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Rosiglitazone pretreatment protects against lipopolysaccharide-induced fetal demise through inhibiting placental inflammation

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ABSTRACT

Peroxisome proliferator-activated receptor (PPAR)-γ is highly expressed in human and rodent placentas. Nevertheless, its function remains obscure. The present study investigated the effects of rosiglitazone, a PPAR-γ agonist, on LPS-induced fetal death. All pregnant mice except controls were intraperitoneally injected with LPS (150 μg/kg) daily from gestational day (GD)15 to GD17. As expected, maternal LPS injection caused placental inflammation and resulted in 63.6% fetal death in dams that completed the pregnancy. Interestingly, LPS-induced fetal mortality was reduced to 16.0% when pregnant mice were pretreated with RSG. Additional experiment showed that rosiglitazone pretreatment inhibited LPS-induced expressions of tumor necrosis factor (*Tnf*)-α, interleukin (*Il*)-1β, *Il-6*, macrophage inflammatory protein (*Mip*)-2 and keratinocyte-derived chemokine (*Kc*) in mouse placenta. Although rosiglitazone had little effect on LPS-evoked elevation of IL-10 in amniotic fluid, it alleviated LPS-evoked release of TNF-α and MIP-2 in amniotic fluid. Further analysis showed that pretreatment with rosiglitazone, which activated placental PPAR-γ signaling, simultaneously suppressed LPS-evoked nuclear factor kappa B (NF-κB) activation and blocked nuclear translocation of NF-κB p65 and p50 subunits in trophoblast giant cells of the labyrinth layer. These results provide a mechanistic explanation for PPAR-γ-mediated anti-inflammatory activity in the placentas. Overall, the present study provides additional evidence for roles of PPAR-γ as an important regulator of placental inflammation.

Lipopolysaccharide (LPS) is a toxic component of cell walls in gram-negative bacteria and is widely present in the digestive tracts of humans and animals (Jacob et al., 1997). Humans are constantly exposed to low levels of LPS through infection. Gastrointestinal inflammatory diseases and excess alcohol intake are known to increase permeability of LPS from gastrointestinal tract into blood (Zhou et al., 2003). LPS has been associated with adverse developmental outcomes. According to several earlier reports, maternal LPS exposure at early gestational stage induced embryonic resorptions and fetal death in rodent animals (Gendron et al., 1990; Ogando et al., 2003). Moreover, maternal LPS exposure at middle gestational stages caused fetal death and abortion (Leazer et al., 2002). Several studies demonstrated that maternal LPS exposure during organogenesis induced fetal malformations and skeletal abnormalities in rats, golden hamsters and mice (Ornoy and Altshuler, 1976; Lanning et al., 1983; Collins et al., 1994; Zhao et al., 2008, 2014; Fu et al., 2014; Chen et al., 2015a). Increasing evidence demonstrated that maternal LPS exposure at late gestational stage caused preterm delivery, fetal death, fetal growth restriction and skeletal development retardation (Rivera et al., 1998; Buhimschi et al., 2003; Xu et al., 2005, 2006a, 2007a; Guo et al., 2013). Moreover, maternal LPS exposure during pregnancy caused age- and gender-dependent impairments of neurobehavioral development in offspring (Romero et al., 2007; Wang et al., 2010; Xia et al., 2014; Solati et al., 2015; Zager et al., 2015). In addition, maternal LPS exposure at late gestational stage permanently impaired steroidogenesis and spermatogenesis in male offspring (Wang et al., 2014).

Numerous studies demonstrate that inflammatory cytokines

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and chemokines, such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-8, are associated with adverse pregnant outcomes including preterm delivery, fetal death and fetal growth restriction (Leazer et al., 2002; Xu et al., 2006b; Rode et al., 2012; Cotechini et al., 2014). Indeed, maternal LPS exposure elevated the levels of inflammatory cytokines in maternal serum, amniotic fluid, fetal liver and fetal brain (Xu et al., 2007b; Ning et al., 2008; Li et al., 2008). Several studies found that some chemicals alleviated LPS-induced abortion, preterm birth, fetal death, neural tube defects and fetal growth restriction through their anti-inflammatory activity (Chen et al., 2006, 2012; Awad et al., 2011; Zhao et al., 2013; Zhao et al., 2014; Fu et al., 2014; Chen et al., 2015b).

Peroxisome proliferator-activated receptor (PPAR)-γ is a nuclear receptor with an immune modulation and antiinflammatory activity (Ahmadian et al., 2013). An early report showed that rosiglitazone (RSG), an agonist of PPAR-γ, inhibited the release of inflammatory cytokines during LPS-evoked acute kidney injury (Lee et al., 2005). An in vitro study found that RSG inhibited LPS-induced up-regulation of inflammatory cytokines in HK-2 cells (Wang et al., 2011a). Indeed, PPAR-γ is highly expressed in human and rodent placentas (Wang et al., 2002; Capobianco et al., 2013). In the present study, we investigated the effects of RSG pretreatment on LPS-induced fetal death. We demonstrated that pretreatment with RSG protected mice from LPS-induced fetal death through inhibiting placental inflammation. Our results provide additional evidence for roles of PPAR-γ as an important regulator of placental inflammation.

1. Materials and methods

1.1. Chemicals and reagents

Lipopolysaccharide (*Escherichia coli* LPS, serotype 0127:B8) and RSG were purchased from Sigma Chemical Co. (St. Louis, MO). Nuclear factor-kappa B p65 (NF-κB p65) and Lamin antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). NF-κB p50 antibodies were from Cell Signaling Technology (Beverley, MA). PPAR-γ, Phosphor-inhibitor of kappa B (p-IκB) and IκB antibodies were purchased from Abcam (Cambridge, MA). Chemiluminescence (ECL) detection kit was from Pierce Biotechnology (Rockford, IL). TRI reagent was from Molecular Research Center, Inc (Cincinnati, Ohio). RNase-free DNase was from Promega Corporation (Madison, WI). All the other reagents were from Sigma or as indicated in the specified methods.

1.2. Animals and treatments

The ICR mice (8–10 week-old; male mice: 28–30 g; female mice: 24–26 g) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc. 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 week before use. For mating purposes, four females were housed overnight with two males starting at 9:00 P M. Females were checked by 7:00 A M. the next morning, and the presence of a vaginal plug was designated as gestational day (GD) 0. The present study consisted of two separate experiments.

1.2.1. Experiment 1

To investigate the effects of RSG on LPS-induced fetal demise, forty pregnant mice were divided into four groups randomly. In the RSG alone group and the RSG + LPS group, pregnant mice were orally administered with RSG (10 mg/kg) daily from GD13 to GD17. The dose of RSG used in the present study referred to others (Hwang et al., 2012). In the LPS alone group and the RSG + LPS group, pregnant mice were intraperitoneally (i.p.) injected with LPS (150 μg/kg) daily from GD15 to GD17. The dose of LPS used in the present study referred to others with minor modulation (Xu et al., 2007a). In control group, pregnant mice were i.p. injected with saline daily from GD15 to GD17. All dams were sacrificed 24 h after the last LPS injection (on GD18). For each litter, the number of live fetuses, dead fetuses and resorption sites were counted. Live fetuses were weighed and crown-rump lengths were measured in control group and RSG alone group.

1.2.2. Experiment 2

To investigate the effects of RSG on LPS-induced placental inflammatory signaling, twenty-four pregnant mice were divided into four groups randomly. In the RSG alone group and the LPS + RSG group, pregnant mice were orally administered with RSG (10 mg/kg) daily from GD13 to GD15. In the LPS alone group and the RSG + LPS group, pregnant mice were i.p. injected with LPS (150 μg/kg) on GD15. In control group, pregnant mice were i.p. injected with saline on GD15. Preliminary experiment showed that the expressions of most inflammatory cytokines and chemokines in the placentas were highest at 1.5 h after LPS injection. Thus, all dams were sacrificed at 1.5 h after LPS injection. Maternal serum and amniotic fluid were collected for measurement of TNF-α, MIP-2 and IL-10. The placentas were collected for real-time RT-PCR and immunoblots. Some placentas were collected for histopathology and 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.

1.3. Isolation of total RNA and real-time RT-PCR

Total RNA was extracted using TRI reagent. RNase-free DNase-treated total RNA (1.0 μg) was reverse-transcribed with AMV (Pregmega). Real-time RT-PCR was performed with a LightCycler 480 SYBR Green I kit (Roche Diagnostics GmbH) using gene-specific primers as listed in Table 1. The amplification reactions were carried out on a LightCycler 480 Instrument (Roche Diagnostics GmbH) 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).

1.4. Immunoblots

For nuclear protein extraction, total lysate from the placental tissues was suspended in hypotonic buffer (50 mM Tris–HCl,

Table 1
Oligonucleotide sequences and size of primers.

Genes	Sequences	Sizes (bp)
18S	Forward: 5′- GTAACCCGTTGAACCCCATT-3′ Reverse: 5′- CCATCCAATCGGTAGTAGCG-3′	151
Ppar-γ	Forward: 5′- GGGCTGAGGAGAAGTCACAC-3′ Reverse: 5′- TCAGTGGTTCACCGCTTCTT-3′	144
Tnf-α	Forward: 5′- CCCTCCTGGCCAACGGCATG -3′ Reverse: 5′- TCGGGGCAGCCTTGTCCCTT -3′	109
Il-1β	Forward: 5′- GCCTCGTGCTGTCGGACCCATAT-3′ Reverse: 5′- TCCTTTGAGGCCCAAGGCCACA -3′	143
Il-6	Forward: 5′- AGACAAAGCCAGAGTCCTTCAGAGA -3′ Reverse: 5′- GCCACTCCTTCTGTGACTCCAGC -3′	146
Kc	Forward: 5′-ACTCAAGAATGGTCGCGAGG-3′ Reverse: 5′-GTGCCATCAGAGCAGTCTGT-3′	123
Mip-2	Forward: 5′- TTGCCTTGACCCTGAAGCCCCC -3′ Reverse: 5′- GGCACATCAGGTACGATCCAGGC -3′	175

150 mM NaCl, 1% Sodium deoxycholate, 1% Triton x-100, 0.1% SDS, 1 mM EDTA and 100 mM PMSF) 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 cocktail, incubated for 30 min on ice, and centrifuged for 10 min at 14,000 × g. Protein concentrations were determined with the bicinchoninic acid (BCA) protein assay reagents (Pierce, Rockford, IL) according to the manufacturer's instructions. For immunoblots, 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 antibodies: NF-κB p65 (1:1000), p50 (1:3000), PPAR-γ (1:3000), p-IκB (1:3000) and IκB (1:3000). For nuclear protein, lamin (1:1000) was used as a loading control. For total proteins, β-actin (1:3000) was used as a loading control. After washes in DPBS containing 0.05% Tween-20 four times for 10 min each, the membranes were incubated with goat anti–rabbit IgG (1:80000) or goat anti–mouse antibody (1:80000) for 2 h. The membranes were then washed for four times in DPBS containing 0.05% Tween-20 for 10 min each, followed by signal development using an ECL detection kit.

1.5. Immunohistochemistry

Placental tissues were fixed in 4% paraformaldehyde and embedded in paraffin according to the standard procedure. Paraffin embedded tissues were cut 5 μm thick and stained with hematoxylin and eosin (H & E) for morphological analysis. Mononuclear sinusoidal trophoblast giant cells in the labyrinth zone can be readily identified based on position and morphology (Chen et al., 2012). Sinusoidal trophoblast giant cells have large round nuclei and are easily distinguished from other cell types such the fetal endothelium or the syncytium. For immunohistochemistry, paraffin-embedded placental sections were deparaffinized and rehydrated in a graded ethanol series. Antigen retrieval was achieved by microwave method using sodium citrate solution with pH 6.0. After antigen retrieval and quenching of endogenous peroxidase, sections were incubated with anti-p65, anti-p50 or anti-PPAR-γ monoclonal antibodies (1:200 dilution) at 4 °C overnight. The color reaction was developed with HRP-linked polymer detection system (Golden Bridge International, WA) and counterstaining with hematoxylin (Xu et al., 2015a).

1.6. Enzyme-linked immunosorbent assay

Commercial enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, Oxon, UK) kits were used to determine levels of TNF-α, MIP-2 and IL-10 in maternal serum and amniotic fluid according to the manufacturer's protocol.

1.7. 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 per litter and then averaged per group. Quantified data were expressed as means ± S.E.M. at each point. $P < 0.05$ was considered statistically significant. ANOVA and the Student-Newmann-Keuls post hoc test were used to determine differences between the treated animals and the control and statistical significance.

2. Results

2.1. RSG pretreatment alleviates LPS-induced fetal death in mice

RSG had no effect on fodder consumption and weight gain of pregnant mice (data not shown). No abortion was observed before LPS injection. No dams died throughout the pregnancy. In control group and RSG alone group, all pregnant mice completed pregnancy. The mean fetal weights per litter did not differ between control group (1.37 ± 0.074 g) and RSG alone group (1.38 ± 0.181 g). Moreover, there is no difference in the mean fetal crown-rump length per litter between control group (25.16 ± 0.670 mm) and RSG alone group (25.12 ± 1.165 mm). The rate of litters with dead fetuses and the number of dead fetuses per litter were analyzed. As expected, RSG alone did not elevate fetal mortality. As expected, an

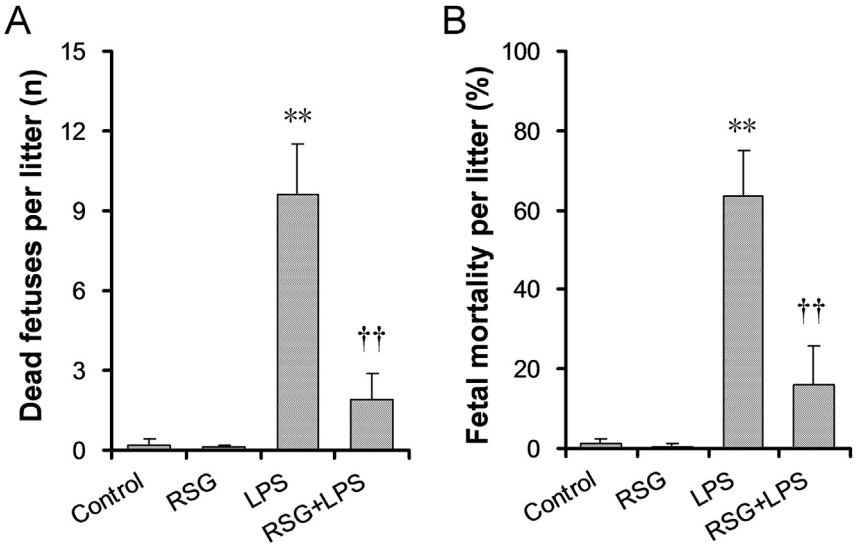

Fig. 1. RSG pretreatment alleviates LPS-induced fetal demise. In the LPS alone and the RSG + LPS groups, pregnant mice were i.p. injected with LPS (150 μg/kg) daily from GD15 to GD17. In the RSG alone and the RSG + LPS groups, pregnant mice were orally administered with RSG (10 mg/kg) daily from GD13 to GD17. All dams were sacrificed on GD18. The numbers of live fetuses, dead fetuses and resorption sites were counted. (A) The mean number of dead fetuses per litter. (B) The mean fetal mortality per litter. All data were expressed as means ± S.E.M (N = 10). $^{**}P < 0.01$ vs control group. $\dagger\dagger P < 0.01$ vs LPS group.

intraperitoneal injection with LPS daily from GD15 to GD17 resulted in 100% (10/10) of pregnant mice with dead fetuses. The mean number of dead fetuses per litter was 9.6 in LPS-treated mice (Fig. 1A). Further analysis showed that maternal LPS injection resulted in 63.6% fetal death in dams that completed the pregnancy (Fig. 1B). Interestingly, the mean number of dead fetuses per litter was reduced to 1.9 in RSG-pretreated mice (Fig. 1A). Moreover, RSG pretreatment significantly reduced LPS-induced fetal mortality (Fig. 1B).

2.2. RSG pretreatment promotes nuclear PPAR-γ translocation in mouse placenta

The effects of RSG on placental PPAR-γ signaling are presented in Fig. 2. As shown in Fig. 2A, RSG pretreatment had little effect on the expression of placental *Ppar-γ* mRNA. Interestingly, the level of nuclear PPAR-γ was significantly elevated in the placentas of RSG-pretreated mice (Fig. 2B). Immunohistochemistry showed that nuclear translocation of PPAR-γ was mainly observed in mononuclear sinusoidal trophoblast giant cells of the labyrinth zone (Fig. 2C).

2.3. RSG pretreatment inhibits LPS-evoked proinflammatory cytokines and chemokines in maternal serum, placenta and amniotic fluid

The effects of RSG on LPS-induced expression of proinflammatory cytokines and chemokines were analyzed. As shown in Fig. 3A–C, the levels of placental *Tnf-α*, *Il-1β* and *Il-6* mRNAs, three proinflammatory genes, were significantly elevated at 1.5 h after LPS injection. In addition, the levels of placental *Kc* and *Mip-2* mRNAs, two chemokine genes, were significantly up-regulated 1.5 h after LPS injection (Fig. 3D and E). Interestingly, RSG pretreatment inhibited LPS-induced up-regulation of placental *Tnf-α*, *Il-1β* and *Il-6* mRNAs. Moreover, RSG pretreatment blocked LPS-induced elevation of placental *Kc* and *Mip-2* mRNAs. The effects of RSG on LPS-induced TNF-α and MIP-2 in maternal serum and amniotic fluid were then analyzed. As expected, the levels of TNF-α and MIP-2 in maternal serum were significantly elevated 1.5 h after LPS injection (Fig. 3F and G). In addition, the levels of TNF-α and MIP-2 in amniotic fluid were significantly increased in LPS-treated mice (Fig. 3H and I). Although RSG had no effect on LPS-induced elevation of TNF-α in maternal serum (Fig. 3F), it significantly attenuated LPS-induced release of TNF-α in amniotic fluid (Fig. 3H). Moreover, RSG pretreatment

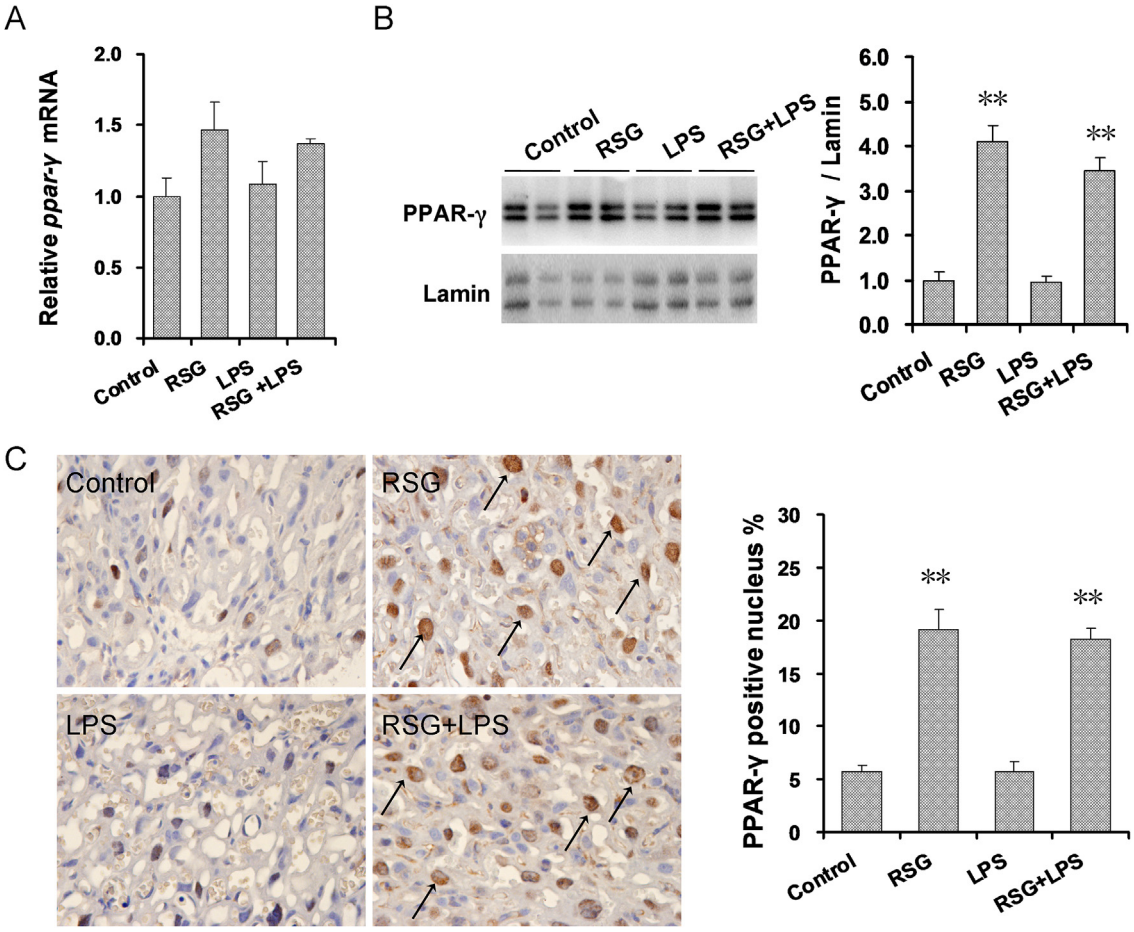

Fig. 2. RSG pretreatment promotes nuclear PPAR-γ translocation in mouse placenta. In the LPS alone and the RSG + LPS groups, pregnant mice were i.p. injected with a single dose of LPS (150 μg/kg) GD15. In the RSG alone and the RSG + LPS groups, pregnant mice were orally administered with RSG (10 mg/kg) daily from GD13 to GD15. Mouse placentas were collected 1.5 h after LPS injection. (A) Placental ppar-γ mRNA was measured using real-time RT-PCR. All data were expressed as means ± S.E.M of six samples from six different pregnant mice. (B) Nuclear PPAR-γ was measured using immunoblot. A representative gel for PPAR-γ (upper panel) and Lamin (lower panel) was shown. All data were expressed as means ± S.E.M of six samples from six different pregnant mice. (C) Nuclear translocation of PPAR-γ was analyzed using IHC. Representative photomicrographs of placental histology from mice treated with saline (as control), RSG alone, LPS alone and RSG + LPS are shown. Original magnification: 400 × . Nuclear translocation of PPAR-γ was observed in mononuclear sinusoidal trophoblast giant cells of the labyrinth zone (arrows). $^{**}P < 0.01$ vs controls. $\dagger\dagger P < 0.01$ vs LPS group.

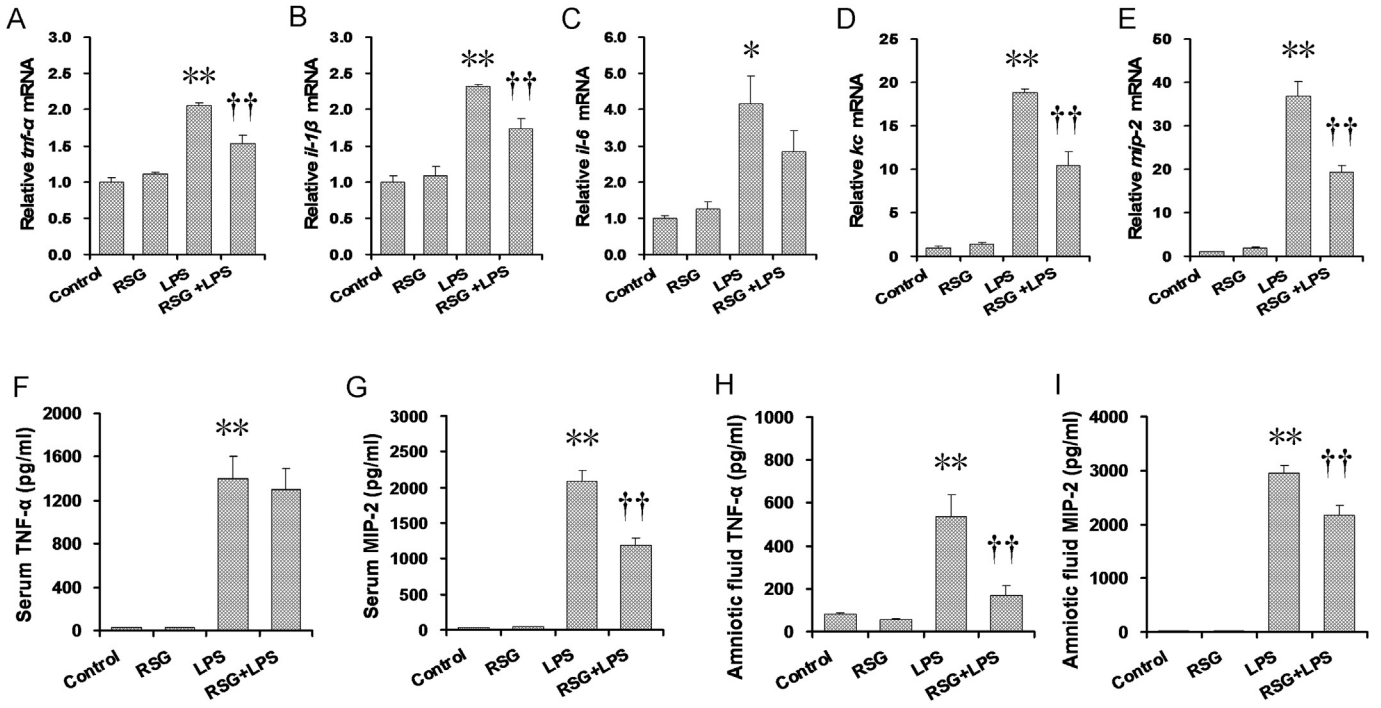

Fig. 3. RSG pretreatment inhibits LPS-induced proinflammatory cytokines and chemokines. In the LPS alone and the RSG + LPS groups, pregnant mice were i.p. injected with a single dose of LPS (150 μg/kg) GD15. In the RSG alone and the RSG + LPS groups, pregnant mice were orally administered with RSG (10 mg/kg) daily from GD13 to GD15. (A–E) Mouse placentas were collected 1.5 h after LPS injection. Placental *Tnf-α*, *Il-1β*, *Il-6*, *Kc* and *Mip-2* mRNAs were measured using real-time RT-PCR. (A) *Tnf-α*. (B) *Il-1β*. (C) *Il-6*. (D) *Kc*. (E) *Mip-2*. (F–I) Maternal serum and amniotic fluid were collected 1.5 h after LPS injection. TNF-α and MIP-2 were measured using ELISA. (F) TNF-α in maternal serum. (G) MIP-2 in maternal serum. (H) TNF-α in amniotic fluid. (I) MIP-2 in amniotic fluid. All data were expressed as means ± S.E.M of six samples from six different pregnant mice. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control group. ††$P < 0.01$ vs LPS group.

attenuated LPS-induced elevation of MIP-2 in maternal serum and amniotic fluid (Fig. 3G and I).

2.4. Effects of RSG pretreatment on LPS-induced anti-inflammatory cytokines in maternal serum and amniotic fluid

The effects of RSG pretreatment on antiinflammatory cytokines in maternal serum and amniotic fluid were analyzed. As shown in Fig. 4A, the level of IL-10 in maternal serum, an anti-inflammatory cytokine, was elevated at 1.5 h after LPS injection. Correspondingly, the level of IL-10 in amniotic fluid was elevated at 1.5 h after LPS injection (Fig. 4B). Although RSG pretreatment slightly attenuated LPS-induced release of IL-10 in maternal serum (Fig. 4A), it had little effect on LPS-induced elevation of placental IL-10 in amniotic fluid (Fig. 4B).

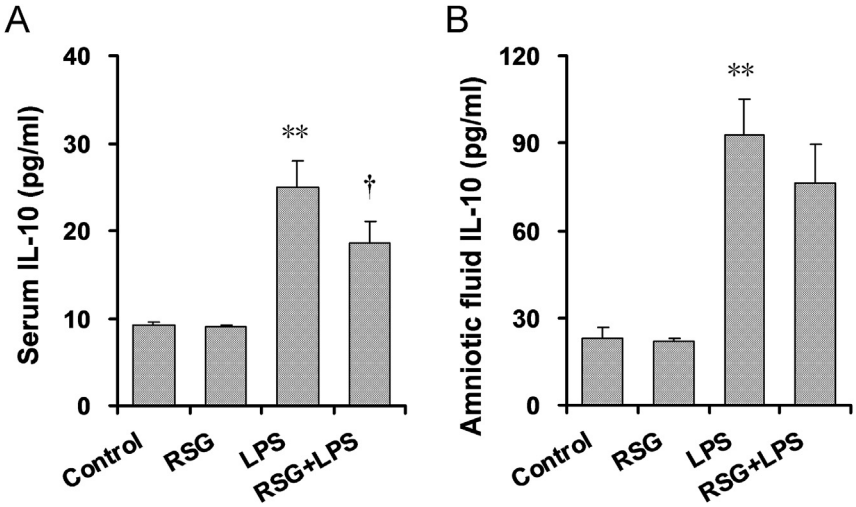

Fig. 4. Effects of RSG on LPS-induced IL-10 in maternal serum and amniotic fluid. In the LPS alone and the RSG + LPS groups, pregnant mice were i.p. injected with a single dose of LPS (150 μg/kg) GD15. In the RSG alone and the RSG + LPS groups, pregnant mice were orally administered with RSG (10 mg/kg) daily from GD13 to GD15. Maternal serum and amniotic fluid were collected 1.5 h after LPS injection. IL-10 were measured using ELISA. (A) IL-10 in maternal serum. (B) IL-10 in amniotic fluid. All data were expressed as means ± S.E.M of six samples from six different pregnant mice. $^{**}P < 0.01$ vs control group. †$P < 0.05$ vs LPS group.

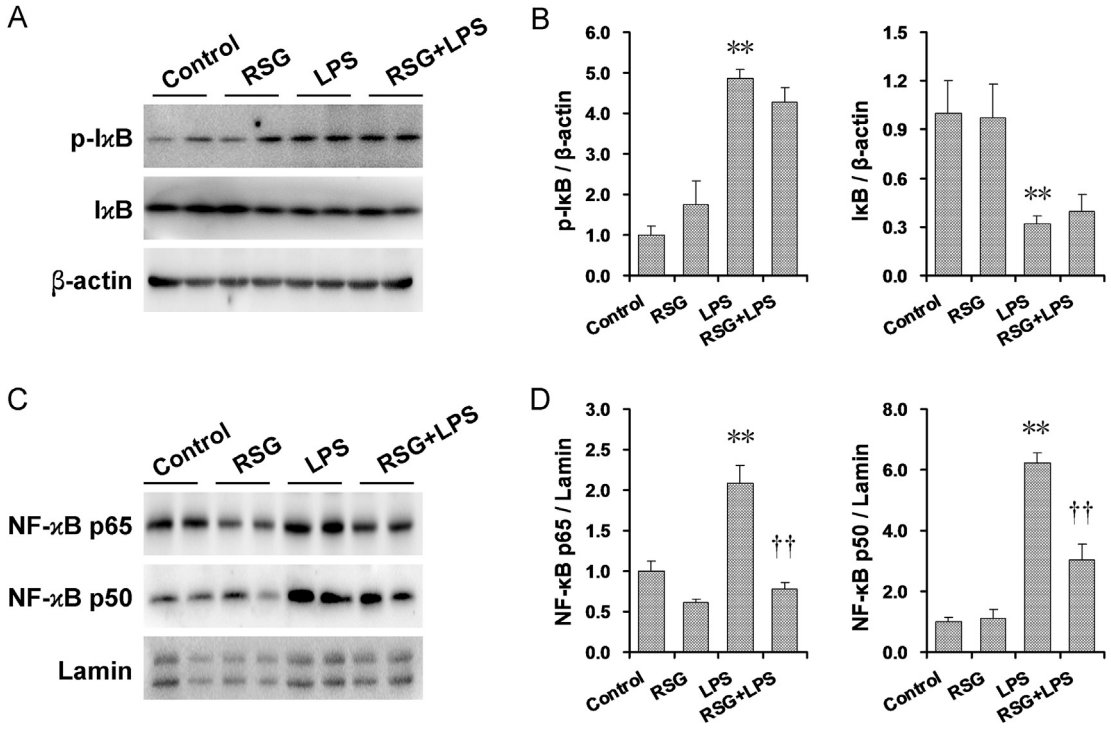

Fig. 5. RSG pretreatment inhibits LPS-induced placental NF-κB activation. In the LPS alone and the RSG + LPS groups, pregnant mice were i.p. injected with a single dose of LPS (150 μg/kg) GD15. In the RSG alone and the RSG + LPS groups, pregnant mice were orally administered with RSG (10 mg/kg) daily from GD13 to GD15. Mouse placentas were collected 1.5 h after LPS injection. (A) Placental p-IκB and IκB were measured using immunoblots. A representative gel for p-IκB (upper panel), IκB (middle panel) and β-actin (lower panel) was shown. (B) All experiments were repeated for three times. Quantitative analyses of scanning densitometry on six samples from six different litters were performed. (C and D) The 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 (lower panel) was shown. (D) All experiments were repeated for three times. Quantitative analyses of scanning densitometry on six samples from six different litters were performed. All data were expressed as means ± S.E.M. $^{**}P < 0.01$ vs control group. $\dagger\dagger P < 0.01$ vs LPS group.

2.5. RSG pretreatment inhibits LPS-evoked placental NF-κB activation

To investigate the effects of RSG pretreatment on LPS-activated placental NF-κB signaling, the levels of placental p-IκB and IκB were measured. As expected, the level of p-IκB was significantly increased at 1.5 h after LPS injection (Fig. 5A and B). In the contrary, the level of IκB was significantly decreased at 1.5 h after LPS injection (Fig. 5A and B). Unexpectedly, RSG pretreatment had no effect on LPS-induced placental IκB phosphorylation. Correspondingly, RSG pretreatment did not attenuate LPS-induced reduction of IκB in the placentas. The levels of nuclear NF-κB p65 and p50 subunits in the placenta were then measured. As expected, the levels of nuclear NF-κB p50 and p65 subunits were significantly increased at 1.5 h after LPS injection (Fig. 5C and D), suggesting that maternal LPS exposure promotes translocation of placental NF-κB p65 and p50 subunits from cytoplasm to nucleus. Immunohistochemistry showed that nuclear translocation of NF-κB p65 and p50 was mainly observed in mononuclear sinusoidal trophoblast giant cells of the labyrinth zone (Fig. 6A and B). The effects of RSG pretreatment on LPS-induced nuclear translocation of NF-κB p65 and p50 were analyzed. Interestingly, RSG pretreatment significantly attenuated LPS-induced elevation of placental nuclear NF-κB p65 and p50 (Fig. 5C and D). Immunohistochemistry showed that RSG almost completely inhibited LPS-induced nuclear translocation of placental NF-κB p65 and p50 in mononuclear sinusoidal trophoblast giant cells of the labyrinth zone (Fig. 6A and B).

3. Discussion

A recent study showed that PPAR-γ level in placentas from small-for-gestational-age (SGA) infants was lower than in placentas from AGA and LGA infants and placental PPAR-γ expression was associated positively to placental and fetal weight at birth, particularly within the SGA subpopulation (Díaz et al., 2012). According to another recent report, reduced expression of 11β-hydroxysteroid dehydrogenase in preeclamptic placentas was associated with decreased PPAR-γ level (He et al., 2014). The present study investigated the effect of pretreatment with RSG, a PPAR-γ agonist, on LPS-induced fetal death. Our results demonstrated that RSG pretreatment protected mice from LPS-induced fetal death.

Increasing evidence demonstrates that maternal LPS injection stimulates the release of proinflammatory cytokines in maternal serum and amniotic fluid (Gayle et al., 2004; Xu et al., 2007b), of which TNF-α was the major mediator leading to fetal death (Leazer et al., 2002). Indeed, RSG has an antiinflammatory activity (Lee et al., 2005). An early in vitro report showed that RSG inhibited LPS-induced release of TNF-α, IL-1β and IL-6 in microglia and astrocytes (Storer et al., 2005). According to another in vitro study, RSG suppressed production of TNF-α in LPS-stimulated monocyte leukemia cells (Wang et al., 2011b). The present study found that pretreatment with RSG significantly attenuated LPS-induced upregulation of TNF-α, IL-1β and IL-6 in mouse placenta. In addition, RSG pretreatment attenuated LPS-induced release of TNF-α in maternal serum and amniotic fluid. The chemokines, a superfamily of small chemotactic cytokines, contribute to inflammation-associated miscarriage, fetal death and preterm delivery (Du et al., 2014; Jaiswal et al., 2015). According to an early report, the chemokine decoy receptor D6 protects against inflammation-caused fetal loss (Martinez de la Torre et al., 2007). In addition, pretreatment with a chemokine inhibitor alleviates LPS-induced preterm birth (Shynlova et al., 2014). A recent study found that

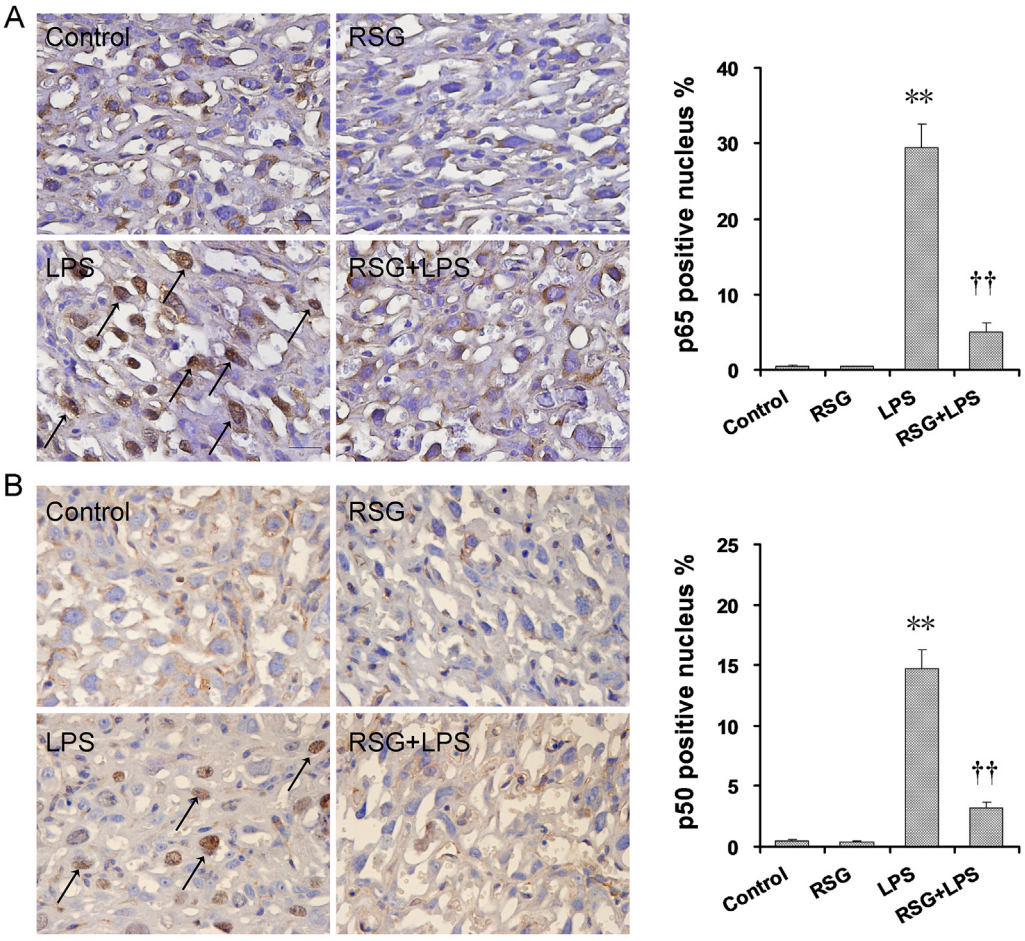

Fig. 6. Effects of RSG pretreatment on LPS-induced nuclear translocation of placental NF-κB p65 and p50 subunits. In the LPS alone and the RSG + LPS groups, pregnant mice were i.p. injected with a single dose of LPS (150 μg/kg) GD15. In the RSG alone and the RSG + LPS groups, pregnant mice were orally administered with RSG (10 mg/kg) daily from GD13 to GD15. Mouse placentas were collected 1.5 h after LPS injection. (A) Nuclear translocation of placental NF-κB p65 subunit was analyzed using IHC. Representative photomicrographs of placental histology from mice treated with saline (as control), RSG alone, LPS alone and RSG + LPS are shown. Original magnification: 400 × . Nuclear translocation of placental NF-κB p65 was observed in mononuclear sinusoidal trophoblast giant cells of the labyrinth zone (arrows). (B) Nuclear translocation of NF-κB p50 subunit was analyzed using IHC. Representative photomicrographs of placental histology from mice treated with saline (as control), RSG alone, LPS alone and RSG + LPS are shown. Original magnification: 400 × . NF-κB p50 subunit was mainly distributed in the labyrinth zone (brown). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CXCR3 blockade protected against Listeria monocytogenes infection–induced fetal wastage (Chaturvedi et al., 2015). The present study showed that mRNA levels of placental KC and MIP-2, two chemokines, were significantly up-regulated 1.5 h after LPS injection. Correspondingly, the level of MIP-2 in maternal serum and amniotic fluid was markedly elevated in LPS-treated mice. Interestingly, RSG pretreatment attenuated LPS-induced up-regulation of KC and MIP-2 in the placenta. In addition, RSG pretreatment attenuated LPS-induced release of MIP-2 in maternal serum and amniotic fluid. Taken together, these results suggest that RSG-mediated protection against LPS-induced fetal death might be partially attributed to its antiinflammatory effects.

IL-10 is an important antiinflammatory cytokine. According to an earlier report, the level of IL-10 was significantly lower in the placentas of women undergoing preterm delivery as compared with women undergoing normal delivery at term (El-Shazly et al., 2004). An experimental report from rodent animals showed that IL-10 protected against LPS-induced fetal death and fetal growth restriction (Rivera et al., 1998). The present study showed that the levels of IL-10 in maternal serum and amniotic fluid were significantly elevated 1.5 h after LPS injection. Moreover, the level of IL-10 in amniotic fluid was much higher than in maternal serum, suggesting its important role in protecting against inflammation-associated fetal death. Although RSG significantly attenuated LPS-induced proinflammatory cytokines and chemokines in the placentas and amniotic fluid, it had little effect on LPS-induced IL-10 in maternal serum and amniotic fluid. These results suggest that RSG selectively inhibits LPS-induced proinflammatory cytokines and chemokines but not anti-inflammatory cytokines in the placentas and amniotic fluid.

NF-κB plays important roles in the regulation of LPS-induced inflammatory genes. Under unstimulated conditions, NF-κB is usually retained in the cytoplasm by binding to the I-κB. LPS can cause I-κB phosphorylation and evoke NF-κB translocation from the cytoplasm to the nucleus. The present study observed that the level of pI-κB was significantly increased in placentas of mice treated with LPS. In contrast, the level of I-κB was decreased in placentas of mice treated with LPS. Correspondingly, the level of nuclear NF-κB p65 and p50 was increased in placenta of mice treated with LPS. An earlier report showed that RSG pretreatment inhibited nuclear localization of pulmonary NF-κB during LPS-induced acute lung injury (Liu et al., 2005). The present study investigated the effects of RSG pretreatment on placental NF-κB signaling. Unexpectedly, RSG pretreatment had no effect on LPS-induced placental I-κB

phosphorylation. Correspondingly, RSG pretreatment did not inhibit LPS-induced reduction of placental I-κB. Interestingly, RSG pretreatment significantly attenuated LPS-induced elevation of nuclear NF-κB p65 and p50 in mouse placentas. Moreover, RSG pretreatment almost completely inhibited LPS-induced nuclear translocation of NF-κB p65 and p50 subunits in mouse placentas.

The mechanism through which RSG inhibits LPS-activated placental NF-κB signaling remains obscure. Several earlier reports have demonstrated that activated nuclear receptors, such as liver X receptor and pregnane X receptor, repress NF-κB signaling in macrophages (Joseph et al., 2003; Zhou et al., 2006). Recently, two reports from our laboratory indicate that pretreatment with vitamin D3, which activates placental and renal VDR, simultaneously blocks LPS-evoked nuclear NF-κB p65 translocation in mouse placentas and kidneys through their interaction between VDR and NF-κB p65 subunit (Chen et al., 2015b; Xu et al., 2015b). Indeed, PPAR-γ is also a nuclear receptor and is highly expressed in human and rodent placentas (Wang et al., 2002; Schaiff et al., 2006; Díaz et al., 2012). The present study showed that RSG, which activated placental PPAR-γ, simultaneously blocked LPS-evoked nuclear translocation of placental NF-κB p65 and p50 subunits. Moreover, LPS-evoked nuclear translocation of NF-kB p65 and p50 subunits was mainly observed in mononuclear sinusoidal trophoblast giant cells of the labyrinth zone. Correspondingly, RSG-induced nuclear translocation of PPAR-γ was observed in mononuclear sinusoidal trophoblast giant cells of the labyrinth zone. Thus, we guess that the activated PPAR-γ inhibits LPS-activated placental NF-κB signaling through its interaction with NF-κB p65 and p50 subunits. Additional work is necessary to demonstrate whether RSG pretreatment reinforces the interaction between PPAR-γ and NF-κB p65 and p50 subunits in the placentas. It is well known that inflammatory cells express PPAR-γ (Uchimura et al., 2001; Jung et al., 2012). Thus, the present study does not exclude the role of PPAR-γ in inflammatory cells on RSG-mediated anti-inflammatory effect during LPS-induced fetal demise. Additional work is required to measure inflammatory infiltrates present in the mouse placentas and to determine the role of immune cells in RSG-mediated protection against LPS-induced fetal demise.

The inhibitive effects of RSG on placental inflammation may have preventive and therapeutic implications. Numerous studies have demonstrated that maternal type II diabetes is associated with the increased risks of spontaneous abortions, malformations, stillbirth and perinatal mortality (Persson and Fadl, 2014; McGrogan et al., 2014; Tennant et al., 2014). On the other hand, several reports have shown increased markers of prooxidant and proinflammatory states in the placenta from type II diabetic patients (Lyall et al., 1998; Coughlan et al., 2004; Pantham et al., 2015). Indeed, PPAR-γ is a target for the treatment of metabolic disorders including maternal type II diabetes. According to an early report, RSG reduces plasma levels of MCP-1 and C-reactive protein in patients with type II diabetes (Mohanty et al., 2004). The present study demonstrates that RSG pretreatment represses placental proinflammatory cytokines and protects mice from LPS-induced fetal death. Thus, PPAR-γ agonists may be used as pharmacological agents to prevent placentas and fetuses from inflammation-associated developmental damage.

In summary, the present study investigated the effects of RSG pretreatment on LPS-induced fetal death. We found that pretreatment with RSG protected mice from LPS-induced fetal death through its anti-inflammatory activity. Moreover, we demonstrate that pretreatment with RSG, which activates placental PPAR-γ signaling, simultaneously inhibites LPS-induced NF-κB activation and almost completely blocks nuclear translocation of NF-κB p65 and p50 subunits in mouse placentas. These results provide a mechanistic explanation for RSG-mediated anti-inflammatory activity. Overall, the present study provides additional evidence for roles of PPAR-γ as an important regulator of placental inflammation.

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