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# Inhibition of liver fibrosis by solubilized coenzyme Q10: Role of Nrf2 activation in inhibiting transforming growth factor-β1 expression

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ABSTRACT

Coenzyme Q10 (CoQ10), an endogenous antioxidant, is important in oxidative phosphorylation in mitochondria. It has anti-diabetic and anti-cardiovascular disease effects, but its ability to protect against liver fibrosis has not been studied. Here, we assessed the ability of solubilized CoQ10 to improve dimethylnitrosamine (DMN)-induced liver fibrogenesis in mice. DMN treatments for 3 weeks produced a marked liver fibrosis as assessed by histopathological examination and tissue 4-hydroxyproline content. Solubilized CoQ10 (10 and 30 mg/kg) significantly inhibited both the increases in fibrosis score and 4-hydroxyproline content induced by DMN. Reverse transcription-polymerase chain reaction and Western blot analyses revealed that solubilized CoQ10 inhibited increases in the transforming growth factor-β1 (TGF-β1) mRNA and α-smooth muscle actin (α-SMA) protein by DMN. Interestingly, hepatic glutamate-cysteine ligase (GCL) and glutathione S-transferase A2 (GSTA2) were up-regulated in mice treated with CoQ10. Solubilized CoQ10 also up-regulated antioxidant enzymes such as catalytic subunits of GCL and GSTA2 via activating NF-E2 related factor2 (Nrf2)/antioxidant response element (ARE) in H4IIE hepatoma cells. Moreover, CoQ10's inhibition of α-SMA and TGF-β1 expressions disappeared in Nrf2-null MEF cells. In contrast, Nrf2 overexpression significantly decreased the basal expression levels of α-SMA and TGF-β1 in Nrf2-null MEF cells. These results demonstrated that solubilized CoQ10 inhibited DMN-induced liver fibrosis through suppression of TGF-β1 expression via Nrf2/ARE activation.



## Introduction

Coenzyme Q10 (CoQ10), an endogenous antioxidant, regulates oxidative phosphorylation in mitochondria and subsequent ATP production (Crane, 2001). CoQ10 content gradually decreases during aging (Pignatti at al., 1980; Sohal and Forster, 2007). CoQ10 localized in the mitochondria and plasma membrane functions as an efficient antioxidant either by direct conjugation with reactive oxygen species (ROS) or by regeneration of cellular antioxidants such as tocopherol and ascorbic acid (Crane, 2001). Moreover, tissue or blood levels of CoQ10 are significantly lower in patients with cardiovascular disease, including chronic heart failure and type II diabetes (Mortensen et al., 1990; Molyneux et al., 2008; Miyake et al., 1999). Decreased CoQ10 levels are a pathological marker of the increased oxidative stress in congestive heart failure, coronal artery disease (Chagan et al., 2002; Sarter, 2002), and diabetes (Hodgson et al., 2002).

Liver fibrosis, which arises from overproduction of the extracellular matrix, including collagens, is an early stage of cirrhosis. Therapeutic management of liver fibrosis and cirrhosis is still an unsolved clinical problem. Although silymarin is hepatoprotective in different experimental conditions, it has no effect on survival and the clinical course in liver cirrhosis (Pares et al., 1998). Hepatocyte growth factor (HGF) secreted from stellate cells significantly improves hepatic function and reverses liver cirrhosis in diverse animal models (Matsuda et al., 1995; Ueki et al., 1999), but the clinical efficacy of HGF for the treatment of liver fibrosis and cirrhosis is still uncertain. We have also reported that oltipraz inhibits liver fibrosis and cirrhosis in DMN-treated rat models (Kang et al., 2002a, 2002b), and clinical evaluation is now progressing. The lack of treatments for liver fibrosis or cirrhosis makes identifying a safe drug candidate interesting.

*Abbreviations:* CoQ10, CoenzymeQ10; DMEM, Dulbecco's modified Eagle's medium; DMN, dimethylnitrosamine; TGF-β1, transforming growth factor β1; α-SMA, α-smooth muscle actin; GST, glutathione S-transferase; GCL, glutamate-cysteine ligase; GCLC, catalytic subunit of glutamate-cysteine ligase; HO-1, heme oxygenase-1; ARE, antioxidant response element; Nrf2, NF-E2 related factor-2; MEF, mouse embryonic fibroblast; ROS, reactive oxygen species.

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CoQ10 treatment can inhibit lipid peroxidation and hepatotoxicity induced by chemical exposure (Sugino et al., 1987; Amimoto et al., 1995), but the effect of CoQ10 on hepatic fibrosis is unclear. Despite many *in vivo* or cell-based reports using CoQ10 (Sugino et al., 1987; Amimoto et al., 1995; Kooncumchoo et al., 2006), the pharmacological effect of solubilized CoQ10 would be different from the normal insoluble CoQ10. Here we investigated ability of solubilized CoQ10 to protect against dimethylnitrosamine (DMN)-induced liver fibrosis in mice, and examined its mechanism of action by, focusing on its NF-E2 related factor-2 (Nrf2) activation and the subsequent inhibition of transforming growth factor-β1 (TGF-β1) and α-smooth muscle actin (α-SMA) expression.

## Materials and methods

*Preparation of solubilized CoQ10.* We used two different methods to solubilize CoQ10: poloxamer-based solid dispersion (for cell culture study) and nano-emulsion preparation (for animal study). To prepare solid dispersion, coenzyme Q10 (CoQ10) and poloxamer 407 were mixed at 1:5 weight ratio with a mortar and pestle. The physical mixture was then placed in the oven at 70 °C for 15 min. During this process, CoQ10 was dissolved in melted poloxamer and then cooled to room temperature. To prepare a self-emulsifying nano-emulsion, CoQ10, Witepsol® H35, Solutol® HS15, and Lauroglycol® FCC were mixed in a weight ratio 1:0.7:4:2 to form self-emulsifying nano-emulsion. The constituents were mixed in the required weight ratio in an oven at 37 °C. When the mixture became a homogenous oily liquid, CoQ10 was added. The mixture was continuously stirred with a magnetic bar to dissolve CoQ10. The single phase nano-emulsion base was emulsified with distilled water to 10 mg/ml.

*Animals.* The Institutional Animal Care and Utilization Committee of Chosun University approved all the animal procedures used in this study. ICR male mice (25–27 g) were provided from Joong-Ang Experimental Animals Co. (Seoul, Korea) and acclimatized for 1 week before use. Animals were caged with commercial rat chow (Purina, Korea) and water *ad libitum* at a temperature between 20 °C and 23 °C, a 12-h light-dark cycle, and relative humidity of 50%. DMN was intraperitoneally injected, dissolved in sterile saline (10 μl/kg body weight) 3 times per week for 3 weeks. Control animals received vehicle. Nano-emulsified CoQ10 (10 and 30 mg/kg) was orally administered 24 h before treatment with DMN (3 times per week for 3 weeks). Animals were euthanized on day 23 and the liver was excised and subjected to histopathological examinations and 4-hydroxyproline determination. For the determination of liver TGF-β1 mRNA, mice were administered with solubilized CoQ10 at 10 and 30 mg/kg 18 h before an injection of DMN (10 μl/kg) and euthanized 6 h after DMN treatment.

*Histopathology.* Hepatic morphology was assessed by light microscopy. The left lateral lobe of the liver was sliced (3 slices per rat) and tissue slices were fixed in 10% buffered-neutral formalin for 6 h. Fixed tissue slices were processed and embedded in a paraplast automatic tissue processor, Citadel 2000 (Shandon Scientific, Cheshire, UK). Sections of 6 μm in thickness were subjected to hematoxylin and eosin and Masson's trichrome staining prior to examination (Kang et al., 2002a, 2002b). A certified pathologist scored samples in a blinded fashion. An arbitrary scope was given to each microscopic field viewed at magnifications of 100–200×. A minimum of 10 fields was scored per liver slice to obtain the mean value. Fibrosis extent was graded as 0, absent; 1, trace; 2, mild; 3, moderate; and 4, severe.

*Assay of 4-hydroxyproline in liver tissues.* The 4-hydroxyproline content in the liver was determined by the methods described by Kondo et al. (1997), with modifications. Briefly, the right lobe of the liver (0.25 g) was homogenated with deionized water, and 0.2 ml of the liver homogenate was hydrolyzed in 1 ml of 9 M HCl. The samples were incubated at 110 °C for 24 h. After cooling, the hydrolysate was diluted with 1.5 ml of 0.26 M borate buffer (pH, 9.5) and centrifuged at 10,000 ×*g* at room temperature. The pellet was discarded. The supernatant was diluted with 4 times its volume of 0.26 M borate buffer (pH, 9.5). A high-performance liquid chromatographic method was used for determination of hydroxyproline. First, the primary amino group in liver homogenate was blocked with O-phthalaldehyde, then histidinohydroxylysinonorleucine in the hydrolysate was labeled with 9-fluorenylmethyl chloroformate. The samples were then analyzed by reverse-phase high-performance liquid chromatography at 40 °C [Kromasil C18 column, 25 cm, 5 mM; flow rate, 1.5 ml/min; Mobile phase A, 0.08 M NaCl with 0.3% acetic acid: acetonitrile (70:30): Mobile phase B, 0.08 M NaCl with 0.3% acetic acid: acetonitrile (30:70) with gradient] with a fluorescence detector (260, 310 nm). The retention time of 4-hydroxyproline was ~6.1 min.

*Reverse transcription-polymerase chain reaction (RT-PCR).* The total RNA was isolated using a total RNA isolation kit (RNAgents®, Promega, Madison, WI). The total RNA (1.0 μg) obtained from the cells was reverse-transcribed using an oligo(dT) 18mer as a primer and M-MLV reverse transcriptase (Bioneer, Eumsung, Korea) to produce the cDNAs. PCR was performed using the selective primers for TGF-β1 (sense: CTTCAGCTCCACAGAGAAGAACTGC, antisense: CACGATCATGTTGGACAACT GCTCC) and S16 ribosomal protein (S16r) genes (sense: 5′-TCCAAGGGTCCGCTGCAGTC-3′, antisense: 5′-CGTTCACCTTGATGAGCCCATT-3′). PCR was performed for 42 cycles using the following conditions: denaturation at 98 °C for 10 s, annealing at 54 °C for 0.5 min, and elongation at 72 °C for 1 min. The band intensities of the amplified DNAs were compared after visualization using FLA-7000 (Fuji film, Tokyo, Japan).

*Cell culture.* H4IIE (a rat hepatoma cell line) cells and wild-type- and Nrf2-null mouse embryonic fibroblast (MEF) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and Dr. MK Kwak (Yeungnam University, Daegu, South Korea), respectively. H4IIE cells were cultured at 37 °C in 5% $CO_2$/95% air in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

*Preparation of nuclear extract and Western blot analysis.* Cells were removed using a cell scraper and centrifuged at 2500 ×*g* for 5 min at 4 °C. The cells were then swollen with 100 μl of lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet-P40, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonylfluoride]. Tubes were vortexed to disrupt cell membranes, and samples were incubated for 10 min on ice and then centrifuged for 5 min at 4 °C. Pellets containing crude nuclei were resuspended in 100 μl of extraction buffer [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride], incubated for 30 min on ice, and centrifuged at 15,800 ×*g* for 10 min; the supernatants containing the nuclear extracts were collected and stored at −80 °C until required. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analyses were performed as described previously (Lee et al., 2007). Cell lysates were fractionated by 10% gel electrophoresis, and electrophoretically transferred to nitrocellulose membranes. The membranes were subsequently incubated with primary antibody [anti-α-SMA (Neomarkers, Fremont, CA); anti-glutathione S-transferase A2 (GSTA2, Detroit R&D, Detroit, MI); anti-catalytic subunit of glutamate-cysteine ligase (GCLC, Neomarkers, Fremont, CA); anti-quinone oxidoreductase (form Dr. Sang Geon Kim in Seoul National University, Seoul, Korea); anti-Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-β-actin (Sigma, St. Louis, MO)], and then with alkaline phosphatase- or horseradish peroxidase-conjugated secondary

antibodies. Finally, the membranes were developed using either 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium or an ECL chemiluminescence detection kit.

*Reporter gene assays.* The firefly luciferase-reporter gene construct, pGL-797, was generated by ligating pGL3-basic vector (Promega, Madison, WI) with the antioxidant response element (ARE)-containing 797 bp promoter region of the *GSTA2* gene (Lee et al., 2007; Kang et al., 2003). pGL3-ARE minimal reporter containing 3 copies of quinone oxidoreductase ARE sequence was kindly donated from Dr. Kwak (Yeungnam University, South Korea). To determine promoter activity, we used a dual luciferase-reporter assay system (Promega, Madison, WI). Briefly, cells were cultured up to 70% confluency in 12-well plates and transiently transfected with luciferase-reporter constructs and phRL-SV plasmid (*hRenilla* luciferase expression for normalization) (Promega, Madison, WI) using Hillymax® reagent (Dojindo Molecular Tech., Gaithersburg, MD). The firefly and *hRenilla* luciferase activities in the cell lysates were measured using a luminometer (Berthold Tech., Bad Wildbad, Germany). The relative luciferase activities were calculated by normalizing the promoter-driven firefly luciferase activity to *hRenilla* luciferase.

*Data analysis.* Paired Student's *t*-test was used to examine the significant inter-group differences. Statistical significance was accepted at either $p<0.05$ or $p<0.01$.

## Results

### *Inhibition of DMN-induced liver fibrosis by solubilized CoQ10*

Metabolic activation of DMN by CYP2E1 causes liver-specific tissue injury and multiple DMN treatments lead to hepatic necrosis and chronic fibrosis (Tsukamoto et al., 1990; George and Chandrakasan, 1996). We evaluated the extent of liver fibrosis in mice histologically after 3 weeks of DMN treatments (Fig. 1A). Masson's trichrome staining to assess extracellular matrix showed thick fibrotic nodules, ballooning degeneration, and thick fibrous bands

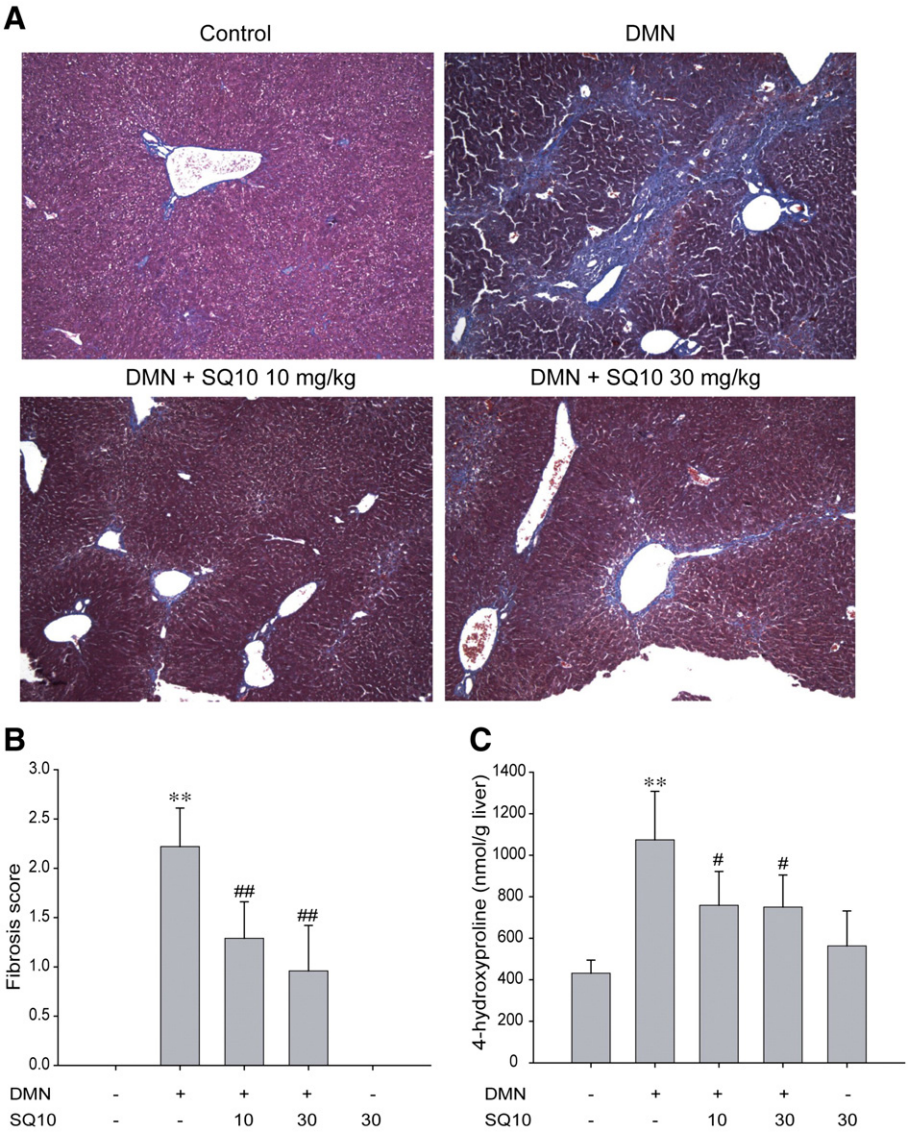


**Fig. 1.** (A) Representative Masson's trichrome staining (×100) of liver sections from mice treated with vehicle control, DMN (10 μl/kg, 3 times per week for 3 weeks) and nano-emulsified CoQ10 (SQ10, 10 and 30 mg/kg) + DMN. (B) Fibrosis score. Fibrosis score was evaluated in the livers of surviving animals by certified pathologist in a blinded fashion. Data represent the mean ± SD ($n=5$–7) (significant as compared to vehicle-treated control, $^{**}p<0.01$; significant as compared to DMN-treated group, $^{\#\#}p<0.01$). (C) 4-hydroxyproline levels. The homogenized liver samples were subjected to the analyses of 4-hydroxyproline. Data represent the mean ± SD ($n=5$–7) (significant as compared to vehicle-treated control, $^{**}p<0.01$; significant as compared to DMN-treated group, $^{\#}p<0.05$).

in the liver (Fig. 1A). Our nanoparticle-emulsified CoQ10 preparation showed a 12.5-fold increase in peak plasma CoQ10 levels compared with normal CoQ10 in oral pharmacokinetics study (Han et al., unpublished results). The nano-emulsified CoQ10 (10 and 30 mg/kg) significantly decreased fibrosis scores (Fig. 1B) and the intensity of fibrotic nodules after DMN treatment, but did not completely bock ballooning degeneration at 10 mg/kg (detected in 4/7 mice; Fig. 1A). In the CoQ10 alone-treated group (30 mg/kg), no pathological changes were found.

We further measured liver 4-hydroxyproline levels to indicate collagen accumulation (Toyoki et al., 1998). DMN increased 4-hydroxyproline content 2.5-fold, and CoQ10 (10 or 30 mg/kg) significantly blocked this induction (Fig. 1C).

### *Effect of solubilized CoQ10 on the production of α-smooth muscle actin and TGF-β1*

Transactivation of quiescent stellate cells contributes to liver fibrosis (Toyoki et al., 1998). α-SMA expression is a marker of activated stellate cells. α-SMA expression was not or slightly detected in control mice, but DMN increased α-SMA expression (Fig. 2A). CoQ10 (10 and 30 mg/kg) blocked this increase in α-SMA expression (Fig. 2A).

Transforming growth factor-β1 (TGF-β1), a key fibrosis mediator produced in activated stellate cells, regulates cell growth and differentiation and synthesis of extracellular matrix proteins (Freidman, 1993). TGF-β1 expression can be controlled by translational efficiency and post-translational modifications, including proteolytic cleavage of latent TGF-β1 to the active from, but quantification of TGF-β1 mRNA can indicate TGF-β1 activity (Krüse et al., 1999). Because TGF-β1 mRNA could not be detected after long-term DMN treatments due to the excess fibrosis, TGF-β1 mRNA levels were determined 18 h after DMN injection. A single injection of 10 μl/kg DMN increased TGF-β1 mRNA, and pretreatment with CoQ10 (30 mg/kg) prevented this induction (Fig. 2B).

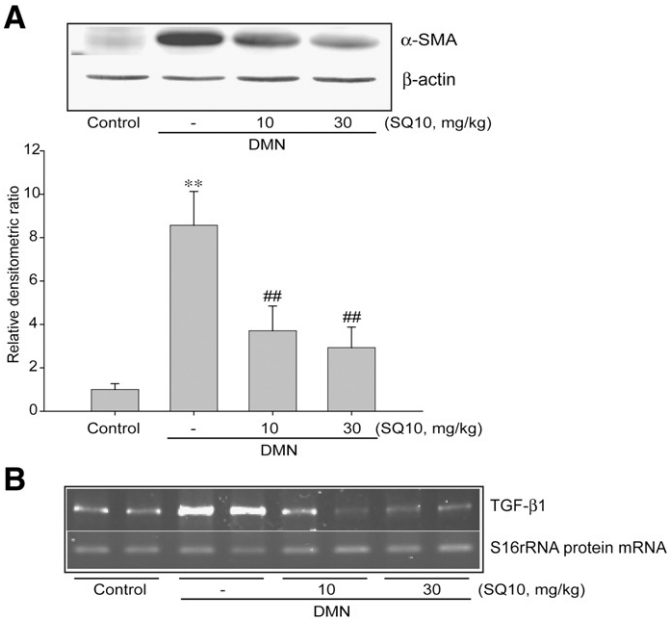


**Fig. 2.** Inhibition of stellate cell activation by solubilized CoQ10. (A) Western blot analysis of α-SMA in liver homogenates. The levels of α-SMA were immunochemically determined in the livers of control mice or fibrotic mice treated with vehicle or 10 and 30 mg/kg nano-emulsified CoQ10 (SQ10). The relative α-SMA levels were assessed by scanning densitometry. Data represent the mean ± SD ($n=4$) (significant as compared to vehicle-treated control, $^{**}p<0.01$; significant as compared to DMN-treated group, $^{\#\#}p<0.01$). (B) RT-PCR analysis of TGF-β1 mRNA in the liver of fibrotic mice. ICR mice were administered with SQ10 at 10 and 30 mg/kg 18 h before an injection of DMN (10 μl/kg) and euthanized 6 h after DMN treatment. Each lane represents RNA sample from different animals.

To assess whether CoQ10 directly suppresses the expressions of α-SMA and TGF-β1 in fibroblast cell-type like activated hepatic stellate cells, we used MEF cells. For *in vitro* experiments with MEF cells, CoQ10 solubilized in dimethylsulfoxide was precipitated in culture medium (shown as blue circle in UV field, Fig. 3A). Hence, we used poloxamer-based solubilized CoQ10 for cell culture studies. This CoQ10 solution was completely dissolved in medium and taken up by MEF cells (Fig. 3A). The solubilized CoQ10 [1–100 ng/ml for 24 h (α-SMA protein) or 4 h (TGF-β1 mRNA)] concentration-dependently decreased basal expression of α-SMA protein and TGF-β1 mRNA (Figs. 3B and C). We then determined phosphorylated Smad2/Smad3 in MEF cells after solubilized CoQ10 treatment, since TGF-β1 mainly acts by stimulating activated Smads (Smad2 and 3). The basal Smad3 phosphorylation was distinctly reduced by solubilized CoQ10 in MEF cells, but Smad2 phosphorylation was not changed by solubilized CoQ10 (Fig. 3D).

### *Nrf2/antioxidant response element (ARE) activation by solubilized CoQ10*

Recent animal and clinical studies show that stellate cell activation during the progress of hepatic fibrosis is associated with oxidative stress (Vendemiale et al., 2001; Friedman, 2008). Scavenging of ROS can be accelerated by induction of cellular detoxifying enzymes such as glutamate-cysteine ligase (GCL) and glutathione S-transferases (GST). Single administration of 10 or 30 mg/kg nanoparticle-emulsified CoQ10 enhanced GSTA2 and GCLC expressions in mouse liver samples (Fig. 4A). Nrf2/ARE is crucial for the expression of antioxidant proteins such as GCLC, GSTA2 and quinone oxidoreductase (McMahon et al., 2001; Balogun et al., 2003). We showed that H4IIE cells derived from rat hepatoma are useful hepatocyte models for the expression of Nrf2/ARE-dependent phase II detoxifying enzymes (Lee et al., 2007; Kim et al., 2006; Kang et al., 2001). Solubilized CoQ10 increased the reporter activity of pGL-797 containing *GSTA2* gene ARE as well as the nuclear translocation of Nrf2 (Fig. 4B). Moreover, expression levels of GSTA2, GCLC and quinone oxidoreductase were enhanced in CoQ10-treated H4IIE cells (Fig. 4C). CoQ10 could increase the Nrf2/ARE activity, putatively from marginal reactive oxygen species (ROS) generation by quinone redox cycling of CoQ10. In fact, CoQ10 treatment increased fluorescence from dichlorofluorescein-hydrogen (DCFH) oxidation (Fig. 4D). Thus, CoQ10 up-regulates antioxidant enzymes via Nrf2/ARE activation in hepatocytes, which may be associated with its anti-fibrotic effect.

### *Inhibition of Nrf2 activation in the downregulation of TGF-β1 and α-SMA*

We then examined the functional roles of CoQ10-inducible Nrf2 activation in α-SMA and TGF-β1 expressions using Nrf2 wild-type and Nrf2-null MEF cells. The basal Nrf2 expressions in both the cell types were determined by using specific Nrf2 antibody (Fig. 5A). In comparison to Nrf2 wild-type cells (Figs. 3B and C), treatment of Nrf2-null cells with solubilized CoQ10 did not reduce the basal α-SMA and TGF-β1 expressions (Fig. 5B). Vice versa, overexpression of mouse Nrf2 in Nrf2-null cells diminished the basal expression of α-SMA and TGF-β1 (Fig. 5C). α-SMA expression and transformation of stellate cells to myofibroblast-like cells are controlled by TGF-β1 secretion (Gressner, 1996). We found that Nrf2 may regulate TGF-β1 expression. Thus, Nrf2 activation by CoQ10 could block TGF-β1 expression in stellate cells to produce its anti-fibrogenic effect.

It has been recently reported that antioxidant, N-acetyl-L-cysteine suppresses TGF-β1-mediated signalings and consequently blocks the expression of several fibrogenic genes (Kopp et al., 2006). However,

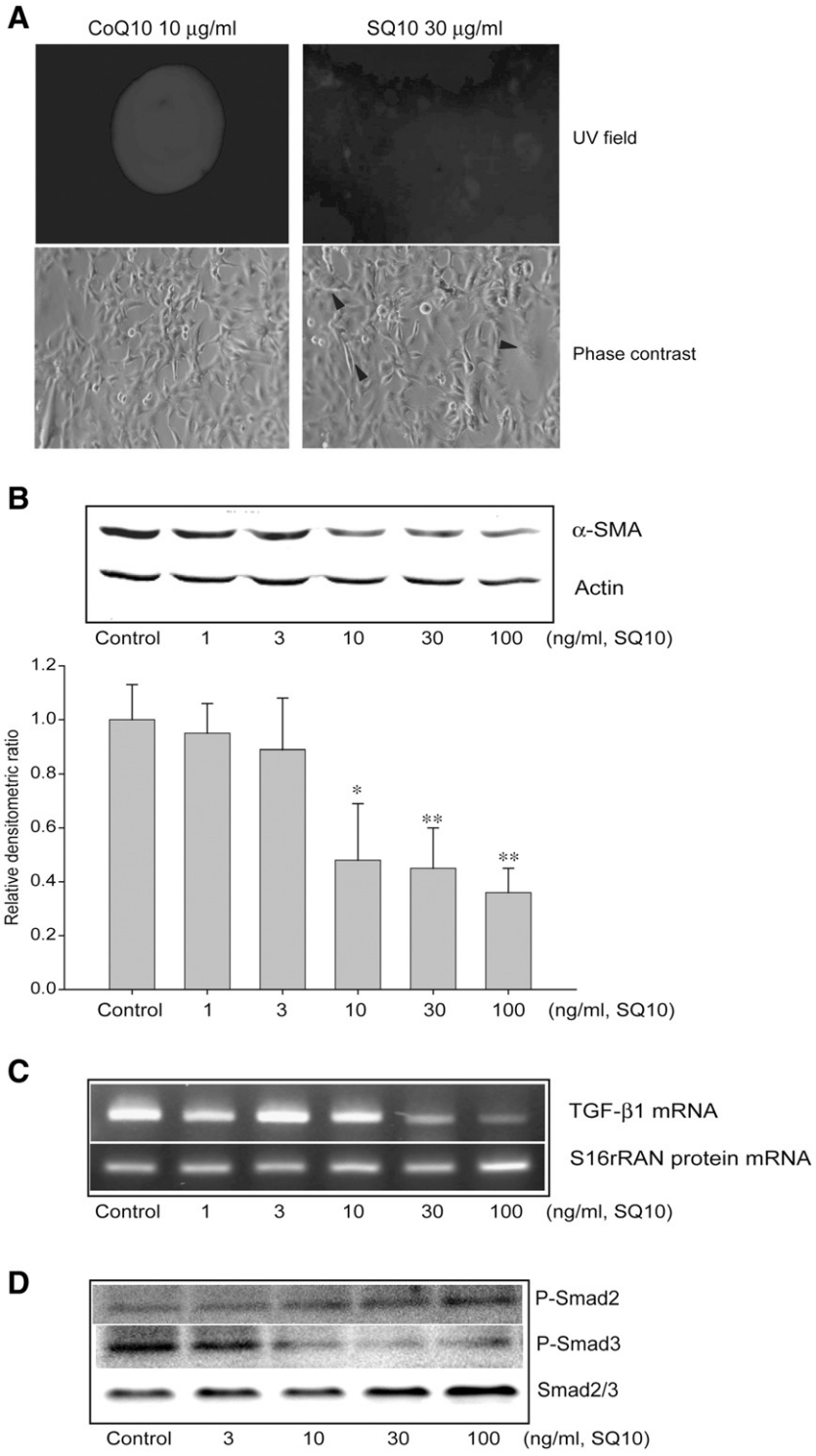


**Fig. 3.** Inhibition of α-SMA and TGF-β1 expressions by solubilized CoQ10 in MEF cells. (A) Enhanced cellular uptake of poloxamer-based solubilized CoQ10 (SQ10) in H4IIE cells. CoQ10 is seen as blue color in UV field. (B) Effect of SQ10 on α-SMA expression in MEF cells. The levels of α-SMA protein were measured in the lysates of cells treated with 1–100 ng/ml SQ10 for 24 h. Data represent the mean ± SD ($n = 3$)(significant as compared to poloxamer-vehicle-treated control, *$p<0.05$; **$p<0.01$). (C) Effect of SQ10 on TGF-β1 expression in MEF cells. Total RNA prepared from MEF cells treated with SQ10 (1–100 ng/ml) for 4 h was used for cDNA synthesis, and TGF-β1 mRNA expression was assessed by RT-PCR. (D) Effect of SQ10 on Smad2/3 phosphorylation in MEF cells. The levels of phosphorylated Smads protein were measured in the lysates of cells treated with 3–100 ng/ml SQ10 for 12 h.

we found that co-treatment of MEF cells with 5 mM N-acetyl-L-cysteine slightly reversed 100 ng/ml solubilized CoQ10's repressive effect on TGF-β1 expression (Fig. 5D). This may be due to the N-acetyl-L-cysteine's blocking effects on marginal ROS generation and subsequent Nrf2 activation by solubilized CoQ10.

## Discussion

CoQ10 is an essential component of the mitochondrial respiratory chain, and also has direct antioxidant properties (Ernster and Forsmark-Andrée, 1993). CoQ10's anti-cardiovascular and anti-

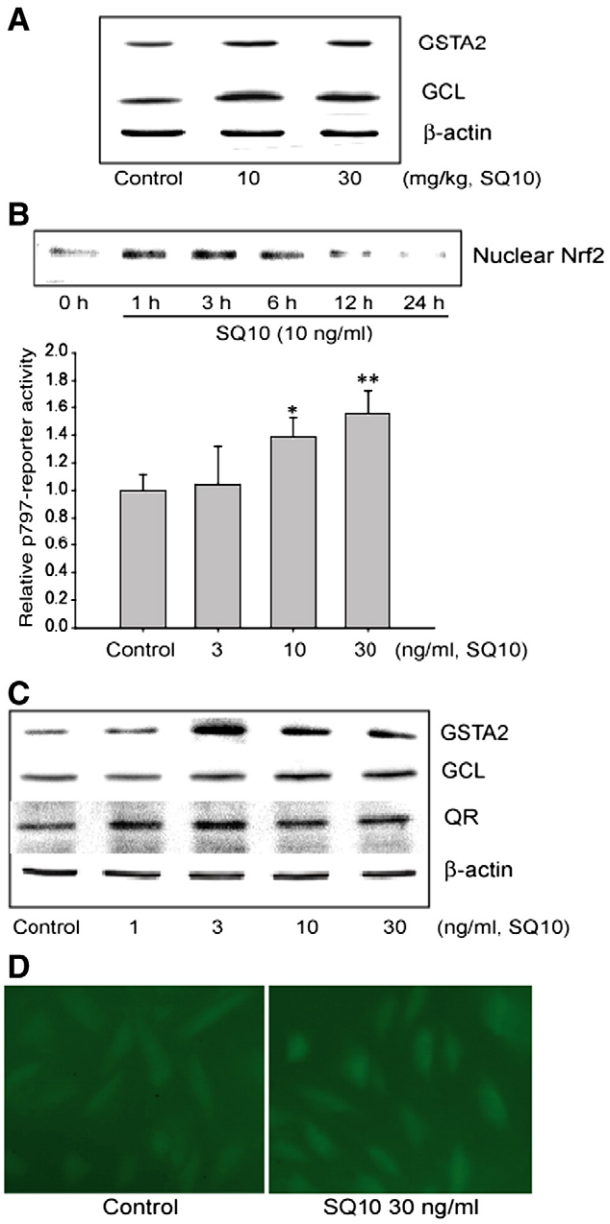


**Fig. 4.** Nrf2 activation by solubilized CoQ10 and subsequent induction of antioxidant enzymes. (A) Nrf2-dependent antioxidant enzyme expression in the liver tissues from mice orally administered with nano-emulsified CoQ10 (SQ10, 10 or 30 mg/kg). ICR male mice (25–27 g) were gavaged with SQ10 (10 or 30 mg/kg) and euthanized 24 h after SQ10 treatment for the determination of hepatic expression levels of antioxidant enzymes. Three individual animals were used to confirm the data. (B) Upper panel; Nrf2 activation by solubilized CoQ10. The nuclear localization (active form) of Nrf2 was immunochemically assessed in H4IIE cells treated with poloxamer-based CoQ10 (SQ10, 10 ng/ml) for 0–24 h. (B) Analysis of antioxidant response element (ARE) reporter activity. Induction of luciferase activity by SQ10 in H4IIE cells transiently transfected with the GSTA2 chimeric gene construct pGL-797 containing the ARE element. Dual luciferase-reporter assays were performed on lysed H4IIE cells co-transfected with pGL-797 (firefly luciferase) and phRL-SV (h*Renilla* luciferase) after exposure to SQ10 (3–30 ng/ml) for 18 h. Data represent the mean ± SD ($n=3$) (significant as compared to poloxamer-vehicle-treated control, $*p<0.05$; $**p<0.01$). (C) Induction of antioxidant enzymes by solubilized CoQ10 in H4IIE cells. H4IIE cells were treated with SQ10 (1–30 ng/ml) for 18 h and the total cell lysates were subjected to immunoblottings of GSTA2, GCLC and quinine oxidoreductase (QR). Results were confirmed by repeated experiments. (D) DCF fluorescence change by solubilized CoQ10. H4IIE cells were loaded with DCFH-DA (5 μM) and 30 ng/ml SQ10 was added. Fluorescence was monitored using fluorescence microscope (Axiovert 200M; Carl Zeiss, Hamburg, Germany; supported from Chosun University).

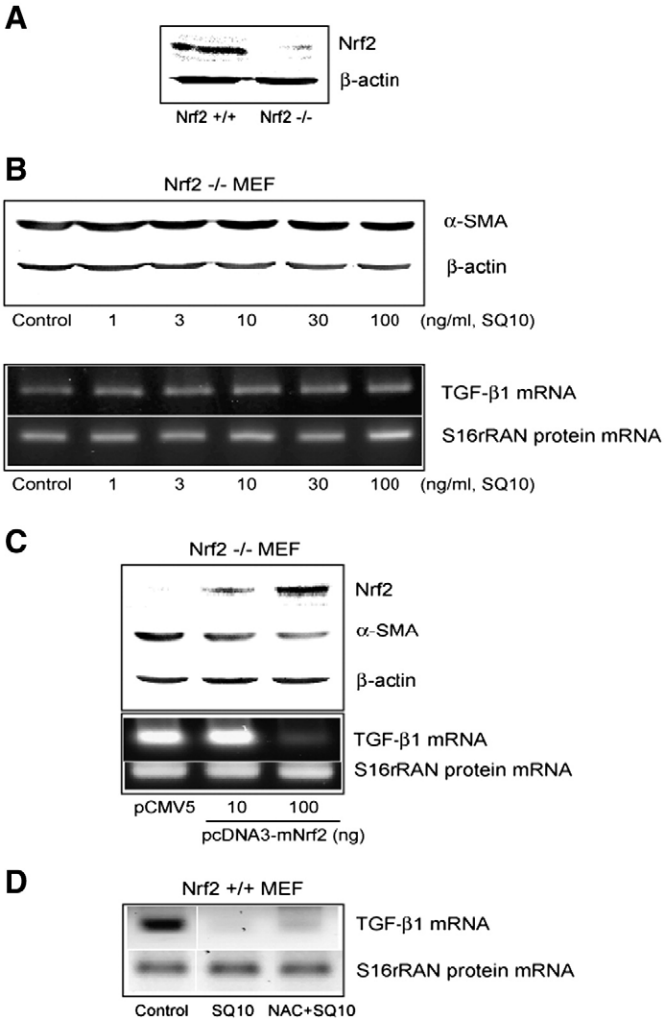


**Fig. 5.** Negative role of Nrf2 in TGF-β1 and α-SMA expression. (A) Nrf2 expression in Nrf2 wild-type (Nrf2 +/+) and Nrf2-null (Nrf2 −/−) MEF cells. (B) Expression changes of α-SMA and TGF-β1 by poloxamer-based solubilized CoQ10 (SQ10) in Nrf2-null MEF cells. α-SMA protein and TGF-β1 mRNA expression levels were measured as described in Figs. 3(B) and (C). (C) Down-regulation of α-SMA and TGF-β1 expressions by Nrf2 overexpression. Nrf2-null MEF cells cultured in 6-well plate were transfected with pcDNA3-mNrf2 (10 and 100 ng/well) and then incubated for 24 h (α-SMA protein) and 6 h (TGF-β1 mRNA). (D) Effect of N-acetyl-L-cysteine (NAC) on the SQ10-mediated TGF-β1 down-regulation. NAC (5 mM) was pretreated in Nrf2 wild-type MEF cells for 30 min and the cells were incubated with SQ10 (100 ng/ml) for 4 h. Total RNA was isolated and subjected to RT-PCR analysis for TGF-β1.

neurodegenerative activities (Singh et al., 2007; Chaturvedi and Beal, 2008) have not been extended to liver fibrosis. In the present study, we showed that solubilized CoQ10 protected against liver fibrosis induced by DMN. Solubilized CoQ10 (10 and 30 mg/kg) decreased both the fibrosis score and the 4-hydroxyproline levels after DMN treatment in mice. Considering used dosage regimen for this study, the daily intake (500 mg/day) of CoQ10 as a food supplement may be sufficient to protect against liver fibrosis if its solubility is enough.

Hepatic fibrosis is marked by hepatocyte damage, leading to the recruitment of inflammatory cells and platelets and subsequent release of cytokines, including TGF-β1, a key fibrogenic mediator (Bauer and Schuppan, 2001). This activity affects the inflammatory and repair phase of liver fibrosis by activating stellate cells (Gianneli et al., 2005). During activation, stellate cells transit into myofibroblast-like cells that express α-SMA and these activated stellate cells excrete extracellular matrix proteins in hepatic fibrosis (Pinzani et al., 1998). Hence, α-SMA is a useful marker for the earliest stages of hepatic fibrosis, as well as for monitoring therapeutic efficacy (Carpino et al., 2005). Here, we hypothesized that CoQ10 may affect the activation of hepatic stellate cells. In fact, solubilized CoQ10 administration potently inhibited α-SMA expression in fibrotic livers

as well as in mouse MEF cells. These data indicate that CoQ10 may block fibrosis by blocking hepatic stellate cell activation.

Water-soluble CoQ10 has a direct antioxidation function that is important for its pharmacological effects. Water-soluble formulation of CoQ10 prevents oxidative stress and cell damage induced by paraquat in human neuroblastoma cells (McCarthy et al., 2004). A solublilized CoQ10 also inhibits hydrogen peroxide or Bax-induced destabilization of mitochondria in mammalian cells (Naderi et al., 2006). ROS removal can be improved by induction of endogenous antioxidant enzymes, such as GCL, heme oxygenase-1 (HO-1) and GST (Pokharel et al., 2006; Kim et al., 2008). We found that administration of nano-emulsified CoQ10 in mice increased hepatic GSTA2 levels, as well as increasing GCLC. Moreover, in H4IIE cell culture model, poloxamer-based solubilized CoQ10 induced GSTA2, GCLC and quinone oxidoreductase. Nrf2/ARE is crucial for the expression of diverse antioxidant enzymes including GSTA2, GCL, and HO-1 (Kang et al., 2002a; Clouthier et al., 2000). In fact, CoQ10 increased both the reporter activities of pGL-797 (reporter containing *GSTA2* gene ARE) (Fig. 4B) and pGL3-ARE (minimal reporter containing 3 copies of *quinone oxidoreductase* gene ARE) (data not shown). CoQ10-mediated Nrf2 activation and subsequent induction of antioxidant enzymes may result from the sustained auto-oxidation of the quinone moiety of CoQ10, as shown by the DCFH oxidation assay.

TGF-β1 causes stellate cell activation and the formation of liver fibrosis (Freidman, 1993). TGF-β1 can enhance extracellular matrix deposition and inhibit collagenase activity in stellate cells (George and Chandrakasan, 1996; Clouthier et al., 2000; Qi et al., 1999). Solubilized CoQ10 suppressed the expression of TGF-β1 induced by DMN in mouse liver and MEF cells. These results demonstrate that solubilized CoQ10 blocks TGF-β1 expression in stellate cells to inhibit fibrosis development. It has been revealed that TGF-β1 down-regulates the expression levels and activity of antioxidant enzymes, including GST, GCL, superoxide dismutase, and glutathione peroxidase (White et al., 1992; Kayanoki et al., 1994; Arsalane et al., 1997). TGF-β1 inhibits antioxidant enzyme expression through Smad3/ATF-dependent Nrf2 inactivation (Bakin et al., 2005). However, it has been still unclear whether Nrf2 activation affects TGF-β1 expression. Here, we showed that CoQ10 decreased α-SMA and TGF-β1 expressions in Nrf2 wild-type MEF cells, but not in Nrf2-null MEF cells, indicating that CoQ10 requires Nrf2 activation to down-regulate TGF-β1 and subsequent inactivate stellate cells. Furthermore, we found that Nrf2 overexpression itself reduced the basal expression of α-SMA and TGF-β1. Hence, CoQ10-induced Nrf2 activation may suppress TGF-β1 expression and stellate cell inactivation. Many natural antioxidants such as quercetin and resveratrol inhibit stellate cell activation (Kawada et al., 1998) and these phytochemicals are also potent inducers of phase II antioxidant enzymes through Nrf2/ARE activation (Kim et al., 2006; Chen et al., 2005). In the present study, we raise a possibility that Nrf2 activators function as a suppressor of stellate activation during liver fibrogenesis. A recent microarray analysis revealed that mRNA expression of several growth factors including TGF-β2 and neuregulin-1 was decreased in Nrf2-deficient type II alveolar epithelial cells compared to wild-type cells and suggested that Nrf2 could initiate signal transduction pathways that regulate cell proliferation during development and in response to injury (Reddy et al., 2007). However, our data imply that Nrf2 negatively act against fibrotic TGF-β1 signaling. Hence, the differential role of Nrf2 between the expression of TGF-β1 and TGF-β2 may determine the recovery direction after tissue damage.

There is still no report showing that Nrf2 binding site exists in TGF-β1 promoter region. Thus, direct binding of Nrf2 to TGF-β1 promoter may not be possible; rather it could be plausible that Nrf2 regulates other transcription factors that affect TGF-β1 expression. Transcriptional regulation of *TGF-β1* gene is dependent on the activities of several transcription factors. In human TGF-β1 promoter region, two AP-1 sites located between −453 bp and −323 bp play a key role in target gene expression (Kim et al., 1989). Proximal promoter bindings of Zf9 and Sp1 are also required for the transactivation of *TGF-β1* gene (Kim et al., 1998). Hence, Nrf2 itself or Nrf2-dependent induction of antioxidant proteins could dominate AP-1, Zf9 or Sp1 activity, which may be critical for the down-regulation of TGF-β1 by Nrf2. In fact, it has been reported that Nrf2 modulates the expression of redox-sensitive transcription factor family, AP-1 and NF-κB (Yang et al., 2005).

In summary, solubilized CoQ10 protects against hepatic fibrosis by diminishing TGF-β1 expression and subsequently blocking the activation of hepatic stellate cells. Nrf2 activation by CoQ10 is important for either the induction of antioxidant enzymes or the blocking of TGF-β1-mediated stellate cell activation. Thus, a solubilized formulation of CoQ10 may have therapeutic potential for liver fibrosis.

**Conflict of interest statement**

The authors declare that there are no conflicts of interest.

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