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Melatonin alleviates lipopolysaccharide-induced placental cellular stress response in mice

Abstract: Melatonin protects mice from lipopolysaccharide (LPS)-induced fetal death and intra-uterine growth retardation. Nevertheless, its molecular mechanism remains obscure. In the present study, we investigated the effects of melatonin on LPS-induced cellular stress in placenta. Pregnant mice were given with melatonin [5.0 mg/kg, intraperitoneal (i.p.)] 30 min before and 150 min after LPS (300 μg/kg, i.p.) on gestational day 15. Oxidative stress, endoplasmic reticulum (ER) stress, hypoxic stress, and heat stress in placenta were analyzed at 4 hr after LPS. As expected, maternal LPS administration resulted in placental glutathione (GSH) depletion and up-regulated the expression of placental antioxidative enzymes. In addition, LPS significantly increased the level of inducible nitric oxide synthase (iNOS) and enhanced the intensity of placental 3-nitrotyrosine residues. An ER stress, as determined by a decreased GRP78 expression, an obvious eIF2α and JNK phosphorylation, and an increased CHOP expression, were observed in placenta of pregnant mice injected with LPS. In addition, LPS significantly increased mRNA level of placental HIF-1α, VEGF, and ET-1, the markers of hypoxic stress. Heme oxygenase (HO)-1, a marker of heat stress, was also up-regulated in placenta of LPS-treated pregnant mice. Interestingly, LPS-induced placental oxidative stress, hypoxic stress, and ER stress were significantly alleviated when pregnant mice were given with melatonin, whereas melatonin had little effect on LPS-evoked placental HO-1 expression. In conclusion, maternally administered melatonin alleviates LPS-induced cellular stress in the placenta. Melatonin may be useful as pharmacological agents to protect the fetuses against LPS-induced intra-uterine fetal death and intra-uterine growth restriction.

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Key words: endoplasmic reticulum stress, heat stress, hypoxic stress, lipopolysaccharide, melatonin, oxidative stress, placenta

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Introduction

LPS is a toxic component of cell walls of gram-negative bacteria that are widely present in the digestive tracts of humans and animals [1]. Humans are constantly exposed to low levels of LPS through infection. Gastrointestinal distress and alcohol consumption often increase the uptake of LPS from the gastrointestinal tract into blood [2]. LPS has been associated with adverse developmental outcomes, including embryonic resorption, fetal death, intra-uterine growth restriction, skeletal development retardation, and preterm labor and delivery in rodents [3–6]. Several earlier studies found that maternal LPS exposure caused the development of malformed fetuses in rats [7] and golden hamsters [3, 8]. Recently, we found that maternal LPS exposure from gestational day (gd) 8 to 12 resulted in external malformations and skeletal abnormalities in mice [9]. In addition, maternal LPS exposure during pregnancy resulted in age- and gender-dependent impairments of neurobehavioral development in offspring [10].

Melatonin (*N*-acetyl-5-methoxytryptamine, melatonin), the major secretory product of the pineal gland, plays a fundamental role in the neuroimmuno-endocrine system. As a potent antioxidant, melatonin and its metabolites directly scavenge a variety of free radicals [11–15]. In addition, melatonin scavenges peroxynitrite anion ($ONOO^-$), the highly destructive product of the interaction between the superoxide anion radical ($O_2\cdot^-$) and nitric oxide (NO) [14]. On the other hand, melatonin also reduces free radical levels via stimulating the activities of antioxidative enzymes [16–18]. As an immunomodulator, melatonin regulates the release of pro-inflammatory and anti-inflammatory cytokines [19, 20]. In vivo, melatonin prevents LPS-induced circulatory failure and improves survival in rats administered a lethal dose of LPS through the repression of plasma TNF-α release and hepatic inducible nitric oxide synthase (iNOS) expression [21, 22]. Our earlier studies demonstrated that melatonin protected mice from LPS-induced fetal death and intra-uterine growth retardation [23].

In the present study, we investigated the effects of maternally administered melatonin on LPS-induced cellular stress in mouse placenta. We found that maternal LPS exposure during pregnancy resulted in oxidative damage,

endoplasmic reticulum (ER) stress, hypoxia-mediated stress and heat stress response in placenta. Maternally administered melatonin alleviated LPS-mediated changes.

Materials and methods

Chemicals and reagents

Lipopolysaccharide (*Escherichia coli* LPS, serotype 0127:B8) and melatonin (melatonin) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3-nitrotyrosine (3-NT), p-JNK, heme oxygenase (HO)-1 and β-actin antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Phosphor-eIF2α antibody was from Cell Signaling Technology (Beverley, MA, USA). Chemiluminescence (ECL) detection kit from Pierce Biotechnology (Rockford, IL, USA). TRIzol reagent was from Invitrogen (Carlsbad, CA, USA). RNase-free DNase and RT and PCR kits were from Promega Corporation (Madison, WI, USA). All the other reagents were from Sigma 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, Inc (Wilmington, MA, USA). The animals were allowed free access to food and water at all times and were maintained on a 12-hr 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 21:00 hr. Females were checked by 7:00 hr the next morning, and the presence of a vaginal plug was designated as gd 0. The pregnant mice were divided randomly into four groups. All pregnant mice except controls (saline and melatonin) received an intraperitoneal (i.p.) injection of LPS (300 μg/kg) between 08:00 and 09:00 hr on gd 15. In melatonin + LPS group, pregnant mice received two doses of melatonin, one (5 mg/kg) injected 30 min before LPS, the second injected 150 min after LPS. The control mice received two doses of NS or melatonin. All pregnant mice were sacrificed at 4 hr after LPS administration. Placentas were collected for measurement of cellular stress. All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

Isolation of total RNA and RT-PCR

Fifty milligrams of placenta was collected from each mouse. Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions. RNase-free DNase was used to remove genomic DNA. The integrity and concentration of RNA was determined by measuring absorbance at 260 nm followed by electrophoresis on agarose gels. For the synthesis of cDNA, 1.0 μg of total RNA from each sample was resuspended in a 20-μL final volume of reaction buffer, which contained 25 mM Tris–HCl, pH 8.3, 37.5 mM KCl, 10 mM dithiothreitol, 1.5 mM $MgCl_2$, 10 mM of each dNTP and 0.5 mg oligo$(dT)_{15}$ primer. After the reaction mixture reached 42°C, 20 units of RT was added to each tube and the sample was incubated for 60 min at 42°C. Reverse transcription was stopped by denaturing the enzyme at 95°C. The final PCR mixture contained 2.5 μL of cDNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP mixture, 1 U of Taq DNA polymerase, 1 μM sense and antisense primers, and of sterile water to 50 μL. The reaction mixture was covered with mineral oil. PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed on each individual sample as an internal positive-control standard. Specific primers were synthesized by Shanghai Sangon Biological Engineering Technology (Shanghai, China). Oligonucleotide sequence, annealing temperature, and the number of cycles were listed in Table 1. The amplified PCR products were subjected to electrophoresis at 75 V through 1.5% agarose gels (Sigma) for 45 min. The pBR322 DNA digested with Alu I was used for molecular markers (MBI Fermentas, Vilnius, LT, USA). Agarose gels were stained with 0.5 mg/mL ethidium bromide (Sigma) TBE buffer.

Immunoblotting

Total lysate from placenta was prepared by homogenizing 50 mg of placental tissue in 300 μL of 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 dodecylsulfate, 1 mM phenylmethylsulfonyl fluoride) supplemented with a cocktail of protease inhibitors (Roche, Indianapolis, IN, USA). Total lysate was separated electrophoretically by SDS–PAGE and transferred onto a polyvinylidene fluoride membrane. The membranes were incubated for 2 hr with the following antibodies: p-eIF2α, HO-1, 3-NT, and p-JNK. β-actin was used as a loading control for total proteins. After washes in DPBS containing 0.05% Tween-20 four times for 10 min each, the membranes were incubated with goat anti-rabbit IgG or goat anti-mouse antibody for 2 hr. 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.

Glutathione measurement

The glutathione (GSH) was determined by the method of Griffith [24]. Proteins of 0.4 mL placenta homogenates were precipitated by the addition of 0.4 mL of a metaphosphoric acid solution. After 40 min, the protein precipitate was separated from the remaining solution by centrifugation at 4000 g at 4°C for 5 min. Four hundred microlitres of the supernatant was combined with 0.4 mL of 300 mM Na_2HPO_4, and the absorbance at 412 nm was read against a blank consisting of 0.4 mL supernatant plus 0.4 mL H_2O. Then, 100 μL DTNB (0.02%, w/v; 20 mg DTNB in 100 mL of 1% sodium citrate) was added to the blank and sample, and absorbance of the sample was read against the blank at 412 nm. The GSH content was determined using a calibration curve prepared with an authentic sample. GSH values were expressed as nanomole per milligram protein.

Table 1. Primers, the number of cycles, and annealing temperature for RT-PCR

Name	Sequence	Size (bp)	Annealing (°C)	Number of cycles (n)
GAPDH	Forward: 5′-GAGGGGCCATCCACAGTCTTC-3′ Reverse: 5′-CATCACCATCTTCCAGGAGCG-3′	340	56°C	28
Catalase	Forward: 5′-AATCCTACACCATGTCGGACA-3′ Reverse: 5′-CGGTCTTGTAATGGAACTTGC-3′	726	55°C	29
SOD2	Forward: 5′-AGCGGTCGTGTAAACCTCA-3′ Reverse: 5′-AGACATGGCTGTCAGCTTC-3′	439	55°C	29
GSHPx	Forward: 5′-CCTCAAGTACGTCCGACCTG-3′ Reverse: 5′-CAATGTCGTTGCGGCACACC-3′	197	57°C	28
GSH-Rd	Forward: 5′-CTTCCTTCGACTACCTGG-3′ Reverse: 5′-ATGCCTGCGATCTCCACA-3′	400	55°C	29
iNOS	Forward: 5′-GCTGCCTTCCTGCTGTCGCA-3′ Reverse: 5′-GGAGCCGCTGCTGCCAGAAA-3′	316	60°C	30
HIF-1α	Forward: 5′-GGCGTTTAGGCCCGAGCGAG-3′ Reverse: 5′-CCCCCGGCTTGTTAGGGTGC-3′	602	63°C	30
VEGF	Forward: 5′-GCCGCTGCCTGCAACAAGTG-3′ Reverse: 5′-AAGGGTTGGCCAGGCTGGGA-3′	324	61°C	40
ET-1	Forward: 5′-ACTTGCTGAGGACCGCGCTG-3′ Reverse: 5′-GCTCCGGTGCTGAGTTCGGC-3′	266	60°C	36
ETA	Forward: 5′-CACGGAACGGAGCAGCCACA-3′ Reverse: 5′-CCGGTCCACCACGTGCTGTT-3′	201	60°C	36
CHOP	Forward: 5′-GGTGGCAGCGACAGAGCCAG-3′ Reverse: 5′-GATCAGAGCCCGCCGTGTGG-3′	592	63°C	35
ATF4	Forward: 5′-CTTGCGGCCACCATGGCGTA-3′ Reverse: 5′-ACCCGACTGGTCGAAGGGGG-3′	280	63°C	33
TRB3	Forward: 5′-GGGCTTGTCTTGCGCGACCT-3′ Reverse: 5′-GCCCTCAGGCAGGGCAAAGG-3′	321	63°C	35
GRP78	Forward: 5′-GCGTGTGTGTGAGACCAGAACCG-3′ Reverse: 5′-TGCGTCCGATGAGGCGCTTG-3′	413	63°C	30
HO-1	Forward: 5′-CCACAGCCCGACAGCATGCC-3′ Reverse: 5′-GCAGCTCAGGGTGAGTGCGC-3′	376	55°C	32

Lipid peroxidation assay

Hepatic lipid peroxidation was quantified by measuring malondialdehyde (MDA) as described previously [25]. Placental tissue was homogenized in 9 volumes of 50 mM Tris–HCl buffer (pH 7.4) containing 180 mM KCl, 10 mM EDTA, and 0.02% butylated hydroxytoluene. To 0.2 mL of the tissue homogenate, 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid, 1.5 mL of 0.9% thiobarbituric acid, and 0.6 mL of distilled water were added and vortexed. The reaction mixture was placed in a water bath at 95°C for 40 min. After cooling on ice, 1.0 mL of distilled water and 5.0 mL of butanol/pyridine mixture (15:1, v/v) were added and vortexed. After the reaction mixture was centrifuged at 10,000 g for 10 min, absorbance of the supernatants was determined at 532 nm. MDA values were expressed as nmol/g placenta.

Statistical analysis

All quantified data were expressed as means ± S.E.M. at each point. ANOVA and the Student-Newmann–Keuls post hoc test were used to determine differences among different groups. Differences were considered to be significant only for $P < 0.05$.

Results

The effects of melatonin on LPS-induced oxidative stress in mouse placenta were analyzed. As shown in Fig. 1A, the level of placental GSH was significantly decreased at 4 hr after the pregnant mice were injected with LPS. Melatonin significantly attenuated LPS-induced GSH depletion. The effects of LPS on the level of placental MDA are presented in Fig. 1B. No significant difference on the level of placental MDA, a marker of lipid peroxidation, was observed after the pregnant mice were administered with LPS for 4 hr. The effects of melatonin on gene expression of antioxidant enzymes in placenta are presented in Fig. 2A–D. As expected, mRNA level of placental catalase, SOD2 and GSH-Px, was significantly increased after the pregnant mice were injected with LPS for 4 hr, whereas LPS had no effect on the expression of placental GSH-Rd. Melatonin significantly attenuated LPS-induced up-regulation of catalase, SOD2 and GSH-Px. The effects of melatonin on placental iNOS expression are presented in Fig. 3A. The level of placental iNOS mRNA was significantly increased in LPS-treated pregnant mice. Melatonin significantly alleviated LPS-induced up-regulation of iNOS in placenta. 3-NT is a specific marker for protein nitration. The effects of melatonin on LPS-induced placental protein nitration are presented in Fig. 3B. As expected, placental 3-NT intensity was significantly enhanced in LPS-treated mice. Melatonin significantly attenuated LPS-induced protein nitration in placenta.

The effects of maternal melatonin administration on LPS-induced placental hypoxic stress are presented in Fig. 4. As expected, HIF-1α was significantly up-regulated in placenta of pregnant mice injected with LPS. Correspondingly, mRNA level of VEGF, a target gene of

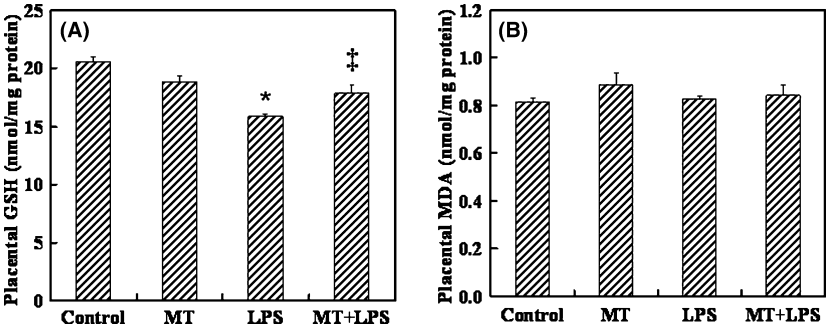

Fig. 1. The effects of melatonin on GSH and malondialdehyde (MDA) content in placenta. All pregnant mice except controls received an intraperitoneal injection of lipopolysaccharide (LPS, 300 μg/kg) on gestational day 15. In MT + LPS group, pregnant mice received two doses of melatonin, one (5 mg/kg) injected 30 min before LPS, the second injected 150 min after LPS. Placenta was collected at 4 hr after LPS. (A) GSH; (B) MDA. Data were expressed as means ± S.E.M. of twelve samples from twelve pregnant mice. *$P < 0.05$ when compared with controls. ‡$P < 0.05$ when compared with LPS group.

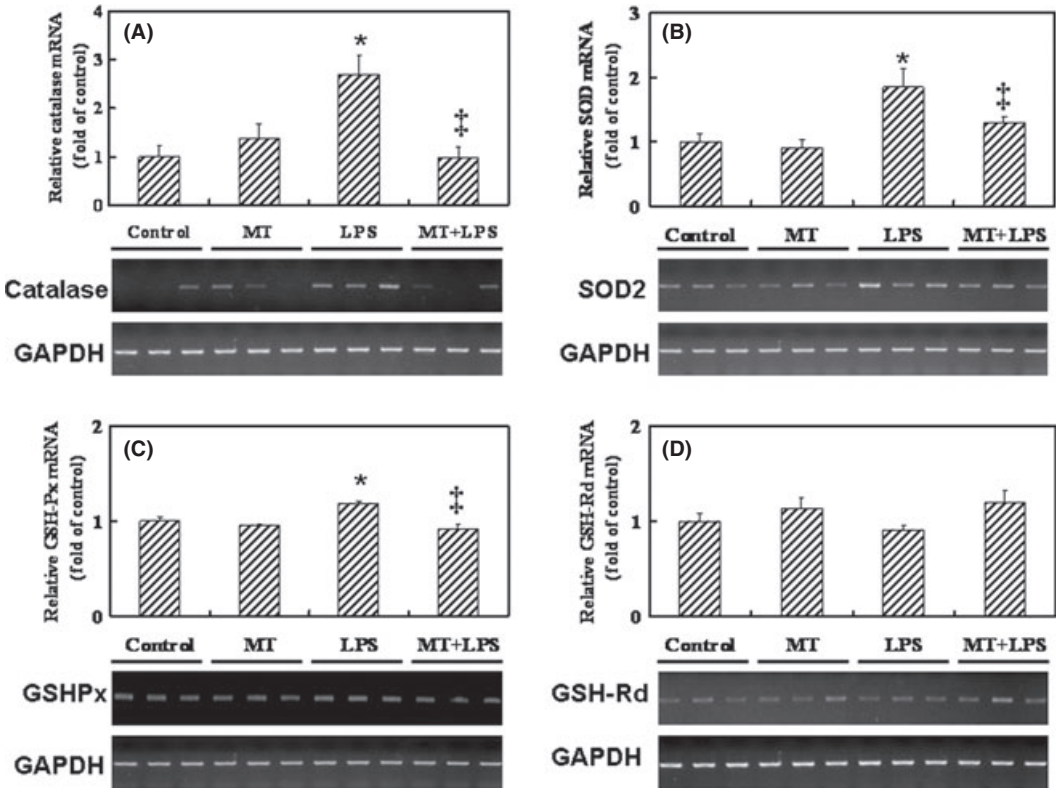

Fig. 2. The effects of melatonin on lipopolysaccharide (LPS)-induced expression of antioxidative enzymes in mouse placenta. All pregnant mice except controls received an intraperitoneal injection of LPS (300 μg/kg) on gestational day 15. In MT + LPS group, pregnant mice received two doses of melatonin, one (5 mg/kg) injected 30 min before LPS, the second injected 150 min after LPS. Placenta was collected at 4 hr after LPS. The expression of SOD2, catalase, GSH-Px, and GSH-Rd in placenta was determined using RT-PCR. Data were expressed as means ± S.E.M. of six samples from six pregnant mice. *$P < 0.05$ when compared with controls. ‡$P < 0.05$ when compared with LPS group.

HIF-1α, was significantly increased in placenta of LPS-treated pregnant mice. Melatonin significantly attenuated LPS-induced up-regulation of placental HIF-1α and VEGF (Fig. 4A,B). The effects of melatonin on placental ET-1 are presented in Fig. 4C. As expected, the level of placental ET-1 mRNA was significantly increased in LPS-treated mice. Melatonin significantly attenuated LPS-induced up-regulation of ET-1 in mouse placenta. The effects of melatonin on placental ETA were analyzed. As shown in Fig. 4D, LPS had no effect on the expression of placental ETA.

To examine whether maternal LPS administration induces placental ER stress, the expression of ER chaperones was analyzed in placenta of mice injected with LPS. The expression of placental GRP78, an ER chaperone and

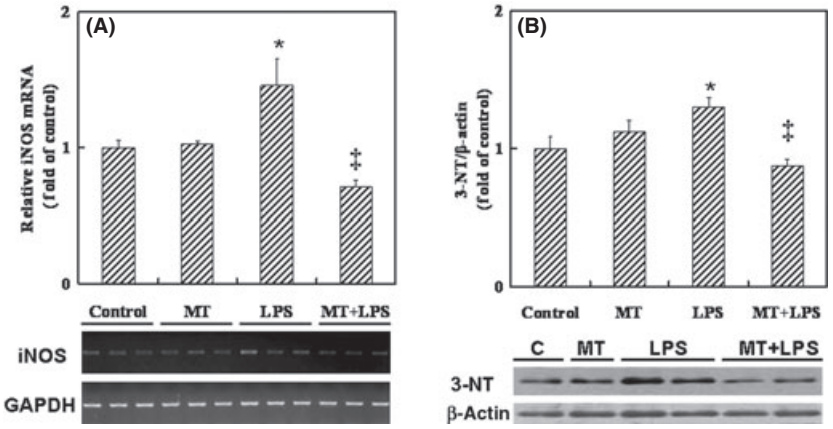

Fig. 3. The effects of melatonin on lipopolysaccharide (LPS)-induced expression of inducible nitric oxide synthase (iNOS) and protein nitration in mouse placenta. All pregnant mice except controls received an intraperitoneal injection of LPS (300 μg/kg) on gestational day 15. In MT + LPS group, pregnant mice received two doses of melatonin, one (5 mg/kg) injected 30 min before LPS, the second injected 150 min after LPS. Placenta was collected at 4 hr after LPS. (A) The expression of iNOS in placenta was determined using RT-PCR. Data were expressed as means ± S.E.M. of six samples from six pregnant mice. (B) 3-nitrotyrosine was measured using immunoblotting. Placental samples in each group were from six pregnant mice. Blots are representative of three independent experiments. $*P < 0.05$ when compared with controls. $‡P < 0.05$ when compared with LPS group.

ATF6 target, was significantly decreased in LPS-treated mice. Melatonin counteracted LPS-induced down-regulation of GRP78 in mouse placenta (Fig. 5D). Next, the level of phosphorylated eIF2α (p-eIF2α), a downstream target of the PERK pathway, was measured in placenta of mice injected with LPS. As expected, the level of p-eIF2α was significantly increased in placenta of mice administered with LPS. Melatonin significantly attenuated LPS-induced placental eIF2α phosphorylation (Fig. 5E). The expression of CHOP, a downstream target of the PERK and ATF6 pathways, was analyzed. As shown in Fig. 5A, the level of CHOP mRNA was significantly increased in placenta of LPS-administered pregnant mice. Melatonin significantly alleviated LPS-induced up-regulation of placental CHOP. The effects of LPS on the expression of placental TRB3 and ATF4 are presented in Fig. 5B,C. No significant difference on mRNA level of TRB3 and ATF4 was observed among different groups. The effects of melatonin on placental JNK phosphorylation are presented in Fig. 5F. As expected, the level of phosphorylated JNK (p-JNK) was significantly increased in placenta of mice injected with LPS. Melatonin significantly attenuated LPS-induced placental JNK phosphorylation.

The expression of HO-1, a marker of heat stress, was analyzed in placenta of LPS-administered mice. As shown in Fig. 6A, maternal LPS administration significantly up-regulated the expression of HO-1 mRNA in placenta. Correspondingly, the level of HO-1 protein was significantly increased in placenta of mice administered with LPS (Fig. 6B). Interestingly, maternal administered melatonin had no effect on LPS-induced up-regulation of placental HO-1 expression.

Discussion

LPS is associated with adverse developmental outcomes, including embryonic resorption, fetal death, intra-uterine growth restriction, external malformations and skeletal abnormalities, and preterm labor and delivery in rodents [6, 9, 26–30]. Several studies have demonstrated that reactive oxygen species (ROS) might be involved in LPS-induced embryolethality and teratogenesis [9, 29, 30]. The placenta performs key transport, metabolic and secretory functions to support normal fetal development. The present study shows that the expression of antioxidative enzymes, a marker of oxidative stress, was significantly increased in placenta of mice injected with LPS during pregnancy. In addition, maternal LPS administration during pregnancy caused placental GSH depletion. NO is also a free radical. NO interacts with superoxide anion radical and forms $ONOO^-$, highly destructive oxidizing agent that induces protein nitration. An earlier study demonstrated that NO fulfills a fundamental role in LPS-induced embryonic resorption [5]. The present study showed that placental iNOS expression was up-regulated in LPS-administered mice. Importantly, the intensity of 3-NT, a marker of protein nitration, was obviously enhanced in placenta when pregnant mice were administered with LPS. Melatonin is a powerful endogenous antioxidant [13–18, 31]. Our earlier study found that melatonin effectually protected mice from LPS-induced fetal death and intra-uterine growth restriction [23]. A recent study reported that melatonin preserved fetal growth in rats through protecting the placenta from ischemia/reperfusion-induced oxidative and nitrosative stress [32]. In addition, melatonin improves placental efficiency and birth weight and increases the placental expression of antioxidant enzymes in undernourished pregnancy [33]. In the present study, we documented that melatonin significantly attenuated LPS-induced placental GSH depletion. In addition, melatonin obviously counteracted LPS-induced iNOS expression and alleviated placental protein nitration. Therefore, it would be reasonable to speculate that the protection of melatonin against LPS-induced fetal death and intra-uterine growth restriction is,

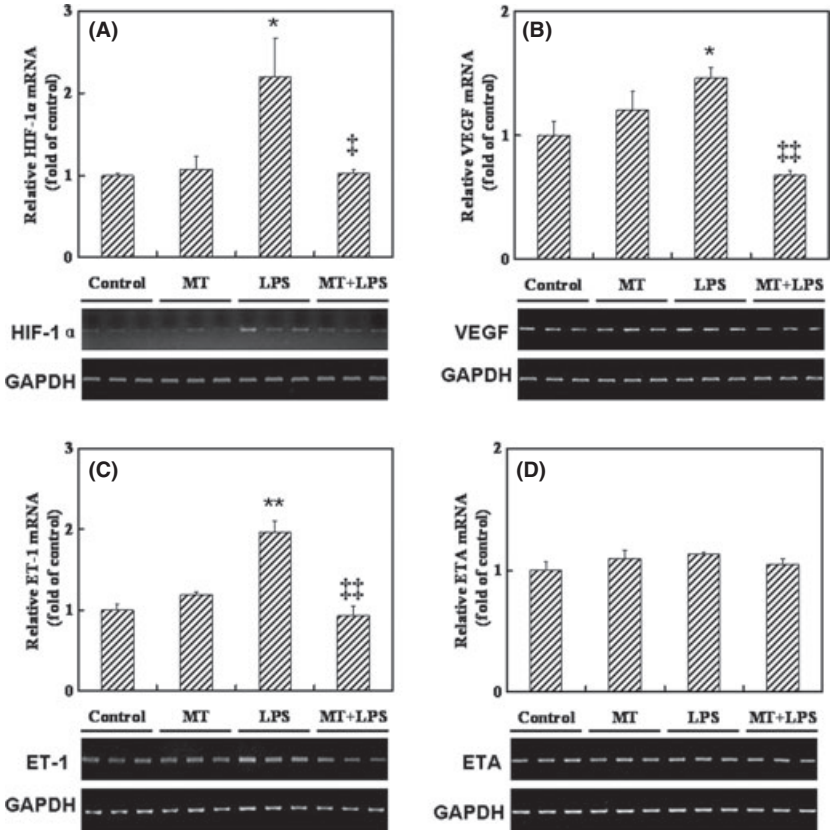

Fig. 4. The effects of melatonin on lipopolysaccharide (LPS)-induced hypoxic stress in mouse placenta. All pregnant mice except controls received an intraperitoneal injection of LPS (300 μg/kg) on gestational day 15. In MT + LPS group, pregnant mice received two doses of melatonin, one (5 mg/kg) injected 30 min before LPS, the second injected 150 min after LPS. Placenta was collected at 4 hr after LPS. The expression of HIF-1α, VEGF, ET-1, and ETA in placenta was determined using RT-PCR. (A) HIF-1α; (B) VEGF; (C) ET-1; (D) ETA. Data were expressed as means ± S.E.M. of six samples from six pregnant mice. $^{*}P < 0.05$, $^{**}P < 0.01$ when compared with controls. $\ddagger P < 0.05$, $\ddagger\ddagger P < 0.01$ when compared with LPS group.

at least partially, attributed to its reduction of oxidative stress in placenta.

In human pregnancies, an impairment in the process of trophoblast differentiation and invasion into maternal decidua is an important cause of placental insufficiency leading to fetal death and intra-uterine growth restriction. Increasing evidence has demonstrated that hypoxia hinders trophoblast differentiation and invasion into maternal deciduas [34]. HIF-1α is a transcriptional factor that activates the transcription of its targets including VEGF and ET-1 in response to hypoxia [35]. In the present study, we found that placental HIF-1α and its targets VEGF and ET-1, markers of hypoxic stress, were significantly up-regulated in LPS-administered pregnant mice. These results support the idea that reduced oxygen tension and hypoxia might play a key role in LPS-induced fetal death and intra-uterine growth restriction. Indeed, increasing data have demonstrated that hypoxia promotes oxidative stress in placenta [36]. As an antioxidant, melatonin has been shown to protect tissues from ischemia-induced damage in the brain as well as in other tissues [37–42]. Interestingly, the present study found that LPS-induced up-regulation of HIF-1α and its target VEGF and ET-1 in placenta was significantly alleviated in melatonin-administered pregnant mice, indicating that the protection of melatonin against LPS-induced fetal death and intra-uterine growth restriction might be not only attributed to its antioxidant effect, but also to its direct alleviation of LPS-induced placental hypoxic stress.

A recent study reported that inositol requiring enzyme-1 (IRE1), a protein located on the ER membrane, is essential for placental development and embryonic viability [43]. In addition, ER stress plays key roles in the pathophysiology of intra-uterine growth restriction [44]. In the present study, we investigated LPS-induced ER stress in placenta. We found that the expression of placental GRP78, an ER chaperone and ATF6 target, was significantly down-regulated in LPS-administered mice. Moreover, p-eIF2α, a downstream target of the PERK pathway, was significantly increased in placenta of mice administered with LPS. In addition, CHOP, a downstream target of the PERK and ATF6 pathways, was up-regulated in placenta of LPS-treated pregnant mice. Finally, the level of placental pJNK was increased in pregnant mice injected with LPS. Interestingly, melatonin significantly attenuated LPS-induced down-regulation of placental GRP78. In addition, melatonin alleviated LPS-evoked induction of CHOP and repressed eIF2α and JNK phosphorylation in placenta. Therefore, melatonin-mediated protection against LPS-induced fetal death and intra-uterine growth restriction might be associated with its alleviation of ER stress in placenta.

Heme oxygenase (HO) catalyzes the rate-limiting step in the degradation of heme to yield equimolar amounts of biliverdin, carbon monoxide, and iron. There is increasing evidence that heme oxygenases play important roles in the cellular defense against oxidative stress [45]. Three isoforms of the HO protein have been identified. HO-1, a marker of heat stress, is expressed in placenta [46]. An earlier study showed that inhibition of placental HO-1 resulted in fetal intra-uterine growth restriction [47]. Conversely, the induction of placental HO-1 during implantation window

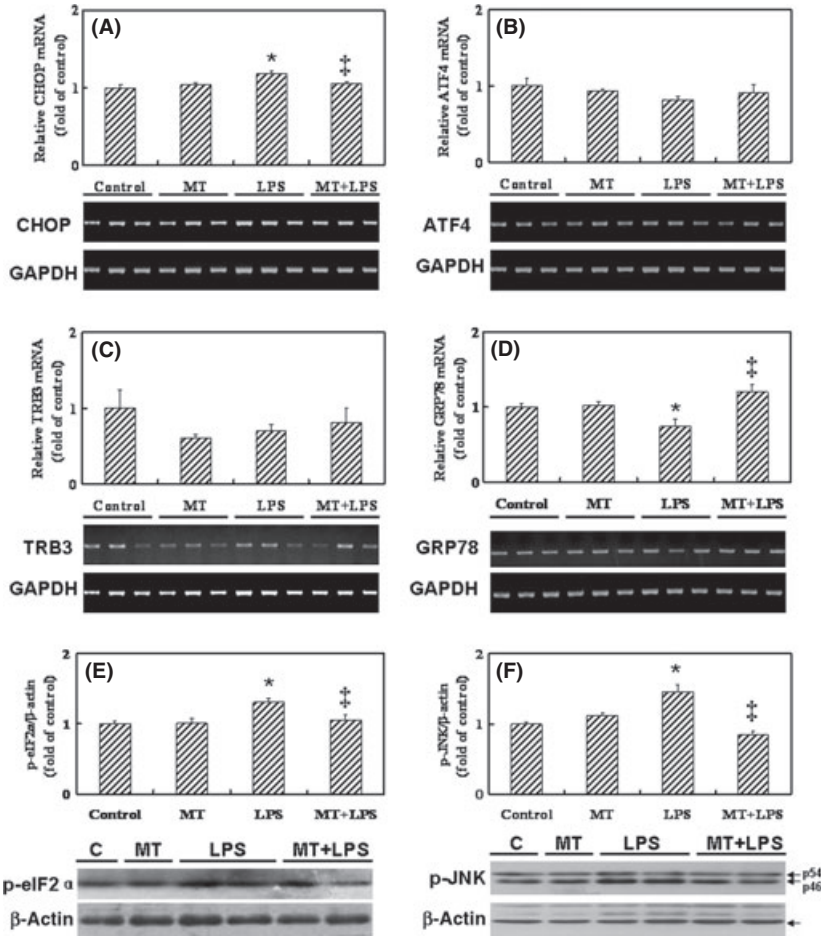

Fig. 5. The effects of melatonin on lipopolysaccharide (LPS)-induced endoplasmic reticulum stress in mouse placenta. All pregnant mice except controls received an intraperitoneal injection of LPS (300 μg/kg) on gestational day 15. In MT + LPS group, pregnant mice received two doses of melatonin, one (5 mg/kg) injected 30 min before LPS, the second injected 150 min after LPS. Placenta was collected at 4 hr after LPS. (A–D) The expression of CHOP, ATF4, TRB3, and GRP78 in placenta was determined using RT-PCR. Data were expressed as means ± S.E.M. of six samples from six pregnant mice. (E and F) The level of p-eIF2α and p-JNK in placenta was measured using immunoblotting. Placental samples in each group were from six pregnant mice. Blots are representative of three independent experiments. $^*P < 0.05$ when compared with controls. $\ddagger P < 0.05$ when compared with LPS group.

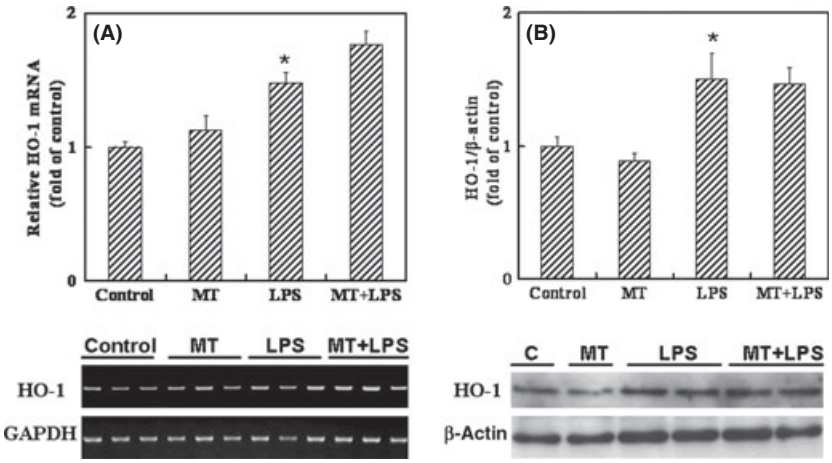

Fig. 6. The effects of melatonin on lipopolysaccharide (LPS)-evoked induction of placental heme oxygenase (HO)-1. All pregnant mice except controls received an intraperitoneal injection of LPS (300 μg/kg) on gestational day 15. In MT + LPS group, pregnant mice received two doses of melatonin, one (5 mg/kg) injected 30 min before LPS, the second injected 150 min after LPS. Placenta was collected at 4 hr after LPS. (A) The level of placental HO-1 mRNA was determined using RT-PCR. Data were expressed as means ± S.E.M. of six samples from six pregnant mice. (B) The level of placental HO-1 protein was measured using immunoblotting. Placental samples in each group were from six pregnant mice. Blots are representative of three independent experiments. $^*P < 0.05$ when compared with controls.

rescued mice from abortion [48]. A recent study found that HO-1 might play an important role on placental development [46]. HO-1 is a 32-kDa inducible protein. Numerous studies demonstrated that a variety of stimuli, such as ROS, metalloporphyrins, transition metals, ischemia-reperfusion, cytokines, and its substrate, hemin, induce HO-1 expression [45]. Our earlier study showed that the expression of placental HO-1 was increased when the pregnant mice were

challenged with LPS [49]. Indeed, the induction of HO-1 is associated with oxidative stress and hypoxic stress [49, 50]. Interestingly, the present study showed that melatonin did not repress LPS-evoked induction of placental HO-1, although this indole significantly counteracted LPS-induced placental oxidative stress and hypoxic stress. Actually, LPS-evoked induction of placental HO-1 mRNA was aggravated in mice administered with melatonin. The effects of melatonin on placental HO-1 expression might be beneficial for its protecting fetuses from LPS-induced fetal death and intra-uterine growth restriction.

In summary, the present study indicates that maternal LPS exposure during pregnancy results in cellular stress response in placenta, which may contribute to LPS-induced intra-uterine fetal death and intra-uterine growth restriction. Maternally administered melatonin alleviates LPS-induced oxidative stress, hypoxic stress, and ER stress. Thus, melatonin may be used as pharmacological agents to protect the fetuses from LPS-induced intra-uterine fetal death and intra-uterine growth restriction.

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