Melatonin protects liver against ischemia and reperfusion injury through inhibition of toll-like receptor signaling pathway

Abstract: This study investigated the immunomodulating effect of melatonin on toll-like receptor (TLR)-stimulated signal transduction. Rats were subjected to 60 min of ischemia followed by 1 or 5 hr of reperfusion. Melatonin (10 mg/kg) or the vehicle was administered intraperitoneally 15 min prior to ischemia and immediately before reperfusion. Melatonin treatment significantly reduced the level of serum alanine aminotransferase activity. Increased levels of TLR3 and TLR4 protein expression induced by ischemia/reperfusion (I/R) were attenuated by melatonin. Serum level of high-mobility group box 1 (HMGB1), a potent alarmin of the TLR system, increased significantly in the I/R group, and melatonin inhibited this release. Melatonin suppressed the increase in myeloid differentiation factor 88 (MyD88) protein expression, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) phosphorylation and nuclear translocation of nuclear factor κB (NF-κB) and phosphorylated c-Jun, a component of activator protein 1. The increased level of toll-receptor-associated activator of interferon (TRIF) expression, phosphorylation of interferon (IFN) regulatory factor 3 (IRF3) and serum IFN-β was attenuated by melatonin. Melatonin attenuated the levels of tumor necrosis factor alpha (TNF-α), interleukin (IL)-6 and inducible nitric oxide synthase (iNOS) protein and mRNA expression, while the level of heme oxygenase-1 (HO-1) was augmented. Our results suggest that melatonin ameliorates I/R-induced liver damage by modulation of TLR-mediated inflammatory responses.

Introduction

Ischemia/reperfusion (I/R) injury develops in the absence of exogenous antigen (Ag), and innate immunity has been recognized as playing a dominant role in its pathology [1]. It has been implicated in the pathogenesis of a variety of clinical conditions, including liver transplantation, liver resection, hypovolemic shock, and trauma [2]. Mechanisms underlying hepatic I/R involve leukocyte accumulation and activation, pro-inflammatory cytokine and chemokine secretion, and vascular cell adhesion molecule activation [3].

Toll-like receptors (TLRs) are pattern-recognition receptors (PRRs) that recognize conserved pathogen-associated molecular patterns (PAMPs). Activation of the sentinel TLR system plays an important role in infectious and inflammatory disease states [4]. It is now apparent that TLRs can also be activated by endogenous ligands. In particular, hepatic I/R injury is exacerbated by activation of TLR4 by high-mobility group box 1 (HMGB1), a damage-associated molecular pattern (DAMP) protein released from dying cells [5]. Myeloid differentiation factor 88 (MyD88)-dependent signaling in the TLR4 activation pathway leads to direct nuclear factor κB (NF-κB) activation and induction of pro-inflammatory cytokines, whereas MyD88-independent signaling mediated by interferon regulatory factor 3 (IRF3) induces type I interferon (IFN) (IFN-α/β) and IFN-inducible genes, such as chemokine (C-X-C motif) ligand 10 (CXCL-10) [6]. TLR3 regulates amplification events of inflammation during experimental polymicrobial septic peritonitis and ischemic gut injury [7]. Recent studies have shown that TLR3 also plays important roles in a variety of liver diseases, including fibrosis, viral hepatitis, and primary biliary cirrhosis [8].

Melatonin (N-acetyl-5-methoxytryptamine) is an indole-amine produced by the pineal gland in a circadian rhythm, and it is one of the most powerful antioxidants known. Melatonin as well as its metabolites exerts direct antioxidative effects via scavenging of radicals [9–11] and act as indirect antioxidants by stimulation of antioxidative enzymes, including glutathione reductase and superoxide dismutase [12]. In addition, melatonin abates hepatic I/R injury by inhibition of hepatic necrosis and apoptosis, improving the balance between nitric oxide and endothelin, and suppressing the c-Jun N-terminal kinase (JNK) pathway [13, 14]. However, the molecular target of melatonin in TLR signaling pathway has not been fully identified in hepatic I/R.

This study examined the protective mechanisms of melatonin in hepatic I/R, particularly focusing on TLR3 and TLR4 innate signaling, potent ligands of TLRs, and further inflammatory cascades.
Materials and methods

Hepatic ischemia procedure

All animal procedures were approved by the Sungkyunkwan University Animal Care Committee and were performed in accordance with the guidelines of the National Institutes of Health. Male Sprague-Dawley rats (270–300 g) were obtained from Orient Bio Inc. (Sungnam, Korea). Rats were fasted for 18 hr before the experiments but were provided with tap water ad libitum. Under ketamine (55 mg/kg, i.p.) and xylazine (7 mg/kg, i.p.) anesthesia, a midline incision was made to the abdomen, and the left branches of the portal vein and hepatic artery were clamped to induce complete ischemia of the median and left hepatic lobes. The right lobes remained perfused to prevent intestinal congestion. After 60 min of ischemia, the clip around the left branches of the portal vein was removed to allow reperfusion. Vehicle- and melatonin-treated control rats were prepared in a similar manner; however, a clip was not placed on the vasculature leading to the median and left lobes. After 1 or 5 hr of reperfusion, the rat was sacrificed and blood and ischemic liver tissue were collected. Liver tissue was analyzed immediately (aliquots from the left lobe), and the remaining major portions of the liver tissues were frozen immediately in liquid nitrogen and kept at −75°C until biochemical analyses.

Administration of melatonin

Melatonin (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in a 5% ethanol solution in saline (vehicle) and administered intraperitoneally (10 mg/kg), 15 min prior to ischemia and immediately before reperfusion. The dosage and time point of melatonin administration were selected based on a previous published report [15]. Four treatment groups were examined: (a) vehicle-treated sham (sham), (b) melatonin-treated sham (MLT), (c) vehicle-treated I/R (I/R), and (d) melatonin-treated I/R (MLT + I/R).

Serum alanine aminotransferase activity

The level of serum alanine aminotransferase (ALT) was determined by standard spectrophotometric procedures using the ChemiLab ALT assay kit (IVDLab Co., Ltd., Uiwang, Korea).

Histologic analysis

Liver tissues were removed from a portion of the left lobe, fixed immediately in 10% neutral-buffered formalin (Sigma Chemical Co.), embedded in paraffin, and cut serially into 5-μm sections. Hematoxylin- and eosin (H&E)-stained sections were evaluated using an optical microscope (Olympus Optical Co., Tokyo, Japan).

Isolation of cytosolic and nuclear proteins

NEPER® (Pierce Biotechnology, Rockford, IL, USA) was used for extraction of nuclear and cytosolic proteins in accordance with the manufacturer’s instructions. Protein concentrations were determined using the BCA Protein Assay kit (Pierce Biotechnology).

HMGB1 analysis

Blood samples were collected from abdominal aorta and centrifuged at 500 g for 10 min at 4°C. Following centrifugation, serum samples were filtered and concentrated through Centricron YM-100 and YM-10 (Millipore, Billerica, MA, USA) with fixed-angle (35°), 7500 g for 15 min at 4°C, respectively. The concentrated samples were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Western blot analysis

Twenty micrograms of whole protein was used for determination of the content of TLR3, TLR4, MyD88, phosphorylated (p-)total forms of p38, extracellular signal-regulated kinase (ERK) and JNK, IRF3, p-IRF3, toll-receptor-associated activator of interferon (TRIF), inducible nitric oxide synthase (iNOS), and heme oxygenase-1 (HO-1). Fifteen micrograms of nuclear protein was used for determination of the content of the inhibitor of κB (IκB)-α. Fifteen micrograms of serum protein was used for determination of the content of HMGB1. ImageQuant™ TL software (Amersham Biosciences/GE Healthcare, Piscataway, NJ, USA) was used for densitometric evaluation of visualized immunoreactive bands. The following primary antibodies were used: TLR3 (Abcam, Cambridge, UK; 1:1000), TLR4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000), HMGB1 (Abcam; 1:1000), MyD88 (Santa Cruz Biotechnology; 1:1000), p-p38, p-JNK, total p38 and total JNK (Cell Signaling Technology Inc., Beverly, MA, USA; 1:1000), p-ERK and total ERK (Cell Signaling Technology Inc.; 1:2000), phosphoryl NF-κB/p65 (Santa Cruz Biotechnology; 1:1000), IκB-α (Santa Cruz Biotechnology; 1:500), c-Jun p39 phosphorylated on serine-63 (Santa Cruz Biotechnology; 1:500), TRIF (Abcam; 1:1000), p-IRF3 (Cell Signaling Technology Inc.; 1:1000), IRF3 (Cell Signaling Technology Inc.; 1:1000), iNOS (Transduction Lab., CA, USA; 1:1000), HO-1 (StressGen Biotechnologies, Vic., Canada; 1:1000) were used, and the signals were normalized to those of β-actin (Sigma Chemical Co.; 1:1000) or lamin B1 (Abcam; 1:2500).

Serum cytokine levels

A commercial tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6 enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences Co., San Jose, CA, USA) was used for quantification of the serum levels of TNF-α and IL-6, respectively. A commercial rat IFN-β ELISA kit (USCN Life Science Inc., Wuhan, China) was used for quantification of the serum level of IFN-β.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted, and the first strand of cDNA was synthesized by reverse transcription of total RNA using oligo(dT)_{12-18} primer and SuperScript™ II RNase
H-Reverse Transcriptase (Invitrogen Tech-Line™, Carlsbad, CA, USA). PCR was carried out in a 20 µL reaction volume with a diluted cDNA sample. Final reaction concentrations were as follows: sense and antisense primers, 10 pm; dNTP mix, 250 µm; 10× PCR buffer and Ex Taq DNA polymerase, 0.5 U/reaction. PCR was performed with an initial denaturation step at 94°C for 5 min, and a final extension step at 72°C for 7 min in the GeneAmp 2700 thermocycler (Applied Biosystems, Foster City, CA, USA). Gene-specific primers used are listed in Table 1. Amplification cycling conditions were as follows: 32 cycles of 94°C (30 s), 56°C (30 s), and 72°C (30 s) for TNF-α and HO-1; 32 cycles of 94°C (30 s), 58°C (45 s) and 72°C (30 s) for IL-6; 32 cycles of 94°C (45 s), 65°C (45 s) and 73°C (60 s) for iNOS and 35 cycles of 94°C (30 s), 62°C (30 s), and 72°C (60 s) for β-actin. Following RT-PCR, 10 µL samples of the PCR products were visualized by ultraviolet illumination after electrophoresis through 1.5% agarose gel and ethidium bromide staining. The intensity of each PCR product was analyzed semiquantitatively using a digital camera (DC120; Eastman Kodak, New Haven, CT, USA) and analysis software.

Statistical analysis
All results are reported as the mean ± S.E.M. The overall significance of the data was examined by two-way analysis of variance using the SPSS ver.12.0 statistical software package (SPSS, Chicago, IL, USA). Differences between the groups were considered statistically significant at P < 0.05 with appropriate Bonferroni corrections made for multiple comparisons.

Results
The level of serum ALT activity, which is a serum marker of hepatocyte necrosis, in the vehicle-treated sham group was 34.7 ± 5.5 U/L at 1 hr after reperfusion and 40.9 ± 2.3 U/L at 5 hr after reperfusion, respectively. The level of serum ALT activity was similar in the vehicle- and melatonin-treated sham groups. In the I/R group, the level of serum ALT activity showed a significant increase to 2839.9 ± 257.1 U/L at 1 hr after reperfusion and 4707.4 ± 538.7 U/L at 5 hr after reperfusion, respectively. These increases were attenuated by melatonin (63.1% of I/R group, P < 0.05 and 55.3% of I/R group, P < 0.01, respectively) (Table 2).

The histologic features shown in Fig. 1 indicate normal lobular architecture and cell structure in the livers of the vehicle- and melatonin-treated sham groups. However, livers exposed to I/R showed apparent broad hemorrhagic necrosis, extensive areas of portal inflammation, and moderate increase in inflammatory cell infiltration at 5 hr after reperfusion. This histologic damage was ameliorated by melatonin.

In the I/R group, the level of TLR3 protein expression showed a significant increase to 1.7-fold of that of the sham group at 1 hr after reperfusion and 25.1-fold of that of the sham group at 5 hr after reperfusion, respectively. This increase was attenuated by melatonin at 5 hr after reperfusion (39.8% of I/R group, P < 0.01). At 1 and 5 hr after reperfusion, the level of TLR4 protein expression showed a significant increase to 3.8-fold and 8.6-fold of that of the sham group, respectively. These increases were attenuated by melatonin at both 1 and 5 hr after reperfusion (53.9% of I/R group, P < 0.01 and 55.5% of I/R group, P < 0.05, respectively) (Fig. 2). The level of serum HMGB1 protein showed a significant increase to 2.8-fold of that of the sham group at 5 hr after reperfusion. Melatonin inhibited the release of HMGB1 to the circulatory system (55.2% of I/R group, P < 0.05) (Fig. 3).

At 5 hr after reperfusion, the level of MyD88 protein expression showed a significant increase to 10.2 times the sham value at 5 hr after reperfusion and melatonin suppressed this increase (40.2% of I/R group, P < 0.05) (Fig. 3). After 1 hr of reperfusion, phosphorylation of p38, ERK, and JNK showed a significant increase to 3.6 times, 5.3 times, and 77.1 times the sham value, respectively. Melatonin attenuated phosphorylation of JNK and ERK (79.5% of I/R group, P < 0.01 and 48.5% of I/R group, P < 0.01, respectively), whereas increased phosphorylation of p38 was not affected by melatonin treatment (99.0% of I/R group) (Fig. 4). We also examined NF-κB activation by measuring the protein level of the NF-κB /p65 subunit in the nucleus. To elucidate the mechanism of NF-κB activation during I/R and its modulation by melatonin, cytosolic protein level of IκB-α, an endogenous NF-κB inhibitor, was also examined. The nuclear level of p65, a subunit of NF-κB, showed a significant increase to 4.2 times the sham value at 5 hr after reperfusion. This increase was attenuated by melatonin (42.1% of I/R group, P < 0.01). Consistent with this result, the cytosolic level of IκB-α decreased to 6.9 times the sham value in the I/R group and melatonin attenuated this decrease (413.7% of I/R group, P < 0.05).

Table 1. RT-PCR primers used in study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Primer sequences (5' → 3')</th>
<th>Product length (bp)</th>
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<tr>
<td>TNF-α</td>
<td>NM_012675</td>
<td>Sense: GTA GCC CAC GTC GTA GCA AA</td>
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<tr>
<td></td>
<td></td>
<td>Anti-sense: CCC TTC TCC AGC TGG AAG AC</td>
<td>347</td>
</tr>
<tr>
<td>IL-6</td>
<td>NM_012589</td>
<td>Sense: GAA AGT CAA CTC CAT CTC CC</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Anti-sense: CAT AGC ACA CTA CGT TTG CC</td>
<td>679</td>
</tr>
<tr>
<td>iNOS</td>
<td>NM_012611</td>
<td>Sense: TTC TTT GCT TCT GTG CTT AAT GCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-sense: GTT GTT GCT GAA CTT CCA ATC GT</td>
<td>3793</td>
</tr>
<tr>
<td>HO-1</td>
<td>NW_047532</td>
<td>Sense: AAG GAG TTT CAT CAC ATC CTT GCA</td>
<td></td>
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<td>Anti-sense: ATG TTT AGC AGG AAG GCC GTC</td>
<td>1395</td>
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<tr>
<td>β-Actin</td>
<td>NM_031144</td>
<td>Sense: TTG TAA CCA ACT GGG ACG ATA TGG</td>
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<td></td>
<td></td>
<td>Anti-sense: GAT CTT GAT CTT CAT GGT GCT AG</td>
<td>764</td>
</tr>
</tbody>
</table>
The nuclear protein level of p-c-Jun in the I/R group showed a marked increase to 90.3 times the sham value. This increase in p-c-Jun was attenuated by melatonin (20.9% of I/R group, \( P < 0.01 \)) (Fig. 5).

The level of TRIF protein expression showed a significant increase to 4.2-fold that of the sham group at 5 hr after reperfusion and melatonin attenuated this increase (45.6% of I/R group, \( P < 0.05 \)). To elucidate the effect of melatonin on IRF3 activation during I/R, the level of p-/total-IRF3 was measured. After 5 hr of reperfusion, phosphorylation of IRF3 resulted in a significant increase to 3.9-fold that of the sham group and melatonin attenuated this enhancement (35.6% of I/R group, \( P < 0.01 \)) (Fig. 6). The level of serum IFN-\( \beta \), the subsequently induced product of IRF3 activation, in the sham group was 41.3 ± 1.3 pg/mL at 5 hr after reperfusion. In the I/R group, the level of serum IFN-\( \beta \) showed a significant increase to 95.5 ± 10.9 pg/mL at 5 hr after reperfusion and melatonin attenuated this increase (50.6% of I/R group, \( P < 0.01 \)) (Table 2).

Table 2. Effects of melatonin on serum alanine aminotransferase activity, serum TNF-\( \alpha \), IL-6 and IFN-\( \beta \) levels

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT (U/L)</th>
<th>TNF-( \alpha ) (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
<th>IFN-( \beta ) (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
<td>5 hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>34.7 ± 5.5</td>
<td>40.9 ± 2.3</td>
<td>38.8 ± 5.3</td>
<td>78.1 ± 0.5</td>
</tr>
<tr>
<td>MLT</td>
<td>29.4 ± 2.4</td>
<td>49.8 ± 2.3</td>
<td>37.2 ± 4.0</td>
<td>57.6 ± 0.5</td>
</tr>
<tr>
<td>I/R</td>
<td>2839.9 ± 257.1**</td>
<td>4707.4 ± 538.7****</td>
<td>122.8 ± 16.3**</td>
<td>298.9 ± 12.6****</td>
</tr>
<tr>
<td>MLT + I/R</td>
<td>1793.1 ± 223.2***</td>
<td>2601.2 ± 346.0***</td>
<td>53.8 ± 3.0**</td>
<td>159.2 ± 22.6****</td>
</tr>
</tbody>
</table>

Liver damage was assessed at 1 and 5 hr after reperfusion by measurement of circulating serum ALT activity. The serum levels of TNF-\( \alpha \), IL-6 and IFN-\( \beta \) were determined at 5 hr after reperfusion using an ELISA kit, respectively. *, ** Denote significant differences (\( P < 0.05 \), \( P < 0.01 \)) versus sham group; +, ++ Denote significant differences (\( P < 0.05 \), \( P < 0.01 \)) versus I/R group. ALT, alanine aminotransferase; IFN-\( \beta \), interferon-\( \beta \); IL-6, interleukin-6; I/R, ischemia/reperfusion; MLT, melatonin; TNF-\( \alpha \), tumor necrosis factor alpha. The results are presented as mean ± S.E.M. of 8–10 animals per group.

Serum levels of TNF-\( \alpha \) and IL-6 in the sham group were 38.3 ± 5.3 pg/mL and 78.1 ± 0.5 pg/mL, respectively. After 5 hr reperfusion, serum levels of TNF-\( \alpha \) and IL-6 showed a significant increase to 122.8 ± 16.3 pg/mL and 298.9 ± 12.6 pg/mL, respectively. Melatonin treatment attenuated these increases (43.8% of I/R group, \( P < 0.01 \) and 53.3% of I/R group, \( P < 0.01 \)) (Table 2). In addition, levels of iNOS and HO-1 protein expression showed a significant rise to 3.6-fold and 2.0 times the sham values at 5 hr after reperfusion, respectively. Melatonin treatment attenuated the level of iNOS, while the level of HO-1 was augmented by melatonin (35.5% of I/R group, \( P < 0.01 \) and 148.2% of I/R group, \( P < 0.01 \), respectively) (Fig. 7). These results also corresponded with the levels of mRNA expression. In the I/R group, levels of TNF-\( \alpha \), IL-6, iNOS, and HO-1 mRNA expression had increased to 3.6-fold, 2.7-fold, 3.8-fold, and 2.7-fold of that of the sham group at 5 hr after reperfusion, respectively. Melatonin attenuated the increased levels of TNF-\( \alpha \), IL-6, and iNOS mRNA expression, while the level of HO-1
mRNA expression was augmented by melatonin (73.8% of I/R group, \( P < 0.05 \), 59.4% of I/R group, \( P < 0.05 \), 47.9% of I/R group, \( P < 0.05 \) and 123.5% of I/R group, \( P < 0.01 \), respectively) (Fig. 8).

**Discussion**

I/R, an exogenous Ag-independent inflammatory event, remains the major problem in clinical transplantation. The pathophysiology of I/R includes both direct cellular damage as a result of ischemic insult and delayed dysfunction following reperfusion resulting from activation of the immune system.

TLRs are ubiquitously expressed PRRs that are central to the inflammatory response in a broad array of species and become novel therapeutic targets for a range of inflammatory and autoimmune diseases [16]. There is considerable evidence showing that I/R, specific to the liver, can induce TLR4-dependent inflammation and organ injury [5, 17]. Antioxidative or anti-inflammatory agents, including bicyclol, \( N \)-acetylcysteine, and neutrophil elastase inhibitor (Sivelestat), showed protective effects in hepatic I/R by downregulation of TLR4 [18–20].

Melatonin exerted protective effects in various in vivo I/R models, such as middle cerebral artery occlusion stroke [21, 22], testicular torsion/detorsion-induced I/R [23], and partial hepatic I/R [15, 24]. The pineal gland, which produces and releases melatonin, was demonstrated to transduce LPS stimulation, trigger the NF-\( \kappa \)B pathway, and produce TNF-\( \alpha \) by possessing TLR4 and CD14 receptors, implicating its constitutive role in the innate immune response [25]. However, specific molecular mechanisms for the in vivo effect of melatonin on a
TLR-dependent inflammatory response during hepatic I/R have not yet been elucidated.

In our study, TLR4 protein expression showed a marked increase at 1 hr after reperfusion and further increased at 5 hr after reperfusion. This pattern coincided with the results of serum ALT activity, indicating that TLR4 overexpression is highly linked to liver damage and dysfunction. Melatonin markedly attenuated the increased level of TLR4 protein expression at both 1 and 5 hr after reperfusion, suggesting that a protective effect of melatonin in hepatic I/R might be associated with its suppression on TLR4 overexpression.
TLR3, located on the endolysosomal membrane, originally recognizes viral double-stranded RNA and is known to lead to aggravation or potentiation of preexisting inflammation in organs, including kidney, rheumatic synovium, and gastrointestinal tract [26–28]. Anti-TLR3 antibody attenuated the tissue injury associated with gut ischemia [7]. Recently, melatonin decreased TLR3-mediated inflammatory factors and NF-κB activation in respiratory syncytial virus (RSV)-infected macrophages [29]. For the first time, we found that hepatic I/R stimulates TLR3 protein expression at both 1 and 5 hr after reperfusion. Marked suppression of TLR3 overexpression by melatonin was observed only at 5 hr after reperfusion.

Nuclear factor HMGB1 is passively released during cell injury and necrosis, or actively secreted during immune cell activation, positioning at the intersection of sterile and infection-associated inflammation and ultimately exerts its pro-inflammatory effect through TLRs and other PRRs [30–32]. Inhibition of HMGB1 activity with neutralizing antibody decreased liver damage after I/R and TLR4 is known to be involved in this process [5]. In our study, melatonin diminished the increased serum release of HMGB1 induced by I/R, which translates into a protective effect.

TLRs can activate two branches of downstream signaling pathways, MyD88- and TRIF-dependent pathways, culminating in expression of inflammatory gene products, including cytokines and chemokines. MyD88 is a common downstream adaptor molecule leading to activation of mitogen-activated protein kinases (MAPKs) and NF-κB transcription factor [33]. MAPK signal transduction pathways represent one of the most widespread mechanisms of cell regulation in response to oxidative and other environmental stress, leading to upregulation of pro-inflammatory mediators, including TNF-α, IL-6, and iNOS [34]. MAPK activation also contributes to the inflammatory response following hepatic I/R [35]. Selective pharmacologic inhibitors of c-Jun, a substrate of JNK, have been shown to improve survival in experimental hepatic warm I/R resection and transplantation, representing a promising target for reduction of hepatic I/R injury [36]. NF-κB is implicated in regulation of many genes coding various mediators of inflammatory response and inhibition of NF-κB activation ameliorates hepatic I/R injury [37]. In particular, previous studies using TLR4 chimeric mice have demonstrated involvement of JNK and NF-κB activation in TLR4-mediated hepatic I/R injury [17]. In the present study, the level of MyD88 protein expression increased at 5 hr of reperfusion and melatonin attenuated this increase. Activation of the MyD88 pathway is also presented by activation of MAPKs. Our data show that melatonin blunted I/R-induced JNK and ERK phosphorylation, whereas p38 activation was not affected by melatonin at 1 hr of reperfusion. In addition, melatonin attenuated elevated nuclear accumulation of NF-κB/p65 and p-c-Jun and decreased cytosolic IκB-α at 5 hr after reperfusion.
Increased levels of serum TNF-α, IL-6, and iNOS protein expression and their mRNA expression were also attenuated by melatonin. Therefore, it is possible that the inhibitory effects of melatonin on the MyD88 signaling pathway of the TLR system may be associated with suppression of activation of MAPKs, NF-κB, and inflammatory mediators.

TRIF, another adapter molecule of TLRs, is mainly responsible for regulation of MyD88-independent pathways [38]. TRIF activates the downstream kinases, TBK1 and IKKe, leading to phosphorylation and activation of IRF3 and the consequent expression of type I IFNs and IFN-inducible genes [39]. In particular, the TLR4-dependent inflammatory response to hepatic I/R seems to be independent of the MyD88 pathway and activation of TRIF/IRF3-dependent signaling seems to be critical in hepatic I/R injury [40]. Luteolin, a flavonoid compound, inhibited TRIF protein expression, phosphorylation of IRF3 and IFN-β expression, in addition to the MyD88 pathway, providing evidence of its dual role in the TLR downstream signaling pathway [41, 42]. In the present study, increased levels of TRIF protein expression and phosphorylation of IRF3 were observed during I/R, and melatonin attenuated these alterations. In addition, serum level of IFN-β showed a marked increase at 5 hr after reperfusion and melatonin attenuated this increase. Collectively, our results suggest that melatonin modulates the TRIF-dependent signaling pathway as well as the MyD88-dependent pathway in hepatic I/R.

HO-1, a rate-limiting enzyme in the catabolism of heme, is a critical protective mechanism activated during cellular stress and is thought to play a key role in hepatic I/R via its antioxidative and anti-inflammatory functions [43]. The importance of cross-talk between HO-1 and the TLR4 system has already been established, and TLR4 was found to be one of the putative HO-1 repressors in noninfectious hepatic I/R [44]. CoPP-induced HO-1 overexpression ameliorates hepatic I/R injury by downregulating signal transduction and activator of transcription (STAT)-1 phosphorylation in type-1 IFN downstream of TLR4, with resultant inhibition of CXCL-10 [45]. The results of our study demonstrated that HO-1 protein and mRNA expression showed an increase at 5 hr after reperfusion and that melatonin augmented these increases. These results suggest that melatonin ameliorates hepatic I/R injury by upregulation of HO-1, as an adaptive antioxidative and anti-inflammatory mechanism.

In summary, this study highlights the cross-talk between melatonin and the TLR system in hepatic I/R, and the overall hepatoprotective effect of melatonin is related to its suppression of TLR3 and TLR4 overexpression, HMGB1 release, and further inflammatory signaling cascades. Effective immunomodulation of melatonin on hepatic I/R through inhibition of TLR signaling warrants further investigation of the possible use of melatonin as a therapeutic strategy for treatment of organ I/R injury.

**Acknowledgement**

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0028646).

**Author contributions**

Jung-Woo Kang: Concept/design, acquisition of data, data analysis/interpretation, drafting of the manuscript; Eun-Ji Koh: Acquisition of data; Sun-Mee Lee: Concept/design, data analysis/interpretation, critical revision of the manuscript.

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