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Molecular, Biological, Physiological and Clinical Aspects of Melatonin

Melatonin downregulates nuclear erythroid 2-related factor 2 and nuclear factor-kappaB during prevention of oxidative liver injury in a dimethylnitrosamine model

Abstract: Melatonin has potent hepatoprotective effects as an antioxidant. However, the signaling pathway of melatonin in the induction of antioxidant enzymes against acute liver injury is not fully understood. The study aimed to determine whether melatonin could prevent dimethylnitrosamine (DMN)-induced liver injury through nuclear erythroid 2-related factor 2 (Nrf2) and inflammation. Liver injury was induced in rats by a single injection of DMN (30 mg/kg, i.p.). Melatonin treatment (50 mg/kg/daily, i.p.) was initiated 24 hr after DMN injection for 14 days, after which the rats were killed and samples were collected. Serum and antioxidant enzyme activities improved in melatonin-treated rats, compared with DMN-induced liver injury group ($P < 0.01$). Melatonin reduced the infiltration of inflammatory cells and necrosis in the liver, and increased the expression of NADPH: quinone oxidoreductase-1, heme oxygenase-1, and superoxide dismutase-2, which were decreased by DMN. Melatonin increased expression of novel transcription factor, Nrf2, and decreased expression of inflammatory mediators including tumor necrosis factor-alpha, interleukin (IL)-1β, IL-6, and inducible nitric oxide synthase. The increased nuclear binding of nuclear factor-kappa B (NF-κB) in the DMN-induced liver injury group was inhibited by melatonin. Our results show that melatonin increases antioxidant enzymes and Nrf2 expression in parallel with the decrease of inflammatory mediators in DMN-induced liver injury, suggesting that melatonin may play a role of antioxidant defense via the Nrf2 pathway, by reducing inflammation by NF-κB inhibition.

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Key words: inflammation, liver injury, melatonin, nuclear erythroid 2-related factor 2, oxidative stress

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Introduction

Dimethylnitrosamine (DMN) is a representative chemical of a family of *N*-nitroso compounds that is present in processed meats and industrial products. It is a potent hepatotoxin, carcinogen, and mutagen [1]. DMN exerts carcinogenic effects, inflammation, hemorrhages and induces hepatic necrosis through metabolic activation by CYP2E1 in experimental animals [2]. Activation of nitrosamine by CYP2E1 in mouse liver stimulates Kuffer cells leading to generation of O_2^- and other reactive oxygen species (ROS) capable of damaging liver cells. Also, single and repeated exposure of DMN causes acute and chronic liver injury with necrosis, fibrosis, and nodular regeneration [3, 4].

Reactive oxygen species cause inflammation and contribute to the pathogenesis of various acute and chronic liver diseases such as acetaminophen overdose, alcoholic liver injury, toxin exposures, and viral hepatitis [5–7]. Furthermore, antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx), and heme oxygenase 1 (HO-1) may provide protection against deleterious effects of ROS [8]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor that regulates the cellular antioxidant response against ROS. Nrf2 is normally sequestered in the cytosol as an inactive complex with its suppressor Keap-1. Upon cell stimulation, Nrf2 is translocated from the cytosol to the nucleus, and sequentially binds to a promoter sequence called the antioxidant response (ARE), resulting in a cytoprotective response characterized by upregulation of antioxidant enzymes such as NADPH: quinone oxidoreductase-1 (NQO1), HO-1, SOD, and GPx, and decreased sensitivity to oxidative stress damage [9, 10]. Also, it has been reported that Nrf2 plays a broader role in modulating acute inflammatory responses [11, 12].

Melatonin is a highly lipophilic molecule that crosses cell membranes to easily reach subcellular compartments including mitochondria, where it seems to accumulate in high concentrations [13]. Also, melatonin interacts with lipid bilayers and stabilizes mitochondria inner membranes [14]. Melatonin and its metabolites exert potent hydroxyl and peroxyl radical-scavenging activities [15, 16]

and increases the efficiency of the electron transport chain in the mitochondria, which, as a consequence, reduces electron leakage and generation of free radicals [17]. Finally, melatonin protects nuclear and mitochondrial DNA, membrane lipids, and cytosolic proteins from oxidative stress [18, 19]. In several studies, melatonin has been reported to increase antioxidant enzymes and gives rise to a cascade of antioxidant compounds like cyclic 3-hydroxy-melatonin, N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), and with a highest potency *N*-acetyl-5-methoxykynuramine (AMK), which are metabolites of melatonin [17, 20–23]. In addition, protective effects of melatonin in sepsis has been reported and is attributed to the mediated decrease in the levels of inflammatory cytokines and oxidative stress [17, 24]. It was shown that melatonin protects against a number of hepatotoxic agents including nitrosamines [25]. However, the mechanism by which melatonin elicits hepatoprotective and antioxidant effects in association with Nrf2 is unclear. Here, we report that melatonin attenuates DMN-induced liver injury in rats via the induction of Nrf2-mediated antioxidant enzymes and the attenuation of inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, and inducible nitric oxide synthase (iNOS) by inhibition of nuclear factor kappaB (NF-κB).

Materials and methods

DMN-induced liver injury

Male 6-wk-old Sprague–Dawley rats weighing 180–200 g were used for the experiments. Animal care and all experimental procedures were conducted in accordance with the Guide for animal experiments edited by the Korea Academy of Medical Science. The animals were housed in an air-conditioned room at 25°C with a 12-hr dark/light cycle. All animals received humane care during the study with unlimited access to chow and water. Forty rats were divided to four groups of 10. A single injection of 30 mg/kg DMN (Sigma-Aldrich, St Louis, MO, USA) intraperitoneally was given to 10 rats and another 10 rats received melatonin (Sigma-Aldrich; 50 mg/kg/day) along with DMN. The control rats received saline or melatonin only. Melatonin was given daily at 17:00 hr for 14 days, whereas DMN-treated groups were given physiological saline. On day 14, all the rats were killed under ketamine anesthesia, and blood was collected and centrifuged at 500 g for 25 min at 4°C. Liver samples were quickly obtained. Biochemical parameters were studied immediately. For the remaining studies, serum and liver tissue samples were stored at −70°C.

Preparation of liver homogenate

Liver samples were weighed and homogenized in 0.15 M NaCl for lipid peroxidation parameters and the other studies. Tissue homogenates were sonicated two times at 30-s intervals. Homogenizatioin and sonication were performed at 4°C. After sonication, homogenates for lipid peroxidation and biochemical studies were centrifuged at 500 g for 10 min and 12,500 g for 15 min, respectively. Aliquots of the supernatants were used for other studies.

Determination of MDA, SOD, and GPx

Malondialdehyde (MDA) was measured using the thiobarbituric acid (TBA) method [26], with 1,1,3,3,-tetramethoxypropane as a standard. Briefly, 2 mL of fresh solution with 15% w/v trichloroacetic acid, 0.375% w/v TBA, and 0.25 mL/L HCl were added to 1 mL of 10% liver homogenate with 1.15% KCl. The mixture was heated at 95°C for 15 min. The solution was cooled to room temperature using tap water and centrifuged at 300 g for 5 min. The supernatant was collected in plastic tubes and centrifuged at 9000 g in a Sorvall centrifuge (Thermo Scientific, Newington, NH, USA) for 30 min. Absorption of the pink supernatant was determined spectrophotometrically at 532 nm. The amount of MDA was expressed as nmol/g wet tissue. SOD levels of homogenized liver tissue were measured using a commercial kit (Cayman Chemical, Ann Arbor, MI, USA) and homogenized GPx levels were measured using a commercial kit (Cayman Chemical).

Biochemical measures

Serum alanine transaminase (ALT), aspartate transaminase (AST), albumin, total protein, alkaline phosphatase (ALP), total bilirubin (T-bilirubin), and direct bilirubin (D-bilirubin) levels were analyzed by Green Gross Reference Lab (Seoul, Korea).

Liver histology

Liver samples were fixed in 10% buffered formaldehyde solution, embedded in paraffin, and sectioned. The 8-μm thick sections were stained with hematoxylin and eosin (H&E) for routine histology. For H&E staining, sections were stained with hematoxylin for 3 min, washed, and stained with 0.5% eosin for an additional 3 min. After an additional washing step with water, the slides were dehydrated in 70%, 96%, and 100% ethanol, and then in xylene. The degree of liver injury was examined using a light microscope (Olympus, Hamburg, Germany) by a pathology specialist who was blind to the nature of the sample.

Fluorescence-immunohistochemistry

Ten-micrometer thick frozen sections were incubated overnight at 4°C with 1:500 dilutions of rabbit anti-Nrf2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-NQO1 antibody (ABR, Golden, CO, USA). After washing three times with phosphate-buffered saline (PBS), detection of Nrf2 primary antibody was performed using a 1:1000 dilution of rabbit cy5-labeled secondary antibody raised in a mouse (Vector Laboratories, Burlingame, CA, USA). NQO1 was detected using a 1:1000 dilution of fluorescein isothiocyanate-labeled anti-mouse secondary antibody raised in a horse (Vector Laboratories). After washing with PBS three times, each slide was occluded with 50% glycerin buffer and was observed using a confocal laser scanning microcope (Olympus).

RNA Extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from a liver sample with Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. An aliquot of total RNA was reverse transcribed and amplified using reverse transcriptase and *Taq* DNA polymerase (Promega, Madison, WI, USA), respectively. The expression level of all transcripts was normalized to that of glyceraldehyde 3-phosphate dehydrogenase mRNA in the same tissue. The PCR product was electrophoresed on a 1.5% agarose gel, results were recorded by an imaging system (Kodak Molecular Imaging Systems, New Haven, CT, USA), and bands were quantitated using densitometry.

Western blot

Forty micrograms of liver protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the resolved proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Middlesex, UK). The membrane was blocked with 5% skim milk in 10 mM Tris–HCl containing 150 mM NaCl and 0.5% Tween 20 [tris-buffered saline (TBS)-T]. After washing with TBS-T, the membrane was then incubated with a 1:1000 dilution of primary antibody. After thorough washing with TBS-T, a 1:2000 dilution of horseradish peroxidase (HRP)-conjugated secondary antibody (New England Biolabs, Beverly, MA, USA) in TBS-T was applied to the membrane and the blot was developed using an enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ, USA).

Immunohistochemistry

Immunostaining was performed on 4-μm sections after deparaffinization. Microwave antigen retrieval was performed in citrate buffer (pH 6.0) for 10 min prior to peroxidase quenching with 3% H_2O_2 in PBS for 10 min. Sections were then washed in water and preblocked with normal goat or rabbit serum for 10 min. In the primary antibody reaction, slides were incubated for 1 hr at room temperature in a 1:500 dilution of antibody. The sections were then incubated with biotinylated secondary antibodies (1:1000) for 1 hr. Following a washing step with PBS, the streptodavidin-HRP was applied. Finally, the sections was developed with diaminobenzidine tetrahydrochloride substrate for 10 min, and counterstained with hematoxylin. At least three random fields of each section were examined at a magnification of ×200 and analyzed by a computer image analysis system (Media Cybernetics, Silver Spring, MD, USA).

Electrophoresis mobility shift assay (EMSA)

Nuclear extracts from liver were prepared by treating samples with 1 mL of lysis buffer (10 mM Tris–HCl, 10 mM NaCl, 3 mM $MgCl_2$, 1% NP-40, pH 7.9) on ice for 4 min. After centrifugation at 1500 g for 10 min, samples were resuspended in 200 μL of extraction buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid, 1 mM dethiothreitol, 1 mM phenylmethyl sulfonyl fluoride, pH 7.9) and incubated on ice for 30 min. After centrifugation at 12,000 g for 5 min, the supernatant containing the nuclear protein extract was harvested and stored at −70°C. Protein concentration was determined with a Lowry protein assay reagent from Bio-Rad (Hercules, CA, USA). Ten micrograms of the nuclear proteins were incubated with [γ-^{32}P] ATP-labeled ARE and NF-κB probe. A 100-fold excess of unlabeled oligonucleotide (competitor) was added where necessary. After 30-min incubation at room temperature, samples were electrophoresed through a 5% polyacylamide gel at 100 V for 2 hr. The gel was dried and exposed to an X-ray film.

Statistical analyses

Data are expressed as mean ± S.E.M. Statistical analysis was performed using ANOVA and unpaired Student's *t*-test. A *P*-value of < 0.05 or < 0.01 was taken to indicate statistical significance. Statistical calculations were performed using SPSS software for Windows (Version 10.0; SPSS, Chicago, IL, USA).

Results

Effect of melatonin in rats with DMN-induced liver injury

The effect of melatonin on DMN-induced liver injury was evaluated by determining the levels of AST, ALT, ALP, total protein, albumin, T-bilirubin, and D-bilirubin. As shown in Table 1, the melatonin-treated group exhibited decreased levels of AST, ALT, ALP, T-bilirubin, and D-bilirubin, and increased levels of albumin and total protein compared with the DMN-induced liver injury group. Among these, AST, ALT, ALP, T-bilirubin, and D-bilirubin were significantly decreased by melatonin ($P < 0.05$). In histological analysis, the saline and melatonin alone-treated groups showed normal liver architecture. However, in DMN-injected rats widespread destruction of liver architecture was evident, which was characterized by massive and severe hepatocyte necrosis as well as infiltration of inflammatory cells (Fig. 1). In melatonin-treated rats, hepatocytes were relatively well preserved.

Hepatic level of MDA, SOD, and GPx by melatonin in rats with DMN-induced liver injury

Oxidative stress was quantified through the levels of MDA, SOD, and GPx in liver tissue homogenates as an indicator of lipid peroxidation and activity of antioxidant enzymes. The liver MDA level of DMN-induced liver injury group was significantly higher compared with the control and melatonin alone-treated groups. However, the level of liver MDA in the melatonin-treated group was decreased compared with the DMN-induced liver injury group (Fig. 2A). The liver SOD level of DMN-induced liver injury group was significantly lower than those of control, whereas the SOD level of melatonin-treated group was 3.5-fold higher than in the DMN-induced liver injury group (Fig. 2B). Also, the liver GPx level in the melatonin-treated group

Table 1. Effects of melatonin on serum parameters in rat with dimethylnitrosamine (DMN)-induced liver injury

Parameter	Group			
	Con	Mel	DMN	DMN + Mel
ALT (IU/L)	74.2 ± 2.8	80.0 ± 0.0	92. 1 ± 4.8†	81.3 ± 3.5*
AST (IU/L)	112.1 ± 8.3	106.2 ± 19.2	182.3 ± 13.1†	140.4 ± 9.4*
Alkaline phophatase (IU/L)	1035.5 ± 18.8	851.0 ± 23.7	1179.1 ± 20.2†	1023.5 ± 15.3*
Total protein (g/dL)	5.82 ± 0.86	5.74 ± 0.00	5.31 ± 1.01	5.46 ± 0.84
Albumin (g/dL)	2.41 ± 0.32	2.35 ± 0.04	2.16 ± 0.22	2.33 ± 0.31
T-bilirubin (mg/dL)	0.053 ± 0.012	0.027 ± 0.010	0.151 ± 0.011†	0.124 ± 0.021*
D-bilirubin (mg/dL)	0.014 ± 0.002	0.03 ± 0.020	0.058 ± 0.004†	0.021 ± 0.001*

Values are expressed as mean ± S.E.M. (n = 8). $^{*}P < 0.05$, compared with dimethylnitrosamine (DMN)-induced group and $^{\dagger}P < 0.05$, compared with control.
Con, control; Mel, melatonin alone-treated group; DMN, DMN-induced liver injury group; DMN + Mel, DMN plus melatonin treated group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; T-bilirubin, total bilirubin; D-bilirubin, direct bilirubin.

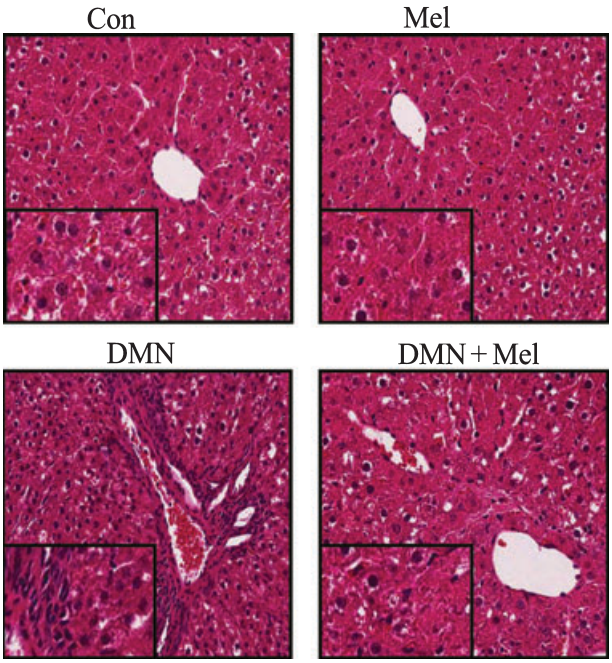

Fig. 1. Histological analysis of liver sections. Con, control; Mel, melatonin alone-treated group; dimethylnitrosamine (DMN), DMN-induced liver injury group; DMN + Mel, DMN plus melatonin treated group. Original magnification ×200 and ×400 (insert).

showed a twofold increase compared with the DMN-induced liver injury group (Fig. 2C).

Involvement of Nrf2 by melatonin in rats with DMN-induced liver injury

Nuclear erythroid 2-related factor 2 is sequestered in the cytoplasm by Keap1 protein under normal conditions and the translocation of Nrf2 into the nucleus is essential for the transactivation of various target genes. To identify whether melatonin is effective on regulation of Nrf2, we investigated mRNA and protein expression of Nrf2 by RT-PCR and Western blot. As shown in Fig. 3A, the mRNA expression level of Nrf2 in the DMN-induced liver injury group was lower than those of control. However, the mRNA expression level of Nrf2 in the melatonin-treated group was threefold higher than that of the DMN-induced liver injury group ($P < 0.05$). Also, the Nrf2 protein expression in the nucleus after melatonin treatment was increased in parallel with the increased Nrf2 protein expression in the cytoplasm. Interestingly, the Nrf2 expression of the nucleus reached nearly twofold as those of cytoplasm (Fig. 3B). This result was confirmed by the results of immunostaining with Nrf2. Melatonin increased the expression of Nrf2 as compared with DMN-induced liver injury group (Fig. 3C). Nrf2 is a major transcription factor regulating ARE-driven antioxidant genes. Therefore, we attempted to determine whether melatonin could activate Nrf2 in rats with DMN-induced liver injury by EMSA. Nuclear extracts from rat liver after melatonin treatment with [γ-^{32}P]-labeled Nrf2 oligonucleotide displayed enhanced ARE-binding Nrf2 activity in DMN-induced liver injury (Fig. 3D) as well as increased ARE-binding Nrf2 activity by melatonin treatment alone.

mRNA and protein expression of NQO1, HO-1, SOD2, and GST-α by melatonin in rats with DMN-induced liver injury

To determine whether Nrf2-regulated genes could be induced by melatonin in DMN-induced liver injury, we examined the mRNA expression of NQO1, HO-1, and SOD2 by RT-PCR. mRNA expression of NQO1 of DMN-induced liver injury group was low compared to control (Fig. 4A). However, NQO1 mRNA expression of the melatonin-treated group was significantly higher than that of DMN-induced liver injury group ($P < 0.01$). Also both HO-1 and SOD2 mRNA expression of the melatonin-treated group were significantly increased compared to the DMN-induced liver injury group ($P < 0.05$). To confirm these results, we examined protein expressions of NQO1, HO-1, and GST-α by melatonin in DMN-induced liver injury using Western blot. As shown in Fig. 4B, melatonin induced various Nrf2-driven proteins and significantly augmented expression of NQO1, HO-1, and GST-α compared to DMN-induced liver injury group ($P < 0.05$).

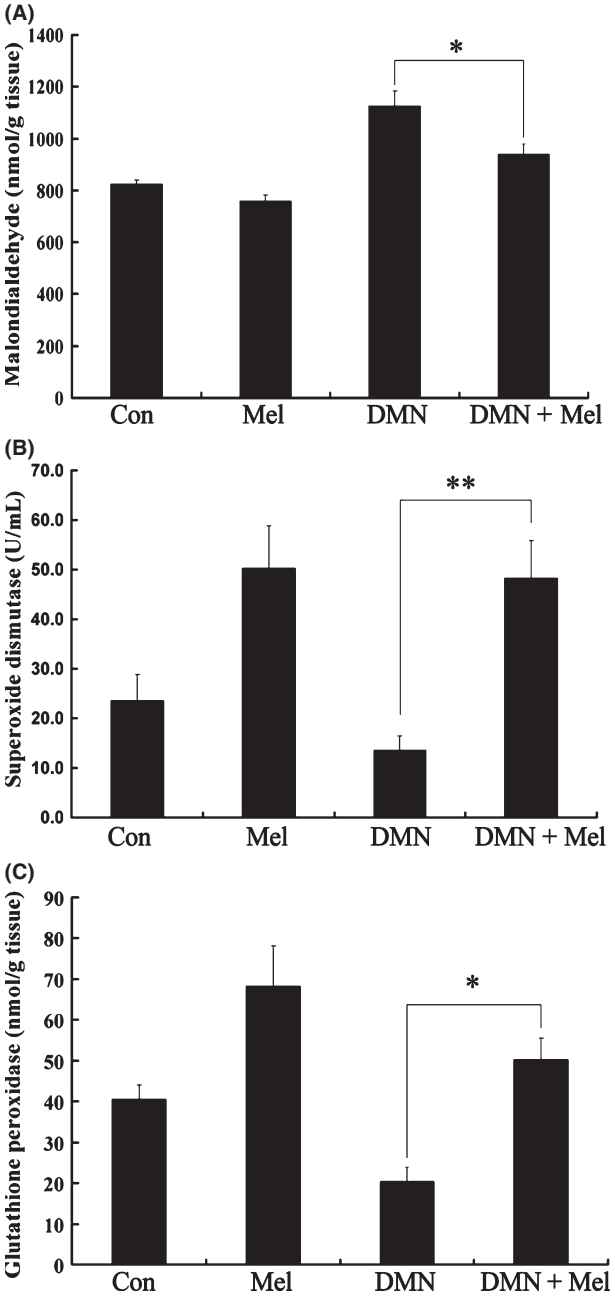

Fig. 2. Hepatic level of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx) after melatonin treatment in rats with dimethylnitrosamine (DMN)-induced liver injury. (A) Hepatic level of MDA (nmol/g tissue); (B) hepatic level of SOD (U/mL); (C) hepatic level of GPx (nmol/g tissue). Each value represents the mean ± S.E.M. of at least three separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to DMN-induced liver injury group. Con, control; Mel, melatonin alone-treated group; DMN, DMN-induced liver injury group; DMN + Mel, DMN plus melatonin treated group.

The expression of NQO1 and HO-1 in rats with DMN-induced liver injury

Antioxidant enzymes such as NQO1 and HO-1 provide significant protection against oxidative and electrophilic stress. To further confirm the effect of melatonin in DMN-induced liver injury, we identified the expression of NQO1 and HO-1 by immunohistochemistry. The effect of melatonin on NQO1 and HO-1 is illustrated in Fig. 5, and the staining was also quantified by metaplus software. A negligible quantity of NQO1 was expressed in the DMN-induced liver injury group. Melatonin treatment resulted in significant elevated expression of NQO1, evident as an intense brown staining ($P < 0.01$). Also, HO-1 expression of the DMN-induced liver injury group was statistically lower than control ($P < 0.05$), whereas there was a significant difference in the level of HO-1 expression between the DMN-induced liver injury group and the melatonin-treated group ($P < 0.05$).

Expression of Nrf2 and NQO1 by melatonin in rats with DMN-induced liver injury

Because multiple ARE contains the NQO1 promoter, we investigated whether melatonin affected the expression of the Nrf2 and NQO1 in DMN-induced liver injury by confocal microscopy (Fig. 6). DMN-induced liver injury group exhibited negligible Nrf2 (red) and NQO1 (green) expression, whereas melatonin treatment in DMN-induced liver injury group produced an increased expression of Nrf2 and NQO1 compared to the DMN-induced liver injury group. Also, treatment with melatonin alone produced more Nrf2 and NQO1 expression than evident in the control group. In addition, red and green fluorescent dots for Nrf2 and NQO1 in the melatonin-treated group were merged within the nuclei, identified by blue fluorescence of 4′,6-diamidino-2-phenylindole staining compared to DMN-induced liver injury group (pink).

The expression of inflammatory mediators and NF-κB activity by melatonin in rats with DMN-induced liver injury

To identify whether melatonin was effective on inflammation, we investigated the expressions of TNF-α, IL-6, IL-1β, and iNOS in rats with DMN-induced liver injury. As shown in Fig. 7A, TNF-α, IL-6, IL-1β, and iNOS mRNA expression in the DMN-induced liver injury group was significantly decreased by melatonin treatment ($P < 0.01$ and $P < 0.05$). As NF-κB is known to regulate the expression of several genes that are essential for initiating and promoting inflammation including TNF and IL-1β, we assessed whether melatonin was effective on NF-κB activation. EMSA showed that DMN-induced NF-κB binding activity was attenuated by melatonin treatment (Fig. 7B). For specificity control, nuclear extracts were exposed to radiolabeled probe in the presence of a 100-fold excess of unlabeled sequences.

Discussion

The present study shows that melatonin prevents liver injury induced by DMN. Melatonin was observed to attenuate severe liver injury and MDA, and increase antioxidant enzymes including SOD, GPx, NQO1, and HO-1 through Nrf2 activation. In addition, melatonin inhibited inflammation mediated by NF-κB in rat with

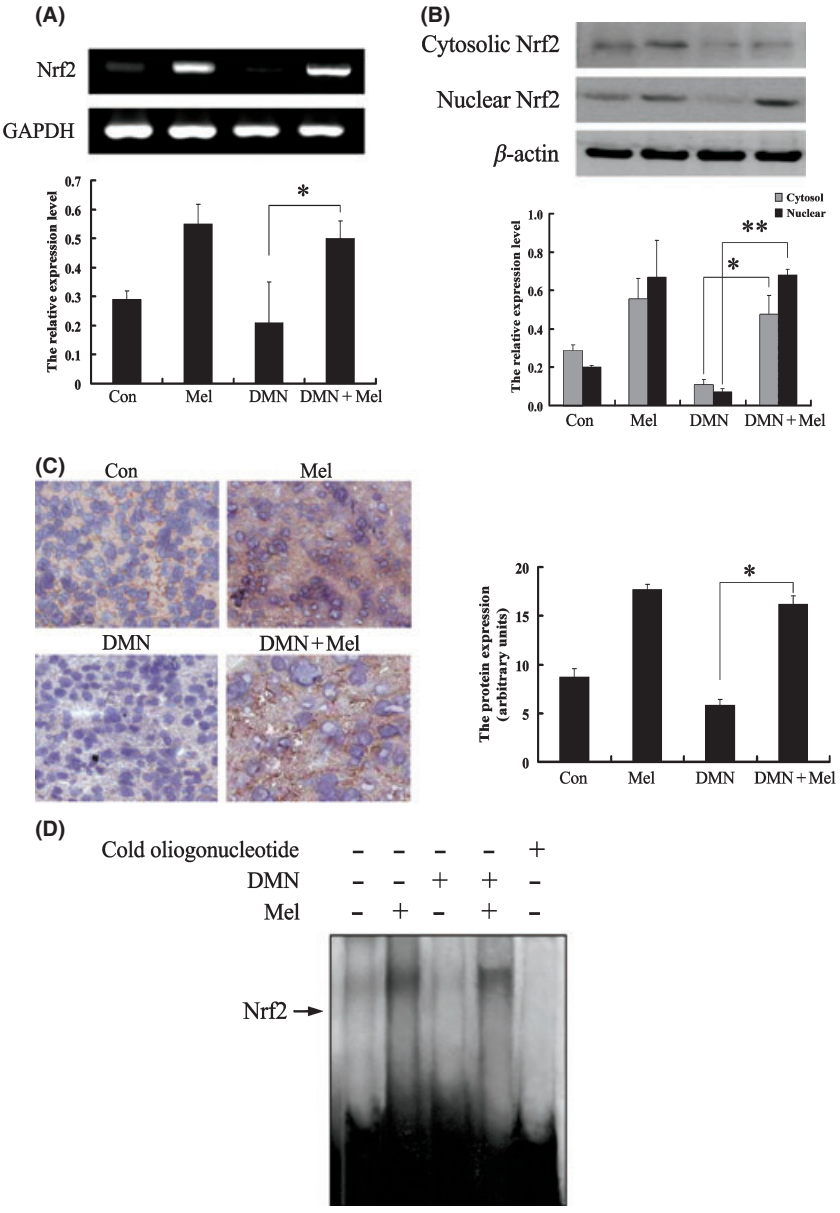

Fig. 3. Effect of melatonin on nuclear erythroid 2-related factor 2 (Nrf2) expression and its transcriptional activity in rats with dimethylnitrosamine (DMN)-induced liver injury. (A) mRNA levels of Nrf2 after melatonin treatment in DMN-induced liver injury by RT-PCR. (B) Cytosolic and nuclear Nrf2 expression after melatonin treatment in DMN-induced liver injury by Western blot. (C) Nrf2 protein expression in DMN-induced liver injury by immunohistochemistry. (D) Transcriptional activity of Nrf2/ARE after melatonin treatment in DMN-induced liver injury by EMSA. Quantification of Nrf2 mRNA and protein expression was performed by densitometry analysis and Metaplus software. Each value represents as mean ± S.E.M. of at least three separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to DMN-induced group. Con, control; Mel, melatonin alone-treated group; DMN, DMN-induced liver injury group; DMN + Mel, DMN plus melatonin treated group.

DMN-induced liver injury. To our knowledge, this is the first report showing that melatonin, via the Nrf2/ARE-mediated pathway, increases antioxidant enzymes. Also, it was confirmed that melatonin has an anti-inflammatory effect by NF-κB inhibition in DMN-induced liver injury.

Reactive oxygen species are a natural byproduct of oxidative energy metabolism and are thought to be a physiologic modulator of a number of intracellular signaling pathways [27]. Thus, the induction of antioxidant enzymes is an important event in the cellular stress response, during which a diverse array of electrophilic and oxidative toxicants can be eliminated or inactivated before they damage critical cellular macromolecules [28]. In addition, enhancement of oxidative stress has been implicated in DMN-induced fibrosis [29]. Indeed, it has been reported that melatonin enhances antioxidant enzymes such as GPx, GST, CAT, and SOD in ischemia/reperfusion-induced cardiac injury and endosulfan-induced oxidative tissue damage, and bile duct ligation-induced hepatic oxidative stress in rats [30–32]. In agreement, we observed that melatonin decreased severe hepatocyte necrosis (Fig. 1) and increased SOD and GPx with concomitant decreases of MDA production in rats with DMN-induced liver injury (Fig. 2).

Melatonin increases antioxidant enzyme induction in various disease models [30–32]. However, the mechanism for induction of antioxidants by melatonin has remained unclear. One mechanism responsible for the antioxidant effect is the Nrf2/ARE pathway. Nrf2 is sequestered in the cytoplasm by the cytosolic repressor Keap 1 and plays a critical role in the maintenance of the cellular redox balance. Keap1 links Nrf2 to the cytoskeleton to retain Nrf2 in the cytoplasm, thereby promoting its degradation. Oxidative stress enables Nrf2 to escape Keap1-mediated proteasomal

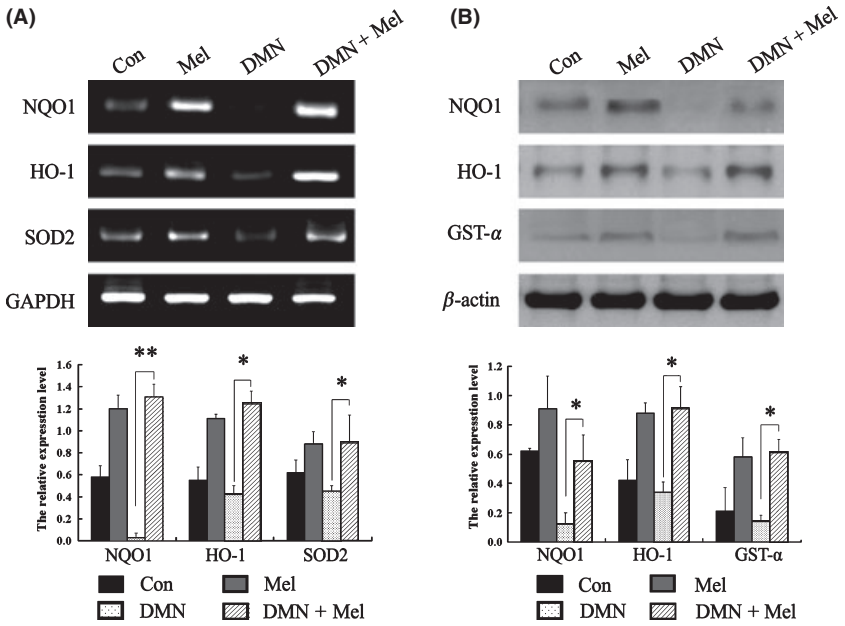

Fig. 4. Expression of antioxidant enzymes after melatonin treatment in rats with dimethylnitrosamine (DMN)-induced liver injury. (A) Expression of mRNA of NADPH: quinone oxidoreductase (NQO1), heme oxygenase 1 (HO-1), and superoxide dismutase 2 (SOD2) after melatonin treatment in rats with DMN-induced liver injury was measured by RT-PCR. (B) Expression of protein of NQO1, HO-1, and glutathione-S-transferase (GST)-α by melatonin in rats with DMN-induced liver injury was measured by Western blot analysis. Quantifications of NQO1, HO-1, SOD2, and GST-α mRNA and protein expressions of those were performed by densitometry analysis. Each value represents the mean ± S.E.M. of at least three separate experiments. $*P < 0.05$ and $**P < 0.01$, compared to DMN-induced group. Con, control; Mel, melatonin alone-treated group; DMN, DMN-induced liver injury group; DMN + Mel, DMN plus melatonin treated group.

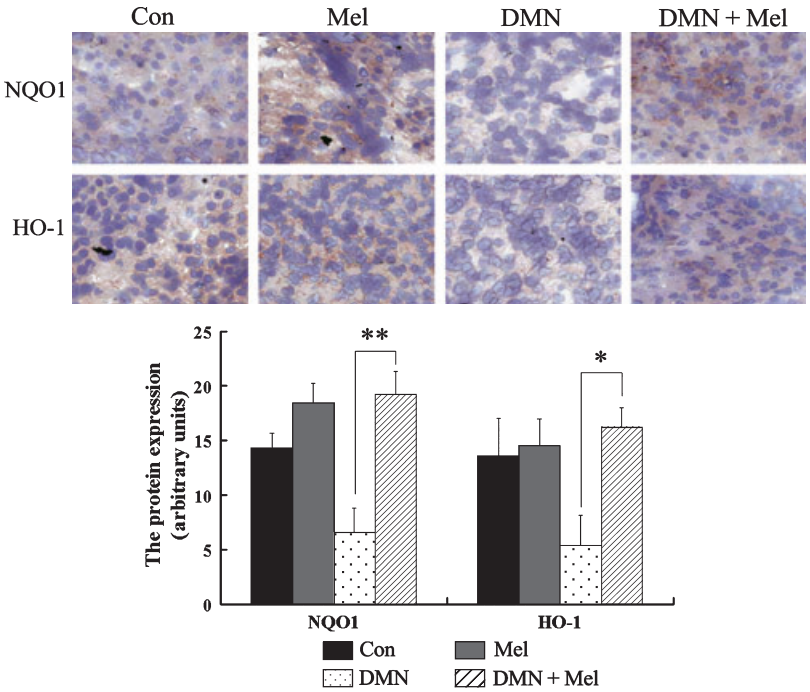

Fig. 5. Expression of NADPH: quinone oxidoreductase (NQO1) and heme oxygenase 1 (HO-1) after melatonin treatment in rats with dimethylnitrosamine (DMN)-induced liver injury by immunohistochemistry. Expressions of NQO1 and HO-1 were quantified using Metaplus software. Values are presented as the mean ± S.E.M. of at least three separate experiments. $*P < 0.05$ and $**P < 0.01$, compared to DMN-induced group. Con, control; Mel, melatonin alone-treated group; DMN, DMN-induced liver injury group; DMN + Mel, DMN plus melatonin treated group. Original magnification ×200.

degradation, leading to Nrf2 stabilization, subsequent nuclear translocation, and binding to ARE [33]. Nrf2-null mice are extremely susceptible to chemical oxidative and electrophilic stress [34], contributing to increased hepatotoxicity by acetaminophen [35], ethanol [36]. In our study, Nrf2 expression in the DMN-induced liver injury group was lower than those of controls, whereas melatonin treatment induced activation of Nrf2 and enhanced nuclear translocation and subsequent ARE binding (Fig. 3). Also, the cytoplasmic stability of Nrf2 was increased by melatonin; the concomitant

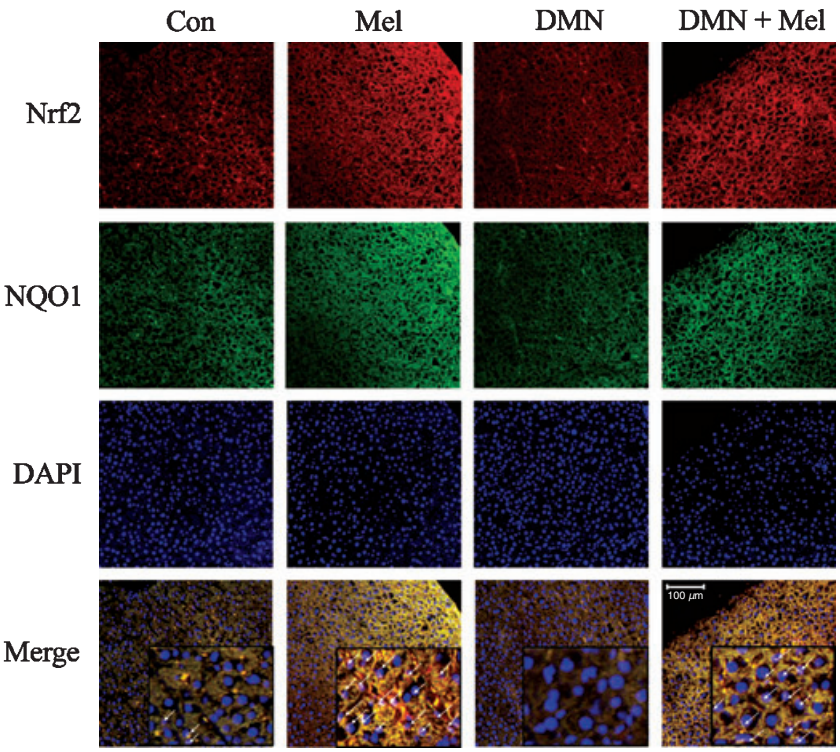

Fig. 6. Confocal microscopy of fluorescent immunohistochemical staining of nuclear erythroid 2-related factor 2 (Nrf2) and NADPH: quinone oxidoreductase (NQO1) after melatonin treatment in rats with dimethylnitrosamine (DMN)-induced liver injury. The cy5-conjugated secondary antibody staining indicates the Nrf2 (red) by anti-Nrf2 antibody and fluorescein isothiocyanate-conjugated secondary antibody staining indicates the NQO1 (green) by anti-NQO1 antibody. 4′,6-diamidino-2-phenylindole staining indicates the nucleus (blue) and merged immunofluorescent images with Nrf2 and NQO1 are also presented. Con, control; Mel, melatonin alone-treated group; DMN, DMN-induced liver injury group; DMN + Mel, DMN plus melatonin treated group. Scale bars represent 100 μm. Original magnifications ×200 and ×400 (insert).

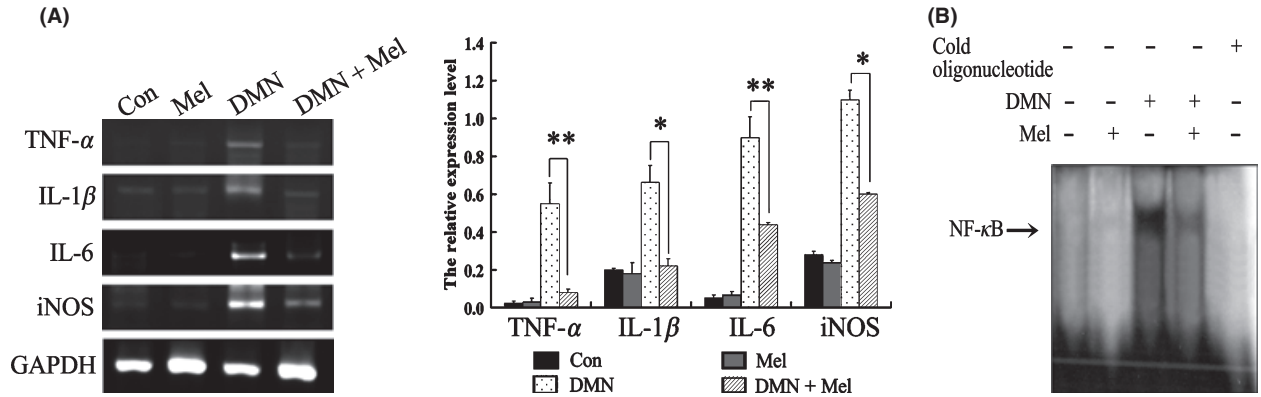

Fig. 7. Expression of inflammatory mediators and nuclear factor kappa B (NF-κB) activity after melatonin treatment in rats with dimethylnitrosamine (DMN)-induced liver injury. (A) Expression of mRNA of TNF-α, interleukin-1β (IL-1β), IL-6, and iNOS after melatonin treatment in rats with DMN-induced liver injury was measured by RT-PCR. (B) Transcriptional activity of NF-κB after melatonin treatment in DMN-induced liver injury by electrophoresis mobility shift assay. Quantifications of TNF-α, IL-1β, IL-6, and iNOS mRNA expressions were performed by densitometry analysis. Each value represents the mean ± S.E.M. of at least three separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to DMN-induced group. Con, control; Mel, melatonin alone-treated group; DMN, DMN-induced liver injury group; DMN + Mel, DMN plus melatonin treated group.

increase in nuclear Nrf2 was similar to other reports [37, 38]. This observation shows that melatonin may be involved in stabilization and activation of Nrf2 in both the cytoplasm and nucleus. In addition, as compared to the localization of Nrf2 in the cytosol and nucleus, melatonin increases the expression of Nrf2 in the nucleus rather than in the cytoplasm. It also seems that melatonin may induce nuclear translocation more than stabilization of Nrf2 in the cytoplasm upon DMN-induced liver injury. This is consistent with a previous study that showed that curcumin attenuates DMN-induced liver injury via Nrf2 in rats [39]. In fact, several dietary phytochemicals such as the triterpenoid compounds isothiocyanates, flavonoids, and curcuminoids, which have antioxidant effect like melatonin, induce Nrf2/ARE-mediated gene expression by increasing the protein levels of Nrf2 or suppressing its turnover by ubiquitination [40, 41]. As previous reports, our study also shows that melatonin activates Nrf2/ARE in DMN-induced liver injury.

Most of the genes encoding antioxidant enzymes have an ARE sequence in their promoter region. Nrf2 is an important transcription factor that regulates ARE-driven NQO1, HO-1, SOD2, and GST gene expression. Thus, we

investigated whether melatonin could induce antioxidant enzymes including NQO1, HO-1, SOD2, and GST-α using Western blot and immunohistochemistry. The increase of Nrf2 by melatonin seemed to induce the increase of NQO1, HO-1, SOD2, and GST-α mRNA and protein expression against DMN-induced liver injury. Hence, melatonin may trigger the translocation of Nrf2 into the nucleus and promote transcription of target genes such as NQO1, HO-1, SOD2, and GST-α.

Activation of the NF-κB signaling pathway is central to the pathophysiology of the inflammatory response, and NF-κB can be activated by oxidative stress, bacterial endotoxin, and cytokines [42]. The functional importance of NF-κB in inflammation is based on its ability to regulate the promoters of multiple inflammatory genes including TNF-α, IL-1β, IL-6, and iNOS. TNF-α is a major initiator of inflammation and is released early after an inflammatory stimulus [43]. IL-1β is regarded as the prototypic 'multifunctional' cytokine and is induced in a multitude of consequences of cell types [44]. IL-6 is increased after TNF-α exposure and is an important proinflammatory cytokine that contributes to morbidity and mortality in 'uncontrolled' inflammation [45]. iNOS produces large amounts of NO, which gives rise to the formation of peroxynitrite [46]. Melatonin reduces the expression of TNF-α, iNOS, IL-1β, and IL-6 in the liver of old and castrated female rats [47]. Also, melatonin inhibits NF-κB binding activity in several tissues and different experimental systems such, primary neurons [48], and cultured murine macrophages [49]. In addition, melatonin inhibits iNOS expression through NF-κB inhibition in murine macrophages and a thioacetamide-induced liver injury rat model [50, 51]. In prostate cancer cells, melatonin significantly enhances TNF-α induced cell death by decreasing NF-κB activation [52]. Recently, it has been reported that melatonin shows strong anti-inflammatory effects by inhibition of NF-κB and TNF-α expression in chronic gastric ulcers [53]. In this study, we investigated whether melatonin had an anti-inflammatory effect in DMN-induced liver injury. We demonstrated that melatonin decreases inflammation mediators including TNF-α, IL-1β, IL-6, and iNOS by NF-κB inactivation in rats with DMN-induced liver injury.

Nuclear factor kappaB and Nrf2 are major transcription factors that are involved in regulating proinflammatory and antioxidant genes, respectively. Recently, studies of interplay between Nrf2 and NF-κB signaling have been reported. Nrf2-deficient mice display increased NF-κB activation in response to lipopolysaccharide [54]. Furthermore, disruption of Nrf2 enhances upregulation of NF-κB activity and proinflammatory cytokines in the brain after traumatic brain injury [55]. Our results show that melatonin increases antioxidant enzymes such as NQO1, HO-1, SOD2, and GST-α by Nrf2 activation and decreases inflammatory mediators by NF-κB inhibition in rats with DMN-induced liver injury. Thus, an anti-inflammation effect by NF-κB inhibition may be achieved by activation of antioxidant machinery through Nrf2 activation, as well as anti-inflammatory effect by melatonin itself.

In conclusion, we showed that melatonin has a hepatoprotective effect and antioxidant defense through the Nrf2 pathway and an anti-inflammation effect by NF-κB inhibition in rats with DMN-induced liver injury. Therefore, we suggest that the Nrf2 activation by melatonin might be a novel mechanism for protecting DMN-induced liver damage.

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