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Melatonin attenuates lipopolysaccharide-induced down-regulation of pregnane X receptor and its target gene CYP3A in mouse liver

Abstract: Pregnane X receptor (PXR) is a member of the nuclear receptor superfamily that regulates target gene transcription in a ligand-dependent manner. Our earlier study indicated that reactive oxygen species contribute to lipopolysaccharide (LPS)-induced down-regulation of PXR and its target gene CYP3A in mouse liver. Melatonin is a powerful endogenous antioxidants. In this study, we investigated the effects of melatonin on LPS-induced down-regulation of PXR and CYP3A in mouse liver. Mice were intraperitoneally administrated different doses of melatonin before and/or after LPS treatment. PXR and CYP3A11 mRNA levels were measured using RT-PCR. Erythromycin *N*-demethylase (ERND) was used as an indicator of CYP3A catalytic activity. Results indicated that melatonin significantly attenuated LPS-induced down-regulation of PXR and CYP3A11 mRNA levels in a dose-dependent manner. Repeated doses of melatonin (10 mg/kg) treatments also significantly attenuated LPS-induced down-regulation of dexamethasone-inducible CYP3A11 mRNA level and ERND activity in mouse liver. In addition, the present study also shows that melatonin significantly increased hepatic superoxide dismutase, Se-dependent glutathione peroxidase, glutathione reductase and catalase activities and glutathione levels in LPS-treated mice. These findings suggest that melatonin may exert its protective effects on LPS-induced down-regulation of PXR and CYP3A via counteracting LPS-induced oxidative stress in mouse liver.

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Introduction

The cytochrome P450 3A (CYP3A) is a member of the cytochrome P-450 monooxygenase superfamily, which is responsible for the oxidative metabolism of numerous clinically used drugs [1]. Gene expression of CYP3A is highly regulated by development, tissue-specific factors, hormonal influences, xenobiotics and pathophysiological mechanisms [2, 3]. Recent studies found that pregnane X receptor (PXR), a member of the nuclear receptor superfamily, regulates CYP3A gene transcription in a ligand-dependent manner [4–6]. CYP3A inducers, such as dexamethasone (DEX), activate PXR and up-regulate CYP3A expressions [7, 8].

Numerous studies indicated that inflammation and infection cause suppression of cytochrome P450 levels in various species including human, rat and mouse [9, 10]. Lipopolysaccharide (LPS)-induced down-regulation of CYP3A has also been demonstrated in mouse model [11, 12]. Furthermore, recent studies have shown that LPS-induced down-regulation of CYP3A is associated with a marked reduction in PXR mRNA and protein levels [13, 14]. Our earlier study indicated that Kupffer cells and reactive oxygen species (ROS) contribute to LPS-induced down-regulation of nuclear receptor PXR and its target gene CYP3A in mouse liver [15].

Melatonin (N-acetyl-5-methoxytryptamine, melatonin), the major product of the pineal gland, plays a fundamental role in the neuroimmuno-endocrine system. As a potent antioxidant, melatonin directly scavenges hydroxyl free radicals ($^{\cdot}OH$) and peroxynitrite anion ($ONOO^{-}$) [16–18]. Melatonin also decreases free radical levels by stimulating the activities of enzymes involved in antioxidative defense [19–21]. Therefore, whether melatonin exerts its protective effects on LPS-induced down-regulation of PXR and CYP3A is of interesting.

In this study, we investigated the effects of melatonin on LPS-induced down-regulation of PXR and CYP3A. Our results demonstrated that melatonin dramatically attenuated LPS-induced down-regulation of PXR and its target gene CYP3A in mouse liver. In addition, the present study also found that melatonin significantly increased hepatic superoxide dismutase (SOD), catalase (CAT), Se-dependent glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Rd) activities and glutathione (GSH) level in LPS-treated mice. These findings suggest that melatonin may exert its protective effects on LPS-induced down-regulation of PXR and CYP3A via

counteracting LPS-induced oxidative stress in mouse liver.

Materials and methods

Chemicals

Lipopolysaccharide (*Escherichia coli* LPS, serotype 0127:B8), melatonin (melatonin) and DEX were purchased from Sigma Chemical Co. (St Louis, MO, USA). All the other reagents were from Sigma or as indicated in the specified methods.

Animals and treatments

Female ICR mice (8–10-week-old, 20–22 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. The present study included two separate experiments.

Experiment 1

To investigate the effects of melatonin on LPS-induced oxidative stress and the constitutive PXR and CYP3A11 mRNAs down-regulation, all groups except controls (saline and melatonin controls) received an intraperitoneal (i.p.) injection of 1.0 mg/kg of LPS between 08:00 and 09:00 hours to minimize diurnal variations. A saline-treated and melatonin-treated groups injected with saline and melatonin served as controls. Melatonin-treated groups were injected intraperitoneally with five doses of melatonin (10 mg/kg, dissolved in 0.2 mL of 0.25% ethanol:saline). The LPS + melatonin group was divided into four subgroups depending on the schedule of melatonin administration: LPS + melatonin(B) received a single dose of melatonin 30 min before LPS; LPS + melatonin(A) received a single dose of melatonin 60 min after LPS; LPS + melatonin(30) received three doses of melatonin, one injected 30 min before LPS, the second injected simultaneously with LPS, the third injected 60 min after LPS; LPS + melatonin(50) received five injections of melatonin: one injection 30 min before LPS, one simultaneous injection with LPS, and three injections beginning 60 min after LPS. Mice were killed at 6 and 12 h after LPS treatment. Mouse livers were excised for measurement of GSH, antioxidative enzymes and total RNA extraction.

Experiment 2

To investigate the effects of melatonin on LPS-induced down-regulation of the DEX-inducible CYP3A11 mRNA and erythromycin *N*-demethylase (ERND) activity. All groups except control received an intraperitoneal injection of DEX (40 mg/kg) for 3 days before LPS treatment. On the fourth day, mice were injected DEX (40 mg/kg, i.p.) and LPS (1 mg/kg i.p.). Melatonin-treated groups received five injections of melatonin: one injection 30 min before LPS, one simultaneous injection with LPS, and three injections beginning 60 min after LPS. Mice were killed at 12 and 18 h after LPS treatment. Mouse livers were excised for total RNA extraction and microsome isolation were performed.

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

Fifty milligrams of liver tissue was collected from each mouse. Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNase-free DNase (Promega, Madison, WI, USA) 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 (Promega). After the reaction mixture reached 42°C, 20 units of RT (Promega) 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.

PCR amplification

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 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. Following primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Company (Shanghai, China), according to sequence designs previously described by others [22, 23]. GAPDH, 5′-GAG GGG CCA TCC ACA GTC TTC-3′ and 5′-CAT CAC CAT CTT CCA GGA GCG-3′; PXR, 5′-GCG CGG AGA AGA CGG CAG CAT C-3′ and 5′-CCC AGG TTC CCG TTT CCG TGT C-3′; CYP3A11, 5′-CTC AAT GGT GTG TAT ATC CCC-3′ and 5′-CCG ATG TTC TTA GAC ACT GCC-3′. The sizes of amplified PCR products were 340 bp for GAPDH, 254 for PXR, 423 bp for CYP3A11, respectively. Number of cycles and annealing temperature were optimized for each primer pair. For GAPDH, amplification was initiated by 3 min of denaturation at 94°C for one cycle, followed by 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. For PXR, amplification was initiated by 3 min of denaturation at 94°C for one cycle, followed by 45 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 1 min. After the last cycle of amplification, samples were incubated for 10 min at 72°C. In preliminary experiments, PXR for 45 cycles were shown to lie in the linear portion of the curve for the amount of PCR

products. For CYP3A11, Number of cycles was distinguished between the constitutive and inducible expressions. For the constitutive expression of CYP3A11, amplification was initiated by 3 min of denaturation at 94°C for one cycle, followed by 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. For the inducible expression of CYP3A11, amplification was initiated by 3 min of denaturation at 94°C for one cycle, followed by 25 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. In preliminary experiments, the constitutive expression of CYP3A11 for 30 cycles and the inducible expression for 25 cycles were shown to lie in the linear portion of the curve for the amount of PCR products. 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). Agarose gels were stained with 0.5 mg/mL ethidium bromide (Sigma) TBE buffer.

Preparation of liver microsomes

Microsomes were isolated from livers by differential centrifugation [24]. All procedures were conducted at 4°C. Tissue was homogenized in four volumes of Tris/chloride buffer, pH 7.4, containing 150 mM potassium chloride and 1 mM ethylenediaminetetraacetic acid (EDTA), with a Polytron homogenizer and centrifuged at 10,000 × g for 20 min. The supernatant was collected and centrifuged at 211,000 × g for 40 min. The microsomal pellet was resuspended and washed in sodium pyrophosphate buffer, pH 7.4, containing 1 mM EDTA and centrifuged again at 211,000 × g for 40 min at 4°C. The washed microsomal pellet was resuspended in a trischloride buffer, pH 7.4, containing 20% glycerol, with a ground glass tissue grinder and stored at −80°C. Protein concentrations of microsome samples were measured according to the method of Lowry et al. [25], using bovine serum albumin as a standard.

CYP3A catalytic activity

Erythromycin *N*-demethylase was used as an indicator of CYP3A catalytic activity in this study. ERND was measured according to the method of Werringloer [26] with a 45-min incubation containing 4 mM erythromycin in the presence of 0.5 mM NADPH and 0.4 mg of microsomal protein in a total assay volume of 1 mL. The rate of formaldehyde formation was determined spectrophotometrically at 412 nm using the Nash reagent. Measurement for ERND catalytic activity was repeated twice for three separately prepared liver microsome samples.

Determination of antioxidative enzyme activities

Liver was homogenized in 50 mM phosphate buffer (pH 7.4) and centrifuged at 3200 g for 20 min at 4°C. According to the method of Geller and Winge [27], supernatant was assayed for SOD activity by following the inhibition of nitroblue tetrazolium (NBT) reduction. SOD was assayed by a reaction mixture containing 985 μL of 100 mM phosphate buffer (pH 7.4), 0.3 mM EDTA, 0.5 mM NBT and 0.1 mM xanthine. The mixture was preincubated for 3 min at 25°C, and 10 mL of 0.02 U/mL xanthine oxidase was added to generate superoxide and to induce NBT reduction. SOD activity was measured at 560 nm by detecting the inhibition of NBT reduction. One SOD U was defined as the enzyme amount causing 50% inhibition in the NBTH2 reduction rate. SOD activity was expressed as U/mg protein. The CAT was measured in Triton X-100 (1%, v/v) treated supernatants by following H_2O_2 disappearance at 240 nm [28]. One CAT U was defined as the amount of the enzyme required for decrease in 1 μmol of H_2O_2/min. CAT activities were expressed as U/mg protein. Se-dependent GSH-Px activity was measured according to the method of Pagia and Valentina [29]. GSH-Px catalyses the oxidation of gluthione in the presence of hydrogen peroxide. Oxidized glutathione is converted into the reduced form in the presence of gluthione reductase and NADPH, while NADPH is oxidized to NADP. Reduction in the absorbance change per minute and by using the molar extinction coefficient of NADPH, the Se-dependent GSH-Px activity of liver tissue was calculated. Se-dependent GSH-Px activity was expressed as substrates (nmol NADPH) transformed / min/mg protein. GSH-Rd activity was analyzed by following NADPH oxidation at 340 nm in the presence of GSSG and expressed as substrate (nmol NADPH) transformed /min/mg protein. All enzyme activities were determined at 25°C. Protein content was measured according to the method of Lowry et al. [25].

Determination of glutathione content

The glutathione (GSH) was determined by the method of Griffith [30]. Proteins of 0.4 mL liver 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 4470 g at 4°C for 5 min. Four hundred microliters 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 nmol/mg protein. Protein content was measured according to the method of Lowry et al. [25].

Statistical analysis

The *PXR* and *CYP3A11* mRNA levels were normalized to GAPDH mRNA level in the same samples. The *PXR* and *CYP3A11* mRNA levels of the control was assigned as 100%. Quantified data from analysis of SOD, CAT, GSH-Px, GSH-Rd, GSH, RT-PCR and ERND assay were expressed as means ± S.E.M. at each point. ANOVA and the Student–Newmann–Keuls post hoc test were used to determine differences between the treated animals and the control and statistical significance.

Results

The effects of melatonin on LPS-induced down-regulation of the constitutive PXR mRNA in mouse liver are presented in Fig. 1. Results showed that melatonin significantly attenuated LPS-induced down-regulation of the constitutive PXR mRNA levels in a dose-respondent manner. One dose of melatonin before or after LPS administration slightly attenuated LPS-induced down-regulation of the constitutive PXR mRNA levels, while five doses of melatonin obviously attenuated LPS-induced down-regulation of the constitutive PXR mRNA levels.

The effects of melatonin on LPS-induced down-regulation of the constitutive CYP3A11 mRNA in mouse liver are shown in Fig. 2. As expected, melatonin significantly attenuated LPS-induced down-regulation of the constitutive CYP3A11 levels in a dose-respondent manner. A single dose of melatonin before or after LPS administration slightly attenuated LPS-induced down-regulation of the constitutive CYP3A11 mRNA levels. Five doses of melatonin significantly attenuated LPS-induced down-regulation of the constitutive CYP3A11 mRNA levels.

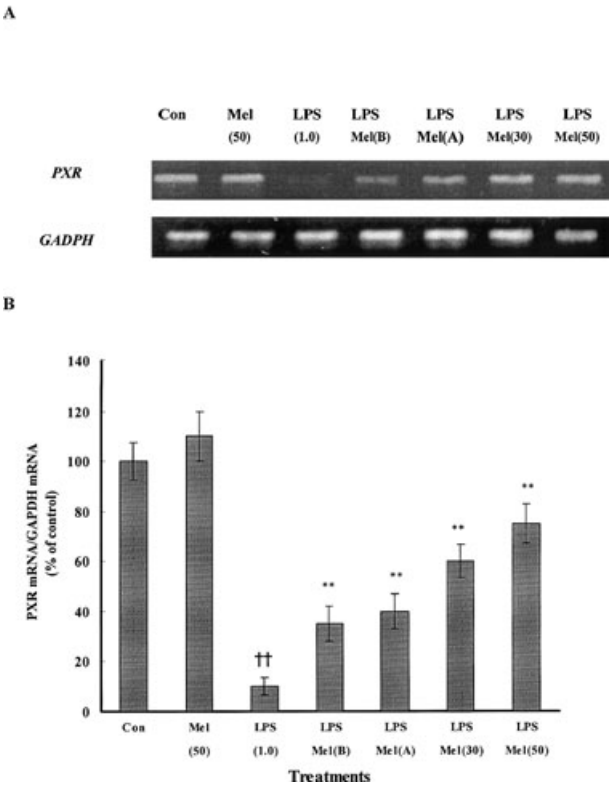

Fig. 1. The effects of melatonin on lipopolysaccharide (LPS)-induced down-regulation of the constitutive pregnane X receptor (PXR) mRNA levels in mouse liver. (A) All groups except control received an intraperitoneal injection of LPS (1.0 mg/kg). Melatonin-treated groups were injected with one to five doses of melatonin (10–50 mg/kg, i.p.). Livers were excised and total RNA was extracted at 12 h after LPS treatment. PXR mRNA levels were determined using RT-PCR. (B) Quantitative analysis of *PXR* mRNA on four individual mouse liver RNA samples at each point was performed. *PXR* mRNA levels were normalized to GAPDH mRNA level in the same samples. *PXR* mRNA levels of the control were assigned as 100%. The number of mice at each point is four. ††$P < 0.01$ as compared with control group. **$P < 0.01$ as compared with LPS-treated group.

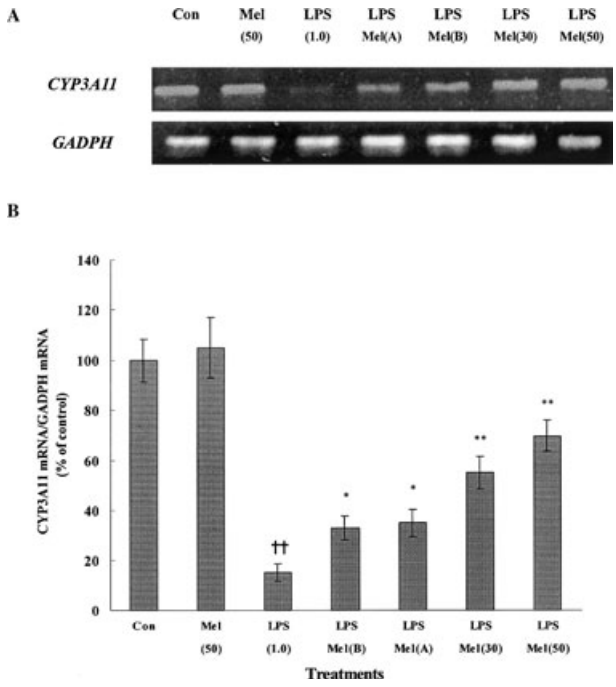

Fig. 2. The effects of melatonin on lipopolysaccharide (LPS)-induced down-regulation of the constitutive CYP3A11 mRNA levels in mouse liver. (A) All groups except control received an intraperitoneal injection of LPS (1.0 mg/kg). Melatonin-treated groups were injected with one to five doses of melatonin (10–50 mg/kg, i.p.). Livers were excised and total RNA was extracted at 12 hr after LPS treatment. CYP3A11 mRNA levels were determined using RT-PCR. (B) Quantitative analysis of *CYP3A11* mRNA on four individual mouse liver RNA samples at each point was performed. *CYP3A11* mRNA levels were normalized to GAPDH mRNA level in the same samples. *CYP3A11* mRNA levels of the control were assigned as 100%. The number of mice at each point is four. ††$P < 0.01$ as compared with control group. *$P < 0.05$, **$P < 0.01$ as compared with LPS-treated group.

The effects of melatonin on LPS-induced down-regulation of the DEX-inducible *CYP3A11* mRNA levels were then analyzed. As shown in Fig. 3, DEX alone caused 3.5-fold induction on *CYP3A11* mRNA levels. LPS repressed the up-regulation of *CYP3A11* mRNA levels in mice pretreated with DEX. Furthermore, LPS-induced down-regulation of the DEX-inducible CYP3A11 mRNA levels was attenuated by melatonin administration.

Effects of melatonin on LPS-induced down-regulation of ERND activity were shown in Fig. 4. Results indicated that DEX caused 10-fold induction on ERND catalytic activity. LPS significantly inhibited the up-regulation of ERND activity in mice pretreated with DEX. Furthermore, LPS-induced down-regulation on DEX-inducible ERND catalytic activities was obviously attenuated in melatonin-treated mice.

The effects of melatonin on antioxidative enzyme activities are presented in Fig. 5. Results showed that LPS slightly decreased SOD activity in mouse liver. Melatonin significantly increased SOD activity in LPS-treated mouse

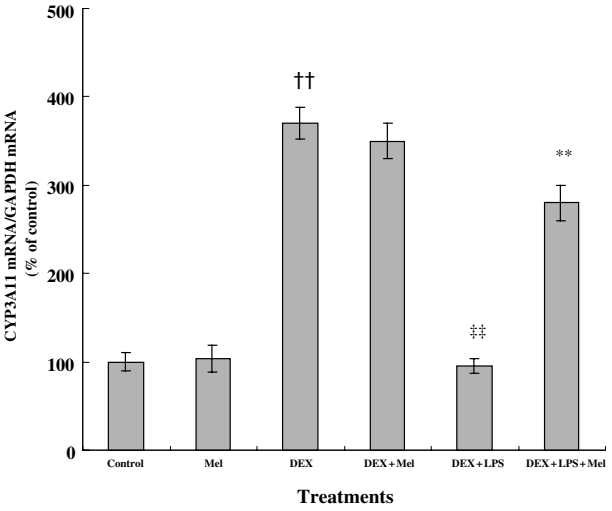

Fig. 3. The effects of melatonin on lipopolysaccharide (LPS)-induced down-regulation of the inducible CYP3A11 mRNA levels in mouse liver. (A) All groups except control received an intraperitoneal injection of DEX (40 mg/kg, i.p.) for 3 days before LPS (1 mg/kg i.p.) treatment. On the fourth day, mice were injected DEX (40 mg/kg, i.p.) and LPS (1 mg/kg i.p.). Melatonin-treated groups received five intraperitoneal injections of melatonin: one injection 30 min before LPS, one simultaneous injection with LPS, and three injections beginning 60 min after LPS. Livers were excised and total RNA was extracted at 12 hr after LPS treatment. CYP3A11 mRNA levels were determined using RT-PCR. (B) Quantitative analysis of *CYP3A11* mRNA on three individual mouse liver RNA samples at each point was performed. *CYP3A11* mRNA levels were normalized to GAPDH mRNA level in the same samples. *CYP3A11* mRNA levels of the control were assigned as 100%. The number of mice at each point is three. ††$P < 0.01$ as compared with control group. ‡‡$P < 0.01$ as compared with DEX-treated group. **$P < 0.01$ as compared with LPS/DEX co-treated group.

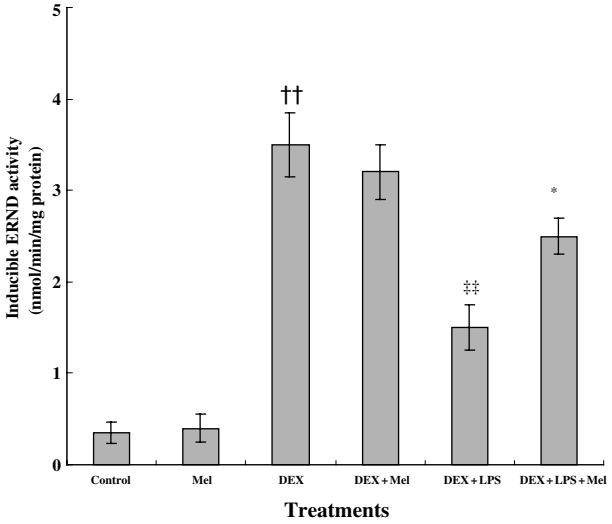

Fig. 4. The effects of lipopolysaccharide (LPS) on the inducible erythromycin *N*-demethylase (ERND) catalytic activities in mouse liver. All groups except control received an intraperitoneal injection of DEX (40 mg/kg, i.p.) for 3 days before LPS (1 mg/kg i.p.) treatment. On the fourth day, mice were injected DEX (40 mg/kg, i.p.) and LPS (1 mg/kg i.p.). Melatonin-treated groups received five injections of melatonin: one injection 30 min before LPS, one simultaneous injection with LPS, and three injections beginning 60 min after LPS. Livers were excised and microsomes were isolated from livers at 18 hr after LPS treatment. ERND catalytic activities in mouse liver were measured as described in Materials and methods. Data were expressed as mean ± S.E.M. (n = 6) ††$P < 0.01$ as compared with control group. ‡‡$P < 0.01$ as compared with DEX-treated group. *$P < 0.05$ as compared with LPS/DEX co-treated group.

liver in a dose-dependent manner (Fig. 5A). LPS significantly decreased CAT activity by 35%. Melatonin significantly stimulated CAT activity in LPS-treated mouse liver in a dose-dependent manner (Fig. 5B). LPS slightly reduced GSH-Px activity in mouse liver. Melatonin significantly increased GSH-Px activity in LPS-treated mouse liver in a dose-dependent manner (Fig. 5C). LPS reduced GSH-Rd activity by about 36%. Melatonin significantly augmented GSH-Rd activity in LPS-treated mouse liver in a dose-dependent manner (Fig. 5D).

The effects of melatonin on GSH content in mouse liver are presented in Fig. 6. As expected, melatonin alone did not increase GSH content in mouse liver. LPS treatment reduced hepatic GSH content by about 30%. Melatonin significantly attenuated LPS-induced decreases in hepatic GSH content.

Discussion

The PXR is a member of the nuclear receptor superfamily that regulates target gene transcription in a ligand-dependent manner. Several studies including our recent report demonstrated that LPS significantly inhibited the constitutive expressions of PXR mRNA in a dose-respondent manner, followed by suppression of CYP3A11 mRNA and ERND catalytic activity in mouse liver. LPS also reversed the up-regulation of CYP3A11 mRNA and ERND catalytic activities in mice pretreated with PXR ligand DEX, RIF, RU486 and PB [13, 15]. Many studies demonstrated that pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, were involved in LPS-induced down-regulation of cytochrome P450s [31, 32]. Our earlier study indicated that ROS contribute to LPS-induced down-regulation of PXR and its target gene CYP3A in mouse liver. In addition, N-acetylcysteine (NAC) and ascorbic acid, two well-known antioxidants, prevented from LPS-induced down-regulation of PXR and *CYP3A11* mRNA levels and ERND catalytic activities in mouse liver [15].

Melatonin is a powerful endogenous antioxidant. In present study, we investigated the effects of melatonin on LPS-induced down-regulation of PXR and CYP3A in mouse liver. Results indicated that melatonin significantly attenuated LPS-induced down-regulation of PXR and CYP3A11 mRNA levels in a dose-dependent manner. Repeated doses of melatonin (10 mg/kg) treatments also significantly attenuated LPS-induced down-regulation of DEX-inducible CYP3A11 mRNA level and ERND activity in mouse liver.

Antioxidative effects of melatonin can occur by at least two mechanisms. In one case, melatonin itself exerts direct antioxidative effects via scavenging free radicals and

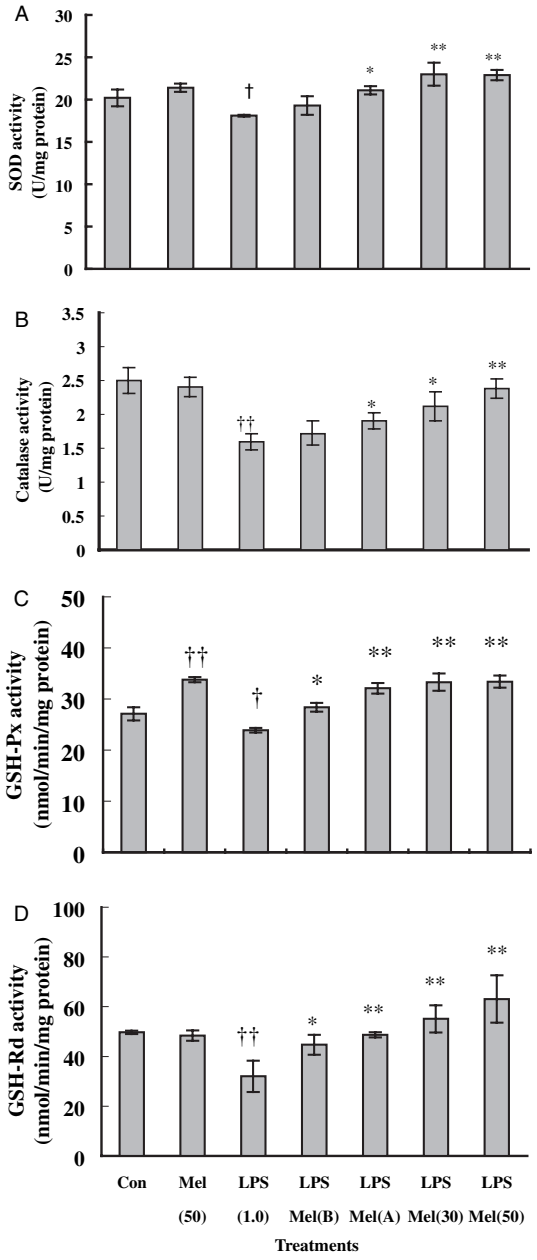

Fig. 5. The effects of melatonin on antioxidative enzyme activities in mouse liver. All groups except control received an intravenous (i.v.) injection of lipopolysaccharide (LPS) (1.0 mg/kg). Melatonin-treated groups received five injections of melatonin: one injection 30 min before LPS, one simultaneous injection with LPS, and three injections beginning 60 min after LPS. Livers were excised and homogenized at 6 hr after LPS treatment. Superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase activities were measured as described in Materials and methods. Data were expressed as mean ± S.E.M. (n = 8) †$P < 0.05$, ††$P < 0.01$ as compared with control group. *$P < 0.05$, **$P < 0.01$ as compared with LPS-treated group.

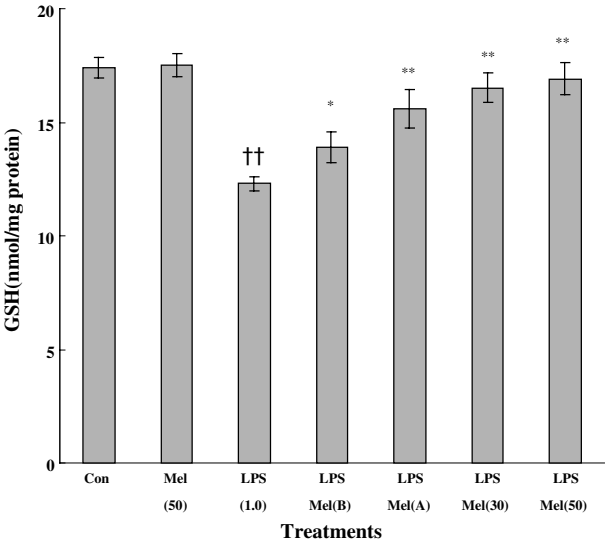

Fig. 6. The effects of melatonin on GSH content in mouse liver. All groups except control received an intravenous (i.v.) injection of lipopolysaccharide (LPS) (1.0 mg/kg). Melatonin-treated groups received five injections of melatonin: one injection 30 min before LPS, one simultaneous injection with LPS, and three injections beginning 60 min after LPS. Livers were excised and homogenized at 6 hr after LPS treatment. Glutathione content was measured as described in Materials and methods. Data were expressed as mean ± S.E.M. (n = 8). ††$P < 0.01$ as compared with control group. *$P < 0.05$, **$P < 0.01$ as compared with LPS-treated group.

inhibiting free radical generation. Additionally, melatonin alters the activities of antioxidative enzymes, which improve the endogenous antioxidative defense capacity of organisms.

The ability of melatonin to scavenge free radicals is undoubtedly an important property in its protection against oxidative stress. First, melatonin directly scavenges hydroxyl free radicals (·OH) to form cyclic 3-hydroxymelatonin (3-OHM) [33, 34]. Secondly, melatonin has been shown to scavenge peroxynitrite anion ($ONOO^-$), the highly destructive product of the interaction between the superoxide anion radical ($O_2^{\bullet -}$) and nitric oxide (NO) [35, 36]. Also, melatonin, as it donates an electron in the process of detoxifying electron-deficient ROS, itself becomes a low reactive melatonyl cation radical [37, 38].

The effects of melatonin on the activities of antioxidative enzymes have been extensively studied. According to Liu's report, a single dose of melatonin (5 mg/kg) enhanced SOD and GSH-Rd activities in rat liver [39]. Melatonin also attenuated the decrease in hepatic Cu,Zn-SOD and GSH-Rd activities without affecting hepatic CAT and GSH-Px activities in alpha-naphthylisothiocyanate-treated rats [40]. Recent study found that melatonin attenuated CCl_4-induced reductions in hepatic SOD, CAT and GSH-Rd activities in a dose-dependent manner without affecting hepatic GSH-Px activity in CCl_4-treated rats [41].

The SOD and CAT are two important antioxidative enzymes. SOD dismutases $O_2^{\bullet -}$ to H_2O_2, decreasing the amount of $O_2^{\bullet -}$ and the formation of $ONOO^-$. CAT then eliminates H_2O_2. In present study, we evaluated the effects of melatonin on hepatic SOD and CAT activities in LPS-treated mice. Results indicated that melatonin significantly increased hepatic SOD and CAT activities in LPS-treated mice in a dose-dependent manner.

On the contrary, GSH-Px and GSH-Rd are involved in GSH metabolism. GSH-Px transforms H_2O_2 to O_2. In this process GSH is oxidized, thus forming oxidized GSSG. The reduced GSH is replenished by the action of GSH-Rd. Present study showed that melatonin significantly increased hepatic GSH-Px and GSH-Rd activities in LPS-treated mice. This study also found that melatonin significantly increased GSH content in LPS-treated mice in a dose-dependent manner. These results indicated that melatonin attenuated LPS-induced down-regulation of PXR and CYP3A via increasing hepatic GSH-Px and GSH-Rd activities and influencing hepatic GSH metabolism in LPS-treated mice. These results are in agreement with earlier work [15], in which NAC, an antioxidant and a precursor for GSH synthesis, has been found to attenuate LPS-induced down-regulation of *PXR* and *CYP3A11* mRNA levels.

Recent studies showed that melatonin decreased the inducible nitric oxide synthase (iNOS) mRNA level and inhibited iNOS activity in LPS-treated rats [42, 43]. However, several studies found that LPS-induced down-regulation of *PXR* and *CYP3A* in mouse liver is independent of NO production [11, 44]. Thus, the effects of melatonin on iNOS in LPS-treated mice have not been evaluated in present study.

In summary, present study found that melatonin significantly attenuated LPS-induced down-regulation of PXR and CYP3A11 mRNA levels and DEX-inducible ERND activity in a dose-dependent manner. In addition, present study also found that melatonin significantly increased hepatic SOD, CAT, GSH-Px and GSH-Rd activities and GSH content in LPS-treated mice. These findings suggest that melatonin may exert its protective effects on LPS-induced down-regulation of PXR and CYP3A via counteracting LPS-induced oxidative stress in mouse liver.

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References

1. Thummel KE, Wilkinson GR. In vitro and in vivo drug interactions involving human CYP3A. Annu Rev Pharmacol Toxicol 1998; **38**:389–430.
2. Quattrochi LC, Guzelian PS. CYP3A regulation: from pharmacology to nuclear receptors. Drug Metab Dispos 2001; **29**:615–622.
3. Goodwin B, Redinbo MR, Kliewer SA. Regulation of cyp3a gene transcription by the pregnane x receptor. Annu Rev Pharmacol Toxicol 2002; **42**:1–23.
4. Kliewer SA, Moore JT, Wade L et al. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell 1998; **92**:73–82.
5. Lehmann JM, McKee DD, Watson MA et al. The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. J Clin Invest 1998; **102**:1016–1023.
6. Bertilsson G, Heidrich J, Svensson K et al. Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. Proc Natl Acad Sci USA 1998; **95**:12208–12213.
7. Moore LB, Parks DJ, Jones SA et al. Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. J Biol Chem 2000; **275**:15122–15127.
8. Xie W, Barwick JL, Simon CM et al. Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. Genes Dev 2000; **14**:3014–3023.
9. Morgan ET. Regulation of cytochrome p450 by inflammatory mediators: why and how? Drug Metab Dispos 2001; **29**:207–212.
10. Morgan ET. Regulation of cytochromes P450 during inflammation and infection. Drug Metab Rev 1997; **29**:1129–1188.
11. Sewer MB, Barclay TB, Morgan ET. Down-regulation of cytochrome P450 mRNAs and proteins in mice lacking a functional NOS2 gene. Mol Pharmacol 1998; **54**:273–279.
12. Li-Masters T, Morgan ET. Effects of bacterial lipopolysaccharide on phenobarbital-induced CYP2B expression in mice. Drug Metab Dispos 2001; **29**:252–257.
13. Beigneux AP, Moser AH, Shigenaga JK et al. Reduction in cytochrome P-450 enzyme expression is associated with repression of CAR (constitutive androstane receptor) and PXR (pregnane X receptor) in mouse liver during the acute phase response. Biochem Biophys Res Commun 2002; **293**:145–149.
14. Sachdeva K, Yan B, Chichester CO. Lipopolysaccharide and cecal ligation/puncture differentially affect the subcellular distribution of the pregnane X receptor but consistently cause suppression of its target genes CYP3A. Shock 2003; **19**:469–474.
15. Xu DX, Wei W, Sun MF et al. Kupffer cells and ROS partially mediate LPS-induced down-regulation of nuclear receptor Pregnane X receptor and its target gene CYP3A in mouse liver. Free Radic Biol Med 2004; **37**:10–22.
16. Matuszak Z, Reszka KJ, Chignell CF. Reaction of melatonin and related indoles with hydroxyl radicals: EPR and spin trapping investigations. Free Radic Biol Med 1997; **23**:367–372.
17. Stasica P, Ulanski P, Rosiak JM. Melatonin as a hydroxyl radical scavenger. J Pineal Res 1998; **25**:65–66.
18. Gilad E, Cuzzocrea S, Zingarelli B et al. Melatonin is a scavenger of peroxynitrite. Life Sci 1997; **60**:PL169–PL174.
19. Hara M, Yoshida M, Nishijima H et al. Melatonin, a pineal secretory product with antioxidative properties, protects against cisplatin-induced nephrotoxicity in rats. J Pineal Res 2001; **30**:129–138.
20. Shen YX, Xu SY, Wei W et al. The protective effects of melatonin from oxidative damage induced by amyloid beta-peptide 25–35 in middle-aged rats. J Pineal Res 2002; **32**:85–89.
21. Rodriguez C, May JC, Sainz RM et al. Regulation of antioxidant enzymes: a significant role for melatonin. J Pineal Res 2004; **36**:1–9.
22. Ueda A, Hamadeh HK, Webb HK et al. Diverse roles of the nuclear orphan receptor CAR in regulating hepatic genes in response to phenobarbital. Mol Pharmacol 2002; **61**:1–6.
23. Li J, Ning G, Duncan SA. Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha. Genes Dev 2000; **14**:464–474.

24. Haugen DA, Coon MJ. Properties of electrophoretically homogeneous phenobarbital-inducible and beta-naphthoflavone-inducible forms of liver microsomal cytochrome P-450. J Biol Chem 1976; **251**:7929–7939.
25. Lowry OH, Rosebrough NJ, Farr AL et al. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; **193**:265–275.
26. Werringloer J. Assay of fomaldehyde generated during microsomal oxidation reactions. Methods Enzymol 1978; **52**:297–302.
27. Geller BL, Winge DR. Subcellular distribution of superoxide dismutases in rat liver. Methods Enzymol 1984; **105**:105–114.
28. Beers RF, Sizer WI. A spectrophotometric method of measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 1952; **195**:133–140.
29. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967; **70**:158–169.
30. Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem 1980; **106**:207–212.
31. Sewer MB, Morgan ET. Nitric oxide-independent suppression of P450 2C11 expression by interleukin-1beta and endotoxin in primary rat hepatocytes. Biochem Pharmacol 1997; **54**:729–737.
32. Monshouwer M, McLellan RA, Delaporte E et al. Differential effect of pentoxifylline on lipopolysaccharide-induced downregulation of cytochrome P450. Biochem Pharmacol 1996; **52**:1195–1200.
33. Matuszak Z, Reszka K, Chignell CF. Reaction of melatonin and related indoles with hydroxyl radicals: EPR and spin trapping investigations. Free Radic Biol Med 1997; **23**:367–372.
34. Tan DX, Manchester LC, Reiter RJ et al. A novel melatonin metabolite, cyclic 3-hydroxymelatonin: a biomarker of in vivo hydroxyl radical generation. Biochem Biophys Res Commun 1998; **253**:614–620.
35. Zhang H, Squadrito GL, Uppu R et al. Reaction of peroxynitrite with melatonin: a mechanistic study. Chem Res Toxicol 1999; **12**:526–534.
36. El-Sokkary GH, Omar HM, Hassanein AF et al. Melatonin reduces oxidative damage and increases survival of mice infected with *Schistosoma mansoni*. Free Radic Biol Med 2002; **32**:319–332.
37. Blanchard B, Pompon D, Ducrocq C. Nitrosation of melatonin by nitric oxide and peroxynitrite. J Pineal Res 2000; **29**:184–192.
38. Poeggeler B, Reiter RJ, Hardeland R et al. Melatonin and structurally related endogenous indoles act as potent electron donors and radical scavengers in vitro. Redox Rep 1996; **2**:179–184.
39. Liu F, Ng TB. Effect of pineal indoles on activities of the antioxidative defense enzymes superoxide dismutase, catalase, and glutathione reductase, and levels of reduced and oxidized glutathione in rat tissues. Biochem Cell Biol 2000; **78**:447–453.
40. Ohta Y, Kongo M, Kishikawa T. Effect of melatonin on changes in hepatic antioxidant enzyme activities in rats treated with alpha-naphthylisothiocyanate. J Pineal Res 2001; **31**:370–377.
41. Ohta Y, Kongo-Nishimura M, Matsura T et al. Melatonin prevents disruption of hepatic reactive oxygen species metabolism in rats treated with carbon tetrachloride. J Pineal Res 2004; **36**:10–17.
42. Crespo E, Macias M, Pozo D et al. Melatonin inhibits expression of the inducible NO synthase II in liver and lung and prevents endotoxemia in lipopolysaccharide-induced multiple organ dysfunction syndrome in rats. FASEB J 1999; **13**:1537–1546.
43. Escames G, Leon J, Macias M et al. Melatonin counteracts lipopolysaccharide-induced expression and activity of mitochondrial nitric oxide synthase in rats. FASEB J 2003; **17**:932–944.
44. Sewer MB, Morgan ET. Down-regulation of the expression of three major rat liver cytochrome P450s by endotoxin in vivo occurs independently of nitric oxide production. J Pharmacol Exp Ther 1998; **287**:352–358.