestinal inflammation in cholestatic and cirrhotic rats (36, 37). This study investigated the effects of pretreatment with OCA on LPS-evoked maternal and placental inflammation. Our results found that pretreatment with OCA repressed LPS-evoked release of proinflammatory cytokine TNF-α in maternal serum and amniotic fluid. In addition, pretreatment with OCA significantly inhibited LPS-induced upregulation of proinflammatory genes in placenta. This study demonstrates for the first time, to our knowledge, that pretreatment with OCA inhibits LPS-induced inflammatory response. These results suggest that OCA protects against LPS-induced fetal death and IUGR partially through its anti-inflammatory activity.

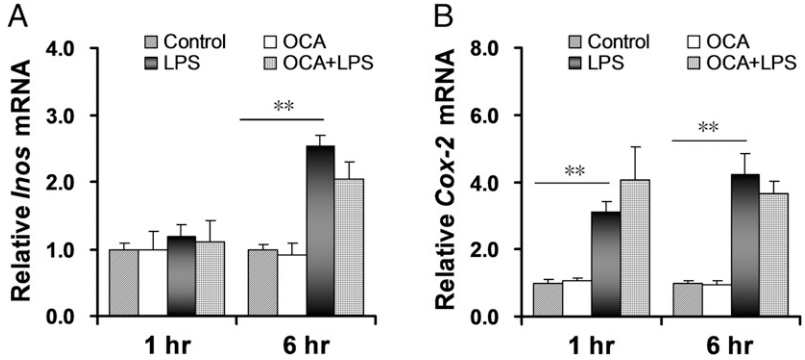

FIGURE 5. Effects of pretreatment with OCA on LPS-induced placental *Inos* and *Cox-2* expression. In LPS alone and OCA+LPS groups, pregnant mice were i.p. injected with a single dose of LPS (100 μg/kg) GD15. In OCA alone and OCA+LPS groups, pregnant mice were administered with OCA (5 mg/kg) by gavage daily from GD13 to GD15. Mouse placentas were collected 1 and 6 h after LPS injection. Placental *Inos* and *Cox-2* mRNAs were measured using real-time RT-PCR. (**A**) *Inos*. (**B**) *Cox-2*. All data were expressed as means ± SE of six samples from six different pregnant mice. $**p < 0.01$.

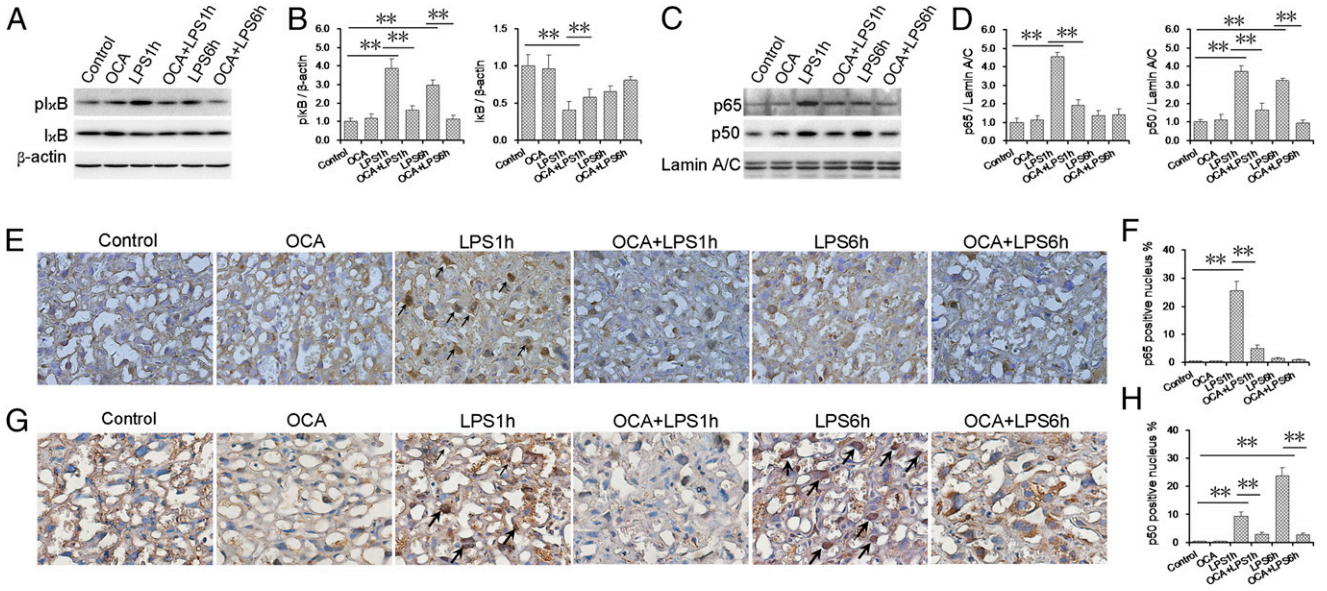

FIGURE 6. OCA pretreatment inhibits LPS-induced placental NF-κB activation. In LPS alone and OCA+LPS groups, pregnant mice were i.p. injected with a single dose of LPS (100 μg/kg) on GD15. In OCA alone and OCA+LPS groups, pregnant mice were administered with OCA (5 mg/kg) by gavage daily from GD13 to GD15. Mouse placentas were collected 1 and 6 h after LPS injection. (**A** and **B**) Placental p-IκB and IκB were measured using immunoblots. (A) A representative gel for p-IκB (upper panel), IκB (middle panel), and β-actin (lower panel) was shown. (B) All experiments were repeated six times. Quantitative analyses of scanning densitometry on six samples from six different litters were performed. (**C** and **D**) Nuclear fractions were prepared from placenta. Placental NF-κB p65 and p50 subunits were measured using immunoblots. (C) A representative gel for p65 (upper panel), p50 (middle panel), and Lamin A/C (lower panel) was shown. (D) All experiments were repeated six times. Quantitative analyses of scanning densitometry on six samples from six different litters were performed. (**E** and **F**) Nuclear translocation of placental NF-κB p65 subunit was analyzed using IHC. (E) Representative photomicrographs of placental histology from mice treated with saline (as control), OCA alone, LPS1h, OCA+LPS1h, LPS6h, and OCA+LPS6h are shown. Original magnification ×400. Nuclear translocation of placental NF-κB p65 was observed in mononuclear sinusoidal TGCs of the labyrinth zone (arrows). (F) $P65^+$ cells were compared among different groups. (**G** and **H**) Nuclear translocation of NF-κB p50 subunit was analyzed using IHC. (G) Representative photomicrographs of placental histology from mice treated with saline (as control), OCA alone, LPS1h, OCA+LPS1h, LPS6h, and OCA+LPS6h are shown. Original magnification ×400. NF-κB p50 subunit was mainly distributed in the labyrinth zone (brown). (H) $P50^+$ cells were compared among different groups. All data were expressed as means ± SE of six samples from six different pregnant mice. $^{**}p < 0.01$.

Increasing evidence demonstrates that inflammatory chemokines, highly expressed in human and mouse placentas, contribute to inflammation-associated early embryo loss, miscarriage, fetal demise, and preterm delivery (38, 39). According to a recent study, pretreatment with broad-spectrum chemokine inhibitor protected against LPS-mediated preterm birth (40). Another recent report showed that CXCR3 blockade protected against *Listeria monocytogenes* infection–induced fetal wastage in mice (41). This study showed that placental *Mip-2*, *Kc*, and *Mcp-1* mRNAs, three inflammatory chemokine genes, were rapidly upregulated 1 h after LPS and remained elevated 6 h after LPS injection. Interestingly, OCA pretreatment inhibited LPS-induced upregulation of placental chemokine genes. Several reports showed that excess NO and PGs were involved in LPS-induced fetal death and preterm delivery (42, 43). Indeed, this study showed that placental *inos* and *cox-2* were significantly upregulated in LPS-treated mice. Unexpectedly, pretreatment with OCA had no effect on LPS-induced upregulation of placental *inos* and *cox-2*. Thus, FXR-mediated protection against LPS-induced fetal death and IUGR is partially attributed to its repressive effect of inflammatory chemokines but independent of NO and PG production.

IL-10 is an important anti-inflammatory cytokine. An early epidemiological report showed that IL-10 was reduced in placentas of women undergoing preterm delivery (44). Similarly, IL-10 mRNA was downregulated in IUGR placentas (45). Indeed, an early report demonstrated that IL-10 protected against LPS-induced preterm birth (46). This study analyzed the effects of pretreatment with OCA on IL-10 in maternal serum, amniotic fluid, and placenta. As expected, placental *Il-10* gene was rapidly upregulated 1 h after LPS and remained elevated 6 h after LPS injection. Correspondingly, IL-10 in maternal serum and amniotic fluid was elevated at 1 h after LPS and remained elevated 6 h after LPS injection. The effects of OCA on LPS-induced IL-10 were then analyzed. Interestingly, pretreatment with OCA significantly elevated LPS-induced release of IL-10 in maternal serum and amniotic fluid. In addition, pretreatment with OCA significantly elevated placental *Il-10* mRNA in LPS-treated pregnant mice. Taken together, these results suggest that OCA selectively inhibits LPS-evoked proinflammatory cytokines and chemokines but elevates anti-inflammatory cytokines in maternal serum, amniotic fluid, and placenta. This study showed that OCA alone had little effect on IL-10 in maternal serum. Moreover, OCA alone only slightly elevated the level of IL-10 in amniotic fluid. In addition, OCA alone did not upregulate placental *Il-10* mRNA. These results are in agreement with a recent study from our laboratory, in which pretreatment with vitamin D3, a vitamin D receptor (VDR) agonist, elevated serum IL-10 in LPS-treated pregnant mice, whereas vitamin D3 alone had no effect on IL-10 in maternal serum (Y. Zhou, unpublished observations). Thus, additional study is necessary to determine the mechanism through which OCA upregulates IL-10 in maternal serum, amniotic fluid, and placenta of LPS-treated mice.

The mechanism through which OCA inhibits LPS-induced placental inflammation remains obscure. NF-κB is an important transcription factor that regulates TLR4-mediated inflammatory genes (47). Indeed, this study showed that the levels of placental nuclear NF-κB p65 and p50 subunits were elevated in LPS-treated mice. Correspondingly, nuclear translocation of NF-κB

p65 and p50 subunits was observed in mononuclear sinusoidal TGCs of the labyrinth zone, indicating that placental NF-κB signaling was activated. Several early studies demonstrate that activated nuclear receptors, such as liver X receptor and pregnane X receptor, repress NF-κB signaling in macrophages (48, 49). Two reports from our laboratory showed that activated VDR suppressed LPS-evoked NF-κB activation in mouse placentas and kidneys through reinforcing the interaction between VDR and NF-κB p65 subunit (30, 50). Recently, we found that activated peroxisome proliferator-activated receptor-γ inhibited LPS-induced placental inflammation through blocking nuclear translocation of NF-κB p65 and p50 subunits in mononuclear sinusoidal TGCs (51). This study analyzed the effects of OCA pretreatment on LPS-induced placental NF-κB activation. Our results showed that pretreatment with OCA, which promoted placental FXR activation, blocked LPS-evoked nuclear translocation of NF-κB p65 and p50 subunits in mononuclear sinusoidal TGCs of the labyrinth zone. These results suggest that OCA inhibits LPS-induced placental inflammation, at least partially, through repressing placental NF-κB activation. Several studies demonstrated that FXR was also highly expressed in liver, intestine, and kidney (25, 29, 52). Moreover, FXR agonist inhibited inflammatory cytokines in mouse colonic mucosa and liver (25, 53). Thus, this study cannot exclude the possibility that OCA protects against LPS-induced fetal death and IUGR through inhibiting NF-κB activation and inflammatory cytokines in liver, intestine, and kidney.

FXR-mediated inhibition on placental inflammation may have preventive and therapeutic implications. According to a population-based cohort study, intrahepatic cholestasis of pregnancy was associated with adverse pregnant outcomes including gestational diabetes, preeclampsia, and preterm delivery (54). Indeed, FXR is a target for the treatment of intrahepatic cholestasis of pregnancy (55). A recent study showed that FXR agonist not only modulated bile acid balance but also protected against placental oxidative stress (29). This study found that pretreatment with OCA, which activated placental FXR signaling, repressed placental inflammation and protected mice from LPS-induced fetal death and IUGR. Thus, FXR agonist may be used as pharmacological agents to prevent inflammation-associated fetal demise and IUGR.

This study placed emphasis on whether pretreatment with OCA protected against LPS-induced fetal death and IUGR. However, this study has several limitations. First, this study had not investigated the effects of posttreatment with OCA on LPS-induced fetal death and IUGR. Second, this study had not investigated the effects of OCA on LPS-induced early pregnancy loss and preterm delivery. Indeed, several studies showed that maternal exposure to higher doses of LPS at late pregnant stage induced preterm delivery (20, 56). In addition, maternal LPS exposure at early gestational stage induced early pregnancy loss (4). Thus, additional experiment is required to investigate whether OCA protects against LPS-induced early pregnant loss and preterm birth in mice.

In summary, this study investigated the effects of pretreatment with OCA on LPS-induced fetal death and IUGR in mice. We found that pretreatment with OCA protected against LPS-induced fetal death and IUGR through its anti-inflammatory activity. We demonstrate for the first time, to our knowledge, that pretreatment with OCA, which activates placental FXR signaling, simultaneously inhibits LPS-evoked activation of NF-κB signaling in placenta. Our results provide a mechanistic explanation for placental FXR-mediated anti-inflammatory activity. Overall, this study provides evidence for roles of FXR as an important regulator of placental inflammation.

Disclosures

The authors have no financial conflicts of interest.

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