

Catalase ameliorates hepatic fibrosis by inhibition of hepatic stellate cells activation

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1. ABSTRACT

Catalase, an endogenous antioxidant enzyme, is thought to have rescue effects on hepatic fibrosis. In this study, the regulation of catalase in CCl₄-induced hepatic fibrogenesis was investigated. Our results indicated that catalase expression was decreased upon CCl₄ treatment in a time-dependent manner, while the expression of several profibrosis and proangiogenic factors, including transforming growth factor (TGF)- β 1, vascular endothelial growth factor (VEGF), and angiopoietin 1 were significantly increased. To assess the role of catalase in hepatic fibrosis, catalase was overexpressed in HSC-T6 cells. This overexpression resulted in the inhibition of cell proliferation, migratory activity, and α -smooth muscle actin (α -SMA) expression, key features that characterize activation of hepatic stellate cells (HSC). Overexpression of catalase led to a decrease in the secretion of collagen type 1 and angiopoietin 1. These results indicate that loss of catalase activity is involved in the pathogenesis of hepatic fibrosis caused by the activation of HSCs.

2. INTRODUCTION

The pathophysiology of liver fibrosis is a complicated biological process featuring an excessive accumulation of extracellular matrix (ECM) and increased neoangiogenesis, caused by the activation of hepatic stellate cells (HSCs) (1-3). In advanced stages, liver fibrosis disrupts the normal liver architecture, leading to portal hypertension and impaired liver function, which are the main risk factors for hepatocellular carcinoma (4). Therefore, development of novel therapies that target critical molecules involved in the progression of fibrosis is an important therapeutic strategy in hepatology.

In the last decade, there have been major advances in our knowledge regarding the pathogenesis of hepatic fibrosis. HSCs have been recognized for their role in contributing to the fibrotic process, regardless of the underlying etiology (3, 5-7). In response to liver damage, HSCs transform to an activated myofibroblast phenotype, displaying enhanced cell migration and adhesion, up-regulated α -SMA expression, increased proliferation, and

secretion of proinflammatory cytokines (8). Quantitative analysis of HSC activation has been shown to be useful in predicting the rate of progression of liver fibrosis in some clinical situations (5). Therefore, HSCs represent an attractive target for the development of antifibrotic therapies. At the molecular level, there is a substantial amount of evidence supporting the hypothesis that increased oxidative stress plays an important role in causing liver damage and initiating hepatic fibrogenesis in humans (9). Evidence of oxidative stress has been detected in almost all cases, clinical or experimental, of chronic liver disease (CLD), independent of their etiologies or rate of fibrotic progression, and often in association with a decreased antioxidant defense (10). A series of *in vivo* and *in vitro* studies have outlined a close relationship between oxidative stress and activation of HSCs in terms of α -SMA expression, proliferation, and invasiveness (11, 12). Various cytokines, chemokines, and growth factors are involved in the development of fibrosis; however, TGF- β is considered the most potent and ubiquitous profibrogenic cytokine and TGF- β 1 is the primary mediator of human fibrogenesis (2). In HSCs, TGF- β initiates the transition to myofibroblast-like cells, stimulating the synthesis of ECM proteins, while simultaneously inhibiting their degradation (3, 13).

Angiogenesis is a dynamic, hypoxia-stimulated, and growth factor-dependent process strongly linked to progressive fibrogenesis. Experimental and clinical studies have unequivocally reported that pathological hepatic angiogenesis, irrespective of etiology, plays a pivotal role in the fibrogenic progression of CLD (1, 14). HSCs play an important pro-angiogenic role in fibrogenesis through the enhanced expression of proangiogenic factors (15). In addition to vascular endothelial growth factor (VEGF), HSCs also secrete Angiopoietin 1 (Ang 1) which behaves as a key contributor to fibrosis-associated vascular change (6, 16). Recent literature has identified a number of molecular and cellular mechanisms that manipulate the cross talk between angiogenesis and fibrogenesis, with a specific focus on the crucial role of oxidative stress and activated HSCs (9, 10, 12, 17-21). Angiogenesis and hepatic fibrosis are thus, mutually stimulatory and their association is regarded as crucial in the evaluation of disease progression and in the search for therapeutic targets.

Catalase, a 240-kDa tetrameric heme protein, is one of the major intracellular antioxidant enzymes. Its function is to protect cells from the accumulation of H_2O_2 by catalyzing its decomposition into water and oxygen. Catalase activity has been reported to diminish in human pulmonary fibrosis; deficiency in catalase activity in the lungs results in a predisposition towards lung inflammation and subsequent fibrosis. Administration of catalase attenuates the development of pulmonary fibrosis in mice (22). In the liver, studies show that catalase expression (both at the mRNA and protein level), and activity, decreases with progressive liver fibrogenesis (17, 18, 21, 23). PPAR α ligands, when introduced in the liver, exhibit antifibrotic action by increasing catalase expression and activity (21). However, the mechanism regulating catalase activity in hepatic fibrosis remains to be determined. Therefore, the aim of this study was to clarify the effects of catalase in the pathogenesis of hepatic fibrosis.

3. MATERIALS AND METHODS

3.1. Reagents

CCl_4 was obtained from Sigma-Aldrich Chemical Co. (St Louis, USA). Antibodies against β -actin, α -SMA, CD31, TGF- β 1, Ang 1, and horseradish peroxidase-conjugated goat anti-mouse IgG antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against VEGF, and tyrosine kinase with immunoglobulin (Ig)G-like and endothelial growth factor-like domain 2 (Tie2) were purchased from Cell Signaling Technology Inc. (Beverly, MA).

3.2. Animal models of liver fibrosis

Sprague-Dawley rats, purchased from the Shanghai laboratory animal center, were caged in an environment with regulated temperature ($23^\circ\text{C} \pm 1.6^\circ\text{C}$), humidity ($45\% \pm 10\%$), and an alternating 12-h light and dark cycle. The animals had free access to water and diet throughout the study. All animal protocols were carried out in accordance with ethical guidelines, and animals had free access to chow and water throughout the experiments. Liver Fibrosis (in 6 rats) was induced by subcutaneous injection of CCl_4 . Briefly, the rats received a single injection of 40% CCl_4 at 5 mL/kg and then 3 mL/kg of 40% CCl_4 dissolved in olive oil twice every week for 8 weeks. Rats in the normal group ($n = 6$) did not receive CCl_4 treatment but they were injected with the vehicle olive oil. At the time of killing, hepatectomy was performed and liver tissue samples were either frozen immediately in liquid nitrogen and stored at -70°C or fixed in 10% buffered formalin and embedded in paraffin.

3.3 Pathological examination

Liver specimens were preserved in 4% formaldehyde and dehydrated in a graded alcohol series. The specimens were then embedded in paraffin blocks, cut into 4 μm thick sections and placed on glass slides. Sections were then stained with Hematoxylin-eosin (HE).

3.4. Western blotting analyses

Liver tissues were homogenized in RIPA lysis buffer containing 150 mM NaCl, 50 mM Tris, 0.1% SDS, 1% Nonidet P-40, and 0.5% deoxycholate supplemented with protease inhibitors. The supernatants were collected by centrifugation at $10,000 \times g$ at 4°C for 15 min.

HSC-T6 cells were harvested and lysed on ice for 30 minutes in lysis buffer containing 150 mM NaCl, 100 mM Tris (pH 8.0), 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 5 mM EDTA, 10 mM sodium formate, 1 mM sodium orthovanadate, 2 mM leupeptin, 2 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 2 mM pepstatin A. After centrifugation at $14,000 \times g$ for 15 minutes at 4°C , the supernatant was collected as the total cellular protein extract.

Protein concentration was determined using the BCA Protein Assay Kit according to the protocol provided by the manufacturer (Pierce Chemical, Rockford, IL). Equal amounts of protein were separated using 10% SDS gel electrophoresis (SDS-PAGE) under denaturing and

nonreducing conditions, and then transferred to a nitrocellulose membrane. The membrane was blocked with 3% BSA in TBST at room temperature for 1 h and then incubated with primary antibody at 4°C overnight. After washing in TBST, the blots were incubated with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). β -Actin was probed as an internal control. Protein bands were visualized by using chemiluminescence reagent (Amersham, Chalfont St. Giles, Buckinghamshire, UK). The signals were visualized using the enhancement system (ECL).

3.5. Recombinant adenovirus production

The plasmid shuttle vectors were constructed by inserting full-length rat catalase cDNA into pShuttle-CMV-EGFP. pAdeno-GFP and pShuttle-catalase were double-cut and recombined to build the pAdeno-GFP-catalase vector, which was transfected into human embryonic kidney 293 cells for viral replication. After transfection, plates were overlaid with agar, and initial plaques were harvested, amplified, and screened for enzymatic activity. Adenovirus containing no cDNA (AdGFP) was used as a control.

Virus possessing cytosolic catalase (AdCat) was plaque-purified two times and amplified in HEK293 cells. Purified high titer stocks of recombinant adenovirus were purified by double cesium gradient ultracentrifugation. The preparation was dialyzed against 10 mmol/L Tris, 1.0 mmol/L $MgCl_2$, 1.0 mmol/L HEPES, and 10% glycerol, and stored at $-80^\circ C$. Viral titer was determined by plaque assay. The concentration of recombinant adenovirus was quantified by absorbance, and the ratio of particles to plaque-forming units was confirmed to consistently range between 20 and 30.

3.6. Cell culture and infection

HSC-T6 cells, an immortalized rat HSC line, was gifted by Scott L Friedman (Division of Liver Diseases, Mount Sinai School of Medicine, New York, NY, USA), were routinely cultured in Dulbecco's-modified Eagle's medium (DMEM; Gibco, USA United States) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 $\mu g/mL$ streptomycin. Cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at $37^\circ C$; 0.25% trypsin was used for cell passage. Before infection, HSC-T6 cells were seeded onto dishes, grown to 70% confluence, infected with AdCat, and AdGFP at m.o.i. = 100. Forty-eight hours after infection, cells were collected and assayed for catalase expression.

3.7. Cell viability assay

Cell proliferation was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Roche, Mannheim, Germany), according to the manufacturer's protocols. After treatment, cells were harvested and washed in PBS, and 200 μL DMEM without phenol red, containing 5 mg/mL MTT, was added to each well. Four hours later, the supernatant was discarded and 100 μL DMSO was added to each well. The mixture was shaken and OD values were measured at 570 nm using a

Universal Microplate Reader (EL800, BIO-TEK Instruments Inc).

3.8. HSC-T6 migration assay

The migratory capacity of HSC-T6 cells was investigated by using a BioCoat Matrigel Invasion Chamber (Becton Dickinson, San Jose, CA, USA). Confluent HSC-T6 cells at the top chamber were incubated in serum free medium, and medium with 10% FBS was used as a chemoattractant in the bottom chamber. After incubation for 12 h, cells from the upper surface of the membrane were completely removed by gentle swabbing. The remaining cells that migrated to the lower surface of the membrane were fixed and stained with H&E. Cellular migration was determined by counting the number of stained cells on membranes in five randomly selected fields. HSC-T6 cells migrating through the membrane were photographed using an inverted microscope.

3.9. Enzyme-linked immunosorbent assay

HSC-T6 cells were incubated with serum free media for 24 h and the supernatants were collected. The secretion of collagen type I and Ang 1 into the culture supernatant was assessed by ELISA using a commercially available kit (R&D Systems, Minneapolis, MN), following the manufacturer protocol.

3.10. Statistical Analysis

Results are depicted as mean \pm standard error of the mean (SEM) from three or more independent experiments. Statistical significance was assessed by One-way ANOVA or two-tailed Student's t-test where appropriate. A P-value of <0.05 was considered statistically significant.

4. RESULTS

4.1. Catalase was decreased in liver fibrosis induced by CCl_4

The CCl_4 -induced fibrotic model has obvious characteristics of fibrosis and angiogenesis in the liver. CCl_4 administration for 8 weeks caused extensive fibrosis, which was visually assessed by H&E staining (Figure 1A). The histological analysis of livers from the control group indicated a normal liver lobular architecture with central vein and radiating hepatic cords. CCl_4 -induced liver damage resulted in marked morphological alterations and fibrosis, as evidenced by disruption of the tissue architecture, large fibrous septa formation, pseudo-lobe separation, and fibrous accumulation. TGF- $\beta 1$, widely known as an important mediator for progressing fibrosis, was upregulated. Because neo-angiogenesis is a common feature of most fibrogenic disorders including hepatic fibrosis, we examined specific markers of angiogenesis, such as CD31, VEGF, Tie 2, and Ang 1. All four markers in the CCl_4 -induced fibrotic liver were upregulated when compared with the control groups (Figure 1B). The level of catalase protein in the fibrotic liver tended to be lower than in the control liver. As shown in Figure 2, the expression of catalase was decreased after 4 weeks of CCl_4 administration in a time-dependent manner.

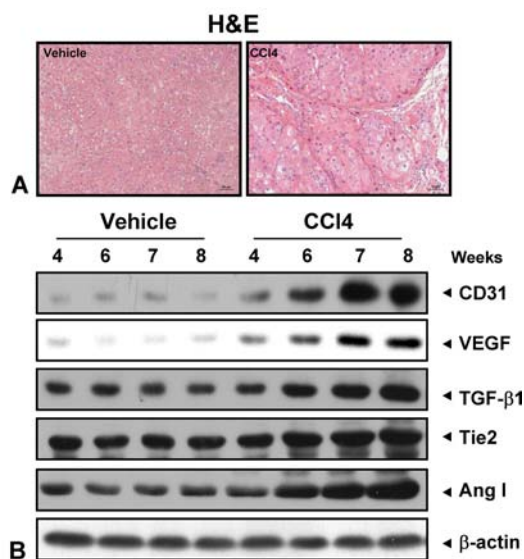


Figure 1. Liver fibrosis induced by CCl₄. A) Histopathological examination of rat livers revealed fibrosis. CCl₄ led to pronounced morphological alterations and fibrosis, as evidenced by disruption of the tissue architecture, extension of fibers, large fibrous septa formation, pseudo-lobe separation, and fibers accumulation. B). Increased expressions of fibrosis-related molecules in CCl₄-induced fibrotic liver tissue in rats. Compared to the normal group, the expression of CD31, VEGF, TGF- β 1, Angiopoietin 1 and Tie 2 protein significantly increased in fibrotic livers detected by western blot. β -actin was used as a control.

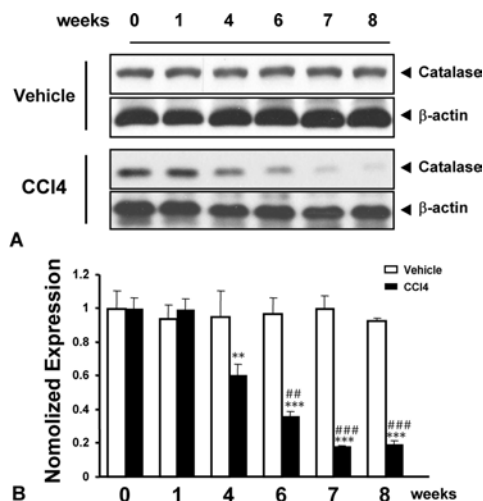


Figure 2. Expression of catalase in CCl₄-induced fibrotic liver tissue in rats. The expression of catalase protein was detected by western blot. Compared to the normal group, the expression of catalase was significantly decreased in fibrotic livers. The proteins were semi-quantified by densitometric analysis and expressed as the mean \pm SEM for three separate experiments. ** $P < 0.01$, *** $P < 0.001$ versus week 0, ### $P < 0.01$, #### $P < 0.001$ versus corresponding vehicle control.

4.2. Catalase inhibits activation of HSC-T6 cells

To explore the function of catalase in liver fibrogenesis, we developed stable clones overexpressing catalase in HSC-T6 cells. Successful infection of the AdCat virus was indicated by a fluorescent signal under a microscope, and expression of catalase was confirmed to be markedly increased in the total cell extract, as confirmed by Western blot analysis (Figure 3A). One of the features of activated HSCs is their increased proliferation. Overexpression of catalase inhibited cell proliferation as indicated by the MTT assay (Figure 3B). HSC-T6 cells infected with AdCat also exhibited less migratory activity compared to cells infected with the AdGFP virus (Figure 3C).

4.3. The expression of fibrosis-associated molecules in AdCat-infected HSC-T6 were decreased

After showing that catalase inhibits the activation of HSC-T6 cells, we investigated if catalase has an effect on the cytokines involved in the activation of HSCs. α -SMA, a reliable indicator of HSC activation, was significantly downregulated in cells infected with AdCat (Figure 4A). Collagen type I expression was also significantly reduced. (Figure 4B). Ang 1 levels in the conditioned media was also downregulated by catalase (Figure 4C).

5. DISCUSSION

Liver fibrosis is the result of the wound-healing response of the liver to repeated injury and it affects roughly 100 million people worldwide. The liver fibrosis model caused by chronic CCl₄ administration has been used for decades to investigate the response of the liver to injury, since the elementary lesions caused by this hepatotoxin replicates the pathogenesis seen in most cases of human and animal liver injury (24). We observed fibrous expansion of portal areas with portal-to-portal bridging in the mice treated with CCl₄, confirming that liver damage was induced by CCl₄. Furthermore, we showed that the expression of hepatic catalase protein in the CCl₄ group was decreased. This observation is in accordance with some previous studies (17, 21, 23). Collectively, these findings suggest that the loss of catalase activity may have an important role in the pathogenesis of liver fibrosis induced by chronic CCl₄ administration.

Decrease of catalase suggests the presence of intracellular oxidative stress in fibrotic livers. ROS such as hydrogen peroxide (H₂O₂) act to promote liver fibrosis (9, 10, 12). Catalase is the most important enzyme involved in the detoxification of H₂O₂ and protection of hepatocytes from oxidative stress (17). Accordingly, catalase treatment has been shown to block the effect of enhanced TGF- β 1 production, in response to extrinsic H₂O₂, in HSCs (18). ROS also directly activate HSCs, resulting in the initiation of fibrosis (11). It is well known that HSC activation plays a major role in fibrosis due to its involvement in depositing excess collagen in the liver in response to various stimuli (7, 9, 25). In this study, we demonstrated that catalase effectively inhibits the activation of the immortalized rat hepatic stellate cell line HSC-T6 into myofibroblast-like

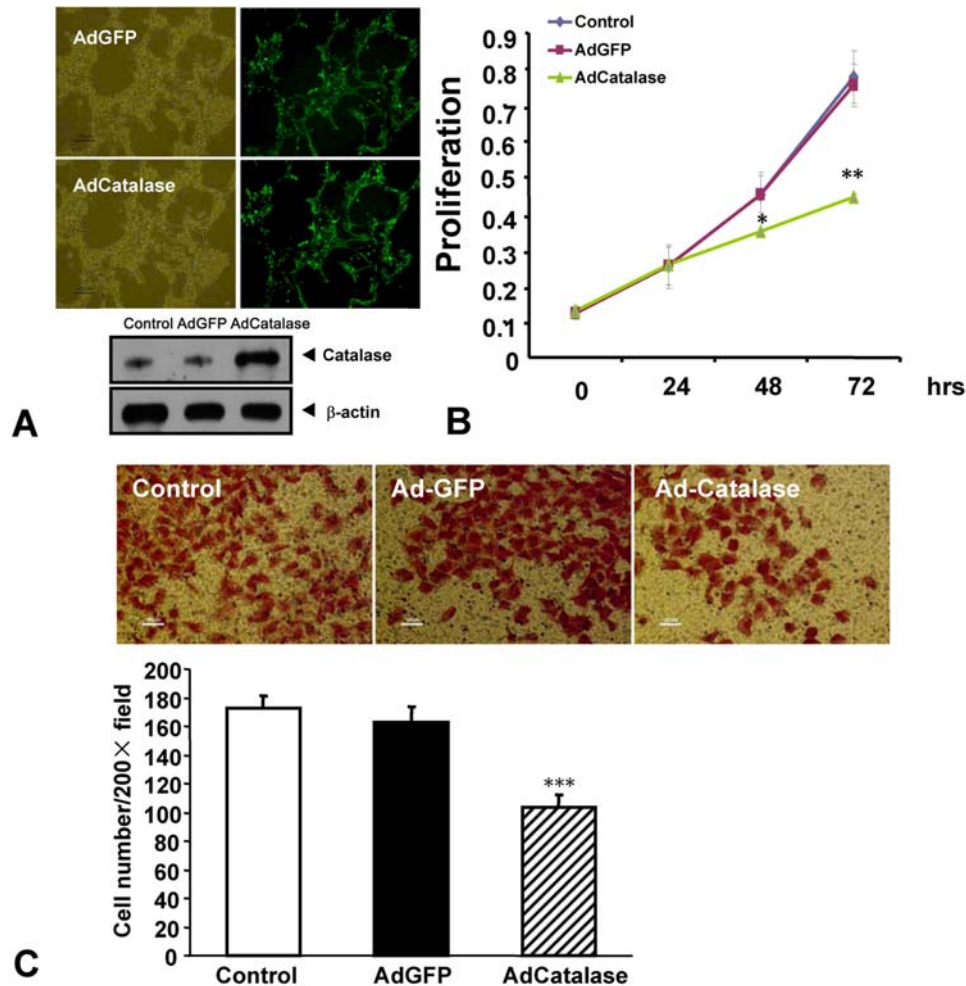


Figure 3. Catalase inhibited proliferation and migration of HSC-6T. A) Schematic representation of adenoviruses encoding GFP (Ad-GFP) and catalase (Ad-catalase GFP); Lower panel, immunoblotting analysis of catalase protein level. β -actin was used as a control. B) HSC-T6 proliferation. The MTT assay was used to measure the relative growth of the cells. Three independent experiments were performed, and the results are shown as the mean \pm SEM. C) Invasion assay. After 12 hours of incubation, filters were stained and migrating cells that adhered to the underside of the membrane were counted (upper panel; original magnification \times 200). Data are expressed as the mean \pm SEM from four experiments. ** P < 0.01, *** P < 0.001 versus control.

cells, by reducing α -SMA expression, inhibiting cell proliferation, invasion, and collagen I secretion *in vitro*.

Activated HSCs accumulate at the sites of tissue repair and regulate other key processes in fibrogenesis such as angiogenesis and ECM degradation. Neoangiogenesis and the development of an abnormal angioarchitecture in the liver are strongly linked to progressive fibrogenesis (1, 14), although the direct interaction between both processes is not yet fully understood. Furthermore, angiogenesis is proportional to the increase in the stage of fibrosis. Anti-angiogenic drugs have been suggested as a new therapeutic strategy for treating chronic liver disease (26). HSCs play a central role in the development of liver fibrosis by secreting proinflammatory and profibrogenic cytokines (5). Among the factors secreted by activated HSCs, Ang 1 is involved

in angiogenesis, myofibroblast proliferation, and collagen synthesis (6). Increased mRNA expression of the Ang 1 gene has been observed in fibrotic livers from patients and animal models (6). Ang 1 and its receptor tyrosine kinase with IgG-like and endothelial growth factor-like domain 2 (Tie 2) have been implicated in hepatic fibrogenesis with angiogenesis (19). It has been reported that blocking Ang 1-signaling in murine models of hepatic fibrosis inhibits both angiogenesis and fibrosis (6). Therefore, Ang 1 inhibitors are potentially useful targets for anti-angiogenic therapy in the treatment of liver fibrosis. In the present study, we show that Ang 1 secretion is significantly decreased in catalase-overexpressing HSC-T6 cells, corroborating reports that implicate Ang 1 in hepatic fibrogenesis. These findings indicate that catalase might ameliorate hepatic fibrosis through the inhibition of Ang 1 production.

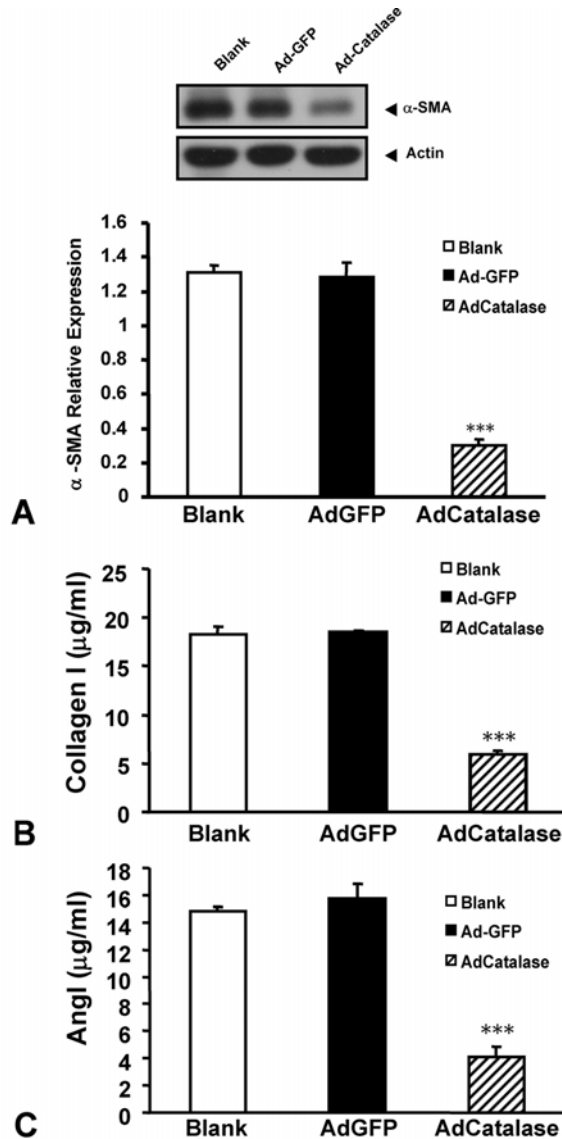


Figure 4. The abolishment of fibrosis-related molecules by catalase overexpression. A) After transfection for 24 h, cell lysate preparation was subjected to Western blotting with an antibody against α-SMA. Representative data from three independent experiments are shown. The histogram summarizes densitometric data from all the experiments undertaken. B, C) Collagen type I and Ang I secretion was measured in conditioned media from cultures of HSC-T6 using an ELISA kit. Data are expressed as the mean ± SEM from three experiments, each of which was performed in duplicate. *** $P < 0.001$ versus control

In conclusion, we have shown that reduction of catalase in the liver is associated with CCL₄-induced liver injury in rats. Additionally, catalase prevents the activation of HSCs and decreases the expression of fibrosis-related molecules. Therefore, we propose that stimulating the expression and activity of catalase in the liver might be an effective therapy for treating liver fibrosis.

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Abbreviations: ROS, Reactive oxygen species; HSCs, hepatic stellate cells; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; ECM, extracellular matrix; CLD, chronic liver diseases; HE, Hematoxylin-eosin; FBS, fetal bovine serum; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

Key Words: ROS, Hepatic Stellate Cells, VEGF, Chronic Liver Diseases, TGF-beta 1

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