Melatonin protects hepatocytes against bile acid-induced mitochondrial oxidative stress via the AMPK-SIRT3-SOD2 pathway

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Abstract
Mitochondrial oxidative damage is hypothesized to contribute to the pathogenesis of chronic cholestatic liver diseases. Melatonin, an indolamine synthesized in the pineal gland, shows a wide range of physiological functions, and is under clinical investigation for expanded applications. Melatonin has demonstrated efficient protective effects against various types of oxidative damage in the liver system. This study investigates the protective effects of melatonin pretreatment on glycochenodeoxycholic acid (GCDCA)-induced hepatotoxicity and elucidates the potential mechanism of melatonin-mediated protection. Melatonin markedly decreased mitochondrial ROS (mROS) production in L02 cells treated with 100 μM GCDCA, and inhibited GCDCA-stimulated cytotoxicity. Notably, melatonin exerted its hepatoprotective effects by upregulating sirtuin 3 (SIRT3) activity and its expression level, thus regulating superoxide dismutase 2 (SOD2) acetylation and inhibiting the production of mROS induced by GCDCA. Moreover, siRNA targeting SIRT3 blocked the melatonin-mediated elevation in mitochondrial function by inhibiting SIRT3/SOD2 signaling. Importantly, melatonin-activated SIRT3 activity was completely ablated by AMP-activated, alpha 1 catalytic subunit (AMPK) siRNA transfection. Similar results were obtained in rat with bile duct ligation or BDL. In summary, our findings indicate that melatonin is a novel hepatoprotective small molecule that functions by elevating SIRT3, stimulating mitochondrial function, and may also promote liver tumorigenesis [2]. Mitochondrial oxidative stress, which can be defined as an imbalance between the production of mitochondrial reactive oxygen species (mROS) and the presence of antioxidant molecules, is one of the most important mechanisms contributing to the progression of cholestatic liver diseases [3,4]. For example, our previous study showed that excess mROS induce oxidative damage to unsaturated fatty acids, proteins, and mitochondrial DNA (mtDNA) in the mitochondria of extrahepatic cholestatic patients [5]. Glycochenodeoxycholic acid (GCDCA) is the main toxic component of bile acid in patients with extrahepatic cholestasis [6], and the toxicity of GCDCA is thought to be mediated through the stimulation of lipid peroxidation and the induction of mitochondrial oxidative damage in a variety of liver cells [7]. Thus, the development of drugs, which maintain mROS homeostasis, could be crucial for preventing GCDCA-induced liver injury.

Keywords: AMPK, GCDCA, hepatotoxicity, mitochondrial ROS, SIRT3, SOD2

Abbreviations: AMPK, AMP-activated, alpha 1 catalytic subunit; AST, aspartate aminotransferase; BDL, bile duct ligation; GCDCA, glycochenodeoxycholic acid; Mel, melatonin; mROS, mitochondrial reactive oxygen species; SIRT1-7, sirtuin 1-7; SOD2, superoxide dismutase 2

Introduction
Cholestasis, impairment in bile formation, occurs in a wide variety of human liver diseases [1]. High concentrations of certain bile acids induce rapid hepatocellular injury, inflammation, bile duct proliferation, and fibrosis, and may also promote liver tumorigenesis [2]. Mitochondrial oxidative stress, which can be defined as an imbalance between the production of mitochondrial reactive oxygen species (mROS) and the presence of antioxidant molecules, is one of the most important mechanisms contributing to the progression of cholestatic liver diseases [3,4]. For example, our previous study showed that excess mROS induce oxidative damage to unsaturated fatty acids, proteins, and mitochondrial DNA (mtDNA) in the mitochondria of extrahepatic cholestatic patients [5]. Glycochenodeoxycholic acid (GCDCA) is the main toxic component of bile acid in patients with extrahepatic cholestasis [6], and the toxicity of GCDCA is thought to be mediated through the stimulation of lipid peroxidation and the induction of mitochondrial oxidative damage in a variety of liver cells [7]. Thus, the development of drugs, which maintain mROS homeostasis, could be crucial for preventing GCDCA-induced liver injury.

Melatonin, the chief secretory product of the pineal gland, is best known for its free radical scavenging and anti-oxidative properties [8–11]. As a type of antioxidant, melatonin could interact with mitochondria to maintain mitochondrial homeostasis, and it has been found to be protective against mitochondrial oxidative damage under various pathological conditions, including hypoxia-, ischemia-, high-fat-, and metabolic poisons-induced hepatotoxicity [12–14]. In particular, melatonin administered at pharmacological doses exerts a therapeutic effect on cholestatic liver injury in rats with bile duct ligation (BDL) through its antioxidant and anti-inflammatory actions [15,16]. However, the mechanism by melatonin protects against GCDCA-induced oxidative stress and mitochondrial dysfunction in the human liver remains obscure.

Sirtuins (SIRTs) are a family of proteins that act as nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylases [17,18]. Seven SIRTs are present in mammals,
with various subcellular localizations and enzymatic activity, thus influencing their in vivo substrates and cellular functions [19]. Among them, SIRT3 is highly expressed in metabolically active tissues including the liver, is located to mitochondria, and regulates mitochondrial function via the deacetylation of key energy metabolic enzymes as well as antioxidant enzymes, such as superoxide dismutase 2 (SOD2) [20,21]. More importantly, SIRT3 physically interacts with and deacetylates SOD2, increasing SOD2 activity and thereby, inhibiting mitochondrial oxidative damage and maintaining mitochondrial function [22]. Recently, changes in SIRT3 expression and activity have been linked to various human liver diseases, including acute liver injury and hepatic lipid homeostasis [21,23].

Considering the profound impact of SIRT3 on mitochondrial function, we hypothesized that melatonin might attenuate oxidative injury in liver cells via mROS homeostasis regulation through the AMPK/SIRT3/SOD2 signaling pathway. Our results indicate that SIRT3 is required for melatonin’s hepatoprotection, as SIRT3 inhibition abolished its observed protection in GCDCA-induced hepatotoxicity.

Methods

Cell culture

The human normal liver cell line, L02, was purchased from the cell bank of the Institute of Biochemistry and Cell Biology (Shanghai, China). The L02 cells were cultured in 1640 medium (HyClone) supplemented with 10% heat-inactivated fetal calf serum or FCS (HyClone) and 1% (v/v) penicillin/streptomycin (Sigma, St Louis, MO, USA) in a 5% CO₂ humidified atmosphere at 37°C. At 80% confluence, the cells were treated with 1 μM melatonin for 2 h and then exposed to 100 μM GCDCA (Sigma, St Louis, MO, USA) for 6 h for the various experiments.

Animal studies

Two-month-old rats were maintained in a 12:12-h light—dark phase and fed ad libitum. They were adapted for 2 weeks to the above conditions before experiments. The rats receiving BDL were randomized into two groups: first group rats underwent BDL and were i.p. injected with melatonin (10 mg/kg), and second group rats underwent BDL and received an equal volume of normal saline. In addition, rats that received sham ligation of the bile duct were included as the sham group. Animals were euthanized under anesthesia at 7 days after surgery [24]. All animal experiments were approved by the Fuzhou General Hospital for accreditation of laboratory animal care.

Mitochondrial oxidative stress determination

Oxidative stress within the mitochondria was determined using MitoSOX Red (Invitrogen Corp., Carlsbad, CA, USA), a mitochondria-targeted fluorescent probe for the highly selective detection of mitochondrial superoxide [25]. After treatment with melatonin and GCDCA, L02 cells were incubated with culture medium containing 5 μM MitoSOX for 10 min at 37°C. Changes in fluorescence intensity were measured using microplate reader at excitation and emission wavelengths of 485 and 530 nm, respectively.

Measurement of mitochondrial membrane potential (ΔΨm)

A mitochondrial membrane potential assay kit including JC-1 (Beyotime Company, Shanghai, China) was used to measure the ΔΨm in L02 cells. Briefly, 1 × 10⁴ cells were seeded in a 96-well plate and incubated with 1xJC-1 in growth medium at 37°C for 20 min. Monomeric JC-1 green fluorescence emission and aggregate JC-1 red fluorescence emission were measured using an Infinite™ M200 Microplate Reader (Tecan, Mannedorf, Switzerland). The ΔΨm in each group was calculated as the fluorescence ratio of red to green and expressed as a multiple of the level in the control groups.

ATP content determination

ATP was measured with an ATP Determination Kit (Beyotime Company, Shanghai, China). Briefly, cell or liver tissue lysates were mixed with a reaction buffer from the kit containing 1 mM dithiothreitol, 0.5 mM luciferin, and 12.5 μg/ml of luciferase. After the solutions were mixed gently, intensity readings for the mixtures were obtained using an Infinite™ M200 microplate reader (Tecan, Mannedorf, Switzerland). The ATP concentrations in the samples were calculated using an ATP standard curve.

Cell viability assay

Cell viability was analyzed using a Cell Counting Kit-8 according to the manufacturer’s instructions (Dojindo Molecular Technologies, Kumamoto, Japan). Briefly, 1 × 10⁴ cells were seeded into 96-well plates. After treatment, 90 μl of medium and 10 μl of CCK-8 solution were added to each well. The cells were then incubated at 37°C for 2 h. After incubation, the absorption at 450 nm was measured using an Infinite™ M200 microplate reader.

SOD2 activity determination

Manganese SOD (SOD2) activity was determined with the SOD1 and SOD2 Assay Kit (Beyotime Company, China). The types of SOD were identified by adding SOD1 inhibitor A and B to inhibit SOD1 activity (i.e., to detect SOD2). The absorption at 450 nm was measured using an Infinite™ M200 Microplate Reader.

SIRT1-7 mRNA determination

Quantitative real-time PCR was applied to detect the content of SIRT3 mRNA level as previously described [7]. The SIRT3 probes were (5’- ACCCAGTGGCATTCCAGAC-3’) and (5’- GGCTTGGGTTGTGAA
AGAAG-3'); and the GAPDH probes were (5′-TGACAAACAGCTCAAGA T-3) and (5′-GAGTCTTT CCACGATACC-3′). The ddCt (SIRT3 to GAPDH) represented the SIRT3 mRNA in the cells. Other probes can be seen in Table I.

**SIRT3 enzymatic activity**

SIRT3 enzymatic activity was assayed using a fluorometric kit (Enzo Life Sciences Inc.) following the manufacturer’s instructions with modifications described below. A 40-μg sample of protein was incubated at 37°C for 45 min with specific substrates. Then, 25 μl of developer was added and the samples were incubated for an additional 45 min. Fluorescence intensity was measured with excitation at 350 nm and emission at 450 nm using an Infinite™ M200 Microplate Reader (Tecan, Mannedorf, Switzerland), as previously described [26].

**RNA interference of SIRT3 or AMPK**

L02 cells (1 × 10⁶) were transfected with either 100 nmol/l SIRT3 or AMPK-targeting small siRNA (Santa Cruz) or a control nonspecific siRNA (Santa Cruz). At 24 h after transfection, the cells were exposed to melatonin and GCDCA. The cells were then collected and processed for immunoblotting and other assays.

**Western blot analysis**

L02 cell or liver tissue lysates were centrifuged for 15 min at 12,000 × g, and the resulting supernatant was transferred to a new tube. The protein concentrations were determined using a Bradford protein assay kit (Beyotime, Shanghai, China). The protein samples were separated by SDS-PAGE. Following protein transfer to PVDF membranes, the membranes were blocked and then incubated overnight at 4°C with antibodies against SIRT3 (Abcam, MA, USA), SOD2 (acetyl K68) (Abcam, MA, USA), SOD2 (Santa Cruz, CA, USA), pAMPK (Santa Cruz, CA, USA), AMPK (Santa Cruz, CA, USA), and β-actin (sigma, St Louis, MO, USA). The protein signals were visualized using an ECL detection system (Thermo Scientific) [27].

**Assays of serum enzymes**

Serum aspartate aminotransferase (AST) was assayed using a commercial test kit (Uscn, SEB214Ra) according to the manufacturer’s instructions. The activity of serum enzyme determined is expressed as an international unit (IU/L).

**Statistical analysis**

Data were analyzed using GraphPad Prism-5 software. All experimental data are expressed as the mean ± SEM, and each experiment was performed a minimum of three times. One-way ANOVA was used to determine statistical significance, and P < 0.05 was considered to be statistically significant.

**Results**

**Melatonin reduces GCDCA-induced mitochondrial oxidative damage in L02 cells**

As GCDCA causes mitochondrial oxidative stress and cytotoxicity in L02 cells, we tested whether melatonin could interfere with this GCDCA-induced hepatotoxicity in vitro. Exposure to 100 μM GCDCA increased mROS production to 2.2-fold of that in control L02 cells. However, pretreatment with 1 μM melatonin successfully reduced this increase in mROS production (Figure 1A). Excess mROS exposure could result in the oxidative damage to mitochondrial function and cell viability. Thus, we analyzed the mitochondrial membrane potential (△Ψm), the ATP content and cell viability to explore the protective effects of melatonin against GCDCA. As expected, melatonin administration significantly reversed observed reductions in mitochondrial function in GCDCA-treated cells (Figure 1B–D).

**Melatonin suppresses GCDCA-induced mitochondrial oxidative damage not by upregulating SOD2 expression but by downregulating acetylated SOD2 expression**

SOD2, the primary mitochondrial oxidative scavenger, plays a crucial role in the regulation of mROS [28]. In an attempt to elucidate the mechanism by which melatonin inhibits mitochondrial oxidative stress, the effects of melatonin on SOD2 activity were investigated. As expected, SOD2 activity was reduced after L02 cells were exposed to 100 μM GCDCA, and melatonin pretreatment reversed this decrease (Figure 2A). SOD2 is regarded as a scavenging enzyme, and its activity is thought to be dependent on its mitochondrial level [29]. However, neither GCDCA nor melatonin treatment had a significant effect on SOD2 protein levels (Figure 2B). Moreover, SOD2 activity is also tightly regulated by acetylation at its lysine residues.

**Table I. Oligonucleotide description.**

| Gene | Sequences of oligonucleotide primers |
|------|-------------------------------------|
| SIRT1 | Sense: GGTATTTATGCTCGCCCTTGCA, Antisense: TGCAGAGAGATGGCTGGAA |
| SIRT2 | Sense: ATAACCACACCAACGGTAG, Antisense: AATGTCTTCTGCCCATCCAG |
| SIRT4 | Sense: GCTTTGCGTTGACTTTCAGG, Antisense: TCCAGAGGAGGACTTGCTG |
| SIRT5 | Sense: CCGAGAGGAGACTTGCTG, Antisense: CCGTGAGAGGACACCTTCTC |
| SIRT6 | Sense: TCTTCCAGTGTGTTGTTCA, Antisense: GTTTCTCAAAAGGTGTTTC |
| SIRT7 | Sense: CCTTTCCAGTTCAAGG, Antisense: CGGTTCCAGTGAAGG |

The sequence of forward and reverse primers used for cDNA amplification.
with K68 being an important acetylation site. We found the GCDCA-induced SOD2(acetyl K68) was reversed by the addition of melatonin (Figure 2C).

The mitochondrial-protective action of melatonin is SIRT3/SOD2 dependent on GCDCA-induced hepatotoxicity

Since SIRT3 function as a key regulator of SOD2 expression via epigenetic regulation of SOD2 gene, we sought to investigate the effect of melatonin on SIRT3. As shown in Figure 3B and C, GCDCA treatment resulted in a significant decrease in SIRT3 expression and activity in L02 cells. Notably, melatonin pretreatment resulted in a significant increase in both SIRT3 expression and activity. To confirm whether melatonin is involved in GCDCA-induced mitochondrial oxidative damage regulated by SIRT3, we used an siRNA targeting SIRT3. SIRT3 siRNA successfully inhibited SIRT3 protein activity and expression (Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1067806). Moreover, SIRT3 siRNA reversed the protective effects of melatonin on GCDCA-induced mROS production, △Ψm decreases, ATP decline, and cytotoxicity (Figure 4A–D). Most importantly, as shown in Figure 4E, the melatonin-induced decrease in acetylated-SOD2 expression was significantly attenuated by SIRT3 siRNA in L02 cells exposed to GCDCA. Together, these data suggest a SIRT3-dependent effect of melatonin on acetylated-SOD2 expression with GCDCA exposure.

AMPK accounts for the key role of the SIRT3/SOD2 pathway in the hepatoprotective effects of melatonin

It was recently reported that AMPK could act upstream of the SIRT3 pathway [31,32]. We assessed AMPK signaling using Western blotting for the total and phosphorylated form of the protein. As shown in Figure 5A, treatment with melatonin increased the expression of p-AMPK. However, AMPK siRNA completely prevented the induction of SIRT3 activity and but did not change SIRT3 expression in L02 cells (Figure 5B and C), indicating that AMPK is required for the activation of SIRT3 by melatonin. As expected, the melatonin-induced decrease in the expression of acetylated-SOD2 as well as the increase of cell viability was also blocked by AMPK siRNA (Figure 5D and Supplementary Figure 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1067806).

Melatonin suppresses mitochondrial oxidative damage in the livers of rats with BDL

To determine whether melatonin suppressed GCDCA-induced mitochondrial oxidative damage through a SIRT3/SOD2-dependent pathway in vivo, we examined the effects of melatonin in a BDL rat model. BDL resulted in

Figure 1. Melatonin reduces GCDCA-induced mitochondrial oxidative damage in L02 cells. (A) mROS production, (B) △Ψm level, (C) ATP level, and (D) cell viability. The results are expressed as a percentage of the control, which was set at 100%. The values are presented as means ± SEM; **p < 0.01 versus control group, *p < 0.05, ***p < 0.01 versus the GCDCA (100 μM) group. (n = 6).
Melatonin protects against GCDCA-induced mROS

Figure 2. Melatonin induces deacetylation of SOD2 after GCDCA treatment. (A) SOD2 activity. (B) The expression of SOD2. (C) The expression of SOD2 (acetyl K68). The results are expressed as a percentage of the control, which was set at 100%. The values are presented as the means ± SEM, **p < 0.01 versus control group, #p < 0.05, ##p < 0.01 versus the GCDCA (100 μM) group. (n = 6).

Liver damage, which was confirmed by increases in plasma AST levels and elevated AST level was partly attenuated by melatonin therapy (Figure 6A). As expected, in the liver, melatonin significantly increased p-AMPK expression (Figure 6B and Supplementary Figure 3A to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1067806). Moreover, SIRT3 protein levels and activity was decreased in BDL groups compared with shams. Interestingly, melatonin only restored SIRT3 activity without significantly affecting SIRT3 protein levels in livers of BDL rats (Figure 6B, C and Supplementary Figure 3B to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1067806). Next, the level of SOD2 (acetyl K68), a downstream target of the SIRT3 pathway, was also measured. Melatonin

Figure 3. Melatonin increases expression and activity of SIRT3 and inhibits acetylated SOD2 expression after GCDCA treatment. Melatonin enhances (A) the mRNA level of SIRT3, and (B) SIRT3 protein expression and (C) its activity at 6 h after exposure to 100 μM GCDCA. The results are expressed as a percentage of the control, which is set at 100%. The values are presented as the means ± SEM, **p < 0.01 versus the control group, and #p < 0.05 versus the GCDCA (100 μM) group. (n = 6).
Figure 4. SIRT3 siRNA pretreatment abolishes melatonin-enhanced mitochondrial function and biogenesis in GCDCA-injured L02 cells. The effects of melatonin and SIRT3 siRNA pretreatment on (A) mROS production, (B) $\Delta$Ψm level, (C) ATP level, (D) cell viability, (E) SOD2 activity, and (F) acetylated SOD2 expression. The results are expressed as a percentage of the control (Control-siRNA/GCDCA group taken as control), which is set at 100%. The values are presented as the means ± SEM, *p < 0.05, **p < 0.01 versus the Control siRNA/GCDCA (100 μM) group, ##p < 0.01 versus the Control siRNA/GCDCA (100 μM) melatonin group, and $p < 0.05 versus the SIRT3 siRNA/GCDCA (100 μM) group (n = 6).

significantly attenuated the upregulation of acetylated SOD2, restored the SOD2 activity, and increased ATP synthesis in the livers of BDL rats (Figure 6B, D, E and Supplementary Figure 3C to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1067806).

Discussion

A major advance of this study is that it is the first to demonstrate that melatonin, a novel small molecule drug, protects liver cells against GDCDA-induced hepatotoxicity by activating the AMPK/SIRT3/SOD2 signaling pathway.
Melatonin protects against GCDCA-induced mROS

Figure 5. AMPK accounts for the key role of the SIRT3/SOD2 pathway in the hepatoprotective effects of melatonin. (A) pAMPK expression. The effects of melatonin and AMPK siRNA treatment on (B) SIRT3 expression, (C) SIRT3 activity, and (D) acetylated-SOD2 expression. The results are expressed as a percentage of the control (Control-siRNA/GCDCA group taken as control), which is set at 100%. The values are presented as the means ± SEM, *p < 0.05 versus the Control siRNA/GCDCA (100 μM) group, **p < 0.05 versus the Control siRNA + GCDCA (100 μM) + melatonin group, and $$$p < 0.01 versus the AMPK siRNA + GCDCA (100 μM) group (n = 6).

and reducing mitochondrial oxidative damage. More importantly, our findings demonstrate a key role for SIRT3 in the inhibitory effects of melatonin on oxidative injury in liver cells, thus providing new insight into the hepatoprotective effects of melatonin.

Chronic liver cholestasis is responsible for the rapid development of progressive liver failure, for which there is still no effective therapy. GCDCA is the main toxic component of bile acid in patients with extrahepatic cholestasis. Experimental evidence indicates that increased mitochondrial oxidative stress plays an important role in the pathogenesis of extrahepatic cholestasis [25,33]. In cholestatic patients, the accumulation of mROS levels would decrease the synthesis of key subunits of respiratory complexes, reduce mtDNA copy number, and affect mitochondrial homeostasis [5,25]. Moreover, toxic bile acids have been found to increase mROS production, disrupt the mitochondrial membrane potential (ΔΨm), and inhibit ATP synthesis in liver cells [34]. Consistent with previous research, we found that GCDCA increased mitochondrial oxidative damage in vitro. Taken together, GCDCA-induced hepatotoxicity may be the consequence of mROS.

Evidence suggests that mitochondria are the primary source of ROS, and the homeostatic regulation of mROS
Figure 6. Melatonin suppresses mitochondrial oxidative damage in the livers of rats with BDL. (A) Serum AST level. (B) p-AMPK/SIRT3/ Ac-SOD2 expression. (C) SIRT3 activity, (D) SOD2 activity, and (E) ATP concentration. The results are expressed as a percentage of the control, which is set at 100%. The values are presented as the means ± SEM, *p < 0.01 versus the control group, and #p < 0.05, ##p < 0.01 versus the GCDCA (BDL) group. (n = 8).

is influenced by various enzymes involved in the tricarboxylic acid or TCA, ETC, OXPHOS, and free-radical-scavenging systems in mitochondria, especially SOD2 [35,36]. Indeed, SOD2 activity is crucial for maintaining mROS balance. In the present study, cadmium treatment resulted in a decrease in the activity of SOD2, an enzyme that is required for scavenging excessive mROS in mitochondria. SOD2 activity is thought to be dependent on its mitochondrial levels; however, SOD2 protein expression was unaffected by GCDCA treatment (Figures 2B, 6B and Supplementary Figure 3D to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1067806). Moreover, SOD2 is also regulated at the post-translational level, as modifications, such as acetylation, significantly influence its activity and the activity of SOD2 is inversely proportional to its acetylation [29,37]. Here, we observed that GCDCA increased the expression of acetylated SOD2 and inhibited SOD2 activity. These data suggest that GCDCA induces mROS production through the upregulation of acetylated SOD2 without affecting SOD2 protein levels.

Sirtuins are NAD⁺-dependent enzymes that have been implicated in a wide range of physiological and pathophysiological conditions, largely through their deacetylation of numerous substrates. Recently, three mitochondrial deacetylation enzymes have been identified, including SIRT3, SIRT4, and SIRT5 [38]. SIRT3 is the most robust mitochondrial deacetylase, and increases in SIRT3 expression were found to be accompanied by decreases in SOD2 deacetylation and increased SOD2 activity, limiting the accumulation of mROS [39]. Importantly, mice lacking SIRT3 showed striking hyperacetylation of mitochondrial
proteins, which was associated with accelerated development of metabolic syndrome, whereas a lack of SIRT4 or SIRT4 caused no change [40–42]. Our study shows that GCDCA decreased SIRT3 protein expression and activity, providing evidence for a detailed mechanism by which GCDCA increases both acetylation of SOD2 and mitochondrial oxidative damage in L02 cells.

Melatonin is an effective free radical scavenger and antioxidant, and it can prevent GCDCA-induced liver damage in young rats [24,43]. However, the hepatoprotective effects of melatonin are not simply related to the direct scavenging of free radicals and reduced inflammation. Considering the profound impact of SIRT3 on mitochondrial function, an important finding is that melatonin can activate SIRT3 and thus downregulate mROS production in a dose- and time-dependent manner (Figure 1A and Supplementary Figure 4 to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1067806). In the present experiments, we clearly show that melatonin can upregulate SIRT3 expression and its activity in liver cells, and in turn protect against GCDCA-induced mitochondrial oxidative damage. Nonetheless, blocking SIRT3 by siRNA could only partly abolish the liver cell protection conferred by melatonin suggesting that melatonin possibly acts via other pathways. One possibility is that melatonin readily enters mitochondria and has a direct role in maintaining SOD2 or other free-radical-scavenging enzymes to counteract the action of GDCCA; however, future studies are required to examine this possibility. Moreover, seven mammalian SIRTs (SIRT1-7) have been now identified with distinct subcellular localization, enzymatic activities, and substrates [44]. In our study, among the seven mammalian sirtuins, only SIRT1 and SIRT3 were inhibited by GCDCA, whereas melatonin increased SIRT3 levels but failed to restore SIRT1 levels (Supplementary Figure 5 to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1067806), which further confirmed the important role of SIRT3 in melatonin-mediated protective effect.

Mechanistically, it is unclear how melatonin increases SIRT3 levels and activities. We can consider two distinct alternatives. First, we found that melatonin-mediated increases in SIRT3 mRNA levels may contribute to changes in SIRT3 levels or activity (Figure 3A). Second, another new finding indicates that AMPK plays a key role in intracellular metabolism and is an attractive therapeutic target, and that switching on/off of AMPK leads to alteration in SIRT3 expression and activity [45]. Our studies demonstrated that melatonin triggers AMPK phosphorylation and activation, which is required for SIRT3 activity but not its expression in GCDCA-induced hepatotoxicity. Moreover, it is noteworthy that AMPK was phosphorylated by LKB1 CaMkkB, etc. [46,47]. Further study is needed to identify mechanisms by which melatonin exerts effects on the AMPK pathway.

In summary, our study clearly demonstrates that melatonin, as mediated by the AMPK-SIRT3-SOD2 axis, protects liver cells from GCDCA-induced mitochondrial oxidative damage. Taken together, our data illustrate a new molecular mechanism underlying the capabilities of melatonin, which should be explored for future clinical treatment of GCDCA-induced hepatotoxicity.

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Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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