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Anti-fibrotic effects of L-2-oxothiazolidine-4-carboxylic acid via modulation of nuclear factor erythroid 2-related factor 2 in rats

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L-2-Oxothiazolidine-4-carboxylic acid (OTC) is a cysteine prodrug that maintains glutathione in tissues. The present study was designed to investigate anti-fibrotic and anti-oxidative effects of OTC via modulation of nuclear factor erythroid 2-related factor 2 (Nrf2) in an *in vivo* thioacetamide (TAA)-induced hepatic fibrosis model. Treatment with OTC (80 or 160 mg/kg) improved serum liver function parameters and significantly ameliorated liver fibrosis. The OTC treatment groups exhibited significantly lower expression of α-smooth muscle actin, transforming growth factor-β1, and collagen α1 mRNA than that in the TAA model group. Furthermore, the OTC treatment groups showed a significant decrease in hepatic malondialdehyde level compared to that in the TAA model group. Nrf2 and heme oxygenase-1 expression increased significantly in the OTC treatment groups compared with that in the TAA model group. Taken together, these results suggest that OTC restores the anti-oxidative system by upregulating Nrf2; thus, ameliorating liver injury and a fibrotic reaction. [BMB Reports 2012; 45(6): 348-353]

INTRODUCTION

Hepatic fibrosis is a wound-healing response to chronic liver injury, including persistent viral infection, alcohol or drug toxicity and hereditary metal overload. Hepatic fibrosis is characterized by excessive production and deposition of extracellular matrix (ECM) (1). Activated hepatic stellate cells (HSCs) have been identified as the primary source of ECM synthesis in patients with liver fibrosis (2). In the normal liver, HSCs are in a quiescent state within the space of Disse, where they primarily store retinoids. However, in response to injury, these cells transform into proliferative, fibrogenic proinflammatory, and contractile myofibroblasts that express α-smooth muscle actin (α-SMA). These activated HSCs also contribute to inflammation by expressing adhesion molecules, cytokines, and chemokines. Transforming growth factor-β1 (TGF-β1) is a major cytokine involved in the activation process and causes enhanced proliferation of HSCs and ECM synthesis (3).

Oxidative stress and reactive oxygen species (ROS) induce liver fibrosis, cholestasis, hepatic inflammation, and necrosis of liver cells (4, 5). ROS are also associated with the activation and proliferation of HSCs and their collagen synthesis. In contrast, antioxidants and glutathione (GSH) precursors inhibit activation and proliferation of HSCs (6-8). The liver has a sophisticated antioxidant defense system to avoid damage by ROS. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that positively regulates basal and inducible expression of a large battery of cytoprotective genes (9-11). Expression of many antioxidant proteins and detoxifying enzymes such as NAD(P)H:quinone oxidoreductase 1, heme oxygenase-1 (HO-1), the glutamate-cysteine ligase catalytic subunit, microsomal epoxide hydrolase, glutathione-S-transferases, and sulfiredoxin 1 are controlled by this transcription factor. The significance of Nrf2 in the liver has been established, as livers of Nrf2-null mice are more susceptible to various oxidative/electrophilic stress-induced pathologies than those of wild-type mice. In contrast, both pharmacological and genetic models of hepatic Nrf2 activation are protective against oxidative/electrophilic stress (10).

L-2-Oxothiazolidine-4-carboxylic acid (OTC), a prodrug of cysteine (12, 13) is a substrate for the ubiquitous intracellular enzyme 5-oxoprolinase that generates cysteine from OTC intracellularly. GSH is synthesized from cysteine and is a vital intracellular and extracellular protective antioxidant. OTC replenishes cellular GSH stores and is more effective than N-acetylcysteine at refilling these stores (14). Using the Tsukamoto- French enteral model of alcohol-induced liver injury, Iimuro *et al.* showed that OTC protects against liver injury by increasing circulating GSH (15). However, the protective role of OTC in hepatic

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fibrosis has not been investigated.

The purpose of the present study was to determine the anti-fibrotic effect of OTC on thioacetamide (TAA)-induced hepatic fibrogenesis and also to investigate the effects of OTC on oxidative stress and the expression of hepatic Nrf-2.

RESULTS

Anti-fibrotic effects of OTC in TAA-induced hepatic fibrosis rats

Rats in the TAA group showed many granulo-nodules on the liver surface. The liver felt hard, and its edges were obtuse. Rats in the OTC (80 or 160 mg/kg) treatment groups showed significant improvement compared that in the TAA model group. There were few granulo-nodules distributed throughout the liver surface, which was relatively smooth compared with that of the TAA group. We also assessed the biochemical liver function parameters, such as serum alanine aminotransferase (ALT) and albumin. The serum ALT level increased significantly in the TAA group compared with that in the control group (360.2 ± 128.0 IU/L vs. 46.8 ± 16.7 IU/L, $P < 0.01$) and was significantly ameliorated with OTC treatment (106.8 ± 30.8 IU/L or 89.3 ± 22.1 IU/L vs. 360.2 ± 128.0 IU/L, $P < 0.01$).

The effects of OTC on TAA-induced liver injury were evaluated by histopathological examination of liver sections using hematoxylin-eosin and Masson's trichrome staining. Rats in the TAA group showed extensive necrosis, marked bridging fibrosis, and thick fibrous septa compatible with cirrhosis, whereas the OTC-treated rats attenuated these alterations and suffered only from mild inflammation and occasional portal-to-portal bridging or periportal fibrosis (Fig. 1A and B). When we assessed the fibrosis stage and grade of inflammatory activities using a pathological score, the OTC-treated rats showed significant reductions in fibrosis and lobular activity scores compared with those in the TAA group (Fig. 1C).

The expression of α-SMA, an indicator of activated HSCs, was analyzed by Western blot analysis. The level of α-SMA expression increased significantly in the TAA-treated rats (Fig. 2A). However, treatment with OTC markedly decreased α-SMA expression. The α-SMA expression level by immunohistochemical analysis exhibited almost the same pattern as the Western blot analysis (Supplementary Fig. 1). TGF-β1 is a key mediator of fibrogenesis. TAA treatment increased the production of TGF-β1 mRNA, whereas OTC reduced the production of TGF-β1 mRNA in the TAA-treated rat liver, which agreed with the reduction in fibrosis (Fig. 2B). Next, we examined the effect of OTC on collagen type I mRNA expression in TAA-treated rat liver. The TAA-induced elevation of collagen type 1 mRNA expression decreased in the OTC-treated rats, consistent with the reduction in fibrosis score seen upon histopathological analysis of the liver (Fig. 2B).

Anti-oxidative effects of OTC via modulation of Nrf2 mediated HO-1 expression

Anti-oxidative stress activity was assayed through the malondialdehyde (MDA) level in liver tissue homogenates. MDA, a product of lipid peroxidation, increased following TAA-induced liver injury. However, the levels of liver MDA in the TAA + OTC 80 and TAA + OTC 160 groups decreased significantly compared to those in the TAA group ($P < 0.01$) (Fig. 3).

Next, we investigated the effects of OTC on expression of hepatic Nrf2 and HO-1. The immunohistochemical assay for Nrf2 revealed that the OTC treatment groups showed significant induction of Nrf2 expression compared with that in the TAA model group (Fig. 4A). Furthermore, hepatic Nrf2 and HO-1 expression was determined by Western blot analyses (Fig. 4B). As a result, Nrf2 and HO-1 expression increased slightly in the TAA group compared with that in the control group. However, the OTC treatment groups showed significant induction of Nrf2 and HO-1 expression compared to that in the TAA group ($P < 0.01$).

DISCUSSION

We made the following observations during this study: (i) OTC significantly attenuated hepatic fibrosis in the TAA-induced *in vivo* model accompanied by the inactivation of HSCs and inhibition of TGF-β1 or collagen type I production; (ii) OTC significantly decreased hepatic MDA levels and markedly increased the expression of transcription factor Nrf2 and its target protein

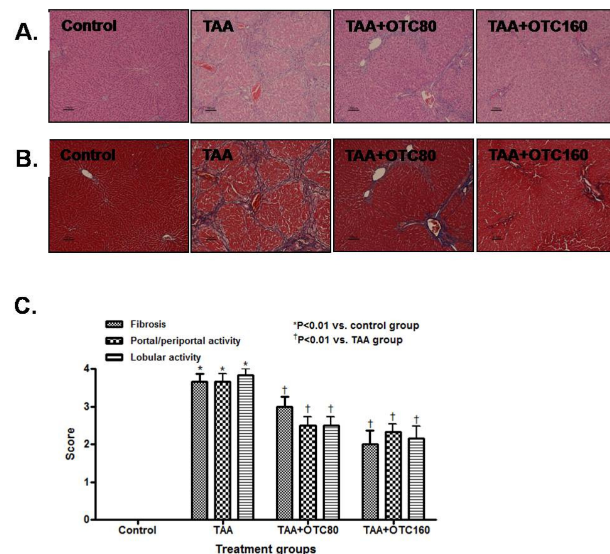
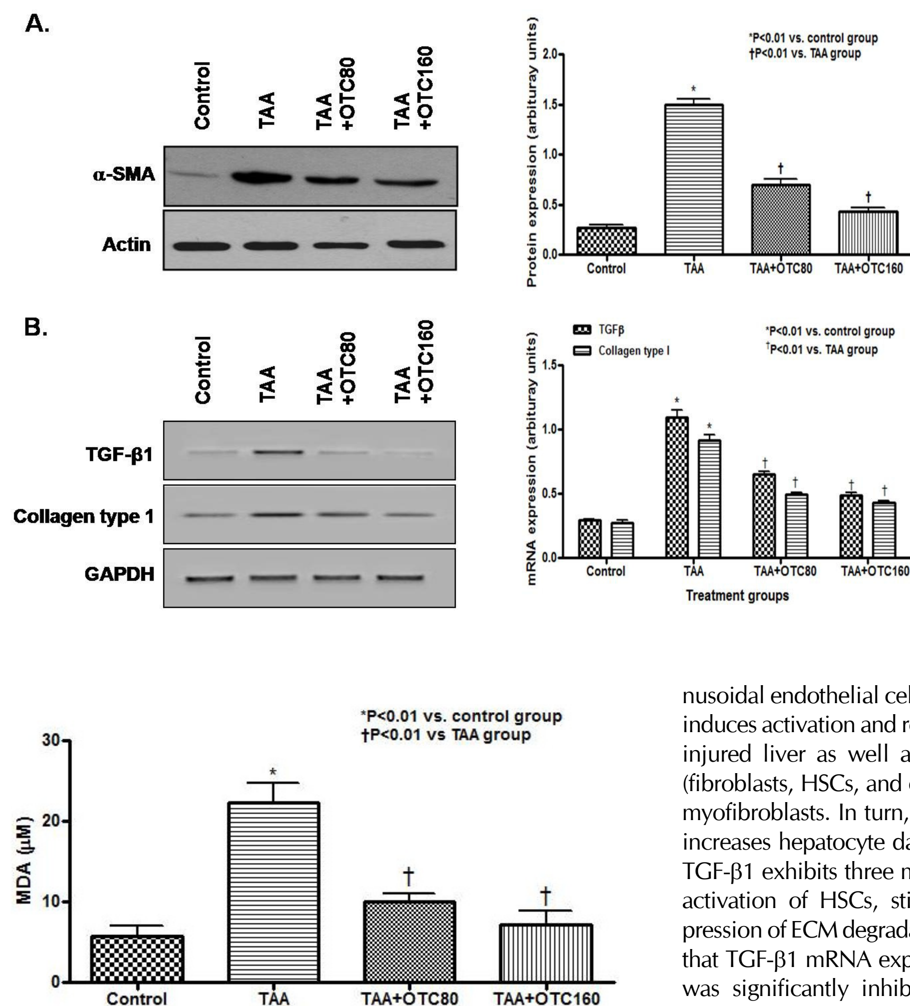


Fig. 1. Histopathological findings and scores. (A) Hematoxylin-eosin staining (magnification, ×100). (B) Masson's trichrome staining (magnification, ×100). (C) Histopathological scoring was conducted with the Scheuer's scoring system. The thioacetamide (TAA) group was given TAA (200 mg/kg body weight, i.p.) three times per week for 8 weeks. The TAA + L-2-oxothiazolidine-4-carboxylic acid (OTC) 80 and TAA + OTC 160 groups received OTC (80 and 160 mg/kg body weight, i.p., respectively) three times per week 30 min before the TAA injections for the same period. Data are mean ± SE (n = 6).



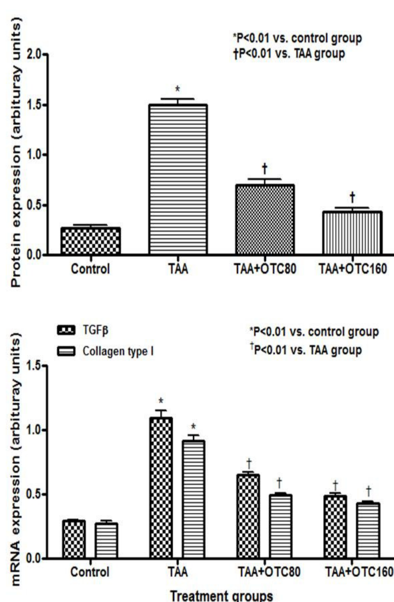


Fig. 2. Effects of L-2-oxothiazolidine-4-carboxylic acid (OTC) on the expressions of α-smooth muscle actin (α-SMA), transforming growth factor (TGF)-β1, and collagen type I in the liver. (A) Western blot analysis and densitometric analysis for α-SMA. (B) Reverse transcription-polymerase chain reaction (RT-PCR) and densitometric analyses for TGF-β1 and collagen type I mRNA. Data are mean ± SE of three determinations.

Fig. 3. Effect of L-2-oxothiazolidine-4-carboxylic acid (OTC) on the hepatic malondialdehyde (MDA) levels. Hepatic MDA level was measured spectrophotometrically. Data are mean ± SE of three determinations.

HO-1. Taken together, our results suggest that OTC restores the anti-oxidative system via upregulating Nrf2; thus, ameliorating liver injury and a fibrotic reaction.

HSCs are regarded as the primary target cells for inflammatory stimuli in the injured liver, and their activation may be initiated by paracrine stimuli from injured neighboring cells, including hepatocytes and Kupffer cells (16). In this study, OTC protected hepatocytes from injury and improved liver function in TAA-treated rats. The hepatoprotective effects of OTC may decrease paracrine stimuli, which can lead to hepatic fibrosis via activated HSCs. TGF-β1 is a well-characterized cytokine that appears to play a central role in fibrosis by mediating cross-talk between parenchymal, inflammatory, and collagen-expressing cells. TGF-β1, which is released by activated Kupffer cells and sinusoidal endothelial cells, triggers apoptosis in hepatocytes and induces activation and recruitment of inflammatory cells into the injured liver as well as differentiation of resident liver cells (fibroblasts, HSCs, and epithelial cells) into collagen-producing myofibroblasts. In turn, activated HSCs secrete TGF-β1, which increases hepatocyte damage and lymphocyte infiltration (16). TGF-β1 exhibits three major molecular effects on liver fibrosis: activation of HSCs, stimulation of ECM synthesis, and suppression of ECM degradation (17, 18). The present study showed that TGF-β1 mRNA expression in the livers of TAA-treated rats was significantly inhibited by OTC. Additionally, OTC significantly inhibited TAA-induced activation of HSCs, as indicated by the markedly decreased α-SMA expression. This finding suggests that the anti-fibrotic effect of OTC may be associated with the inhibition of TGF-β1 production and the inactivation of HSCs.

Oxidative stress, which favors the mitochondrial permeability transition, promotes hepatocyte necrosis and/or apoptosis (19, 20). ROS are generated mainly via the mitochondrial electron transport chain or via activation of cytochrome P450 2E1, NADPH oxidase, xanthine oxidase or mitochondrial damage. Cellular mechanisms to control oxidative stress are critical to cellular homeostasis and include enzyme systems such as catalase, superoxide dismutases, peroxiredoxins, glutathione peroxidase, and a number of thiol reductases that are ultimately linked to either NADH or NADPH as the source of reducing equivalents (21). Non-enzymatic electron receptors such as vitamin E, vitamin C, and glutathione also play a major role in the cellular response to oxidative stress. The ROS generated directly affect the behavior of HSCs and myofibroblasts (19). ROS upregulate the expression of critical fibrosis-associated genes such as collagen type

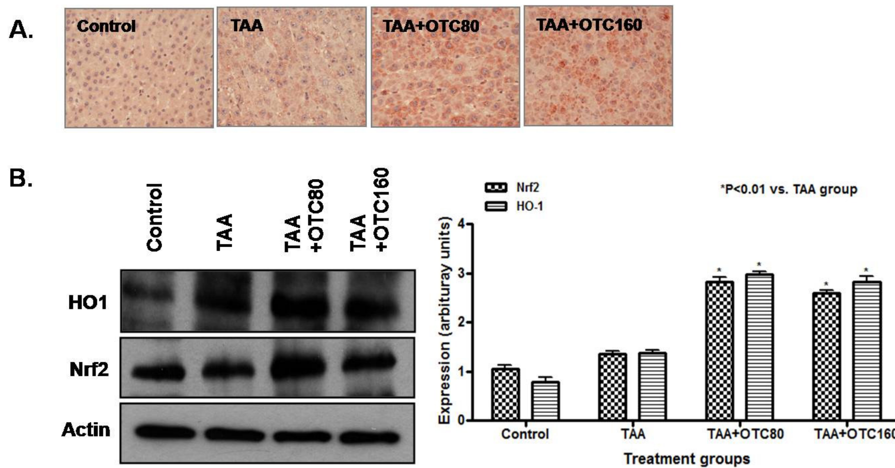


Fig. 4. Effects of L-2-oxothiazolidine-4-carboxylic acid (OTC) on the expressions of hepatic nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1). (A) Immunohistochemical staining for Nrf2 (magnification, × 400). (B) Western blot analysis for hepatic Nrf2 and HO-1. Three independent experiments showed similar profiles, and a representative blot is illustrated. The data are mean ± SE of three determinations.

I, monocyte chemoattractant protein 1, and tissue inhibitor of metalloproteinase 1 via activation of signal transduction pathways and transcription factors, including c-jun N-terminal kinase, activator protein-1, and nuclear factor kappa B (4). ROS generation in HSCs and myofibroblasts occurs in response to several known pro-fibrogenic mediators, including angiotensin II, platelet-derived growth factor, TGF-β1, and leptin. Overall, a decrease in antioxidant defense such as a decrease in GSH, catalase or superoxide dismutase, in conjunction with enhanced lipid peroxidation leads to a pro-fibrogenic response by enhancing collagen type I protein expression. Indeed, various compounds with antioxidant properties are protective against liver disease (22). These include the antioxidant vitamins E and C, glutathione-augmenting agents (e.g., glutathione esters, *N*-acetylcysteine, *S*-adenosyl-L-methionine), sylimaryin, betanine, and polyenylphosphatidylcholine. Our results showed that the TAA-treated rats exhibited increased levels of hepatic MDA and OTC reduced this increase. MDA is a secondary product of oxidative stress formed during lipid peroxidation. The reduction in MDA level in the OTC-treated rats may have been due to its antioxidative and free-radical scavenging effect. In this study, TAA-treated rats exhibited an increased level of liver MDA. However, OTC restored this impairment. OTC is metabolized intracellularly to cysteine, and GSH synthesis increases because g-glyamylcysteine synthetase is limiting (13). OTC replenishes GSH by providing cysteine (14).

The Nrf2 transcription factor was previously shown to protect against ROS- and toxin-induced tissue damage through its capacity to induce the expression of genes encoding ROS- and drug-detoxifying enzymes (11). Under healthy conditions, Nrf2 localizes in the cytoplasm, where it interacts with the actin-binding protein Kelch-like ECH associating protein 1 (Keap1) and is rapidly degraded by the ubiquitin-proteasome pathway. Signals from ROS or electrophilic insults target the Nrf2-Keap1 complex, which dissociates Nrf2 from Keap1. Stabilized Nrf2 then translocates to the nuclei and promotes expression of many antioxidant proteins and phase-II detoxifying enzymes such as Nqo1, GST, and HO-1. Nrf2 activation may be a novel strategy to prevent or ameliorate toxin-induced liver injury and fibrosis (9). Curcumin attenuates dimethylnitrosamine-induced liver injury in rats through Nrf2-mediated induction of HO-1 (23). In this study, the OTC treatment groups showed significant induction of Nrf2 and HO-1 expression compared to that in the TAA group. These results suggest that the mechanism for the anti-fibrotic effects of OTC in TAA-induced liver injury may be related to the reduction of oxidative stress via modulation of Nrf2-mediated induction of detoxifying enzymes.

In summary, the present results demonstrated that OTC exhibited *in vivo* hepatoprotective and anti-fibrotic effects against TAA-induced liver injury. The mechanism appeared mostly to be mediated by the inactivation of HSCs but also could involve inhibition of TGF-β1 production. Additionally, OTC may also exert a beneficial effect on restoring the antioxidant system via Nrf2-mediated induction of HO-1. Our data suggest that OTC may be potentially useful for preventing hepatic fibrosis.

MATERIALS AND METHODS

Animals and reagents

Male Sprague-Dawley (weight, 180-200 g) rats (Charles River Korea, Seoul, Korea) were provided a standard laboratory diet and water *ad libitum* and were cared for under a protocol approved by the Institutional Animal Care and Use Committee of Chonbuk National University Medical School. TAA and OTC were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The antibodies against α-SMA and Nrf-2 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against HO-1 were purchased from Stessgen Biotechnology Co. (Ann Arbor, MI, USA).

TAA-induced hepatic fibrosis and the experimental protocol

Hepatic fibrosis was induced by intraperitoneal (i.p.) injections of TAA dissolved in sterile phosphate-buffered saline (PBS) at a

dosage of 200 mg/kg body weight three times per week for 8 weeks. The OTC solution was freshly prepared by dissolving the chemical in PBS and adjusting the pH to 7.2 with 3 N NaOH. The OTC treatment was performed as a "pre-treatment" with TAA for 8 weeks. Twenty-four rats were divided into control, TAA, TAA + OTC 80, and TAA + OTC 160 groups. The control group received only PBS for 8 weeks. The TAA group was given TAA (200 mg/kg body weight, i.p.) three times per week for 8 weeks. The TAA + OTC 80 group received OTC (80 mg/kg body weight, i.p.) three times per week 30 min before the TAA injections for the same period. The TAA + OTC 160 group received OTC (160 mg/kg body weight, i.p.) three times per week 30 min before the TAA injections for the same period. The dosage of OTC used in this study was determined according to a previous study (24). Rats were anesthetized with ether at the end of treatment, and their body weights were recorded. Blood samples were obtained for testing liver functions. The serum ALT levels were analyzed using an automatic blood chemistry analyzer (Abbott Laboratories, Abbott Park, IL, USA). The liver weight of each rat was recorded after harvesting the liver by laparotomy. The liver specimens were fixed in 10% buffered formalin overnight before being embedded in paraffin in preparation for histopathological and immunohistochemical examinations. The remaining liver was snap-frozen in liquid nitrogen and stored at $-80^{o}C$ for subsequent study.

Histological examination

Liver specimens were preserved in 10% buffered formalin and dehydrated in a graded alcohol series. Following xylene treatment, the specimens were embedded in paraffin blocks and cut into 4-μm-thick sections, which were placed on plain glass microscope slides. The sections were then stained with hematoxylin and eosin or Masson's trichrome staining and observed under a light microscope (Olympus, Tokyo, Japan). The Scheuer's pathological scoring system was used, which was determined by two independent pathologists (25). Fibrosis was staged as follows: stage 0, no fibrosis; stage 1, expansion of the portal tract without linkage; stage 2, portal expansion with portal-to-portal and focal portal-to-central linkage; stage 3, fibrosis with architectural distortion but no obvious cirrhosis; and stage 4, cirrhosis. Portal/periportal activity was graded as follows: grade 0, none or minimal; grade 1, portal inflammation; grade 2, mild piecemeal necrosis; grade 3, moderate piecemeal necrosis; grade 4, severe piecemeal necrosis. Lobular activity was graded as follows: grade 0, none; grade 1, inflammation but no necrosis; grade 2, focal necrosis or acidophil bodies; grade 3, severe focal cell damage; grade 4, damage including bridging necrosis.

Immunohistochemical staining

Formalin-fixed, paraffin-embedded specimens were sectioned at a thickness of 4-μm. Deparaffinization and rehydration were carried out using a graded series of xylene and alcohol, respectively. Sections were then treated with 0.3% hydrogen peroxide for 3 min and then with a blocking antibody for 30 min. Sections were incubated for 1 h with the primary antibodies, anti-α-SMA and anti-Nrf2, which were obtained from Santa Cruz Biotechnology. Detection was performed using the avidin-biotin-peroxidase complex method.

Western blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) assay

Liver samples were homogenized in 500 mM Tris buffer (pH 7.6) containing 1% Triton X-100, 200 mM NaCl, and 10 mM $CaCl_2$. After centrifugation at 14,000 × g at $4^{o}C$ for 30 minutes, supernatant protein concentrations were determined using the Bradford method. Briefly, supernatant samples containing 40 μg of total protein were resolved using 8-15% SDS-PAGE gel and transferred onto Immobilon-P PVDF membranes (Millipore, Billerica, MA, USA) by electroblotting. Samples were then probed with polyclonal anti-α-SMA (Santa Cruz Biotechnology), anti-Nrf2 (Santa Cruz Biotechnology) and anti-HO-1 (Stessgen Biotechnology). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, and blots were developed using an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL, USA). A polyclonal anti-β-actin antibody (Sigma) was used to confirm equal loading of protein.

We carried out RT using 10 μg total RNA, 50 μM decamer and 1 μl (200 units) RT-PCR Superscript II (Invitrogen, Carlsbad, CA, USA) at $37^{o}C$ for 50 min, as described previously, to determine TGF-β1 and collagen α1 mRNA expression. Briefly, total RNA was isolated from liver tissues. The primer sequences for TGF-β1 were 50-CGG CAG CTG TAC ATT GAC TT-30 (sense) and 50-TCA GCT GCA CTT GCA GGA GC-30 (antisense). The primer sequences for collagen α1 were 50-TGC CGT GAC CTC AAG ATG TG-30 (sense) and 50-CAC AAG CGT GCT GTA GGT GA-30 (antisense). The QuantumRNA™ 18S Internal Standard kit (Ambion, Austin, TX, USA) was used as the internal control, according to the manufacturer's instructions. PCR products were separated by electrophoresis on a 2% agarose gel and visualized under ultraviolet light after staining with ethidium bromide. 18S was used as the internal control.

Densitometric data were analyzed using an LAS3000 system (Fuji Photo Film, Tokyo, Japan). Expression of each gene was calculated by normalizing the expression level against the level of the control and then calculating the ratio of expression in the treated groups compared with that in the control group.

Measurement of hepatic MDA level

Hepatic MDA level was measured spectrophotometrically using an OxiSelect™ thiobarbituric acid reactive substances assay kit (Cell Biolabs, San Diego, CA, USA), according to the manufacturer's protocol. Briefly, liver tissue samples were perfused with PBS containing heparin and resuspended at 50-100 mg/mL in PBS containing 1 × butylated hydroxytoluene. The tissue samples were homogenized on ice and centrifuged at 10,000 × g for 5 min to collect the supernatant. The unknown MDA-containing tissue samples or MDA standards were first reacted with thio-

barbituric acid at 95°C. After the 60 min incubation, the samples and standards were spectrophotometrically analyzed at 532 nm. The MDA content in tissue samples was determined by comparison with a predetermined MDA standard curve provided by the manufacturer.

Statistical analysis

The results represent the mean of at least three independent experiments (mean ± SD). Data were analyzed using Instat software (GraphPad Prism4, San Diego, CA, USA). We used Student's *t*-test or A one-way analysis of variance with a post hoc analysis and Tukey's multiple comparison test to analyze parametric data. Statistical significance was accepted for P values $<$ 0.05.

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