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Melatonin modulates TLR4-mediated inflammatory genes through MyD88- and TRIF-dependent signaling pathways in lipopolysaccharide-stimulated RAW264.7 cells

Abstract: Increasing evidence demonstrates that melatonin has an anti-inflammatory effect. Nevertheless, the molecular mechanisms remain obscure. In this study, we investigated the effect of melatonin on toll-like receptor 4 (TLR4)-mediated molecule myeloid differentiation factor 88 (MyD88)-dependent and TRIF-dependent signaling pathways in lipopolysaccharide (LPS)-stimulated macrophages. RAW264.7 cells were incubated with LPS (2.0 μg/mL) in the absence or presence of melatonin (10, 100, 1000 μM). As expected, melatonin inhibited TLR4-mediated tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, IL-8, and IL-10 in LPS-stimulated macrophages. In addition, melatonin significantly attenuated LPS-induced upregulation of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) in macrophages. Further analysis showed that melatonin inhibited the expression of MyD88 in LPS-stimulated macrophages. Although it had no effect on TLR4-mediated phosphorylation of c-Jun N-terminal kinase (JNK), p38, and extracellular regulated protein kinase (ERK), melatonin significantly attenuated the activation of nuclear factor kappa B (NF-κB) in LPS-stimulated macrophages. In addition, melatonin inhibited TLR4-mediated Akt phosphorylation in LPS-stimulated macrophages. Moreover, melatonin significantly attenuated the elevation of interferon (IFN)-regulated factor-3 (IRF3), which was involved in TLR4-mediated TRIF-dependent signaling pathway, in LPS-stimulated macrophages. Correspondingly, melatonin significantly alleviated LPS-induced IFN-β in macrophages. In conclusion, melatonin modulates TLR4-mediated inflammatory genes through MyD88-dependent and TRIF-dependent signaling pathways.

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Key words: Akt, inflammation, lipopolysaccharide, macrophage, melatonin, molecule myeloid differentiation factor 88, nuclear factor kappa B, toll-like receptor

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Introduction

Melatonin is the major product of the pineal gland, with maximal secretion at night [1]. As potent antioxidants, melatonin, and its metabolites can directly scavenge hydroxyl free radicals ($^{\cdot}OH$) [2–4] and peroxynitrite anion ($ONOO^{-}$) [5]. Several reports have demonstrated that melatonin exhibits its indirect antioxidant role through stimulating superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities [6–10]. Importantly, exogenous melatonin upregulates the expression of antioxidant enzymes in different tissues [11–14]. Also, melatonin has anti-inflammatory effects [15, 16]. According to a recent study, melatonin suppressed the production of nitric oxide (NO) and interleukin-6 (IL-6) at both gene transcription and translation levels in lipopolysaccharide (LPS)-activated macrophages [17]. An in vivo report from our laboratory showed that melatonin significantly inhibited LPS-induced tumor necrosis factor alpha (TNF-α), IL-1β, and IL-6 in maternal serum, amniotic fluid, fetal liver, and fetal brain [18]. A recent study found that oral supplementation with melatonin markedly attenuated the elevation of TNF-α caused by strenuous exercise in adult human males [19]. Indeed, melatonin could prevent from circulatory failure in rats, mice, and humans with endotoxemia by repressing the production of inflammatory cytokines [20–22]. Moreover, melatonin alleviated heatstroke-induced systemic inflammation and protected rats from multiple organ dysfunction syndromes [23]. Nevertheless, the molecular mechanism for melatonin-mediated anti-inflammatory role remains obscure.

In mammals, the rapid response to pathogens is called innate immunity. Increasing evidence demonstrates that toll-like receptor 4 (TLR4) plays a critical role in innate immune and inflammatory responses [24]. The TLR4 recognizes its ligand LPS, resulting in recruitment of the adapter molecule myeloid differentiation factor 88 (MyD88), which in turn leads to the activation of the

Table 1. Primers for real-time RT-PCR

Name	Sequences	Sizes (bp)
GAPDH	Forward: 5′- ACCCCAGCAAGGACACTGAGCAAG -3′ Reverse: 5′- GGCCCCTCCTGTTATTATGGGGGT -3′	109
TNF-α	Forward: 5′- CCCTCCTGGCCAACGGCATG -3′ Reverse: 5′- TCGGGGCAGCCTTGTCCCTT -3	109
IL-1β	Forward: 5′- GCCTCGTGCTGTCGGACCCATAT -3′ Reverse: 5′- TCCTTTGAGGCCCAAGGCCACA -3′	143
IL-6	Forward: 5′- AGACAAAGCCAGAGTCCTTCAGAGA -3′ Reverse: 5′- GCCACTCCTTCTGTGACTCCAGC -3′	146
IL-8	Forward: 5′- TTGCCTTGACCCTGAAGCCCCC -3′ Reverse: 5′- GGCACATCAGGTACGATCCAGGC -3′	175
IL-17	Forward: 5′- AACCGTTCCACGTCACCCTGGA -3′ Reverse: 5′- TGGTCCAGCTTTCCCTCCGCA -3′	122
IFN-β	Forward: 5′- GGATCCTCCACGCTGCGTTCC -3′ Reverse: 5′- CCGCCCTGTAGGTGAGGTTGA -3′	153
IFN-γ	Forward: 5′- GCTGTTACTGCCACGGCACAGT -3′ Reverse: 5′- CACCATCCTTTTGCCAGTTCCTCC -3′	129
IL-4	Forward: 5′- GCCATATCCACGGATGCGACA -3′ Reverse: 5′- TCCATCTCCGTGCATGGCGTC -3′	94
IL-10	Forward: 5′- GGCTGGACGAGAGCCGAACG -3′ Reverse: 5′- CCCGGGGTGTAGGCACCACT -3′	124
iNOS	Forward: 5′- GCTCGCTTTGCCACGGACGA -3′ Reverse: 5′- AAGGCAGCGGGCACATGCAA -3′	146
COX-2	Forward: 5′- GGGCTCAGCCAGGCAGCAAAT -3′ Reverse: 5′- GCACTGTGTTTGGGGTGGGCT -3′	187
TLR4	Forward: 5′- TCAGCAAAGTCCCTGATGACATTCC -3′ Reverse: 5′- AGAGGTGGTGTAAGCCATGCCA -3′	180
IRF3	Forward: 5′- ACGGCAGGACGCACAGATGG -3′ Reverse: 5′- TCCAGGTTGACACGTCCGGC -3′	104
IRF7	Forward: 5′- GCTCCAAACCCCAAGCCCTCTG -3′ Reverse: 5′- GACAGCTTCCACCTGCCATGCT -3′	181

iNOS, inducible nitric oxide synthase.

nuclear factor kappa B (NF-κB) and the mitogen-activated protein kinase (MAPK) transduction cascades in macrophages [25]. Under unstimulated conditions, NF-κB is usually retained in the cytoplasm by binding to the inhibitor of kappa B (I-κB). I-κB phosphorylation leads to release and translocation of NF-κB to the nucleus [26]. The MAPK family mainly includes extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) [27]. The NF-κB and MAPK signal transduction pathways are well known for their prominent roles in switching on genes involved in TLR4-mediated inflammatory responses [28]. Recently, numerous studies have demonstrated that TLR4-mediated signaling also leads to rapid activation of the phosphatidylinositol 3-kinase (PI3K) and phosphorylation of Akt in macrophages [29]. Importantly, PI3K/Akt signaling is involved in TLR4-mediated cytokine expression in macrophages [30].

The aim of the present study was to investigate the effect of melatonin on TLR4-mediated inflammatory genes in LPS-stimulated macrophages. In addition, we were to explore whether melatonin modulates MyD88-dependent and MyD88-independent signaling pathways in LPS-stimulated macrophages. Our results found that melatonin inhibited TLR4-mediated inflammatory genes in LPS-stimulated macrophages. We demonstrate for the first time that melatonin modulates TLR4-mediated inflammatory genes through MyD88-dependent and TRIF-dependent signaling pathways in macrophages.

Materials and methods

Chemicals and reagents

Lipopolysaccharide (*Escherichia coli* LPS, serotype 0127:B8) and melatonin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Antibodies against phosphor-p38 (pp38), phosphor-ERK (pERK), phosphor-JNK (pJNK), NF-κB p65, NF-κB p50, inhibitor of κB (I-κB), cyclooxygenase (COX)-2, iNOS and Lamin A/C were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Phosphor-Akt (pAkt), Akt, and MyD88 antibodies were from Cell Signaling Technology (Beverley, MA, USA). β-Actin antibody was from Boster Bio-Technology Co. LTD (Wuhan, China). Chemiluminescence (ECL) detection kit was from Pierce Biotechnology (Rockford, IL, USA). TRI reagent was from Molecular Research Center, Inc. (Cincinnati, OH, 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.

Cell culture and treatments

RAW264.7 cells, a rodent macrophage cell line, were grown in Nunc flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/mL of penicillin, 100 μg/mL streptomycin, 10 mm HEPES, 2 mM L-glutamine,

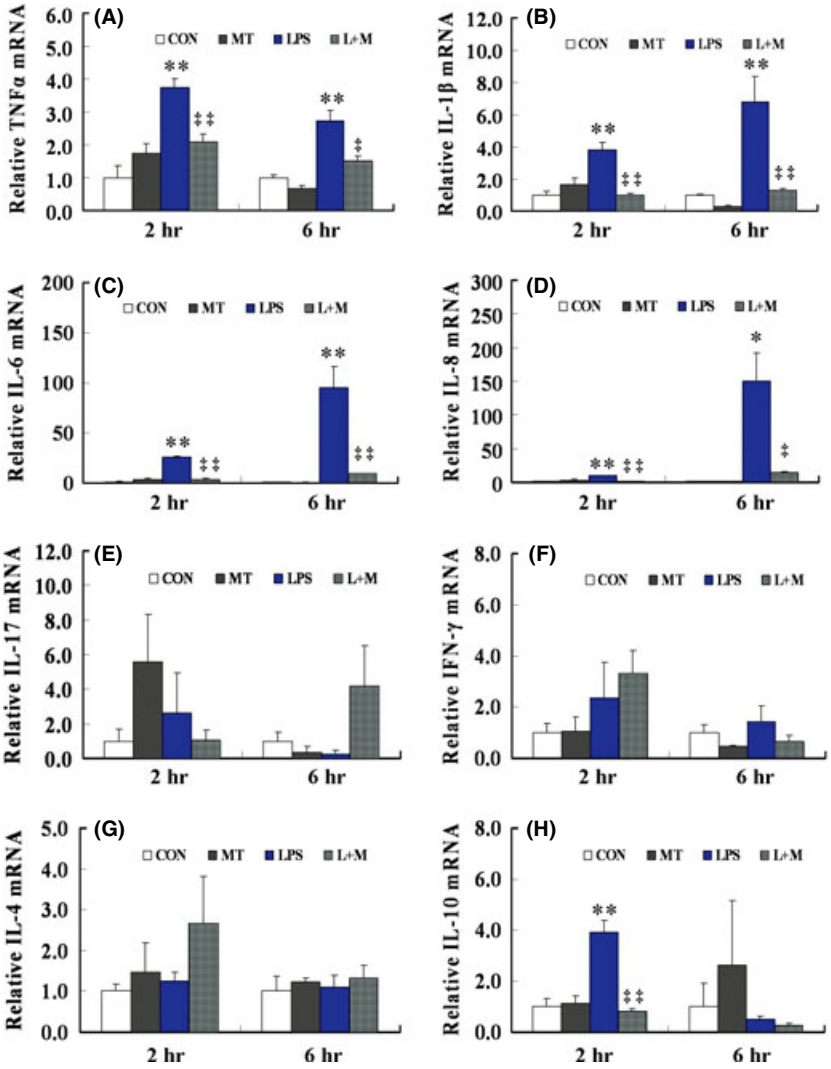

Fig. 1. The effects of melatonin on LPS-induced inflammatory cytokines. RAW264.7 cells were incubated with LPS (2.0 μg/mL) in the absence or presence of melatonin (1.0 mM). The expression of inflammatory cytokines in RAW264.7 cells was measured using real-time RT-PCR at 2 and 6 hr after LPS treatment. (A) TNF-α; (B) IL-1β; (C) IL-6; (D) IL-8; (E) IL-17; (F) IFN-γ; (G) IL-4; (H) IL-10. Data were expressed as means ± S.E.M. of six samples. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with control group. †$P < 0.05$, ††$P < 0.01$ as compared with LPS group.

0.2% $NaHCO_3$, and 10% [v/v] heat-inactivated FBS in a humidified chamber with 5% CO_2/95% air at 37°C. The cells were seeded into 6-well culture plates at a density of 5×10^5 cells/well and incubated for at least 12 hr to allow them to adhere to the plates. After washing three times with medium, the cells were incubated with LPS (2.0 μg/mL) for different times in the absence or presence of different concentration of melatonin (10, 100 or 1000 μM, dissolved in 0.25% ethanol: saline). The cells were washed with chilled PBS for three times and then harvested for real-time RT-PCR and immunoblots.

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 (Promega Corporation). Real-time RT-PCR was performed with a LightCycler® 480 SYBR Green I kit (Roche Diagnostics GmbH, Mannheim, Germany) using gene-specific primers as listed in Table 1. The amplification reactions were carried out on a LightCycler® 480 Instrument (Roche Diagnostics GmbH) with an initial hold step (95°C for 5 min) and 50 cycles of a three-step PCR (95°C for 15 s, 60°C for 15 s, 72°C for 30 s). The comparative C_T-method was used to determine the amount of target, normalized to an endogenous reference (GAPDH) and relative to a calibrator ($2^{-\Delta\Delta C_t}$) using the Lightcycler 480 software (Roche, version 1.5.0). All RT-PCR experiments were performed in triplicate.

Immunoblots

To prepare cell lysates, cells were washed three times with ice-cold PBS and lysed by incubating for 30 min on ice with

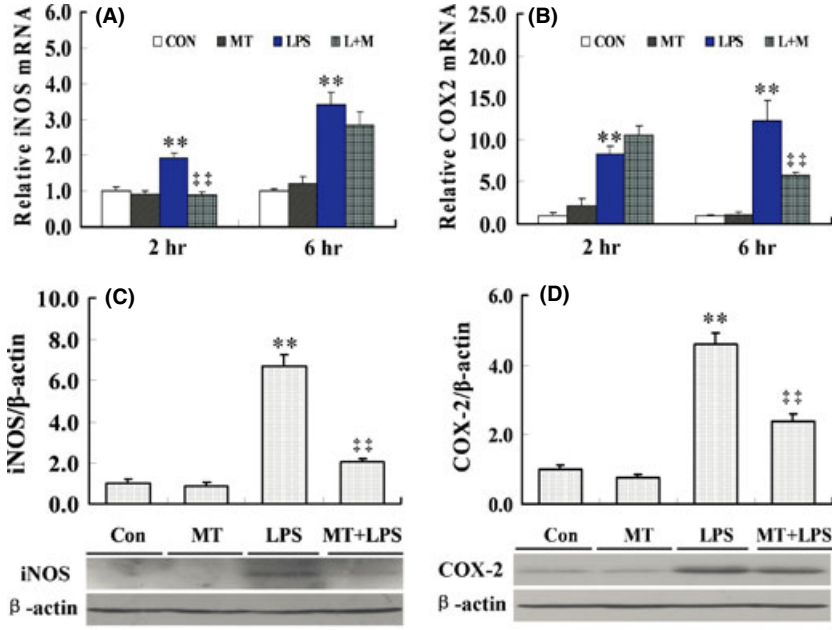

Fig. 2. The effects of melatonin on LPS-induced iNOS and COX-2. RAW264.7 cells were incubated with LPS (2.0 μg/mL) in the absence or presence of melatonin (1.0 mM). (A and B) The levels of iNOS and COX-2 mRNA were measured using real-time RT-PCR at 2 and 6 hr after LPS treatment. (A) iNOS; (B) COX-2. Data were expressed as means ± S.E.M. of six samples. (C and D) The levels of iNOS and COX-2 protein in RAW264.7 cells were measured using immunoblots at 6 hr after LPS treatment. (C) A representative gel for iNOS (upper panel) and β-actin (lower panel) was shown. (D) A representative gel for COX-2 (upper panel) and β-actin (lower panel) was shown. All experiments were repeated for four times. Quantitative analyses of scanning densitometry on four different samples were performed. $^{**}P < 0.01$ as compared with control group. $^{\dagger\dagger}P < 0.01$ as compared with LPS group.

200 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 dodecylsulphate, 1 mM phenylmethylsulfonyl fluoride) supplemented with a cocktail of protease inhibitors (Roche, Indianapolis, IN, USA). The cell lysates were centrifuged at 10,000 g for 10 min to remove insoluble material. For nuclear protein extraction, the cells were washed with ice-cold PBS/phosphatase inhibitors, collected with a cell scraper, and harvested by centrifugation. The cell pellet was then resuspended in hypotonic buffer and then kept on ice for 15 min. The suspension was then mixed with detergent and centrifuged for 30 s at 14,000 g. The nuclear pellet obtained was resuspended in complete lysis buffer in the presence of the protease inhibitor cocktail, incubated for 30 min on ice, and centrifuged for 10 min at 14,000 g. Protein concentrations were determined with the bicinchoninic acid (BCA) protein assay reagents (Pierce) according to the manufacturer's instructions. For immunoblots, same amount of protein (40–80 μg) was separated electrophoretically by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The protein was transferred to a polyvinylidene fluoride membrane by electroblotting. The membranes were incubated for 2 hr with the following antibodies (1:1000 dilution): pAkt, pp38, pERK, pJNK, I-κB, NF-κB p65, NF-κB p50, COX-2, and iNOS. For total protein, β-actin was used as a loading control. For nuclear protein, lamin A/C was used as a loading control. After washes in DPBS containing 0.05% Tween-20 four times for 10 min each, the membranes were incubated with goat anti-rabbit IgG 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.

Statistical analysis

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 between the treated cells and the control. Differences were considered to be significant only for $P < 0.05$.

Results

First, we analyzed the effects of melatonin on LPS-induced proinflammatory cytokines in RAW264.7 cells. In response to LPS, the expression of several proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, were significantly increased at 2 hr after LPS treatment. The expression of these proinflammatory cytokines remained elevated at 6 hr after LPS treatment (Fig. 1A–D), whereas LPS had little effect on the expression of IL-17 and IFN-γ in RAW264.7 cells (Fig. 1E,F). As shown in Fig. 1A–D, melatonin significantly attenuated LPS-evoked upregulation of proinflammatory cytokines. The effects of melatonin on LPS-evoked expression of anti-inflammatory cytokines are presented in Fig. 1G,H. As expected, the level of IL-10 mRNA was obviously increased at 2 hr after LPS treatment (Fig. 1H), whereas LPS had little effect on the expression of

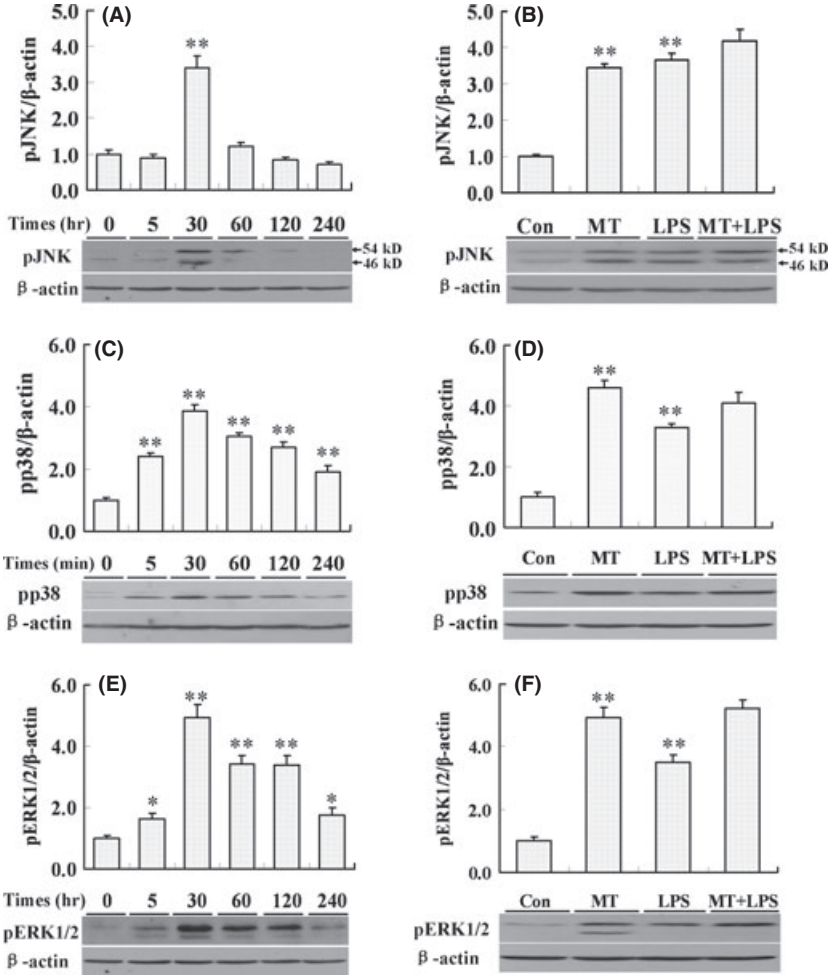

Fig. 3. The effects of melatonin on LPS-induced MAPK signaling pathway. (A, C, and E) RAW264.7 cells were incubated with LPS (2.0 μg/mL) for different times. (B, D, and F) Cells were incubated with LPS (2.0 μg/mL) for 30 min in the absence or presence of melatonin (1.0 mM). (A and B) pJNK was measured using immunoblots. A representative gel for pJNK (upper panel) and β-actin (lower panel) was shown. (C and D) pp38 was measured using immunoblots. A representative gel for pp38 (upper panel) and β-actin (lower panel) was shown. (E and F) pERK was measured using immunoblots. A representative gel for pERK (upper panel) and β-actin (lower panel) was shown. All experiments were repeated for four times. Quantitative analyses of scanning densitometry on four different samples were performed. Data were expressed as means ± S.E.M. of four samples. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with control group.

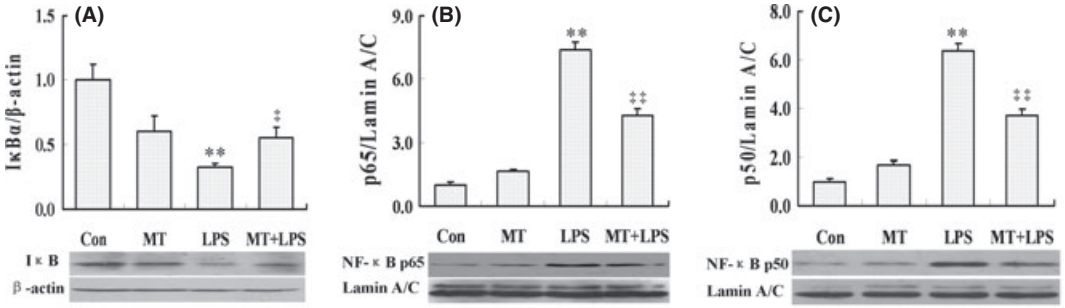

Fig. 4. The effects of melatonin on LPS-induced NF-κB activation. RAW264.7 cells were incubated with LPS (2.0 μg/mL) for (A) 30 min and (B) 1 hr in the absence or presence of melatonin (1.0 mM). (A) IκB was measured using immunoblots. A representative gel for IκB (upper panel) and β-actin (lower panel) was shown. (B) Nuclear NF-κB p65 was measured using immunoblots. A representative gel for p65 (upper panel) and lamin A/C (lower panel) was shown. (C) RAW264.7 cells were incubated with LPS (2.0 μg/mL) for 6 hr in the absence or presence of melatonin (1.0 mM). Nuclear NF-κB p50 was measured using immunoblots. A representative gel for p50 (upper panel) and lamin A/C (lower panel) was shown. All experiments were repeated for four times. Quantitative analyses of scanning densitometry on four different samples were performed. Data were expressed as means ± S.E.M. of four samples. $^{**}P < 0.01$ as compared with control group. † $P < 0.05$, †† $P < 0.01$ as compared with LPS group.

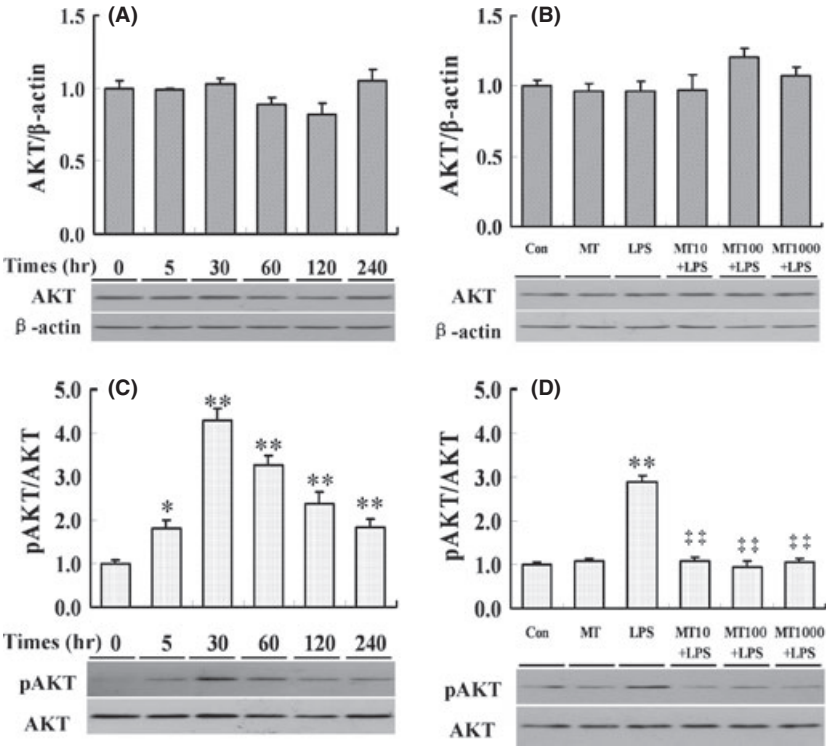

Fig. 5. The effects of melatonin on LPS-induced AKT phosphorylation. (A and C) RAW264.7 cells were incubated with LPS (2.0 μg/mL) for different times. (B and D) RAW264.7 cells were incubated with LPS (2.0 μg/mL) for 30 min in the absence or presence of different concentration of melatonin (10, 100 or 1000 μM). (A and B) AKT was measured using immunoblots. A representative gel for AKT (upper panel) and β-actin (lower panel) was shown. (C and D) pAkt was measured using immunoblots. A representative gel for pAkt (upper panel) and AKT (lower panel) was shown. All experiments were repeated for four times. Quantitative analyses of scanning densitometry on four different samples were performed. Data were expressed as means ± S.E.M. of four samples. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with control group. $\dagger\dagger P < 0.01$ as compared with LPS group.

IL-4 in RAW264.7 cells (Fig. 1G). Melatonin significantly attenuated LPS-evoked upregulation of IL-10 in RAW264.7 cells (Fig. 1H).

The effects of melatonin on LPS-induced expression of iNOS were then investigated. As expected, the level of iNOS mRNA was significantly increased at 2 hr after LPS treatment. The expression of iNOS mRNA remained elevated at 6 hr after LPS treatment (Fig. 2A). Interestingly, melatonin significantly attenuated LPS-evoked upregulation of iNOS mRNA (Fig. 2A). Correspondingly, melatonin significantly attenuated LPS-induced elevation of iNOS protein in RAW264.7 cells (Fig. 2C). Next, we analyzed the effects of melatonin on LPS-induced expression of COX-2 in RAW264.7 cells. As shown in Fig. 2B, the level of COX-2 mRNA was slightly increased at 2 hr after LPS treatment. Further analysis showed that the expression of COX-2 mRNA was significantly upregulated at 6 hr after LPS treatment. Interestingly, melatonin briefly attenuated LPS-evoked upregulation of COX-2 mRNA (Fig. 2B). Interestingly, melatonin almost completely inhibited LPS-induced elevation of COX-2 protein in RAW264.7 cells (Fig. 2D).

To investigate the effects of melatonin on LPS-induced MAPK signaling, the levels of pJNK, pp38, and pERK were measured in LPS-stimulated RAW264.7 cells. As expected, the levels of pJNK, pp38, and pERK were significantly increased in LPS-treated RAW264.7 cells in a time-dependent manner (Fig. 3A,C,E). Unexpectedly, melatonin had no effect on LPS-induced JNK phosphorylation (Fig. 3B). In addition, melatonin did not inhibit LPS-induced p38 and ERK phosphorylation in RAW264.7 cells (Fig. 3D,F). Surprisingly, melatonin alone induced the phosphorylation of JNK, p38, and ERK in RAW264.7 cells (Fig. 3B,D,F).

The effects of melatonin on LPS-induced NF-κB activation are presented in Fig. 4. As expected, the level of I-κB was significantly decreased in LPS-stimulated macrophages (Fig. 4A). By contrast, the levels of nuclear NF-κB p65 and p50 were significantly increased in LPS-stimulated macrophages (Fig. 4B,C). Melatonin significantly attenuated LPS-induced I-κB degradation in rodent macrophages (Fig. 4A). In addition, melatonin significantly inhibited LPS-induced nuclear translocation of NF-κB p65 and p50 (Fig. 4B,C).

The effects of melatonin on LPS-induced AKT phosphorylation were analyzed. As shown in Fig. 5A,B, neither LPS nor melatonin regulated the expression of AKT in RAW264.7 cells. LPS significantly increased the level of pAkt in a time-dependent manner (Fig. 5C). Interestingly, melatonin almost completely inhibited LPS-induced elevation of pAkt in RAW264.7 cells (Fig. 5D).

The effects of melatonin on the expression of TLR4 and MyD88 were then analyzed RAW264.7 cells. As shown in Fig. 6A, LPS alone had no effect on the

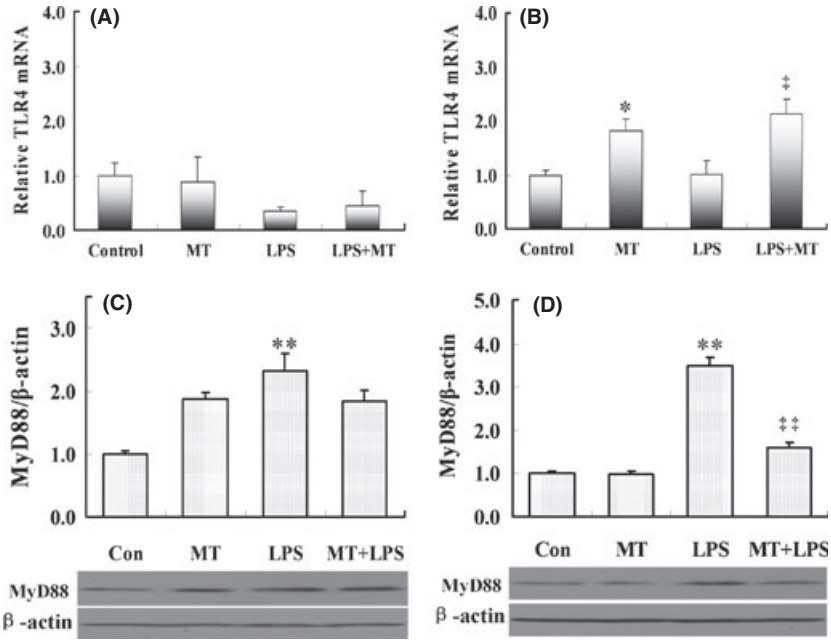

Fig. 6. The effects of melatonin on the expression of TLR4 and MyD88 in RAW264.7 cells. RAW264.7 cells were incubated with LPS (2.0 μg/mL) in the absence or presence of melatonin (1.0 mM). (A and B) The expression of TLR4 mRNA was measured using real-time RT-PCR. (A) TLR4 mRNA at 2 hr after LPS treatment. (B) TLR4 mRNA at 6 hr after LPS treatment. Data were expressed as means ± S.E.M. of six samples. (C and D) The level of MyD88 protein was measured using immunoblots. (C) MyD88 at 1 hr after LPS treatment. (D) MyD88 at 6 hr after LPS treatment. A representative gel for MyD88 (upper panel) and β-actin (lower panel) was shown. All experiments were repeated for four times. Quantitative analyses of scanning densitometry on four different samples were performed. Data were expressed as means ± S.E.M. of four samples. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with control group. $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ as compared with LPS group.

expression of TLR4 mRNA. Interestingly, melatonin slightly upregulated the expression of TLR4 mRNA at 6 hr after LPS treatment (Fig. 6B). The effects of melatonin on the level of MyD88 protein were analyzed. As shown in Fig. 6C, melatonin had little effect on LPS-induced elevation of MyD88 protein at 2 hr after LPS treatment. Interestingly, melatonin significantly alleviated LPS-induced elevation of MyD88 protein at 6 hr after LPS treatment (Fig. 6D).

Finally, the effects of melatonin on the expression of IFN-regulated factor-3 (IRF3), IRF7, and IFN-β were analyzed in LPS-stimulated RAW264.7 cells. As shown in Fig. 7A, LPS significantly upregulated the expression of IRF7 mRNA, whereas the level of IRF3 mRNA was not significantly increased at 6 hr after LPS treatment (Fig. 7B). Interestingly, melatonin significantly attenuated LPS-induced elevation of IRF7 mRNA in macrophages (Fig. 7A). The effects of melatonin on LPS-induced expression of IFN-β mRNA are presented in Fig. 7C. As expected, the level of IFN-β mRNA was significantly increased in LPS-stimulated macrophages. Melatonin significantly attenuated LPS-induced expression of IFN-β mRNA.

Discussion

In the present study, we investigated the effects of melatonin on TLR4-mediated inflammation in macrophages. As expected, melatonin significantly inhibited the expression of IL-6 mRNA in LPS-stimulated macrophages. In addition, melatonin significantly alleviated LPS-evoked upregulation of COX-2 and iNOS mRNA in macrophages. These results are in agreement with those from several others [17, 31]. Several in vivo studies demonstrated that melatonin significantly attenuated LPS-induced elevation of TNF-α in serum [20, 32]. In addition, maternally administered melatonin significantly alleviated LPS-induced TNF-α production in maternal serum, amniotic fluid, fetal liver, and fetal brain [18]. The present study showed that melatonin significantly alleviated LPS-induced expression of TNF-α mRNA in macrophages. In addition, the present study found for the first time that melatonin almost completely inhibited TLR4-mediated elevation of IL-1β and IL-8 mRNA in LPS-stimulated macrophages. These results suggest that melatonin alleviates TLR4-mediated expression of inflammatory genes in LPS-stimulated macrophages.

The MAPK signaling is one of the most important signaling cascades that regulate TLR4-mediated inflammatory genes [33]. The MAPK family mainly includes ERK, p38, and JNK [27]. The effects of melatonin on MAPK signaling remain controversial. An in vivo study showed that melatonin suppressed the increase in ERK and JNK phosphorylation in ischemia/reperfusion-treated rat liver [34]. According to an in vitro report, melatonin increased the phosphorylation of ERK independently from the presence of UVB stress and decreased the UVB-mediated activation of p38 and JNK in U937 cells [35]. A recent study demonstrated that melatonin significantly inhibited

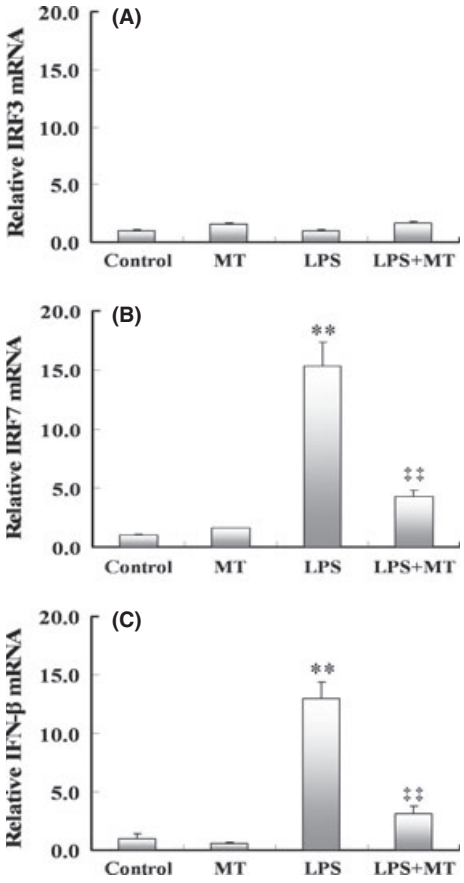

Fig. 7. The effects of melatonin on the expression of IRF3, IRF7, and IFN-β in RAW264.7 cells. RAW264.7 cells were incubated with LPS (2.0 μg/mL) in the absence or presence of melatonin (1.0 mM). The expression of IRF3, IRF7, and IFN-β mRNA was measured using real-time RT-PCR at 6 hr after LPS treatment. (A) IRF3 mRNA. (B) IRF7 mRNA. (C) IFN-β mRNA. Data were expressed as means ± S.E.M. of six samples. $^{**}P < 0.01$ as compared with control group. $^{\dagger\dagger}P < 0.01$ as compared with LPS group.

phosphorylation of ERK but not p38 or JNK in a human osteoblastic cell line 1.19 [36]. Another study found that melatonin significantly increased the level of phosphorylated p38, ERK, and JNK in HepG2 cells [37]. In the present study, we investigated the effects of melatonin on TLR4-mediated MAPK signaling in LPS-stimulated macrophages. As expected, LPS quickly activated MAPK signaling, as determined by the elevation of ERK, p38, and JNK phosphorylation. Interestingly, melatonin had no effect on the phosphorylation of ERK, p38, and JNK in LPS-stimulated macrophages. Unexpectedly, melatonin alone increased the level of ERK, p38, and JNK phosphorylation in macrophages. These results suggest that melatonin inhibits TLR4-mediated expression of inflammatory genes independent of MAPK signaling pathway.

NF-κB plays a central role in TLR4-mediated inflammatory genes in macrophages. Under unstimulated conditions, NF-κB is usually retained in the cytoplasm by binding to the I-κB. I-κB degradation leads to release and translocation of NF-κB p65 and p50 subunits to the nucleus [26]. According to an earlier report, reactive oxygen species is involved in TLR4-mediated NF-κB activation in LPS-stimulated neutrophils [38]. Indeed, melatonin is a potent antioxidant. An earlier study showed that melatonin significantly inhibited TLR3-mediated nuclear translocation of NF-κB p65 subunit in macrophages [39]. The present study showed that melatonin significantly attenuated TLR4-mediated I-κB degradation in LPS-stimulated macrophages. In addition, melatonin significantly inhibited TLR4-mediated nuclear translocation of NF-κB p65 and p50 subunits. These results are in agreement with those from a recent report [17], in which melatonin inhibits nuclear translocation of NF-κB p50 subunit and NF-κB DNA-binding activity in *Prevotella intermedia* LPS-activated macrophages. Thus, we guess that the inhibitive effect of melatonin on TLR4-mediated inflammatory genes might be, at least partially, attributed to the repression of NF-κB activation in macrophages.

Numerous reports have demonstrated that PI3K mediates LPS-induced activation of NF-κB and regulates TLR4-mediated inflammatory genes in LPS-stimulated macrophages [30, 40, 41]. Indeed, Akt is a major downstream target of PI3K and PI3K activation is necessary and sufficient for Akt phosphorylation [42]. Several reports demonstrate that melatonin persistently activates neuronal PI3K/Akt signaling pathway [43–45]. Moreover, melatonin induces tyrosine phosphorylation of insulin growth factor receptor and insulin receptor and activates the PI3K/Akt signaling pathway in isolated rat pancreatic islets [46]. By contrast, melatonin inhibits reactive oxygen species-mediated Akt phosphorylation in H4IIE hepatoma cells [47]. In addition, melatonin suppresses acrolein-induced Akt activation in human pulmonary fibroblasts [48]. In the present study, we investigated the effects of melatonin on TLR4-mediated Akt activation in LPS-stimulated macrophages. Our results found for the first time that melatonin almost completely inhibited TLR4-mediated Akt phosphorylation in LPS-stimulated macrophages. Thus, our results do not exclude that melatonin inhibits TLR4-mediated NF-κB activation and regulates TLR4-mediated inflammatory genes through repressing Akt phosphorylation.

TLR4 modulates the expression of inflammatory genes through at least two branches of downstream signaling pathways, MyD88-dependent and TRIF-dependent signaling pathways [49]. MyD88 is the key signaling adaptor that is involved in TLR4-mediated expression of inflammatory genes in LPS-stimulated macrophages [50]. The present study showed that the level of MyD88 protein was significantly increased in LPS-stimulated macrophages. These results are in agreement with those from others, in which the expression of MyD88 was significantly increased in response to LPS stimulus [51]. An in vivo study demonstrated that melatonin significantly suppressed the elevation of MyD88 in livers of ischemia/reperfusion-treated rats [34]. The present study investigated the effects of melatonin on the expression of MyD88 in LPS-stimulated macrophages. Our results found for the first time that melatonin inhibited the elevation of MyD88 protein in LPS-stimulated macrophages. TRIF, another adapter molecule of TLR4, is mainly responsible for the regulation of TRIF-dependent signaling pathway in macrophages [52].

TLR4-mediated TRIF-dependent signaling, which induces IFN-α/β and IFN-inducible genes, is mediated by IRF3 and IFR7 [53]. The present study showed that the level of IRF7 mRNA was markedly increased in LPS-stimulated macrophages. Interestingly, melatonin inhibited the expression of IRF7 mRNA in LPS-stimulated macrophages. In addition, melatonin significantly attenuated TLR4-mediated expression of IFN-β mRNA in LPS-stimulated microphages. Taken together, these results suggest that melatonin modulates TLR4-mediated inflammatory genes through MyD88-dependent and TRIF-dependent signaling pathways.

In summary, the present study indicates that melatonin inhibits TLR4-mediated inflammatory genes in macrophages. In addition, melatonin alleviates TLR4-mediated NF-κB and Akt activation in macrophages. Importantly, melatonin inhibits not only MyD88, the key signaling adaptor for MyD88-dependent signaling pathway, but also IRF7, which is involved in TRIF-dependent signaling pathway, in LPS-stimulated macrophages. The present study suggests that melatonin modulates TLR4-mediated inflammatory genes through not only MyD88-dependent but also TRIF-dependent signaling pathway. Thus, melatonin may have potential preventive and therapeutic utilities for protecting against TLR4-mediated inflammation.

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References

1. Stehle JH, Saade A, Rawashdeh O et al. A survey of molecular details in the human pineal gland in the light of phylogeny, structure, function and chronobiological diseases. J Pineal Res 2011; **51**:17–43.
2. Tan DX, Chen LD, Poeggeler B, Manchester LC, Reiter RJ. Melatonin: a potent, endogenous hydroxyl radical scavenger. Endocrine J 1993; **1**:57–60.
3. Tan DX, Manchester LC, Hardeland R, Reiter RJ. Melatonin: a hormone, a tissue factor, an autocoid, a paracoid, and an antioxidant vitamin. J Pineal Res 2003; **34**:75–78.
4. Tan DX, Manchester LC, Terron MP, Flores LJ, Reiter RJ. One molecule, many derivatives: a never-ending interaction of melatonin with reactive oxygen and nitrogen species? J Pineal Res 2007; **42**:28–42.
5. Allegra M, Reiter RJ, Tan DX, Gentile C, Tesoriere L, Livrea MA. The chemistry of melatonin's interaction with reactive species. J Pineal Res 2003; **34**:1–10.
6. Rodriguez C, Mayo JC, Sainz RM et al. Regulation of antioxidant enzymes: a significant role for melatonin. J Pineal Res 2004; **36**:1–9.
7. Tomas-Zapico C, Coto-Montes A. A proposed mechanism to explain the stimulatory effect of melatonin on antioxidative enzymes. J Pineal Res 2005; **39**:99–104.
8. Li Z, Nickkholgh A, Yi X et al. Melatonin protects kidney grafts from ischemia/reperfusion injury through inhibition of NF-kB and apoptosis after experimental kidney transplantation. J Pineal Res 2009; **46**:365–372.
9. Crespo I, Miguel BS, Laliena A et al. Melatonin prevents the decreased activity of antioxidant enzymes and activates nuclear erythroid 2-related factor 2 signaling in an animal model of fulminant hepatic failure of viral origin. J Pineal Res 2010; **49**:193–200.
10. Liu Y, Zhang L, Zhang H et al. Exogenous melatonin modulates apoptosis in the mouse brain induced by high-LET carbon ion irradiation. J Pineal Res 2012; **52**:47–56.
11. Mauriz JL, Molpeceres V, García-Mediavilla MV, González P, Barrio JP, González-Gallego J. Melatonin prevents oxidative stress and changes in antioxidant enzyme expression and activity in the liver of aging rats. J Pineal Res 2007; **42**:222–230.
12. Richter HG, Hansell JA, Raut S, Giussani DA. Melatonin improves placental efficiency and birth weight and increases the placental expression of antioxidant enzymes in undernourished pregnancy. J Pineal Res 2009; **46**:357–364.
13. García-Macia M, Vega-Naredo I, De Gonzalo-Calvo D et al. Melatonin induces neural SOD2 expression independent of the NF-kappaB pathway and improves the mitochondrial population and function in old mice. J Pineal Res 2011; **50**:54–63.
14. Kim CH, Kim KH, Yoo YM. Melatonin protects against apoptotic and autophagic cell death in C2C12 murine myoblast cells. J Pineal Res 2011; **50**:241–249.
15. Rodríguez MI, Escames G, López LC et al. Chronic melatonin treatment reduces the age-dependent inflammatory process in senescence-accelerated mice. J Pineal Res 2007; **42**:272–279.
16. Esposito E, Paterniti I, Mazzon E, Bramanti P, Cuzzocrea S. Melatonin reduces hyperalgesia associated with inflammation. J Pineal Res 2010; **49**:321–331.
17. Choi EY, Jin JY, Lee JY, Choi JI, Choi IS, Kim SJ. Melatonin inhibits Prevotella intermedia lipopolysaccharide-induced production of nitric oxide and interleukin-6 in murine macrophages by suppressing NF-κB and STAT1 activity. J Pineal Res 2011; **50**:197–206.
18. Xu DX, Wang H, Ning H, Zhao L, Chen YH. Maternally administered melatonin differentially regulates lipopolysaccharide-induced proinflammatory and anti-inflammatory cytokines in maternal serum, amniotic fluid, fetal liver, and fetal brain. J Pineal Res 2007; **43**:74–79.
19. Ochoa JJ, Díaz-Castro J, Kajarabille N et al. Melatonin supplementation ameliorates oxidative stress and inflammatory signaling induced by strenuous exercise in adult human males. J Pineal Res 2011; **51**:373–380.
20. Wu CC, Chiao CW, Hsiao G, Chen A, Yen MH. Melatonin prevents endotoxin-induced circulatory failure in rats. J Pineal Res 2001; **30**:147–156.
21. Gitto E, Karbownik M, Reiter RJ et al. Effects of melatonin treatment in septic newborns. Pediatr Res 2001; **50**:756–760.
22. Carrillo-Vico A, Lardone PJ, Naji L et al. Beneficial pleiotropic actions of melatonin in an experimental model of septic shock in mice: regulation of pro-/anti-inflammatory cytokine network, protection against oxidative damage and anti-apoptotic effects. J Pineal Res 2005; **39**:400–408.
23. Lin XJ, Mei GP, Liu J et al. Therapeutic effects of melatonin on heatstroke-induced multiple organ dysfunction syndrome in rats. J Pineal Res 2011; **50**:436–444.
24. Erridge C. Endogenous ligands of TLR2 and TLR4: agonists or assistants? J Leukoc Biol 2010; **87**:989–999.
25. Beutler B, Hoebe K, Du X, Ulevitch RJ. How we detect microbes and respond to them: the Toll-like receptors and their transducers. J Leukoc Biol 2003; **74**:479–485.

26. Natoli G, Chiocca S. Nuclear ubiquitin ligases, NF-kappaB degradation, and the control of inflammation. Sci Signal 2008; **1**:pe1.
27. McCubrey JA, Lahair MM, Franklin RA. Reactive oxygen species-induced activation of the MAP kinase signaling pathways. Antioxid Redox Signal 2006; **8**:1775–1789.
28. Ivashkiv LB. Inflammatory signaling in macrophages: transitions from acute to tolerant and alternative activation states. Eur J Immunol 2011; **41**:2477–2481.
29. Laird MH, Rhee SH, Perkins DJ et al. TLR4/MyD88/PI3K interactions regulate TLR4 signaling. J Leukoc Biol 2009; **85**:966–977.
30. Ojaniemi M, Glumoff V, Harju K, Liljeroos M, Vuori K, Hallman M. Phosphatidylinositol 3-kinase is involved in Toll-like receptor 4-mediated cytokine expression in mouse macrophages. Eur J Immunol 2003; **33**:597–605.
31. Deng WG, Tang ST, Tseng HP, Wu KK. Melatonin suppresses macrophage cyclooxygenase-2 and inducible nitric oxide synthase expression by inhibiting p52 acetylation and binding. Blood 2006; **108**:518–524.
32. Wang H, Xu Dx, Lu JW, Ning H, Wei W. Melatonin attenuates lipopolysaccharide (LPS)-induced apoptotic liver damage in D-galactosamine-sensitized mice. Toxicology 2007; **237**:49–57.
33. Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal 2001; **13**:85–94.
34. Kang JW, Koh EJ, Lee SM. Melatonin protects liver against ischemia and reperfusion injury through inhibition of toll-like receptor signaling pathway. J Pineal Res 2011; **50**:403–411.
35. Luchetti F, Betti M, Canonico B et al. ERK MAPK activation mediates the antiapoptotic signaling of melatonin in UVB-stressed U937 cells. Free Radic Biol Med 2009; **46**:339–351.
36. Liu L, Zhu Y, Xu Y, Reiter RJ. Prevention of ERK activation involves melatonin-induced G(1) and G(2)/M phase arrest in the human osteoblastic cell line hFOB 1.19. J Pineal Res 2011; DOI: 10.1111/j.1600-079X.2011.00971.x.
37. Carbajo-Pescador S, García-Palomo A, Martín-Renedo J, Piva M, González-Gallego J, Mauriz JL. Melatonin modulation of intracellular signaling pathways in hepatocarcinoma HepG2 cell line: role of the MT1 receptor. J Pineal Res 2011; **51**:463–471.
38. Asehnoune K, Strassheim D, Mitra S, Kim JY, Abraham E. Involvement of reactive oxygen species in Toll-like receptor 4-dependent activation of NF-kappa B. J Immunol 2004; **172**:2522–2529.
39. Huang SH, Cao XJ, Wei W. Melatonin decreases TLR3-mediated inflammatory factor expression via inhibition of NF-kappa B activation in respiratory syncytial virus-infected RAW264.7 macrophages. J Pineal Res 2008; **45**:93–100.
40. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 1999; **401**:82–85.
41. Li X, Tupper JC, Bannerman DD, Winn RK, Rhodes CJ, Harlan JM. Phosphoinositide 3 kinase mediates Toll-like receptor 4-induced activation of NF-kappa B in endothelial cells. Infect Immun 2003; **71**:4414–4420.
42. Ha T, Liu L, Kelley J, Kao R, Williams D, Li C. Toll-like receptors: new players in myocardial ischemia/reperfusion injury. Antioxid Redox Signal 2011; **15**:1875–1893.
43. Lee SH, Chun W, Kong PJ et al. Sustained activation of Akt by melatonin contributes to the protection against kainic acid-induced neuronal death in hippocampus. J Pineal Res 2006; **40**:79–85.
44. Tajes Orduña M, Pelegrí Gabalda C, Vilaplana Hortensi J, Pallàs Lliberia M, Camins Espuny A. An evaluation of the neuroprotective effects of melatonin in an in vitro experimental model of age-induced neuronal apoptosis. J Pineal Res 2009; **46**:262–267.
45. Kwon KJ, Kim JN, Kim MK et al. Melatonin synergistically increases resveratrol-induced heme oxygenase-1 expression through the inhibition of ubiquitin-dependent proteasome pathway: a possible role in neuroprotection. J Pineal Res 2011; **50**:110–123.
46. Picinato MC, Hirata AE, Cipolla-Neto J et al. Activation of insulin and IGF-1 signaling pathways by melatonin through MT1 receptor in isolated rat pancreatic islets. J Pineal Res 2008; **44**:88–94.
47. Kimball SR, Abbas A, Jefferson LS. Melatonin represses oxidative stress-induced activation of the MAP kinase and mTOR signaling pathways in H4IIE hepatoma cells through inhibition of Ras. J Pineal Res 2008; **44**:379–386.
48. Kim GD, Lee SE, Kim TH, Jin YH, Park YS, Park CS. Melatonin suppresses acrolein-induced IL-8 production in human pulmonary fibroblasts. J Pineal Res 2012; **52**:356–364.
49. O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol 2007; **7**:353–364.
50. O'Neill LA, Fitzgerald KA, Bowie AG. The Toll-IL-1 receptor adaptor family grows to five members. Trends Immunol 2003; **24**:286–290.
51. Oshikawa K, Sugiyama Y. Gene expression of Toll-like receptors and associated molecules induced by inflammatory stimuli in the primary alveolar macrophage. Biochem Biophys Res Commun 2003; **305**:649–655.
52. Yamamoto M, Sato S, Hemmi H et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 2003; **301**:640–643.
53. Fitzgerald KA, Rowe DC, Barnes BJ et al. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J Exp Med 2003; **198**:1043–1055.