Cellular Physiology and Biochemistry

Cell Physiol Biochem 2014;33:23-36
DOI: 10.1159/000356647
Published online: January 02, 2014
Accepted: December 02, 2013

1421-9778/14/0331-0023$39.50/0

Original Paper

Melatonin Inhibits mTOR-Dependent Autophagy during Liver Ischemia/Reperfusion

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Key Words
Autophagy • Calpain • Ischemia/reperfusion • Mammalian target of rapamycin • Melatonin • Oxidative stress

Abstract
Background: Autophagy is a self-digestion system responsible for maintaining cellular homeostasis and interacts with reactive oxygen species produced during ischemia/reperfusion (I/R). Melatonin (MLT) is a potent and endogenous anti-oxidant that has beneficial effects in liver I/R injury. In this study, we examined the cytoprotective mechanisms of MLT in liver I/R, focusing on autophagic flux and associated signaling pathways. ***Methods:*** Male C57BL/6 mice were subjected to 70% liver ischemia for 60 min followed by reperfusion. MLT (10 mg/kg, i.p.) was injected 15 min prior to ischemia and again immediately before reperfusion. Rapamycin (Rapa, 1 mg/kg, i.p.), which induces autophagy, was injected 1.5 h before ischemia. ***Results:*** Liver I/R increased autophagic flux as indicated by the accumulation of LC3-II and degradation of sequestosome1/p62. This increase was attenuated by MLT. Likewise, electron microscopic analysis showed that autophagic vacuoles were increased in livers of mice exposed to I/R, which was attenuated by MLT. I/R decreased phosphorylation of mammalian target of rapamycin (mTOR) and 4E-BP1 and 70S6K, downstream molecules of the mTOR pathway, but increased expression of calpain 1 and calpain 2. MLT attenuated the decrease in mTOR, 4E-BP1 and 70S6K phosphorylation. Pretreatment of Rapa reversed the effect of MLT on autophagic flux as well as mTOR pathway. ***Conclusion:*** Our findings suggest that MLT downregulates autophagy via activation of mTOR signaling, which may in turn contribute to its protective effects in liver I/R injury.

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Introduction

Liver ischemia/reperfusion (I/R) injury is a complex and multifactorial pathophysiological process that is a commonly encountered in a variety of clinical events such as liver transplantation, tumor resection, and traumatic shock. Due to their high metabolic rate, liver cells are vulnerable to the deleterious effects of ischemia including ATP depletion and anoxia. A plethora of studies have shown that excessive production of reactive oxygen species (ROS) followed by reperfusion is the earliest and most important factor for cellular damage in liver I/R [1, 2], because ROS directly attack cellular molecules as well as indirectly recruit and activate pro-inflammatory mediators. Ultimately, these detrimental events during I/R lead to both necrotic and apoptotic cell death [3].

Autophagy is a self-digestion process that is important for maintaining basal homeostasis against intrinsic and extrinsic stress. Autophagic flux is the dynamic process of autophagy in which cytosolic organelles and proteins are sequestered by double membrane structures termed autophagosomes, which are then delivered to lysosomes for the formation of autolysosomes that are subsequently degraded by proteases [4]. Autophagy functions as an adaptive response by which amino acids and fatty acids are recycled for ATP generation and cellular components leading to malfunction are removed. However, excessive activation of autophagy can be toxic and may cause cell death, which is called type II programmed cell death [5]. Oxidative stress was recently shown to activate starvation-induced autophagy, and conversely, autophagy can also play an important role in suppressing ROS production [6, 7]. This conundrum is further complicated by cross-talk and coordinated regulation between autophagy and other types of cell death under oxidative environments.

Although the role of autophagy in I/R injury of various organs has recently been investigated, the exact function of autophagy appears to be model and organ dependent. In the kidneys and heart, autophagy is activated by I/R, with the function of increased autophagy having been reported as both protective and deleterious [8, 9]. In the liver, warm reperfusion injury is involved in the degeneration of hepatocytes, a process that is stimulated by autophagy [10]. Moreover, induction of autophagy is evident in normothermic liver I/R, observed in the form of simultaneous apoptosis and necrosis [11]. In contrast, Kim et al. [12] reported that impaired autophagy occurs in calpain 2-dependent mechanisms that lead to mitochondrial dysfunction in anoxic rat hepatocytes. Most recently, autophagy was shown to suppress ischemic liver damage by reducing ROS-induced necrosis [13].

Melatonin (MLT, *N*-acetyl-5-methoxytryptamine) is a lipophilic indole secreted by pineal and non-pineal cells that has potent anti-oxidant, anti-inflammatory, and anti-apoptotic properties [14]. We previously showed that MLT inhibits necrotic and apoptotic cell death in a rat model of liver I/R by alleviating levels of oxidative stress [15]. Recently, a protective role of MLT has been reported in cold liver I/R through suppression of endoplasmic reticulum (ER) stress and enhanced autophagy [16]. Although the effects of MLT on autophagy have been actively studied, the precise mechanism by which MLT regulates autophagy in liver I/R remains unclear.

This study was designed to investigate the protective mechanism of MLT in liver I/R, focusing particularly on autophagic flux and associated signaling pathways.

Materials and Methods

Chemicals and antibodies

MLT (M5250) and chloroquine (CQ) (C6628) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rapamycin (Rapa, 53123-88-9) was purchased from Calbiochem (Billerica, MA, USA). The following antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA): Beclin-1 (3738), Atg3 (3415), Atg7 (2631), p-mammalian target of rapamycin (mTOR) (Ser2448) (2971), p-4E-BP1 (Ser65) (9451) and p-70S6K (Thr389) (9205). The following antibodies were purchased from Abcam (Cambridge, UK): sequestosome 1 (SQSTM1)/p62 (ab56416), calpain 1 (ab49652) and calpain 2 (ab39168). Microtubule-

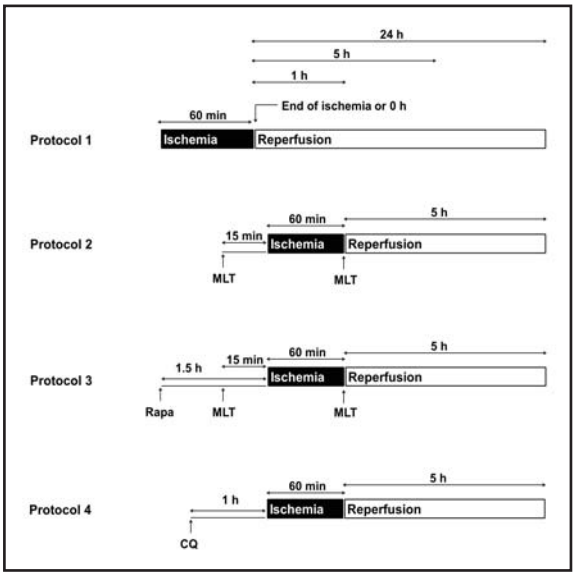

Fig. 1. Schematic presentation of the experimental procedures. Protocol 1- Time course of autophagy changes during liver I/R: After 60 min of ischemia, the clamp was removed to allow reperfusion for 0 (immediately after declamping), 1, 5 and 24 h. Protocol 2- Effects of MLT on hepatocellular damage and autophagic flux during liver I/R: MLT was administered 15 min prior to ischemia and directly before reperfusion. Mice were sacrificed after 5 h of reperfusion. Protocol 3- Effects Rapa on MLT-induced protection during liver I/R: Rapa was injected 1.5 h prior to ischemia and MLT was administered 15 min prior to ischemia and directly before reperfusion. Mice were sacrificed after 5 h of reperfusion. Protocol 4- Effects of CQ on autophagic flux during liver I/R: CQ was injected 1 h prior to ischemia and mice were sacrificed after 5 h of reperfusion.

associated protein 1 light chain 3 (LC3)-II antibody (NB100-2220) was purchased from Novus Biologicals (Littleton, CO, USA) and the β-actin antibody (A5441) was purchased from Sigma-Aldrich.

Animals

All animals received care in compliance with the Principles of Laboratory Animal Care formulated by the National Institutes of Health (NIH publication No.86-23, revised 1985) and the guidelines of the Sungkyunkwan University Animal Care Committee. Male C57BL/6 mice weighing 22 to 24 g were obtained from Orient Bio Inc. (Seongnam, Korea) and were acclimatized to the laboratory conditions at Sungkyunkwan University for at least one week prior to initiating experiments. Mice were maintained in a room with controlled temperature and humidity (25 ± 1°C and 55 ± 5%, respectively) and a 12-h light-dark cycle. The mice were fasted for 18 h prior to experiments and were provided with tap water *ad libitum*.

Liver I/R procedure and drug treatment

I/R experimental protocol is depicted in Figure 1. We randomly assigned 6 animals for vehicle- or drug-treated sham groups and 8 animals for vehicle- or drug-treated I/R groups. Mice were anesthetized with ketamine (100 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.). The body temperature of mice was maintained at 37°C throughout the anesthesia process. The left branches of the portal vein and hepatic artery were clamped to induce complete ischemia of the median and left lobes of the liver. The right lobes remained perfused to prevent venous congestion of the intestine. After 60 min of ischemia, the clamp was removed to allow reperfusion for 0 (immediately after declamping), 1, 5 and 24 h. Sham-operated mice were prepared in a similar manner; however, a clip was not placed on the vasculature leading to the median and left lobes. After I/R injury, mice were sacrificed and blood from the inferior vena cava and liver tissue was collected. Liver tissue was analyzed immediately by histological staining of sections from the left lobe. The remaining portions of the median and left lobes were flash frozen in liquid nitrogen and kept at -75°C until biochemical analyses were performed.

MLT was dissolved in 5% ethanol in saline and administered (10 mg/kg of bw, i.p.) 15 min prior to ischemia and again directly before reperfusion. The dosage and timing of MLT administration were selected based on a previous study [17]. CQ dissolved in phosphate-buffered saline (PBS) was injected (60 mg/kg of bw, i.p.) 1 h prior to ischemia. Rapa was dissolved in dimethyl sulfoxide (DMSO) and diluted with PBS to a final concentration of 1% DMSO in PBS. Rapa was injected (1 mg/kg of bw, i.p.) 1.5 h prior to ischemia. In vehicle-treated sham or I/R mice, 5% ethanol-saline, PBS, or 1% DMSO-PBS were administered in the same volume and route as the respective drug treatments. No effects of these vehicles on liver function were detected. The dosage and timing of injections of CQ and Rapa were determined based on previous studies [18, 19] as well as preliminary investigations in our laboratory.

Serum aminotransferase activities

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured with ChemiLab ALT and AST assay kit (IVDLab Co., Uiwang, Korea), respectively.

Hematoxylin and eosin (H&E) staining and terminal dUTP nick-end labeling (TUNEL) assay

Liver specimens were fixed in 10% buffered formalin, embedded in paraffin, and stained with H&E. The stained sections were blindly examined using a light microscope (Olympus CKX41, Olympus Optical Co., Tokyo, Japan). We have utilized the criteria reported by Suzuki et al. [20]. In this classification, 3 liver injury indices- sinusoidal congestion (score: 0–4), hepatocyte necrosis (score: 0–4), and ballooning degeneration (score: 0–4)- are graded for a total score of 0–12. Apoptotic cells were detected by TUNEL staining with a commercially available kit (ApopTag® *In Situ* Apoptosis Detection Kits, Millipore, Billerica, MA, USA). Apoptosis of hepatocytes in liver sections was quantitated by counting the number of TUNEL-positive cells in random microscopic high-power fields (x100).

Caspase-3 activity

Caspase-3 activity was measured using a fluorogenic peptide substrate, N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-cumarine (DEVD-AFC; Bio-Mol, Plymouth Meeting, PA, USA). A 30 μg sample of the liver cytosolic protein was incubated in a buffer containing 30 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), 0.3 mM EDTA, 100 mM NaCl, 0.15% Triton X-100 and 10 mM dithiothreitol. The samples were incubated at room temperature for 15 min. The caspase reaction was then initiated by adding 200 μM DEVD-AFC, and the resulting mixture was incubated at 37°C. The change in fluorescence (excitation at 400 nm and emission at 490 nm) was monitored after 120 min.

Hepatic lipid peroxidation and GSH/GSSG ratio

The steady-state level of malondialdehyde (MDA), an end-product of lipid peroxidation, was determined in liver tissues by measuring the levels of thiobarbituric acid-reactive substances [21]. Total levels of hepatic glutathione were determined by measuring yeast-glutathione reductase, 5,5'-dithio-bis(2-nitrobenzoic acid), and nicotinamide adenine dinucleotide phosphate (NADPH) in liver homogenates after precipitation with 1% picric acid. Levels of oxidized glutathione (GSSG) were determined by the same method but in the presence of 2-vinylpyridine. The levels of reduced glutathione (GSH) were calculated as the difference between the total levels of glutathione and GSSG [22].

Western blot analysis

Liver tissue protein lysates were prepared using radioimmunoprecipitation assay buffer, separated in SDS-PAGE gels, and transferred to polyvinylidene difluoride membranes as previously described [23]. Western blotting was performed using primary antibodies and horseradish peroxidase-conjugated secondary antibodies suitable for each primary antibody. The WEST-one Western Blot Detection System (iNtRON Biotechnology Inc., Seongnam, Korea) was used for detection according to the manufacturer's instructions. Intensities of the immunoreactive bands were determined using TotalLab TL120 software (IVDLab Nonlinear Dynamics Co. Ltd., Newcastle, UK). β-Actin was used as a loading control and protein levels were normalized relative to corresponding β-actin band intensity.

Transmission electron microscopy (TEM)

Liver tissues were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PBS. Tissues were then fixed with 1% osmium tetroxide, serially dehydrated in ethyl alcohol (50% to 100%), stained with 0.5% uranyl acetate, and embedded in Poly/Bed 812 resin (Pelco, Redding, CA, USA) followed by polymerization. Samples were sectioned using an Ultracut S (Leica, Wetzlar, Germany), and ultrathin sections were stained with 1% uranyl acetate and 0.2% lead citrate. Autophagic vacuoles were observed from blinded samples using a transmission electron microscope (JEM-1010, JEOL, Tokyo, Japan). In randomly selected fields, we captured 4 non-repeating micrographs for each liver sample (n=3-4 per group) and the area of one such micrograph was regarded as the unit area. The numbers of autophagic vacuoles per unit area (400 μm^2) in each sample were counted.

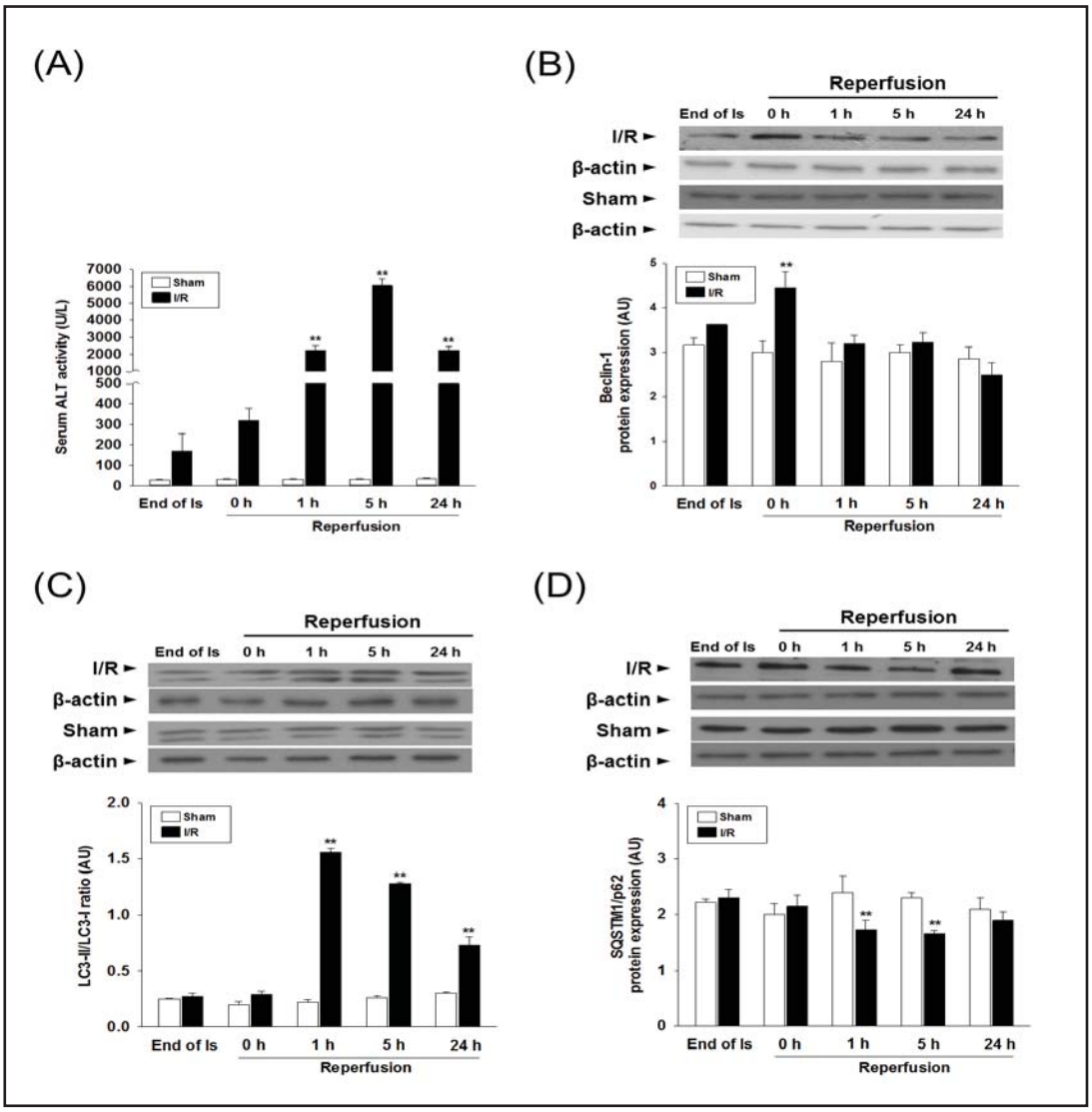

Fig. 2. Time course of hepatic injury and autophagy changes during liver I/R. Serum ALT activity was measured after ischemia for 60-min and reperfusion for 0, 1, 5 or 24 h (A). Western blot analysis was performed to measure proteins levels of beclin-1 (B), LC3-II/I (C) and SQSTM1/p62 (D). Results are presented as the mean ± S.E.M. of 6 to 8 mice per group. For comparison of proteins in different gels, an internal control was used. ** Significantly different ($p < 0.01$) from sham-operated animals.

Statistical analysis

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

Results

Time course of autophagy changes and hepatocellular damage during liver I/R

Serum ALT activity in sham-operated animals remained at basal levels throughout the experimental period. Serum ALT activity was not different from those of sham-operated animals at the end of the ischemic period or immediately after reperfusion (0 h of reperfusion).

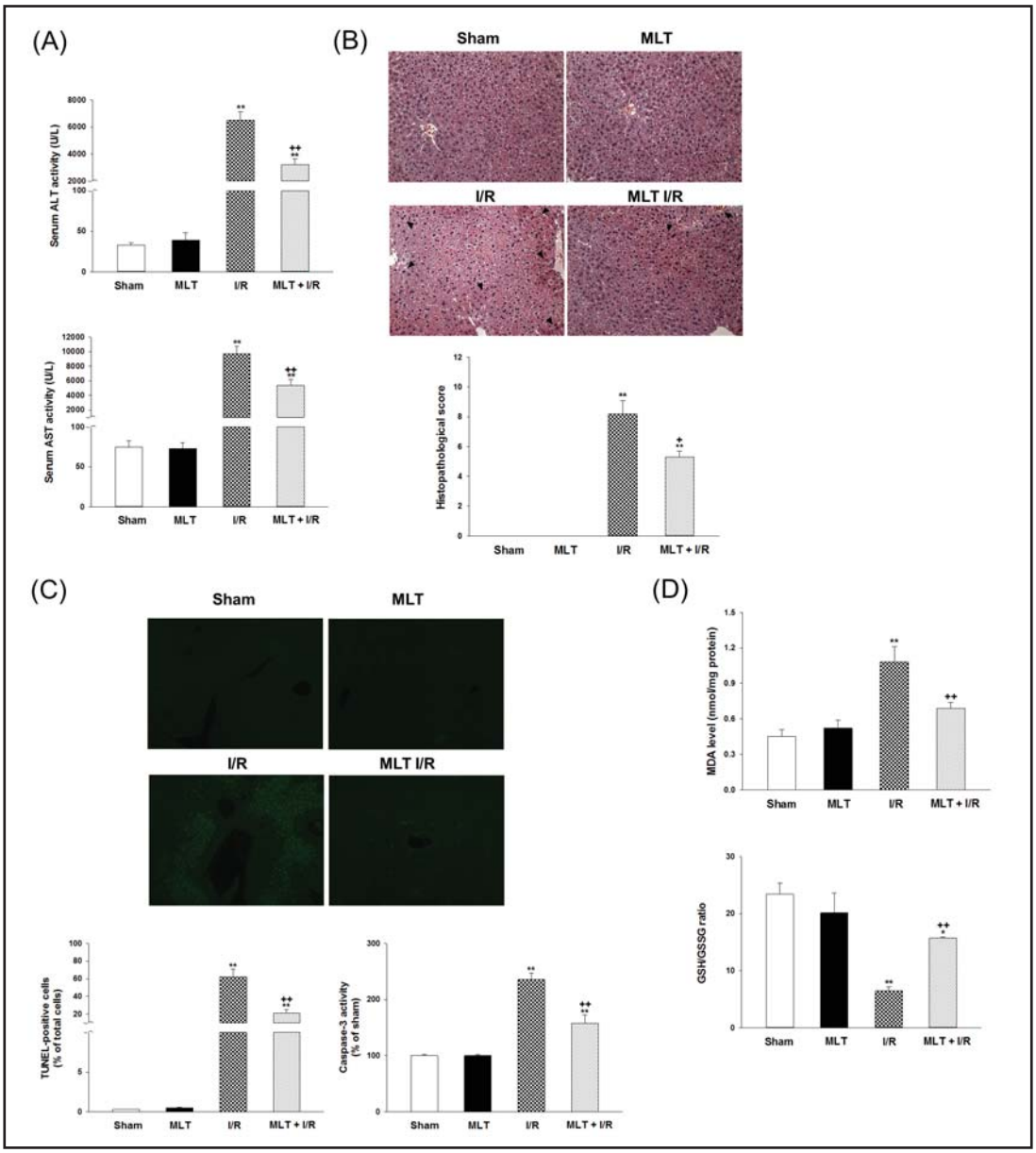

Fig. 3. Effect of MLT on liver injury induced by I/R. MLT was administered 15 min prior to ischemia and directly before reperfusion. After reperfusion for 5 h, serum ALT and AST activities were measured (A). Representative H&E staining (x200) with Suzuki score (B) and TUNEL staining images and caspase-3 activity (C) are shown. Arrow heads indicate necrotic area. Apoptosis in liver sections was quantified by counting the number of TUNEL-positive cells in random microscopic high-power fields (x100). Caspase-3 activity was measured in the cytosolic fraction. Levels of lipid peroxidation and the GSH/GSSG ratio were measured in liver tissue (D). Results are presented as the mean ± S.E.M. of 6 to 8 mice per group. *, **Significantly different ($p < 0.05$, $p < 0.01$) from sham-operated animals. $^{+}$, $^{++}$ Significantly different ($p < 0.05$, $p < 0.01$) from I/R animals, respectively.

However, after reperfusion for 1 h, serum ALT activity increased to 2243.9 ± 272.5 U/L and reached a peak of 6043.1 ± 401.0 U/L after reperfusion for 5 h. Serum ALT then gradually declined to 2251.7 ± 221.1 U/L after reperfusion for 24 h (Fig. 2A).

At the end of the ischemic period, the protein levels of beclin-1 were similar to those of sham-operated animals. However, the levels of beclin-1 protein expression increased

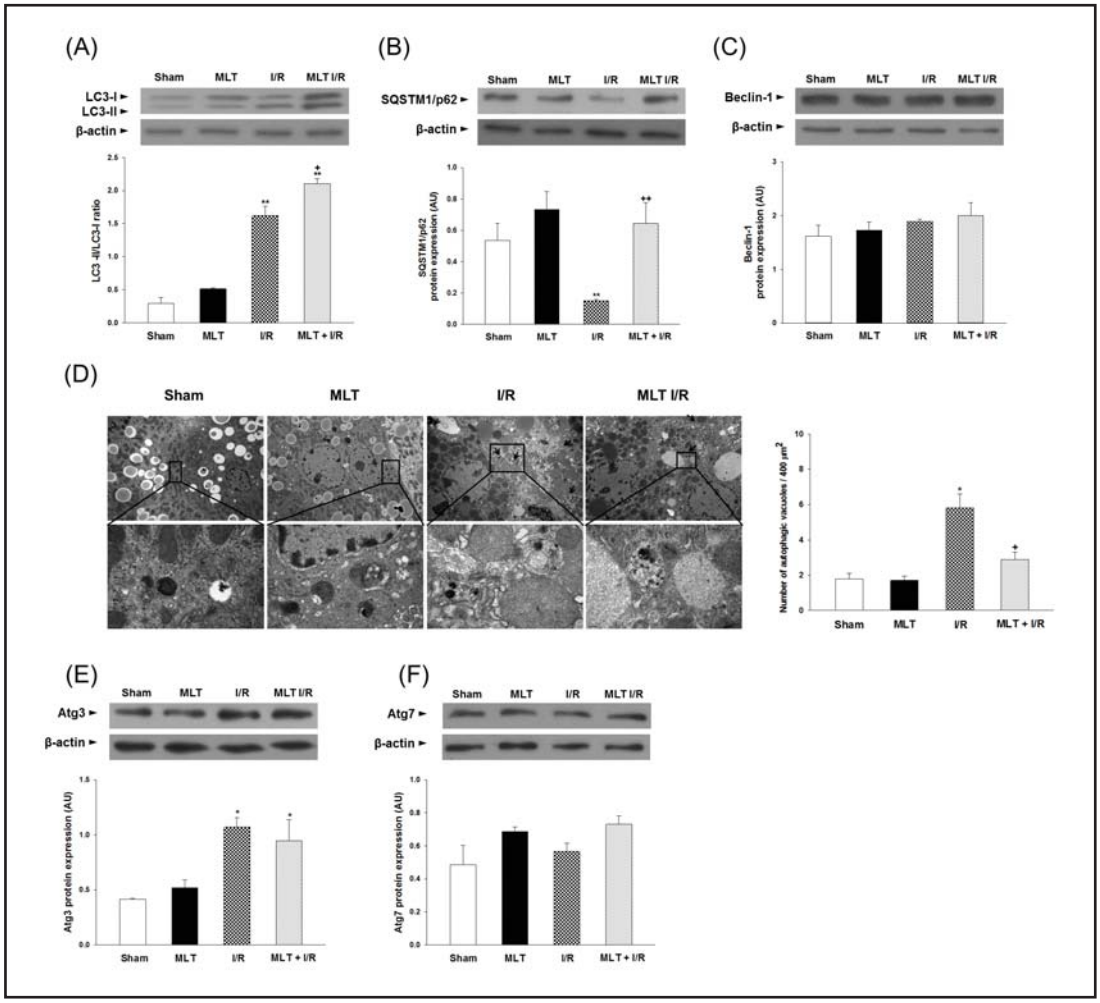

Fig. 4. Effect of MLT on autophagy during liver I/R. MLT was administered 15 min prior to ischemia and again directly before reperfusion. Western blot analysis was performed to measure protein levels of LC3-II/I (A), SQSTM1/p62 (B), beclin-1 (C), Atg3 (E), and Atg7 (F). Autophagic vacuoles (arrows indicated) were observed from TEM images (4 mice per group) (D). Results are presented as the mean ± S.E.M. of 6 to 8 mice per group. *, ** Significantly different ($p < 0.05$, $p < 0.01$) from sham-operated animals. $^{+}$, $^{++}$ Significantly different ($p < 0.05$, $p < 0.01$) from I/R animals, respectively.

significantly immediately after reperfusion (148.3% of sham), but were restored to basal levels after 1 h of reperfusion and maintained thereafter with reperfusion for 24 h (Fig. 2B). To evaluate autophagic flux in the liver, we evaluated changes in levels of LC3-I and LC3-II and protein expression of SQSTM1/p62, which is a polyubiquitin-binding protein known to be sequestered and degraded during autophagy. As shown Fig. 2C, the LC3-II/LC3-I ratio was similar to those of sham-operated animals at the end of the ischemic period and immediately after reperfusion, and was significantly increased after reperfusion for 1 and 5 h, but gradually declined after reperfusion for 24 h (707.0% of sham, 583.1% of sham, and 242.5% of sham, respectively). In contrast, the expression of SQSTM1/p62 was significantly decreased after reperfusion for 1 and 5 h and gradually restored after reperfusion for 24 h (71.9% of sham, 72.1% of sham, and 90.3% of sham, respectively) (Fig. 2D).

Effects of MLT on hepatocellular damage and oxidative stress during liver I/R

Because the most significant changes in LC3-II/LC3-I ratio, SQSTM1/p62 protein expression, and the most severe hepatocellular damage were noted after 5 h of reperfusion,

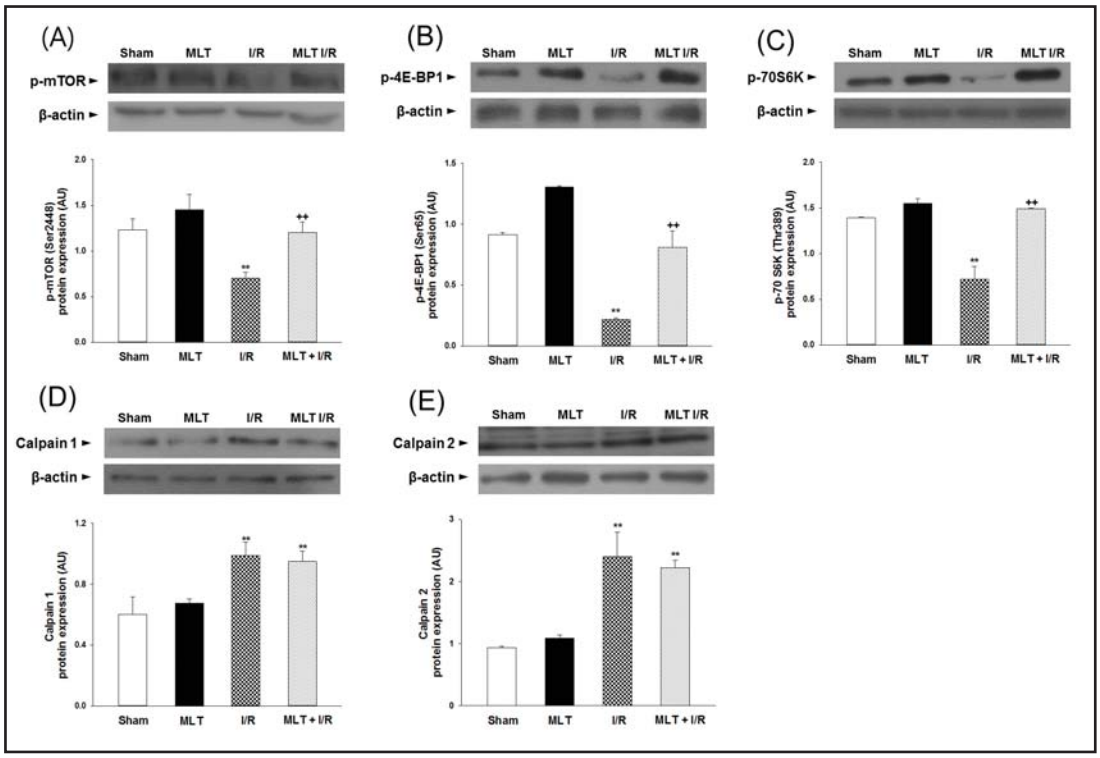

Fig. 5. Effect of MLT on calpains and the mTOR pathway during liver I/R. MLT was administered 15 min prior to ischemia and again directly before reperfusion. Western blot analysis was performed to measure protein levels of p-mTOR (A), p-4E-BP1 (B), p-70S6K (C), calpain 1 (D), and calpain 2 (E). Results are presented as the mean ± S.E.M. of 6 to 8 mice per group. ** Significantly different ($p < 0.01$) from sham-operated animals. ++ Significantly different ($p < 0.01$) from I/R animals.

we analyzed the effect of MLT using a model of ischemia for 60 min followed by reperfusion for 5 h. Serum ALT and AST activities in sham-operated animals were 33.0 ± 3.1 U/L and 74.5 ± 8.0 U/L, respectively. MLT alone did not affect serum ALT and AST activities. After reperfusion for 5 h, serum ALT and AST activities significantly increased to 6504.1 ± 628.2 U/L and 9706.3 ± 1000.1 U/L, which were attenuated by MLT (3207.2 ± 450.8 U/L and 5395.2 ± 820.2 U/L, respectively) (Fig. 3A). H&E stained liver sections were evaluated for the degree of hepatocellular damage using the Suzuki's criteria. The ischemic lobes in I/R animals showed severe necrosis, sinusoidal congestion and hepatocyte vacuolization (Suzuki score: 8.2 ± 0.9). However, MLT treatment attenuated the Suzuki score (5.3 ± 0.4) (Fig. 3B). We also analyzed apoptosis by TUNEL assay, analysis and representative images of which are shown in Fig. 3C. While no TUNEL-positive cells were detected in sham-operated animals, the level of apoptosis induced by I/R was 62.0 ± 9.1%, which was decreased to 21.4 ± 3.7% after treatment with MLT. After 5 h of reperfusion, caspase-3 activity in the cytosolic fraction significantly increased (235.5 ± 11.8 % of sham) and MLT attenuated this increase (157.9 ± 14.6 % of sham).

In the sham group, the level of MDA was 0.45 ± 0.06 nmol/mg protein. Following I/R, the level of MDA was significantly increased to 1.08 ± 0.13 nmol/mg protein. MLT attenuated the increase in MDA levels (0.69 ± 0.05 nmol/mg protein). The GSH/GSSG ratio in the sham animals were 23.4 ± 1.9. After reperfusion, the GSH/GSSG ratio decreased significantly to 6.5 ± 0.7, which was attenuated by MLT (15.7 ± 0.2) (Fig. 3D).

Effects of MLT on autophagic flux during liver I/R

As shown in Fig. 4A, I/R caused a significant increase in the LC3-II/LC3-I ratio compared to that of sham-operated animals (549.5% of sham). MLT augmented the I/R-induced

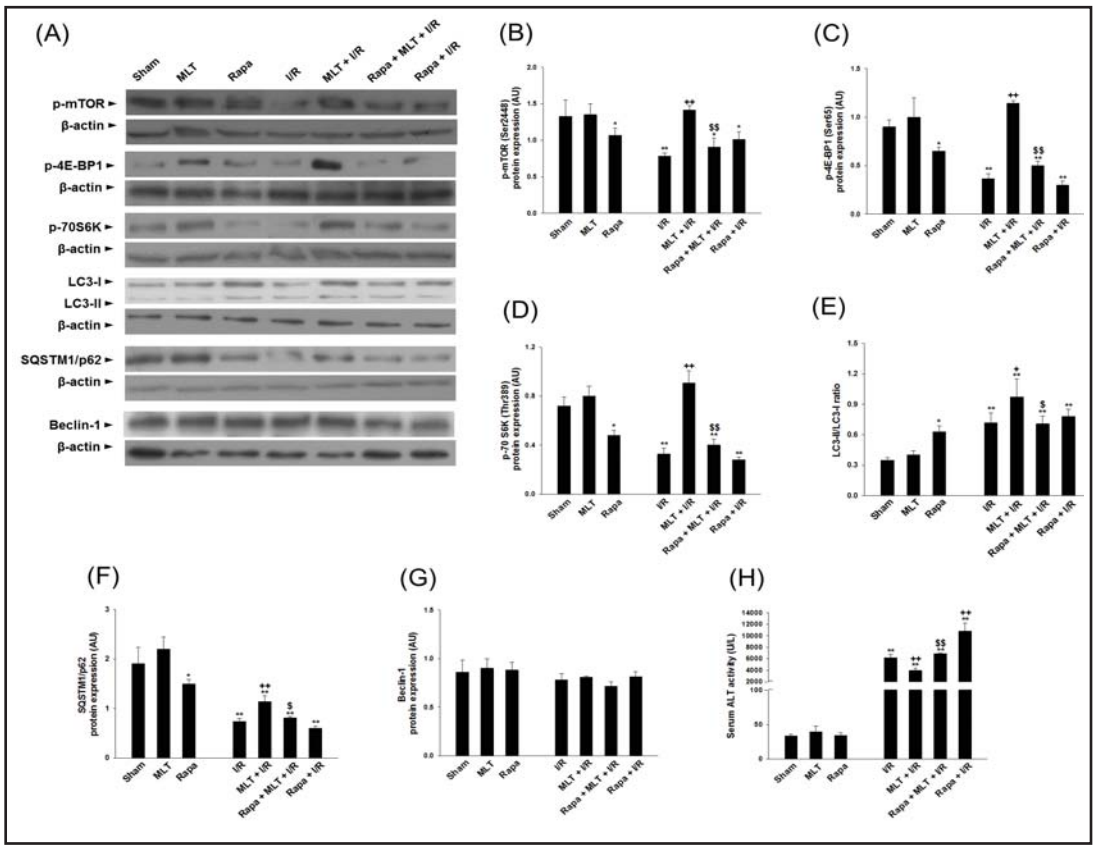

Fig. 6. Effect of rapamycin on hepatoprotection afforded by MLT. MLT was administered 15 min prior to ischemia and directly before reperfusion. Animals were pretreated with Rapa 1.5 h before ischemia. Western blot analysis was performed to measure protein levels of p-mTOR (B), p-4E-BP1 (C), p-70S6K (D), LC3-II/I (E), SQSTM1/p62 (F), and beclin-1 (G). At 5 h of reperfusion, serum ALT activity was measured (H). Results are presented as the mean ± S.E.M. of 6 to 8 mice per group. *, ** Significantly different ($p < 0.05$, $p < 0.01$) from sham-operated animals. $^{+}$, $^{++}$ Significantly different ($p < 0.05$, $p < 0.01$) from I/R animals. $^{\$}$, $^{\$\$}$ Significantly different ($p < 0.05$, $p < 0.01$) from MLT+I/R animals, respectively.

increase in LC3-II/LC3-I ratio (713.8% of sham). On the other hand, SQSTM1/p62 protein expression decreased significantly after reperfusion for 5 h compared to sham-operated animals (28.0% of sham). MLT attenuated this decrease (120.1% of sham), indicating inhibition of autophagic flux by MLT (Fig. 4B); however, protein expression of beclin-1 was not affected by either I/R or treatment with MLT (Fig. 4C).

To confirm our Western blot results, autophagosomes and related autophagic vacuoles were observed by TEM. Autophagosomes are characterized by double- and multiple-membrane structures containing cytoplasm or undigested organelles including mitochondria. Conversely, autolysosomes are identified as single-membrane structures with remnants of cytoplasmic components. Compared with the basal level of autophagic vacuoles in sham-operated animals (1.8 ± 0.3), the number of autophagic vacuoles increased following liver I/R (5.8 ± 0.8), which was attenuated by MLT (2.9 ± 0.4) (Fig. 4D).

LC3 is conjugated to phosphatidylethanolamine (PE) by a sequence of ubiquitination-like reactions that involves the Atg4 and Atg7 proteases as well as a different E2 analogue (Atg3). The lipidated form of LC3 is associated with the autophagosomal membrane, and thus we also assessed two hallmarks of the Atg machinery by measuring the protein expression of Atg3 and Atg7. After reperfusion for 5 h, the protein expression of Atg3 was significantly increased compared with sham-operated animals (257.9% of sham), which was not affected

by MLT. In addition, Atg7 protein expression was not affected by I/R or treatment with MLT (Fig. 4E and 4F).

Effects of MLT on autophagic signaling during liver I/R

To elucidate the mechanisms of MLT inhibitory effects on autophagy during liver I/R *in vivo*, we investigated the involvement of a mTOR-dependent pathway and the calpain system, which is independent of mTOR. The level of p-mTOR protein expression was significantly decreased by I/R (56.9% of sham) and this decrease was attenuated by MLT (97.6% of sham) (Fig. 5A). The phosphorylation status of 4E-BP1 (Ser 65) and 70S6K (Thr 389), two well-known downstream phosphorylation targets of mTOR, was significantly decreased in livers subjected to I/R compared to sham-operated animals (23.5% of sham and 51.5% of sham, respectively). These decreases were attenuated by MLT (88.7% of sham and 107.3% of sham, respectively) (Fig. 5B and 5C). The protein expression of calpain 1 and calpain 2 was significantly increased after reperfusion for 5 h (164.0% of sham and 256.1% of sham, respectively), which was not affected by MLT (Fig. 5D and 5E).

Effects of Rapa on MLT-induced protection during liver I/R

Rapa is a potent inducer of autophagy in various cell lines that functions by suppressing mTORC1, which in turn leads to a reduction in the phosphorylation of the downstream effectors 4E-BP1 and 70S6K. Therefore, we utilized Rapa to confirm the mTOR-dependent inhibition of autophagy by MLT and its possible contribution to amelioration of liver damage triggered by I/R. First, we confirmed the effect of Rapa by Western blot for p-mTOR, p-4E-BP1 and p-70S6K. In the presence of Rapa, phosphorylation of mTOR, 4E-BP1 and 70S6K was significantly lower compared to MLT-treated I/R animals (64.1% of MLT+I/R, 30.7% of MLT+I/R and 33.1% of MLT+I/R, respectively) (Fig. 6B, 6C, and 6D). In addition, treatment with Rapa restored autophagic flux as determined by the LC3-II/LC3-I ratio and protein expression of SQSTM1/p62. Interestingly, accumulation of LC3-II and the increase in SQSTM1/p62 induced by MLT treatment was abolished by Rapa after reperfusion for 5 h (72.7% of MLT+I/R and 70.8% of MLT+I/R, respectively) (Fig. 6E and 6F); however, Rapa did not affect the protein levels of beclin-1 (Fig. 6G). Finally, Rapa reversed the protective effects of MLT against I/R-induced hepatocellular damage as indicated by the significantly higher serum ALT activity compared to MLT-treated I/R animals (MLT + I/R; 3943.6 ± 400.4 U/L *vs.* Rapa + MLT + I/R; 6877.9 ± 112.1 U/L) (Fig. 6H).

Discussion

The pattern changes, functional role, and detailed mechanisms of autophagy in I/R remain controversial. Similarly, it is unclear whether autophagy is hepatoprotective or pro-death in liver I/R injury, and how it varies according to the type, magnitude, and timing of the stimulus. It was recently reported that inhibition of autophagy by chloroquine reduces liver I/R injury at the early phase (0-6 h after reperfusion), but contributes to worsened injury during the late phase (24-48 h after reperfusion) [24].

We initially performed a time course study using 60 min of moderate ischemia with various lengths of reperfusion and measured expression of autophagy-related proteins. Despite the differences in their relative role in the regulation of autophagy, both beclin-1 and LC3 represent core autophagy proteins. Beclin-1 is an upstream signaling mediator of autophagosome nucleation that interacts with a multi-protein regulatory signalosome, whereas LC3 acts downstream of these events by participating in elongation of autophagosomes [25]. In the present study, the level of beclin-1 protein expression increased immediately after reperfusion and recovered to basal levels 1 h after reperfusion, which were maintained up to 24 h of reperfusion. On the other hand, ischemia for 60 min did not affect the LC3-II/LC3-I ratio, while reperfusion for 1 and 5 h resulted in a significant increase in the

LC3-II/LC3-I ratio. This pattern was similar to the results of serum ALT activity, suggesting that increased autophagy is linked to liver damage and dysfunction during I/R.

Oxidative stress may affect autophagy through multiple mechanisms. During starvation, hydrogen peroxide directly oxidizes HsAtg4 and inhibits its cysteine protease activity, resulting in increased LC3 lipidation and autophagy in cancer cells [26]. Other scenarios such as opening of the mitochondrial permeability transition pore and induction of ER stress have been suggested as mediators of oxidative stress-induced autophagy [27]. Previously, anti-oxidant *N*-2-mercaptopropionyl glycine was shown to confer cardioprotection through blockage of the autolysosome formation step [28]. Moreover, MLT suppresses formation of oxygen-glucose deprivation-induced autolysosomes in endothelial cells by inhibiting peroxynitrite formation [29], and also protects against oxidative stress-related diseases such as cerulein-induced pancreatitis by reducing the abundance of autophagosomes [30]. In the present study, MLT exerted a strong protective effect against I/R-induced hepatocellular damage and oxidative stress. Moreover, MLT inhibited the increase in autophagic flux as indicated by the increased LC3-II/LC3-I ratio and SQSTM1/p62 protein expression. These results were supported by TEM images showing that MLT attenuates the increased number of autophagic vacuoles, which correlated with our Western blot results. Interestingly, MLT had similar effects on autophagic flux compared with CQ, a lysosomal protease inhibitor that interferes with lysosomal degradation (data not shown). Turnover of LC3-II in the presence of CQ indicates the delivery of LC3-II to lysosomes for degradation and completion of autophagic flux [31]. In the present study, CQ further increased the LC3-II/LC3-I ratio and blocked SQSTM1/p62 degradation, reflecting the increase of autophagic flux during I/R. In addition, CQ treatment attenuated the increase in serum ALT activity.

To the best of our knowledge, this is the first study demonstrating that the potent anti-oxidant MLT attenuates I/R-induces hepatocellular damage by inhibiting autophagic flux. However, Zaouali et al. [16] reported that autophagy is impaired during cold I/R injury and that a cocktail of MLT and trimetazidine improves steatotic liver graft preservation through activation of autophagy. These differences in results may be due to the different I/R models (warm *vs.* cold) and liver types (normal *vs.* steatotic) employed in the two studies.

LC3 is synthesized as proLC3 and is cleaved by Atg4B to produce LC3-I, which contains an exposed C-terminal Gly. LC3-I is then activated by Atg7, transferred to the E2-like enzyme Atg3, and finally conjugated to PE, where it is then designated as LC3-II [32]. In rat notochordal cells, high levels of glucose evoke ROS-induced autophagy involving up-regulation of Atg3 and Atg7 [33]. Moreover, a deleterious role of Atg7 has been reported due to its presence upstream of neuronal death pathways after hypoxic-ischemic injury [34], whereas studies of Atg3 in ischemic disease are very limited. In the present study, Atg3 protein expression significantly increased after reperfusion for 5 h, while Atg7 protein expression was not affected by I/R. In addition, MLT did not affect protein levels of Atg3 and Atg7. Considering the complexity of Atg machinery during autophagic flux, the precise cross-talk between MLT and the LC3 lipidation should be studied further.

The underlying mechanisms regulating autophagy have been widely studied, with mTOR, intracellular Ca^{2+}, calmodulin-dependent protein kinase kinase-β, and 5' AMP-activated protein kinase having been explored as upstream regulatory mechanisms of autophagy [35]. MLT has been shown to activate mTOR signaling and prevent ischemic brain injury and methamphetamine-induced neurotoxicity [36, 37]. In this study, mTOR activity was decreased in livers subjected to I/R as demonstrated by decreased phosphorylation of mTOR, 4E-BP1 and 70S6K, while MLT treatment attenuated this decrease. In order to further examine the association of mTOR-mediated autophagy suppression and hepatoprotective effects exerted by MLT, we utilized Rapa, which induces autophagy by binding with FKBP1 to specifically inhibit the mTOR complex 1 [19]. Treatment with Rapa alone augmented increased serum ALT activity compared to I/R animals, showing that excessive autophagy activation during I/R could be detrimental. Furthermore, pretreatment with Rapa abrogated the protective effects of MLT against I/R injury.

Abnormal Ca^{2+} influx in cellular compartments causes the activation of a number of Ca^{2+}-dependent degradative processes that are detrimental to cell survival [38]. Calpain is a Ca^{2+}-regulated cytosolic cysteine protease ubiquitously produced in animal cells and plays important roles in Ca^{2+}-dependent cellular events including cytokine processing and apoptosis/necrosis [39]. There are several reports of the MLT-mediated influence on intracellular concentrations of free Ca^{2+} and neuroprotection afforded by inhibition of Ca^{2+}-regulated signaling cascades such as mitochondrial and ER pathways [40]. In addition, MLT alleviates neuronal cell death by reducing calpain expression/activity, while it increases the protein levels of the endogenous calpain inhibitors calpastatin [41, 42]. In our study, protein expression of calpain 1 and calpain 2 in the liver were increased by I/R, but were not affected by treatment with MLT. Collectively, our data demonstrate that MLT inhibits autophagy through an mTOR-dependent mechanism.

In summary, our results suggest that increased autophagic flux might contribute to liver injury during I/R *in vivo.* Moreover, MLT ameliorates I/R-induced hepatocellular damage and inhibits autophagy by activation of mTOR signaling. These findings present a novel therapeutic potential of MLT by pharmacological modulation of autophagy for ischemic liver disease.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2013R1A1A3008145). Jung-Woo Kang (2011-0006724) and Hong-Ik Cho (2012016419) both received 'Global Ph.D. Fellowship Program' support from the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (MEST).

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