Zinc Supplementation during Pregnancy Protects against Lipopolysaccharide-Induced Fetal Growth Restriction and Demise through Its Anti-inflammatory Effect

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LPS is associated with adverse developmental outcomes, including preterm delivery, fetal death, teratogenicity, and intrauterine growth restriction (IUGR). Previous reports showed that zinc protected against LPS-induced teratogenicity. In the current study, we investigated the effects of zinc supplementation during pregnancy on LPS-induced preterm delivery, fetal death and IUGR. All pregnant mice except controls were i.p. injected with LPS (75 μg/kg) daily from gestational day (GD) 15 to GD17. Some pregnant mice were administered zinc sulfate through drinking water (75 mg elemental Zn per liter) throughout the pregnancy. As expected, an i.p. injection with LPS daily from GD15 to GD17 resulted in 36.4% (4/11) of dams delivered before GD18. In dams that completed the pregnancy, 63.2% of fetuses were dead. Moreover, LPS significantly reduced fetal weight and crown–rump length. Of interest, zinc supplementation during pregnancy protected mice from LPS-induced preterm delivery and fetal death. In addition, zinc supplementation significantly alleviated LPS-induced IUGR and skeletal development retardation. Further experiments showed that zinc supplementation significantly attenuated LPS-induced expression of placental inflammatory cytokines and cyclooxygenase-2. Zinc supplementation also significantly attenuated LPS-induced activation of NF-κB and MAPK signaling in mononuclear sinusoidal trophoblast giant cells of the labyrinth zone. It inhibited LPS-induced placental AKT phosphorylation as well. In conclusion, zinc supplementation during pregnancy protects against LPS-induced fetal growth restriction and demise through its anti-inflammatory effect. ***The Journal of Immunology*****, 2012, 189: 454–463.**

Lipopolysaccharide is a toxic component of cell walls in Gram-negative bacteria and is widely present in the digestive tracts of humans and animals (1). Humans are constantly exposed to low levels of LPS through infection. Gastrointestinal inflammatory diseases and excess alcohol intake are known to increase the permeability of LPS from the gastrointestinal tract into blood (2). High levels of LPS have been detected in women with bacterial vaginosis (3). In humans, Gram-negative bacterial infections are a recognized cause of fetal loss and preterm labor (4). Mimicking maternal infection by exposing pregnant rodents to LPS at early gestational stages resulted in embryonic resorption and fetal death (5, 6). Maternal LPS exposure at the middle gestational stages caused fetal death and abortion (7). Several earlier studies showed that maternal LPS exposure resulted in the development of malformed fetuses in rats and golden hamsters (8–10). Recently, we showed that maternal LPS exposure from gestational day (GD) 8 to GD12 caused external malformations and skeletal abnormalities in mice (11). We and others found that maternal LPS exposure at late gestational stages led to fetal death, intrauterine growth restriction (IUGR), skeletal development retardation, and preterm delivery (12–16). In addition, maternal LPS exposure during pregnancy caused age- and gender-dependent impairments of neurobehavioral development in offspring (17).

Numerous reports demonstrate that inflammatory cytokines, such as TNF-α, have been associated with LPS-induced adverse developmental outcomes. Indeed, several studies showed that maternal LPS exposure during pregnancy significantly increased the level of proinflammatory cytokines in maternal serum, amniotic fluid, fetal liver, and fetal brain (18–20). Moreover, pentoxifylline, an inhibitor of TNF-α synthesis, reduced LPS-induced fetal mortality, prevented embryonic resorption and abortion, and reversed LPS-induced IUGR and skeletal development retardation (5, 21). Eicosanoids are also important mediators of LPS-induced adverse developmental outcomes. Silver et al. (22) reported that pregnant C3H/HeN mice injected with LPS showed an increase in decidual eicosanoid production and cyclooxygenase (COX)-2 expression, followed by a dose-dependent increase in embryo death. COX-2 suppressors decreased LPS-induced fetal mortality and prevented preterm delivery (23, 24).

Zinc is a structural constituent essential for cell growth, development, and differentiation (25). Increasing evidence demonstrates that zinc has an anti-inflammatory effect (26, 27). An earlier report showed that injection with zinc sulfate reduced LPS-

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Abbreviations used in this article: COX-2, cyclooxygenase-2; GD, gestational day; IκBα, inhibitor of NF-κB α; I-κB, inhibitor of κB; IUGR, intrauterine growth restriction; pAKT, phosphorylated-AKT; pERK, phosphorylated-ERK; pIκBα, phosphorylated-IκBα; pJNK, phosphorylated-JNK; pp38, phosphorylated-p38.

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Table I. Primers for real-time RT-PCR

Genes	Sequences	Sizes (bp)
gapdh	Forward: 5′-ACCCCAGCAAGGACACTGAGCAAG-3′	109
	Reverse: 5′-GGCCCCTCCTGTTATTATGGGGGT-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-8	Forward: 5′-TTGCCTTGACCCTGAAGCCCCC-3′	175
	Reverse: 5′-GGCACATCAGGTACGATCCAGGC-3′	
il-4	Forward: 5′-GCCATATCCACGGATGCGACA-3′	94
	Reverse: 5′-TCCATCTCCGTGCATGGCGTC-3′	
il-10	Forward: 5′-TTCCCAGTCGGCCAGAGCCA-3′	198
	Reverse: 5′-TGCCTGGGGCATCACTTCTACCA-3′	
cox-2	Forward: 5′-GGGCTCAGCCAGGCAGCAAAT-3′	187
	Reverse: 5′-GCACTGTGTTTGGGGTGGGCT-3′	
mt-1	Forward: 5′-TGCACTTGCACCAGCTCCTGC-3′	123
	Reverse: 5′-GGCGCCTTTGCAGACACAGC-3′	
mt-2	Forward: 5′-GCGGAGCTTTTGCGCTCGAC-3′	210
	Reverse: 5′-CCAGCGCAGGAGCAGGATCC-3′	

induced teratogenicity in mice (28). A recent study found that s.c. injection with zinc sulfate alleviated LPS-induced neurodevelopmental damage in fetal brain (29). Nevertheless, the molecular mechanism of zinc-mediated protection against LPS-induced developmental toxicity remains obscure. In this study, we investigated the effects of zinc supplementation during pregnancy on LPS-induced preterm delivery, fetal death, and IUGR in mice. Our results showed that zinc supplementation protected mice from LPS-induced preterm delivery and fetal death. In addition, zinc supplementation significantly alleviated LPS-induced IUGR and skeletal development retardation. We demonstrated for the first time, to our knowledge, that zinc-mediated protection against LPS-induced developmental toxicity might, at least partially, be due to its anti-inflammatory effects.

Materials and Methods

Chemicals and reagents

LPS (*Escherichia coli* LPS, serotype 0127:B8) and zinc sulfate were purchased from Sigma-Aldrich (St. Louis, MO). COX-2, phosphorylated-p38 (pp38), phosphorylated-ERK (pERK), phosphorylated-JNK (pJNK), NF-κB p65, inhibitor of NF-κB α (IκBα), phosphorylated-IκBα (pIκBα), and lamin A/C Abs were from Santa Cruz Biotechnologies (Santa Cruz, CA). AKT and phosphorylated-AKT (pAKT) Abs were obtained from Cell Signaling Technology (Beverley, MA). β-Actin Ab was from Boster Bio-Technology (Wuhan, China). The chemiluminescence (ECL) detection kit was obtained from Pierce Biotechnology (Rockford, IL). TRI reagent came from Molecular Research Center (Cincinnati, OH). RNase-free DNase was from Promega (Madison, WI). All other reagents came from Sigma-Aldrich or as indicated in the specified methods.

Animals and treatments

The ICR mice (8–10 wk 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 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. The present study consisted of four separate experiments.

Table II. Fetal outcomes among different groups

	Control	Zn	LPS	Zn+LPS
Number of pregnant mice (*n*)	11	11	11	11
Litters of preterm delivery (*n*)	0	0	4*	0†
Litters of term delivery (*n*)	11	11	7	11
Average placental weight (g)	0.106 ± 0.006	0.102 ± 0.003	0.085 ± 0.002**	0.103 ± 0.003††
Dead fetuses per litter (*n*)[a]	0.8 ± 0.4	0.3 ± 0.2	4.0 ± 1.0**	1.5 ± 0.5†
Live fetuses per litter (*n*)[a]	13.1 ± 1.5	13.5 ± 0.9	10.1 ± 0.9	11.8 ± 0.7
Fetal weight (g)	1.42 ± 0.030	1.41 ± 0.018	1.16 ± 0.029**	1.32 ± 0.035††
Crown–rump length (cm)	2.56 ± 0.018	2.57 ± 0.023	2.36 ± 0.031**	2.50 ± 0.020††
Scores				
Supraoccipital bone[b]	1.01 ± 0.01	1.00 ± 0.00	1.48 ± 0.14**	1.11 ± 0.04††
Number ossified				
Sternum (*n*)	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0
Rib (*n*)	26.0 ± 0.0	25.9 ± 0.1	25.7 ± 0.1**	25.9 ± 0.1†
Metacarpus (*n*)	4.0 ± 0.0	4.0 ± 0.0	3.9 ± 0.0	4.0 ± 0.0
Anterior phalanx (*n*)	4.0 ± 0.0	4.0 ± 0.0	3.6 ± 0.1**	3.9 ± 0.1†
Metatarsus (*n*)	5.0 ± 0.0	5.0 ± 0.0	4.8 ± 0.0	4.9 ± 0.0
Posterior phalanx (*n*)	5.0 ± 0.0	5.0 ± 0.0	4.4 ± 0.1**	4.8 ± 0.1††
Caudal vertebrae (*n*)	7.8 ± 0.1	7. 7 ± 0.1	6.3 ± 0.2**	7.1 ± 0.1††

All quantitative data were expressed as means ± SEM.
[a]The number of dead or live fetuses per litter in dams that completed the pregnancy.
[b]Supraoccipital bone scores: 1 = well ossified; 5 = completely unossified.
*$p < 0.05$, **$p < 0.01$ compared with the control group; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ compared with LPS group.

Experiment 1. To investigate the effects of zinc supplementation during pregnancy on LPS-induced preterm delivery, fetal death, and growth restriction, the pregnant mice were divided into four groups randomly. All pregnant mice except controls were i.p. injected with LPS (75 μg/kg) daily from GD15 to GD17. Some pregnant mice were administered zinc sulfate through drinking water (75 mg elemental Zn per liter, dissolved in deionized water) throughout the pregnancy. In the Zn alone group, the pregnant mice were administered zinc sulfate through drinking water (75 mg elemental Zn per liter, dissolved in deionized water) throughout the pregnancy. In the NS group, all pregnant mice were i.p. injected with NS daily from GD15 to GD17. All dams were sacrificed on GD18, and gravid uterine weights were recorded. For each litter, the numbers of live fetuses, dead fetuses, and resorption sites were counted. Live fetuses in each litter were weighed. Crown–rump length was measured. All fetuses were then stored in ethanol for a minimum of 2 wk for subsequent skeletal evaluation.

Experiment 2. To study the effects of zinc supplementation on LPS-induced expression of COX-2 in placenta, the pregnant mice were divided into two groups randomly. In the LPS alone group, the pregnant mice received an i.p. injection of LPS (75 μg/kg) on GD15. In the Zn+LPS group, the pregnant mice were administered zinc sulfate through drinking water (75 mg elemental Zn per liter) from GD0 to GD15 and then received an i.p. injection of LPS (75 μg/kg) on GD15. The pregnant mice were sacrificed at different times (0, 0.5, 1, 2, 6, and 12 h) after LPS injection. Placentas were collected for measurements of COX-2 expression.

Experiment 3. To examine the effects of zinc supplementation on LPS-induced expression of pro- and anti-inflammatory cytokines in placenta, the pregnant mice were divided into four groups randomly. In the LPS group, the pregnant mice received an i.p. injection of LPS (75 μg/kg) on GD15. In the Zn+LPS group, the pregnant mice were administered zinc sulfate through drinking water (75 mg elemental Zn per liter) from GD0 to GD15 and then received an i.p. injection of LPS (75 μg/kg) on GD15. In the Zn alone group, the pregnant mice were administered zinc sulfate through drinking water (75 mg elemental Zn per liter) from GD0 to GD15. In the NS group, the pregnant mice received an i.p. injection of NS on GD15. All dams were sacrificed at 2 h after LPS injection. Maternal sera were collected for measurement of proinflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) and anti-inflammatory cytokines (IL-4 and IL-10). Placentas were collected for measurements of the mRNA level of proinflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) and anti-inflammatory cytokines (IL-4 and IL-10).

Experiment 4. To assess the effects of zinc supplementation on LPS-induced activation of AKT, NF-κB, and MAPK signaling, the pregnant mice were divided into two groups randomly. In the LPS group, the pregnant mice received an i.p. injection of LPS (75 μg/kg) on GD15. In the Zn+LPS group, the pregnant mice were administered zinc sulfate through drinking water (75 mg elemental Zn per liter) from GD0 to GD15 and then received an i.p. injection of LPS (75 μg/kg) on GD15. The pregnant mice were sacrificed at different times (0, 0.5, 1, 2, 6, and 12 h) after LPS injection.

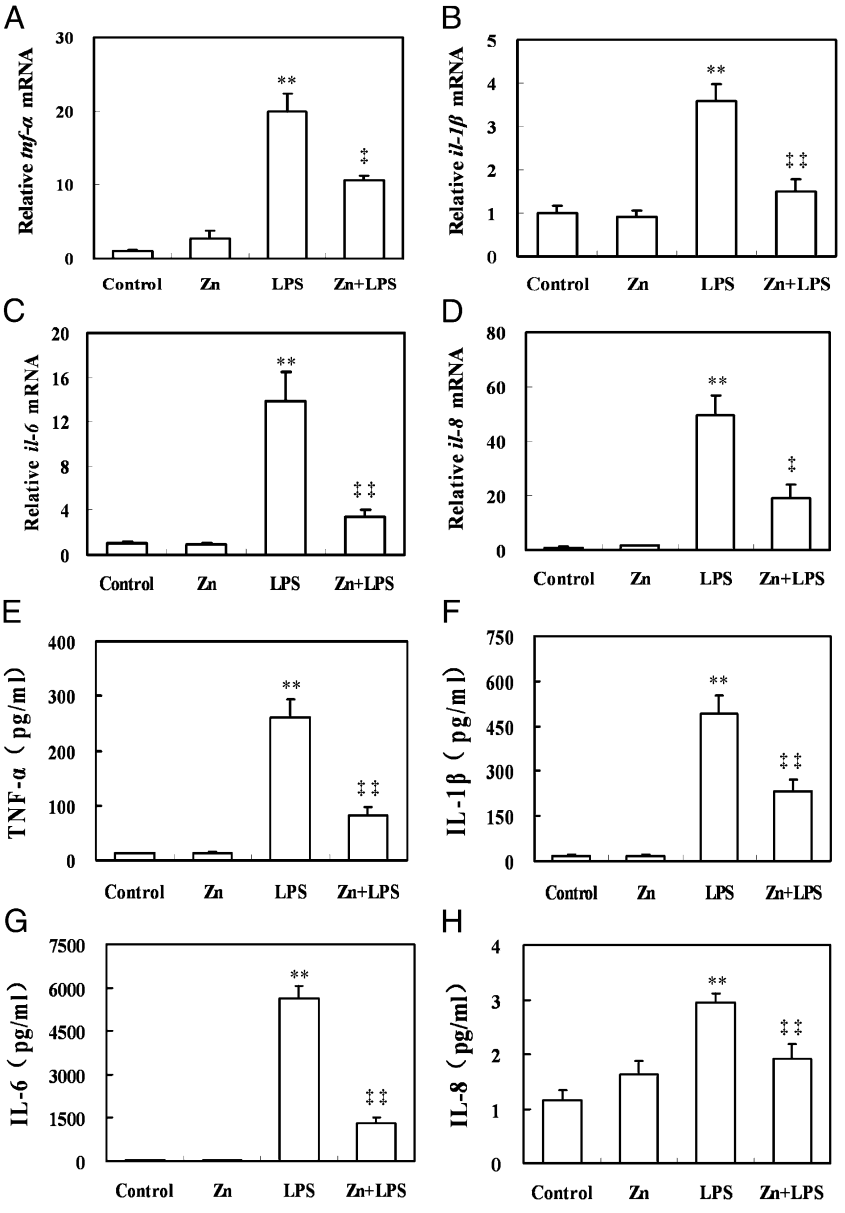

FIGURE 1. Effects of zinc supplementation on LPS-induced proinflammatory cytokines. In the LPS group, the pregnant mice received an i.p. injection of LPS (75 μg/kg) on GD15. In the Zn+LPS group, the pregnant mice drank zinc sulfate (75 mg elemental Zn per liter) dissolved in deionized water from GD0 to GD15. On GD15, the pregnant mice received an i.p. injection of LPS (75 μg/kg). (**A**–**D**) Placentas were collected 2 h after LPS injection. Placental TNF-α, IL-1β, IL-6, and IL-8 mRNA were measured using real-time RT-PCR. (A) TNF-α. (B) IL-1β. (C) IL-6. (D) IL-8. (**E**–**H**) Maternal sera were collected 2 h after LPS injection. TNF-α, IL-1β, IL-6, and IL-8 in maternal serum were measured using ELISA. (E) TNF-α. (F) IL-1β. (G) IL-6. (H) IL-8. All data were expressed as means ± SEM of six samples from six different pregnant mice. $^{**}p < 0.01$ versus control group, $^{\ddagger}p < 0.05$, $^{\ddagger\ddagger}p < 0.01$ versus LPS group.

Placentas were collected for measurements of pAKT, NF-κB p65, IκBα, pIκBα, pp38, pERK, and pJNK.

Experiment 5. To investigate the effects of zinc supplementation on the expression of MT-1 and MT-2, the pregnant mice were divided into four groups randomly. In the LPS group, the pregnant mice received an i.p. injection of LPS (75 μg/kg) on GD15. In the Zn+LPS group, the pregnant mice were administered zinc sulfate through drinking water (75 mg elemental Zn per liter) from GD0 to GD15 and then received an i.p. injection of LPS (75 μg/kg) on GD15. In the Zn alone group, the pregnant mice were administered zinc sulfate through drinking water (75 mg elemental Zn per liter) from GD0 to GD15. In the NS group, the pregnant mice received an i.p. injection of NS on GD15. All dams were sacrificed at 2 h after LPS injection. Maternal liver and placenta were collected for measurement of MT-1 and MT-2 mRNAs, using real-time RT-PCR.

The doses of zinc sulfate used in the current study referred to other studies, in which zinc sulfate was added to the liquid diet at 75 mg elemental Zn per liter for 4 wk (30). All procedures on animals followed the guidelines for humane treatment established by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

Skeletal examination and evaluation

The fetuses stored in ethanol were cleared of skin, viscera, and adipose tissue. Fetuses were then incubated in acetone overnight and subsequently macerated and stained with alizarin red S for 2 d. After an overnight incubation in 70% ethanol/glycerol/benzyl alcohol, the fetuses were stored in glycerol until examination. Skeletal evaluation included determination of the degree of ossification of the phalanges, metacarpals, vertebrae, sternum, and skull. The size of the anterior fontanel and ossification of the supraoccipital were scored.

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) using gene-specific primers as listed in Table I. 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

Total lysate from placenta was prepared by homogenizing 50 mg placental tissue in 300 μl lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsylphate, 1 mM PMSF) supplemented with a mixture of protease inhibitors (Roche). For nuclear protein extraction, total lysate from placenta was suspended in hypotonic buffer and then kept on ice for 15 min. The suspension was 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: pAKT, AKT, pp38, pERK, pJNK, IκBα, pIκBα, NF-κB p65, and COX-2. For total proteins, β-actin was used as a loading control. For nuclear protein, lamin A/C was used as a loading control. After washes in Dulbecco's PBS containing 0.05% Tween 20 four times for 10 min each time, the membranes were incubated with goat anti-rabbit IgG or goat anti-mouse Ab for 2 h. The membranes were then washed in Dulbecco's PBS containing 0.05% Tween 20 four times for 10 min each time, followed by signal development using an ECL detection kit.

Immunohistochemistry

Placental tissues were fixed in 4% formalin and embedded in paraffin according to the standard procedure. Paraffin-embedded tissues were cut 5 μm thick and stained with H&E for morphological analysis. Mononuclear sinusoidal trophoblast giant cells in the labyrinth zone can be readily identified on the basis of position and morphology (31). Sinusoidal trophoblast giant cells have large, round nuclei and are easily distinguished from other cell types such as the fetal endothelium or the syncytium. For immunohistochemical examination, 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 or anti-pp38 mAbs (1:100 dilution) at 4°C overnight. The color reaction was developed with an HRP-linked polymer detection system and counterstaining with hematoxylin.

ELISA

Commercial ELISA (R&D Systems, Abingdon, Oxon, United Kingdom) kits were used to determine levels of TNF-α, IL-1β, IL-6, IL-8, IL-10, and IL-4 in maternal serum 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, crown–rump length, and skeletal evaluation, the means were calculated per litter and then averaged per group. Quantified data were expressed as means ± SEM 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 group, as well as statistical significance.

Results

Effects of zinc supplementation on LPS-induced preterm delivery and fetal death

Zinc had no effect on fodder consumption and weight gain of the pregnant mice (data not shown). No abortion was observed before

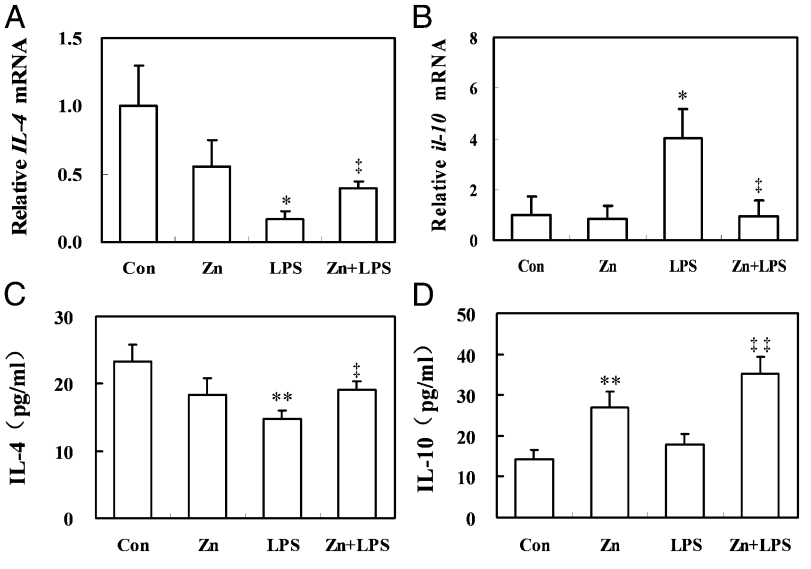

FIGURE 2. Effects of zinc supplementation on LPS-induced anti-inflammatory cytokines. In the LPS group, the pregnant mice received an i.p. injection of LPS (75 μg/kg) on GD15. In the Zn+LPS group, the pregnant mice drank zinc sulfate (75 mg elemental Zn per liter) dissolved in deionized water from GD0 to GD15. On GD15, the pregnant mice received an i.p. injection of LPS (75 μg/kg). (**A** and **B**) Placentas were collected 2 h after LPS injection. Placental IL-4 and IL-10 were measured using real-time RT-PCR. (A) IL-4. (B) IL-10. (**C** and **D**) Maternal sera were collected 2 h after LPS injection. IL-4 and IL-10 in maternal serum were measured using ELISA. (C) IL-4. (D) IL-10. All data were expressed as means ± SEM of six samples from six different pregnant mice. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control group, $^{‡}p < 0.05$, $^{‡‡}p < 0.01$ versus LPS group.

LPS treatment. No dams died throughout the pregnancy. The number of litters, the number of live fetuses per litter and dead fetuses per litter, and the incidence of preterm delivery are presented in Table II. In the control and zinc alone group, all pregnant mice completed pregnancy. An i.p. injection with LPS daily from GD15 to GD17 resulted in 36.4% (4 of 11) of pregnant mice delivered before GD18. Of interest, zinc supplementation during pregnancy significantly alleviated LPS-induced preterm delivery.

The effects of zinc supplementation during pregnancy on LPS-induced fetal death were analyzed. As shown in Table II, maternal LPS administration on GD15 through GD17 resulted in 63.2% fetal deaths in dams that completed the pregnancy. Zinc supplementation during pregnancy almost completely prevented LPS-induced fetal death.

Effects of zinc supplementation on LPS-induced IUGR

The effects of zinc supplementation during pregnancy on LPS-induced IUGR were studied. As shown in Table II, maternal LPS exposure markedly reduced fetal weight and crown–rump length. Zinc supplementation significantly alleviated an LPS-induced decrease in fetal weight and crown–rump length.

Effects of zinc supplementation on LPS-induced skeletal developmental retardation

The effects of zinc supplementation during pregnancy on LPS-induced skeletal development retardation were examined. As expected, the skeletons of fetuses from LPS-treated mice, compared with those from controls, exhibited fewer ossification centers in the caudal vertebrae, as well as the anterior and posterior phalanges. In addition, maternal LPS exposure retarded fetal supraoccipital ossification (Table II). Zinc supplementation during pregnancy sig-

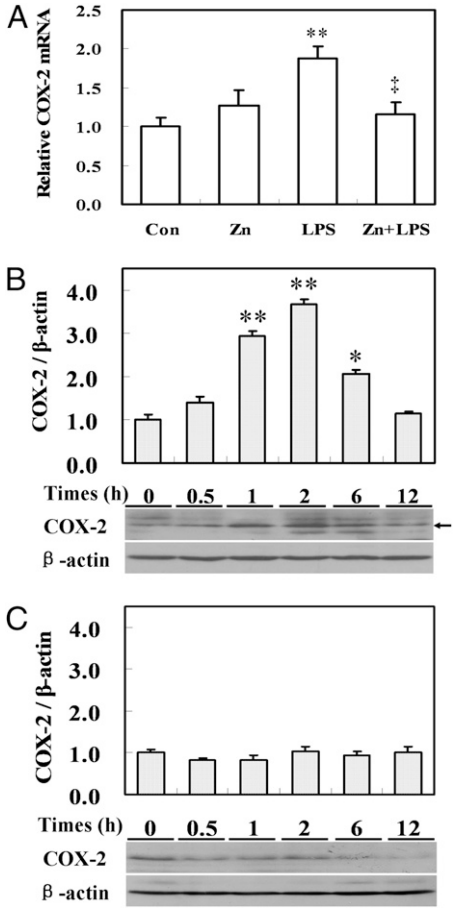

FIGURE 3. Effects of zinc supplementation on LPS-induced placental COX-2 expression. In the LPS group, the pregnant mice received an i.p. injection of LPS (75 μg/kg) on GD15. In the Zn+LPS group, the pregnant mice drank zinc sulfate (75 mg elemental Zn per liter) dissolved in deionized water from GD0 to GD15. On GD15, the pregnant mice received an i.p. injection of LPS (75 μg/kg). (**A**) Placentas were collected 2 h after LPS injection. Placental COX-2 mRNA was measured using real-time RT-PCR. All data were expressed as means ± SEM of six samples from six different litters. (**B** and **C**) Placentas were collected at different times (0, 0.5, 1, 2, 6, 12 h) after LPS injection. Placental COX-2 was measured using immunoblots. A representative gel for COX-2 (*upper panel*) and β-actin (*lower panel*) is shown. (B) The level of COX-2 in the placenta of LPS-treated mice. (C) The level of COX-2 in the placenta of mice treated with LPS plus Zn. All experiments were repeated three times. Quantitative analyses of scanning densitometry on three samples from three different litters were performed. All data were expressed as means ± SEM. $*p < 0.05$, $**p < 0.01$ versus control group, $^{‡}p < 0.05$ versus LPS group.

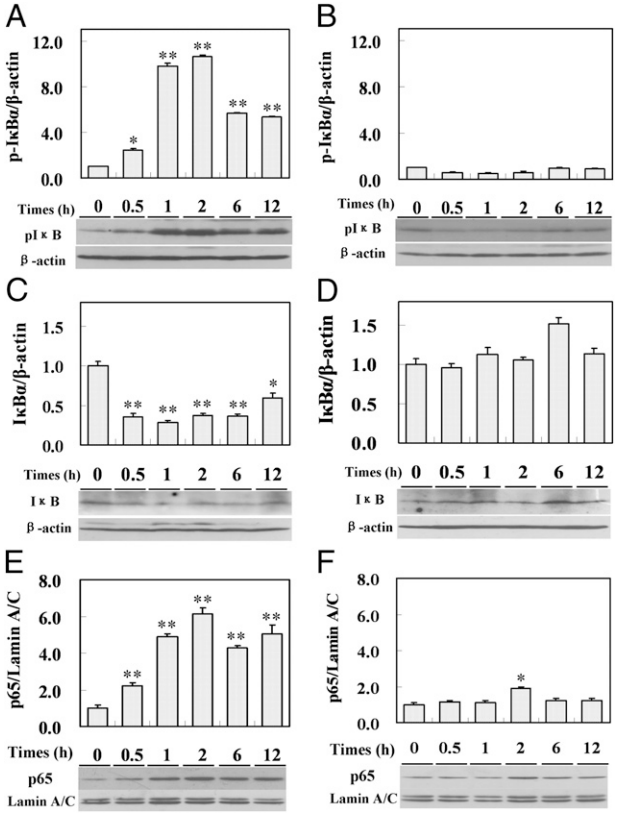

FIGURE 4. Effects of zinc supplementation on LPS-induced placental NF-κB activation. In the LPS group, the pregnant mice received an i.p. injection of LPS (75 μg/kg) on GD15. In the Zn+LPS group, the pregnant mice drank zinc sulfate (75 mg elemental Zn per liter) dissolved in deionized water from GD0 to GD15. On GD15, the pregnant mice received an i.p. injection of LPS (75 μg/kg). Placentas were collected at different times (0, 0.5, 1, 2, 6, 12 h) after LPS injection. (**A** and **B**) Placental pIκBα was measured using immunoblots. A representative gel for pIκBα (*upper panel*) and β-actin (*lower panel*) is shown. (**C** and **D**) Placental IκBα was measured using immunoblots. A representative gel for IκBα (*upper panel*) and β-actin (*lower panel*) is shown. (**E** and **F**) The nuclear fractions were prepared from placenta. Placental NF-κB p65 was measured using immunoblots. A representative gel for p65 (*upper panel*) and lamin A/C (*lower panel*) is shown. (A, C, and E) Placentas were from LPS-treated mice. (B, D, and F) Placentas were from mice treated with LPS plus Zn. All experiments were repeated three times. Quantitative analyses of scanning densitometry on three samples from three different litters were performed. All data were expressed as means ± SEM. $*p < 0.05$, $**p < 0.01$ versus control group.

nificantly attenuated LPS-induced skeletal development retardation.

Effects of zinc supplementation on LPS-induced proinflammatory cytokines

The effects of zinc supplementation during pregnancy on LPS-induced expression of placental proinflammatory cytokines were analyzed. As shown in Fig. 1A, the level of TNF-α mRNA in placenta was significantly increased at 2 h after LPS treatment. In addition, maternal LPS administration significantly upregulated the expression of IL-1β, IL-6, and IL-8 in placenta (Fig. 1B–D). Of interest, zinc supplementation significantly inhibited LPS-induced expression of proinflammatory cytokines in placenta. The effects of zinc supplementation during pregnancy on LPS-induced proinflammatory cytokines in maternal serum were then analyzed. As expected, the levels of TNF-α, IL-1β, IL-6, and IL-8 in maternal serum were significantly increased at 2 h after LPS treatment. Zinc supplementation significantly inhibited LPS-induced release of proinflammatory cytokines in maternal serum (Fig. 1E–H).

Effects of zinc supplementation on LPS-induced anti-inflammatory cytokines

The effects of zinc supplementation during pregnancy on anti-inflammatory cytokines in placenta and maternal serum were analyzed. As shown in Fig. 2A, the level of IL-4 mRNA in placenta was significantly downregulated at 2 h after LPS injection. Correspondingly, the level of IL-4 in maternal serum was significantly increased at 2 h after LPS injection. Interestingly, zinc supplementation significantly attenuated LPS-induced downregulation of IL-4 mRNA in placenta (Fig. 2A). In addition, zinc supplementation significantly attenuated LPS-induced reduction of IL-4 in maternal serum (Fig. 2C). The effects of zinc supplementation on the expression of placental IL-10 are presented in Fig. 2B. As expected, maternal LPS administration significantly upregulated the level of IL-10 mRNA in placenta. Zinc supplementation during pregnancy significantly attenuated LPS-induced IL-10 upregulation in placenta. In addition, zinc supplementation increased the level of IL-10 in maternal serum (Fig. 2D).

Zinc supplementation inhibits LPS-induced expression of COX-2 in placenta

The effects of zinc supplementation during pregnancy on LPS-induced COX-2 expression were studied. As shown in Fig. 3A, maternal LPS exposure significantly upregulated the expression of COX-2 mRNA in placenta. Zinc supplementation significantly attenuated LPS-induced upregulation of COX-2 mRNA in placenta. The effects of zinc supplementation on LPS-induced COX-2 protein are presented in Fig. 3B and 3C. As expected, maternal LPS administration significantly increased the level of placental COX-2 protein in a time-dependent manner (Fig. 3B). Of interest, LPS-induced elevation of COX-2 protein was significantly attenuated in the placenta of mice administered zinc supplementation during pregnancy (Fig. 3C).

Zinc supplementation inhibits LPS-induced placental NF-κB activation

To investigate whether maternal LPS injection activates NF-κB in placenta, the levels of pIκBα, IκBα, and NF-κB p65 were measured. As expected, the level of pIκBα was significantly increased in the placenta of mice treated with LPS (Fig. 4A). By contrast, the level of IκBα was significantly decreased (Fig. 4C), indicating that placental IκB was degraded in LPS-treated mice. The effects of LPS injection during pregnancy on NF-κB p65 are shown in Fig. 4E. As anticipated, the level of placental nuclear NF-κB p65 was significantly increased in a time-dependent manner, suggesting that NF-κB is activated in the placenta of mice treated with LPS. Immunohistochemistry showed that nuclear translocation of NF-κB p65 was mainly observed in mononuclear sinusoidal trophoblast giant cells of the labyrinth zone (Fig. 5G). The effects of zinc supplementation during pregnancy on LPS-induced placental NF-κB activation were then analyzed. Of interest, zinc supple-

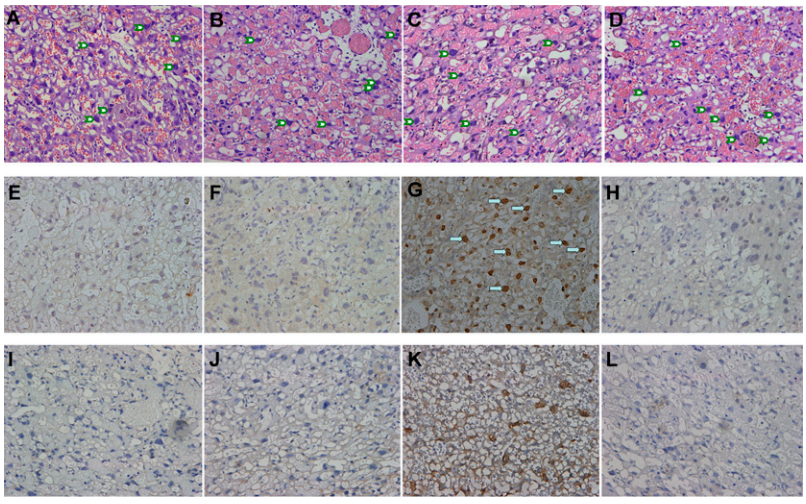

FIGURE 5. Effects of zinc supplementation on LPS-induced nuclear translocation of NF-κB p65 and p38 phosphorylation in placenta. In the LPS group, the pregnant mice received an i.p. injection of LPS (75 μg/kg) on GD15. In the Zn+LPS group, the pregnant mice drank zinc sulfate (75 mg elemental Zn per liter) dissolved in deionized water from GD0 to GD15. On GD15, the pregnant mice received an i.p. injection of LPS (75 μg/kg). Placentas were collected 2 h after LPS injection. (**A**–**D**) Representative photomicrographs of placental histological specimens from mice treated with saline (A as control), zinc alone (B), LPS alone (C), and Zn+LPS (D) are shown (H&E, original magnification ×200). Mononuclear sinusoidal trophoblast giant cells were distributed in the labyrinth zone (arrowheads). (**E**–**H**) Nuclear translocation of NF-κB p65 was analyzed using immunohistochemistry. Representative photomicrographs of placental histological specimens from mice treated with saline (E as control), zinc alone (F), LPS alone (G), and Zn+LPS (H) are shown. Original magnification ×200. Nuclear translocation of NF-κB p65 was observed in mononuclear sinusoidal trophoblast giant cells of the labyrinth zone (arrows). (**I**–**L**) p38 phosphorylation was analyzed using immunohistochemistry. Representative photomicrographs of placental histological specimens from mice treated with saline (I as control), zinc alone (J), LPS alone (K), and Zn+LPS (L) are shown. Original magnification ×200. The pp38 staining was mainly distributed in the labyrinth zone (brown).

mentation significantly attenuated LPS-evoked placental IκBα phosphorylation and degradation (Fig. 4B, 4D). Moreover, zinc supplementation significantly attenuated LPS-induced elevation of placental nuclear NF-κB p65 (Fig. 4F). Correspondingly, zinc supplementation almost completely inhibited LPS-induced nuclear translocation of NF-κB p65 in mononuclear sinusoidal trophoblast giant cells of the labyrinth zone (Fig. 5H).

Zinc supplementation inhibits LPS-induced activation of placental MAPK signaling

To determine whether maternal LPS exposure activates MAPK signaling in placenta, the levels of pJNK, pp38, and pERK were measured. As expected, their levels were significantly increased in the placenta of mice administered LPS (Fig. 6A, 6C, 6E). Immunohistochemistry showed that MAPK p38 phosphorylation was observed in the labyrinth zone (Fig. 5K). The effects of zinc supplementation during pregnancy on LPS-induced activation of placental MAPK signaling were then analyzed. As shown in Fig. 6B, zinc supplementation significantly attenuated LPS-induced JNK phosphorylation. In addition, zinc supplementation almost completely inhibited LPS-induced p38 and ERK phosphorylation in placenta (Figs. 5L, 6D, 6F).

Zinc supplementation inhibits LPS-induced AKT phosphorylation in placenta

As shown in Fig. 7A, maternal LPS exposure had no effect on the expression of AKT in placenta. In addition, zinc supplementation did not affect the level of AKT in placenta (Fig. 7B). The effects of zinc supplementation during pregnancy on LPS-induced placental AKT phosphorylation were analyzed. As shown in Fig. 7C, the level of pAKT was significantly increased in the placenta of mice administered LPS. Interestingly, zinc supplementation completely inhibited LPS-induced AKT phosphorylation in placenta (Fig. 7D).

Effects of zinc supplementation on MT-1 and MT-2 expression in maternal liver and placenta

The effects of zinc supplementation during pregnancy on LPS-induced MT-1 and MT-2 expression were analyzed. As anticipated, the levels of MT-1 and MT-2 mRNA were significantly upregulated in the maternal liver of LPS-treated mice. Zinc supplementation had little effect on LPS-induced upregulation of MT-1 and MT-2 in maternal liver (Fig. 8A, 8C). Unexpectedly, maternal LPS injection during pregnancy had no effect on the expression of MT-1 and MT-2 in placenta (Fig. 8B, 8D).

Discussion

In the current study, we investigated the effect of zinc supplementation during pregnancy on LPS-induced preterm delivery, fetal death, and IUGR. We found that zinc supplementation during pregnancy protected mice from LPS-induced preterm delivery and fetal death. Importantly, such supplementation significantly alleviated LPS-induced IUGR. In addition, it significantly alleviated LPS-induced skeletal development retardation.

Several reports demonstrate that eicosanoids are important mediators for LPS-induced fetal death and preterm delivery (23, 24). The present study showed that zinc supplementation during pregnancy significantly attenuated LPS-induced upregulation of

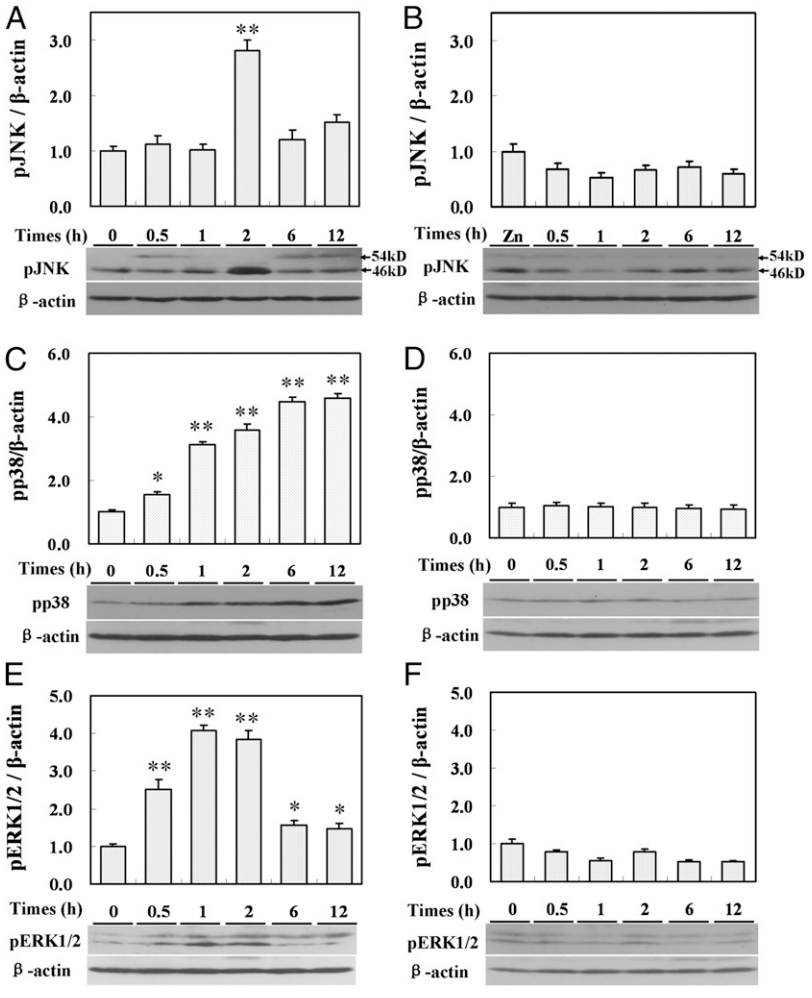

FIGURE 6. Effects of zinc supplementation on LPS-induced activation of MAPK signaling in placenta. In the LPS group, the pregnant mice received an i.p. injection of LPS (75 μg/kg) on GD15. In the Zn+LPS group, the pregnant mice drank zinc sulfate (75 mg elemental Zn per liter) dissolved in deionized water from GD0 to GD15. On GD15, the pregnant mice received an i.p. injection of LPS (75 μg/kg). Placentas were collected at different times (0, 0.5, 1, 2, 6, 12 h) after LPS injection. (**A** and **B**) Placental pJNK was measured using immunoblots. A representative gel for pJNK (*upper panel*) and β-actin (*lower panel*) is shown. (**C** and **D**) Placental pp38 was measured using immunoblots. A representative gel for pp38 (*upper panel*) and β-actin (*lower panel*) is shown. (**E** and **F**) Placental pERK was measured using immunoblots. A representative gel for pERK (*upper panel*) and β-actin (*lower panel*) is shown. (A, C, and E) Placentas were from LPS-treated mice. (B, D, and F) Placentas were from mice treated with LPS plus Zn. All experiments were repeated for three times. Quantitative analyses of scanning densitometry on three samples from three different litters were performed. All data were expressed as means ± SEM. $*p < 0.05$, $**p < 0.01$ versus control group.

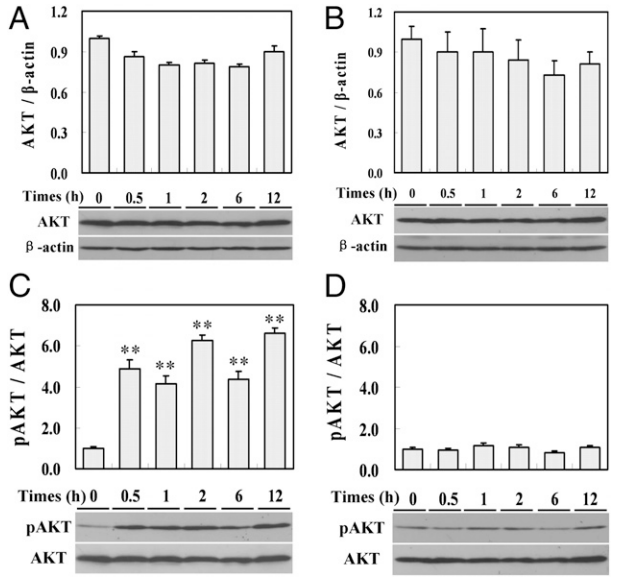

FIGURE 7. Effects of zinc supplementation on LPS-induced AKT phosphorylation in placenta. In the LPS group, the pregnant mice received an i.p. injection of LPS (75 μg/kg) on GD15. In the Zn+LPS group, the pregnant mice drank zinc sulfate (75 mg elemental Zn per liter) dissolved in deionized water from GD0 to GD15. On GD15, the pregnant mice received an i.p. injection of LPS (75 μg/kg). Placentas were collected at different times (0, 0.5, 1, 2, 6, 12 h) after LPS injection. (**A** and **B**) Placental AKT was measured using immunoblots. A representative gel for AKT (*upper panel*) and β-actin (*lower panel*) is shown. (**C** and **D**) Placental pAKT was measured using immunoblots. A representative gel for pAKT (*upper panel*) and AKT (*lower panel*) is shown. (A and C) Placentas were from LPS-treated mice. (B and D) Placentas were from mice treated with LPS plus Zn. All experiments were repeated three times. Quantitative analyses of scanning densitometry on three samples from three different litters were performed. All data were expressed as means ± SEM. $^{**}p < 0.01$ versus control group.

placental COX-2 mRNA. In addition, zinc supplementation almost completely inhibited LPS-induced elevation of COX-2 protein in placenta. Thus, the repression of placental COX-2 might contribute, at least partially, to zinc-mediated protection against LPS-induced developmental toxicity. IL-10 and IL-4 are important anti-inflammatory cytokines. An earlier study showed that IL-10 protected rodents from LPS-induced fetal death and IUGR (12). An additional report found that levels of IL-4 and IL-10 could protect against preterm delivery. In the current study, we showed that zinc supplementation during pregnancy increased the level of IL-10 in maternal serum. We also analyzed the effects of zinc supplementation on IL-4 expression in placenta. Our results showed that zinc supplementation significantly upregulated the expression of IL-4 in the placenta of pregnant mice treated with LPS. Correspondingly, the supplementation significantly increased the level of IL-4 in maternal serum. These findings suggest that IL-10 and IL-4 might play an important role in zinc-mediated protection against LPS-induced preterm delivery, fetal death, and IUGR.

Numerous studies showed that maternal LPS exposure during pregnancy stimulated the production of proinflammatory cytokines (18, 33), of which TNF-α is the major mediator leading to fetal death and IUGR (7, 21). Indeed, zinc has an anti-inflammatory effect (26, 27). According to an earlier report, zinc negatively regulates TNF-α and IL-1β gene expression in the HL-60 monocyte/macrophage cell line (34). Moreover, zinc almost completely blocked LPS-induced expression and release of TNF-α and IL-1β in human PBMCs and monocytes (35). We found that zinc supplementation during pregnancy significantly attenuated LPS-induced upregulation of TNF-α and IL-1β in placenta. Increasing evidence demonstrates that IL-6 and IL-8 are associated with preterm delivery (36, 37). An earlier report showed that treatment of murine decidual explants with LPS resulted in significant increases in IL-6 (38). A recent study demonstrated that intrauterine injection of LPS on GD15 caused a rise of IL-6 levels in amniotic fluid (39). The present study showed that maternal LPS exposure obviously increased the level of IL-6 mRNA in placenta. Moreover, we found for the first time, to our knowledge, that IL-8 mRNA was significantly upregulated in the placenta of pregnant mice treated with LPS. Thus, the effects of zinc supplementation on the expression of IL-6 and IL-8 were analyzed in the placenta of mice treated with LPS. Surprisingly, zinc supplementation almost completely inhibited LPS-evoked elevation of placental IL-6 mRNA. Importantly, zinc supplementation significantly attenuated LPS-induced upregulation of IL-8 in placenta. These results suggest that zinc-mediated protection against LPS-induced preterm delivery, fetal death, and IUGR might, at least partially, be attributed to its anti-inflammatory effects.

MAPK signaling is one of the most important signaling cascades that regulate LPS-induced inflammatory genes. The present study showed that levels of pJNK, pp38, and pERK were significantly increased in the placenta of mice administered LPS. Of interest, MAPK p38 phosphorylation was mainly observed in mononuclear sinusoidal trophoblast giant cells of the labyrinth zone, suggesting

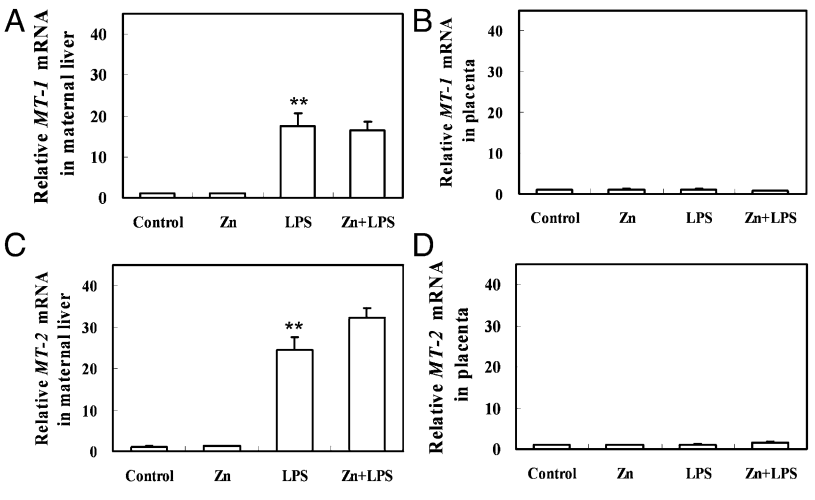

FIGURE 8. Effects of zinc supplementation on LPS-induced MT-1 and MT-2 expression. In the LPS group, the pregnant mice received an i.p. injection of LPS (75 μg/kg) on GD15. In the Zn+LPS group, the pregnant mice drank zinc sulfate (75 mg elemental Zn per liter) dissolved in deionized water from GD0 to GD15. On GD15, the pregnant mice received an i.p. injection of LPS (75 μg/kg). (**A** and **B**) Maternal liver and placenta were collected 2 h after LPS injection. MT-1 and MT-2 mRNAs were measured using real-time RT-PCR. (A) MT-1 mRNA in maternal liver. (B) MT-1 mRNA in placenta. (**C**) MT-2 mRNA in maternal liver. (**D**) MT-2 mRNA in placenta. All data were expressed as means ± SEM of six samples from six different pregnant mice. $^{**}p < 0.01$ versus control group.

that MAPK signaling was activated in these cells. A recent report demonstrates that intracellular zinc is involved in the activation of LPS-induced MAPK signaling pathways in monocytes (40). We investigated the effects of zinc supplementation on LPS-induced placental MAPK signaling. We found that zinc supplementation completely blocked LPS-induced placental JNK and ERK phosphorylation. In addition, supplementation almost completely inhibited LPS-induced p38 activation in sinusoidal trophoblast giant cells. These findings suggest that zinc-mediated downregulation of LPS-induced inflammatory genes might be associated with the repression of MAPK activation in sinusoidal trophoblast giant cells of the labyrinth zone.

NF-κB plays a central role in LPS-induced upregulation of inflammatory genes. Under unstimulated conditions, NF-κB is usually retained in the cytoplasm by binding to the inhibitor of κB (I-κB). I-κB phosphorylation causes translocation of NF-κB to the nucleus. In the current study, we found that the level of phosphor–IκBα was significantly increased in the placenta of mice treated with LPS. Correspondingly, the level of nuclear NF-κB p65 was significantly increased as well. Moreover, nuclear translocation of NF-κB p65 was mainly observed in mononuclear sinusoidal trophoblast giant cells of the labyrinth zone. Several studies demonstrated that zinc abrogated LPS-induced NF-κB activation and subsequent TNF-α production in monocytes and Kupffer cells (41, 42). The present study showed that zinc supplementation significantly inhibited LPS-evoked placental IκBα phosphorylation and degradation. Importantly, zinc supplementation significantly attenuated LPS-induced elevation of placental nuclear NF-κB p65. In addition, zinc supplementation during pregnancy almost completely inhibited LPS-induced nuclear translocation of NF-κB p65 in mononuclear sinusoidal trophoblast giant cells of the labyrinth zone, indicating that zinc could inhibit LPS-induced NF-κB activation in these cells. According to an earlier report, AKT serine–threonine kinase is involved in the activation of NF-κB (43). A recent study showed that lutein, an antioxidant, significantly attenuated LPS-evoked NF-κB activation and inflammatory gene expression through suppressing the PI3K/AKT signaling pathway (44). Indeed, the current study showed for the first time, to our knowledge, that maternal LPS exposure resulted in placental AKT phosphorylation. Importantly, zinc supplementation during pregnancy almost completely inhibited LPS-induced placental AKT phosphorylation. These results suggest that zinc-mediated repression of LPS-induced placental NF-κB activation might be associated with the inhibition of AKT phosphorylation.

The protection offered by zinc supplementation during pregnancy against LPS-induced inflammation may have preventive and therapeutic implications. According to an earlier report, injection with zinc sulfate reduced LPS-induced teratogenicity in mice (28). A recent study found that s.c. injection with zinc sulfate alleviated LPS-induced neurodevelopmental damage in fetal brain (29). Importantly, dietary zinc supplementation during pregnancy could reduce LPS-induced teratogenicity and cognitive impairments in offspring (45, 46). In addition, the current study showed that zinc supplementation during pregnancy protects mice from LPS-induced preterm delivery, fetal death, IUGR, and skeletal development retardation. Thus, zinc may be used as a pharmacological agent to prevent LPS-induced developmental toxicity.

In summary, the current study indicates that zinc supplementation during pregnancy inhibits LPS-induced inflammation. Moreover, such supplementation inhibits LPS-induced activation of NF-κB and MAPK signaling in mononuclear sinusoidal trophoblast giant cells of the labyrinth zone. In addition, it blocks LPS-induced placental AKT phosphorylation. Importantly, zinc supplements administered during pregnancy protect mice from LPS-induced preterm delivery, fetal death, and IUGR. Thus, zinc supplementation during pregnancy may prove useful for protecting against LPS-induced developmental toxicity.

Disclosures

The authors have no financial conflicts of interest.

References

1. Jacob, A. I., P. K. Goldberg, N. Bloom, G. A. Degenshein, and P. J. Kozinn. 1977. Endotoxin and bacteria in portal blood. *Gastroenterology* 72: 1268–1270.
2. Zhou, Z., L. Wang, Z. Song, J. C. Lambert, C. J. McClain, and Y. J. Kang. 2003. A critical involvement of oxidative stress in acute alcohol-induced hepatic TNF-alpha production. *Am. J. Pathol.* 163: 1137–1146.
3. Platz-Christensen, J. J., I. Mattsby-Baltzer, P. Thomsen, and N. Wiqvist. 1993. Endotoxin and interleukin-1 alpha in the cervical mucus and vaginal fluid of pregnant women with bacterial vaginosis. *Am. J. Obstet. Gynecol.* 169: 1161–1166.
4. Romero, R., P. Roslansky, E. Oyarzun, M. Wan, M. Emamian, T. J. Novitsky, M. J. Gould, and J. C. Hobbins. 1988. Labor and infection. II. Bacterial endotoxin in amniotic fluid and its relationship to the onset of preterm labor. *Am. J. Obstet. Gynecol.* 158: 1044–1049.
5. Gendron, R. L., F. P. Nestel, W. S. Lapp, and M. G. Baines. 1990. Lipopolysaccharide-induced fetal resorption in mice is associated with the intrauterine production of tumour necrosis factor-alpha. *J. Reprod. Fertil.* 90: 395–402.
6. Ogando, D. G., D. Paz, M. Cella, and A. M. Franchi. 2003. The fundamental role of increased production of nitric oxide in lipopolysaccharide-induced embryonic resorption in mice. *Reproduction* 125: 95–110.
7. Leazer, T. M., B. Barbee, M. Ebron-McCoy, G. A. Henry-Sam, and J. M. Rogers. 2002. Role of the maternal acute phase response and tumor necrosis factor alpha in the developmental toxicity of lipopolysaccharide in the CD-1 mouse. *Reprod. Toxicol.* 16: 173–179.
8. Ornoy, A., and G. Altshuler. 1976. Maternal endotoxemia, fetal anomalies, and central nervous system damage: a rat model of a human problem. *Am. J. Obstet. Gynecol.* 124: 196–204.
9. Lanning, J. C., D. R. Hilbelink, and L. T. Chen. 1983. Teratogenic effects of endotoxin on the golden hamster. *Teratog. Carcinog. Mutagen.* 3: 145–149.
10. Collins, J. G., M. A. Smith, R. R. Arnold, and S. Offenbacher. 1994. Effects of *Escherichia coli* and *Porphyromonas gingivalis* lipopolysaccharide on pregnancy outcome in the golden hamster. *Infect. Immun.* 62: 4652–4655.
11. Zhao, L., Y. H. Chen, H. Wang, Y. L. Ji, H. Ning, S. F. Wang, C. Zhang, J. W. Lu, Z. H. Duan, D. X. Xu, and D. X. Xu. 2008. Reactive oxygen species contribute to lipopolysaccharide-induced teratogenesis in mice. *Toxicol. Sci.* 103: 149–157.
12. Rivera, D. L., S. M. Olister, X. Liu, J. H. Thompson, X. J. Zhang, K. Pennline, R. Azuero, D. A. Clark, and M. J. Miller. 1998. Interleukin-10 attenuates experimental fetal growth restriction and demise. *FASEB J.* 12: 189–197.
13. Buhimschi, I. A., C. S. Buhimschi, and C. P. Weiner. 2003. Protective effect of N-acetylcysteine against fetal death and preterm labor induced by maternal inflammation. *Am. J. Obstet. Gynecol.* 188: 203–208.
14. Xu, D. X., Y. H. Chen, H. Wang, L. Zhao, J. P. Wang, and W. Wei. 2005. Effect of N-acetylcysteine on lipopolysaccharide-induced intra-uterine fetal death and intra-uterine growth retardation in mice. *Toxicol. Sci.* 88: 525–533.
15. Xu, D. X., Y. H. Chen, L. Zhao, H. Wang, and W. Wei. 2006. Reactive oxygen species are involved in lipopolysaccharide-induced intrauterine growth restriction and skeletal development retardation in mice. *Am. J. Obstet. Gynecol.* 195: 1707–1714.
16. Xu, D. X., H. Wang, L. Zhao, H. Ning, Y. H. Chen, and C. Zhang. 2007. Effects of low-dose lipopolysaccharide (LPS) pretreatment on LPS-induced intra-uterine fetal death and preterm labor. *Toxicology* 234: 167–175.
17. Wang, H., X. H. Meng, H. Ning, X. F. Zhao, Q. Wang, P. Liu, H. Zhang, C. Zhang, G. H. Chen, D. X. Xu, and D. X. Xu. 2010. Age- and gender-dependent impairments of neurobehaviors in mice whose mothers were exposed to lipopolysaccharide during pregnancy. *Toxicol. Lett.* 192: 245–251.
18. Xu, D. X., H. Wang, H. Ning, L. Zhao, and Y. H. Chen. 2007. Maternally administered melatonin differentially regulates lipopolysaccharide (LPS)-induced proinflammatory and antiinflammatory cytokines in maternal serum, amniotic fluid, fetal liver and fetal brain. *J. Pineal Res.* 243: 74–79.
19. Ning, H., H. Wang, L. Zhao, C. Zhang, X. Y. Li, Y. H. Chen, and D. X. Xu. 2008. Maternally-administered lipopolysaccharide (LPS) increases tumor necrosis factor alpha in fetal liver and fetal brain: its suppression by low-dose LPS pretreatment. *Toxicol. Lett.* 176: 13–19.
20. Li, X. Y., C. Zhang, Y. L. Ji, H. Wang, S. F. Wang, L. Zhao, and D. X. Xu. 2008. Maternally derived TNF-α partially contributes to lipopolysaccharide-induced downregulation of CYP3A in fetal liver. *Toxicol. Lett.* 179: 71–77.
21. Xu, D. X., Y. H. Chen, H. Wang, L. Zhao, J. P. Wang, and W. Wei. 2006. Tumor necrosis factor alpha partially contributes to lipopolysaccharide-induced intrauterine fetal growth restriction and skeletal development retardation in mice. *Toxicol. Lett.* 163: 20–29.
22. Silver, R. M., S. S. Edwin, M. S. Trautman, D. L. Simmons, D. W. Branch, D. J. Dudley, and M. D. Mitchell. 1995. Bacterial lipopolysaccharide-mediated fetal death. Production of a newly recognized form of inducible cyclooxygenase (COX-2) in murine decidua in response to lipopolysaccharide. *J. Clin. Invest.* 95: 725–731.

23. Gross, G., T. Imamura, S. K. Vogt, D. F. Wozniak, D. M. Nelson, Y. Sadovsky, and L. J. Muglia. 2000. Inhibition of cyclooxygenase-2 prevents inflammation-mediated preterm labor in the mouse. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 278: R1415–R1423.
24. Sakai, M., K. Tanebe, Y. Sasaki, K. Momma, S. Yoneda, and S. Saito. 2001. Evaluation of the tocolytic effect of a selective cyclooxygenase-2 inhibitor in a mouse model of lipopolysaccharide-induced preterm delivery. *Mol. Hum. Reprod.* 7: 595–602.
25. Hirano, T., M. Murakami, T. Fukada, K. Nishida, S. Yamasaki, and T. Suzuki. 2008. Roles of zinc and zinc signaling in immunity: zinc as an intracellular signaling molecule. *Adv. Immunol.* 97: 149–176.
26. Rink, L., and H. Haase. 2007. Zinc homeostasis and immunity. *Trends Immunol.* 28: 1–4.
27. Haase, H., and L. Rink. 2009. Functional significance of zinc-related signaling pathways in immune cells. *Annu. Rev. Nutr.* 29: 133–152.
28. Carey, L. C., P. L. Berbée, P. Coyle, J. C. Philcox, and A. M. Rofe. 2003. Zinc treatment prevents lipopolysaccharide-induced teratogenicity in mice. *Birth Defects Res. A Clin. Mol. Teratol.* 67: 240–245.
29. Chua, J. S., C. J. Cowley, J. Manavis, A. M. Rofe, and P. Coyle. 2012. Prenatal exposure to lipopolysaccharide results in neurodevelopmental damage that is ameliorated by zinc in mice. *Brain Behav. Immun.* 26: 326–336.
30. Kang, X., W. Zhong, J. Liu, Z. Song, C. J. McClain, Y. J. Kang, and Z. Zhou. 2009. Zinc supplementation reverses alcohol-induced steatosis in mice through reactivating hepatocyte nuclear factor-4α and peroxisome proliferator-activated receptor-α. *Hepatology* 50: 1241–1250.
31. Simmons, D. G., and J. C. Cross. 2005. Determinants of trophoblast lineage and cell subtype specification in the mouse placenta. *Dev. Biol.* 284: 12–24.
32. El-Shazly, S., M. Makhseed, F. Azizieh, and R. Raghupathy. 2004. Increased expression of pro-inflammatory cytokines in placentas of women undergoing spontaneous preterm delivery or premature rupture of membranes. *Am. J. Reprod. Immunol.* 52: 45–52.
33. Gayle, D. A., R. Beloosesky, M. Desai, F. Amidi, S. E. Nuñez, and M. G. Ross. 2004. Maternal LPS induces cytokines in the amniotic fluid and corticotropin releasing hormone in the fetal rat brain. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 286: R1024–R1029.
34. Bao, B., A. S. Prasad, F. W. Beck, and M. Godmere. 2003. Zinc modulates mRNA levels of cytokines. *Am. J. Physiol. Endocrinol. Metab.* 285: E1095–E1102.
35. von Bülow, V., L. Rink, and H. Haase. 2005. Zinc-mediated inhibition of cyclic nucleotide phosphodiesterase activity and expression suppresses TNF-alpha and IL-1 beta production in monocytes by elevation of guanosine 3′,5′-cyclic monophosphate. *J. Immunol.* 175: 4697–4705.
36. Kurkinen-Räty, M., A. Ruokonen, S. Vuopala, M. Koskela, E. M. Rutanen, T. Kärkkäinen, and P. Jouppila. 2001. Combination of cervical interleukin-6 and -8, phosphorylated insulin-like growth factor-binding protein-1 and transvaginal cervical ultrasonography in assessment of the risk of preterm birth. *BJOG* 108: 875–881.
37. Jacobsson, B., I. Mattsby-Baltzer, and H. Hagberg. 2005. Interleukin-6 and interleukin-8 in cervical and amniotic fluid: relationship to microbial invasion of the chorioamniotic membranes. *BJOG* 112: 719–724.
38. Dudley, D. J., C. L. Chen, D. W. Branch, E. Hammond, and M. D. Mitchell. 1993. A murine model of preterm labor: inflammatory mediators regulate the production of prostaglandin E2 and interleukin-6 by murine decidua. *Biol. Reprod.* 48: 33–39.
39. Schmitz, T., E. Souil, R. Hervé, C. Nicco, F. Batteux, G. Germain, D. Cabrol, D. Evain-Brion, M. J. Leroy, and C. Méhats. 2007. PDE4 inhibition prevents preterm delivery induced by an intrauterine inflammation. *J. Immunol.* 178: 1115–1121.
40. Haase, H., J. L. Ober-Blöbaum, G. Engelhardt, S. Hebel, A. Heit, H. Heine, and L. Rink. 2008. Zinc signals are essential for lipopolysaccharide-induced signal transduction in monocytes. *J. Immunol.* 181: 6491–6502.
41. Zhou, Z., L. Wang, Z. Song, J. T. Saari, C. J. McClain, and Y. J. Kang. 2004. Abrogation of nuclear factor-kappaB activation is involved in zinc inhibition of lipopolysaccharide-induced tumor necrosis factor-alpha production and liver injury. *Am. J. Pathol.* 164: 1547–1556.
42. von Bülow, V., S. Dubben, G. Engelhardt, S. Hebel, B. Plümäkers, H. Heine, L. Rink, and H. Haase. 2007. Zinc-dependent suppression of TNF-alpha production is mediated by protein kinase A-induced inhibition of Raf-1, I kappa B kinase beta, and NF-kappa B. *J. Immunol.* 179: 4180–4186.
43. Ozes, O. N., L. D. Mayo, J. A. Gustin, S. R. Pfeffer, L. M. Pfeffer, and D. B. Donner. 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* 401: 82–85.
44. Kim, J. H., H. J. Na, C. K. Kim, J. Y. Kim, K. S. Ha, H. Lee, H. T. Chung, H. J. Kwon, Y. G. Kwon, and Y. M. Kim. 2008. The non-provitamin A carotenoid, lutein, inhibits NF-kappaB-dependent gene expression through redox-based regulation of the phosphatidylinositol 3-kinase/PTEN/Akt and NF-kappaB-inducing kinase pathways: role of H(2)O(2) in NF-kappaB activation. *Free Radic. Biol. Med.* 45: 885–896.
45. Chua, J. S., A. M. Rofe, and P. Coyle. 2006. Dietary zinc supplementation ameliorates LPS-induced teratogenicity in mice. *Pediatr. Res.* 59: 355–358.
46. Coyle, P., N. Tran, J. N. Fung, B. L. Summers, and A. M. Rofe. 2009. Maternal dietary zinc supplementation prevents aberrant behaviour in an object recognition task in mice offspring exposed to LPS in early pregnancy. *Behav. Brain Res.* 197: 210–218.