Geranylgeranylacetone attenuates hepatic fibrosis by increasing the expression of heat shock protein 70

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Abstract. Increasing evidence has demonstrated that the heat shock protein 70 (HSP70) gene may be closely associated with tissue fibrosis; however, the association between HSP70 and liver fibrosis remains to be fully elucidated. The present study hypothesized that geranylgeranylacetone (GGA) exerts beneficial effects on liver fibrosis though upregulation of the expression of HSP70. Liver fibrosis was induced in rats using carbon tetrachloride (CCl_4). The rats were subsequently divided into three groups: Control group, CCl_4 model group and CCl_4 model + GGA group. Liver fibrosis in the rats was evaluated using hematoxylin and eosin staining, Masson's trichrome staining and Sirius red staining. The levels of serum alanine aminotransferase, aspartate aminotransferase and total bilirubin were determined using an automated biochemistry analyzer. The levels of total hepatic hydroxyproline were also determined. The expression levels of α-smooth muscle actin (α-SMA) and transforming growth factor-β1 (TGF-β1) were determined using immunofluorescence staining and western blotting, and the protein expression levels of HSP70 were determined using western blotting. The CCl_4-induced rats exhibited liver fibrosis, increased hydroxyproline content, impaired liver function, upregulated expression levels of the α-SMA and TGF-β1 pro-fibrogenic proteins, and increased expression of HSP70, compared with the control group. These changes were attenuated by treatment with GGA. These results demonstrated that GGA exerted beneficial effects in CCl_4-induced liver fibrosis via upregulating the expression of HSP70.

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Introduction

Liver fibrosis is the common response to chronic liver injury, which is characterized by accumulation of extracellular matrix proteins and can result in the loss of liver function and disruption of liver tissue structure, ultimately leading to irreversible cirrhosis (1,2). Due to the lack of effective therapies, cirrhosis-associated mortality has been increasing annually. It is increasingly considered that the most effective method to prevent and treat cirrhosis is to inhibit the development of hepatic fibrosis (3).

Heat shock proteins (HSPs) are a family of polypeptide proteins consisting of several members, which perform important housekeeping functions and are named according to their molecular weight, including HSP105/110, Hsp90, HSP70, HSP60, HSP40, and other low molecular weight HSPs (4,5). HSP70 chaperones and their co-chaperones comprise a set of abundant cellular machines, which assist a variety of protein folding processes in almost all cellular compartments (6). They provide an essential action through preventing aggregation and assisting refolding of misfolded proteins (7). By regulating cell apoptosis and autophagy, reducing oxidative stress and resisting inflammation, HSP70 protects the body against acute and subacute injury (8). Previous studies have demonstrated that the HSP70 gene may be closely associated with tissue fibrosis: Firstly, high expression levels of HSP70 have been detected in the livers of patients with cirrhosis and of animals with hepatic fibrosis (9); secondly, HSP has been reported to have a protective role against atrial fibrosis in several studies (10,11); thirdly, abnormal expression of HSP70 has been detected in patients with pulmonary fibrosis and in rats with renal fibrosis (12,13); and finally, by increasing the expression of Toll-like receptor 4, HSP70 interacts with vascular smooth muscle cells, which are the major producers of extracellular matrix proteins (14).

Geranylgeranylacetone (GGA), which is a drug used to treat gastric ulcers, has previously been observed to induce the expression of HSP70 in cultured gastric mucosal cells (15,16). GGA-mediated upregulation of HSP70 has been demonstrated to prevent the majority of the important events that occur during fibrosis, including prevention of myofibroblast differentiation and epithelial-to-mesenchymal

transition (EMT) (17,18). In addition, HSP70 has been reported to reduce apoptosis and EMT, which are important contributors to tubular cell injury *in vitro* and *in vivo* (12). Notably, a high dose of GGA has been observed to induce HSP72 in the rat liver (19,20), and treatment with GGA may prevent liver damage through the induction of HSPs (21). Therefore, the present study hypothesized that GGA attenuates CCl$_4$-induced fibrosis by upregulating the expression of HSP70.

The present study aimed to examine the hypothesis that GGA, an HSP70 inducer, may potentiate its biological effects against CCl$_4$-induced liver injury via the upregulation of HSP70.

Materials and methods

Materials. The reagents used in the present study were obtained from the following sources: Geranylgeranylacetone (GGA) was purchased from Eisai China, Inc. (Shanghai, China). Anti-HSP70 was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). CCl$_4$ was purchased from Merck Millipore (Darmstadt, Germany). Transforming growth factor-β1 (TGF-β1; SC-146) antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). α-smooth muscle actin (α-SMA; A2547) and β-actin (A2228) antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animal models. A total of 24 male Sprague-Dawley rats, weighing between 180 and 220 g, were housed under standard animal laboratory conditions, at a controlled temperature (22±1˚C), humidity (65±5%) and 12 h light/dark cycles with free access to food and water, in a specific-pathogen-free-grade animal room at the Experimental Animal Center of Soochow University (Changzhou, China). All the rats were fasted for 2 days prior to the experiment. The rats were randomly divided into three groups: Group I (control group), group II (CCl$_4$ group), and group III (CCl$_4$ + GGA group). The control rats were administered daily with sterile saline orally (n=8). In the CCl$_4$ model group, liver fibrosis was induced in the rats by intraperitoneal injection of 400 ml/l CCl$_4$ salad oil solution, with a single dose of 3 µg/g/rat twice a week (n=8). In the CCl$_4$ model + GGA group, the rats received the same dose of CCl$_4$ as group II, alongside a daily oral dose of an emulsion containing 400 mg/kg GGA, 4 weeks after modeling (n=8). All the rats were sacrificed by injection with pentobarbital (65 mg/kg i.p.; Sigma-Aldrich) after 9 weeks, following which blood samples (1 ml) and liver tissues were obtained. The animal experimental procedures were approved by the Animal Care and Use Committee of Soochow University and the Ethics Committee of the Third Affiliated Hospital of Soochow University (Changzhou, China).

Serological examination. At the end of the ninth week, blood samples were collected from the caudal vein of the rats and analyzed for levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TB) using ALT/AST and TB kits (Nanjing Jiancheng Bioengineering Institue, Nanjing, China), according to the manufacturer's instructions. SPSS 13.0 softwared (SPSS, Inc., Chicago, IL, USA) was used for analysis and these measurements were used to compare the differences in liver function between each group.

Measurement of hepatic hydroxyproline content. Total hepatic hydroxyproline levels were determined in the hydrolysates of the liver samples. Briefly, 100 mg wet liver samples were subjected to acid hydrolysis, and the quantity of hydroxyproline was determined using a Hydroxyproline Testing kit (cat. no. A030-2; Jiancheng, Nanjing, China), according to the manufacturer's instructions.

Histological examination. Liver biopsies from the rats were harvested and tissue fragments were fixed in Bouin's solution (Sigma-Aldrich) overnight, embedded in paraffin and stained with hematoxylin and eosin (HE) for general histopathological examination. Masson's trichrome staining and Sirius red staining were used to assess collagen levels. The red stained areas in the Sirius Red-stained sections were assessed using an image analyzer (Image-Pro Plus, Media Cybernetics, Inc., Rockville, MD, USA) for semiquantitative analysis. The percentage of Sirius Red staining was used to determine the differences in each group.

Immunofluorescence staining. Indirect immunofluorescence staining was performed, according to an established procedure (22). Briefly, 3 µm cryosections were prepared using a Microm HM 440E (Thermo Fisher Scientific, Waltham, MA, USA) and blocked with 3% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at room temperature. Subsequently, the sections were incubated with primary antibodies against TGF-β1 and α-SMA in phosphate-buffered saline (PBS), supplemented with 3% BSA, overnight at 4˚C. The sections were then washed thoroughly in PBS, followed by incubation with tetramethylrhodamine-conjugated secondary antibody (SAB3700867; Sigma-Aldrich), at a dilution of 1:200, in PBS supplemented with 3% BSA, in the dark for 1 h. Following subsequent washing with PBS, the sections on slides were visualized using a Nikon Eclipse 80i Epi-fluorescence microscope equipped with a digital camera (DS-Ri1; Nikon Corporation, Tokyo, Japan).

Western blot analysis. The total proteins were extracted using radioimmunoprecipitation buffer containing a protease inhibitor cocktail (Sigma-Aldrich). The protein concentrations were determined using a bichinchoninic acid method (Beyotime Institute of Biotechnology, Haimen, China). Equal quantities of protein (50 µg) were loaded onto each lane of a polyacrylamide gel, separated by 10% SDS-PAGE (EMD Millipore, Billerica, MA, USA) and transferred to nitrocellulose membranes (EMD Millipore). The membranes were blocked with 5% skimmed milk in Tris-buffered saline (pH 7.6) at room temperature, and were then incubated overnight at 4˚C with the following primary antibodies: Anti-HSP70 (4008; 1:1,000), anti-TGF-β1 (1:200), anti-α-SMA (1:200) and anti-β-actin (1:500). Following further incubation with the corresponding secondary antibody, immune complexes (7074) were detected using enhanced chemiluminescence western blotting reagents (Sinopharm Chemical Reagent, Shanghai, China). The detected proteins were normalized to β-actin or the respective total protein, as appropriate.

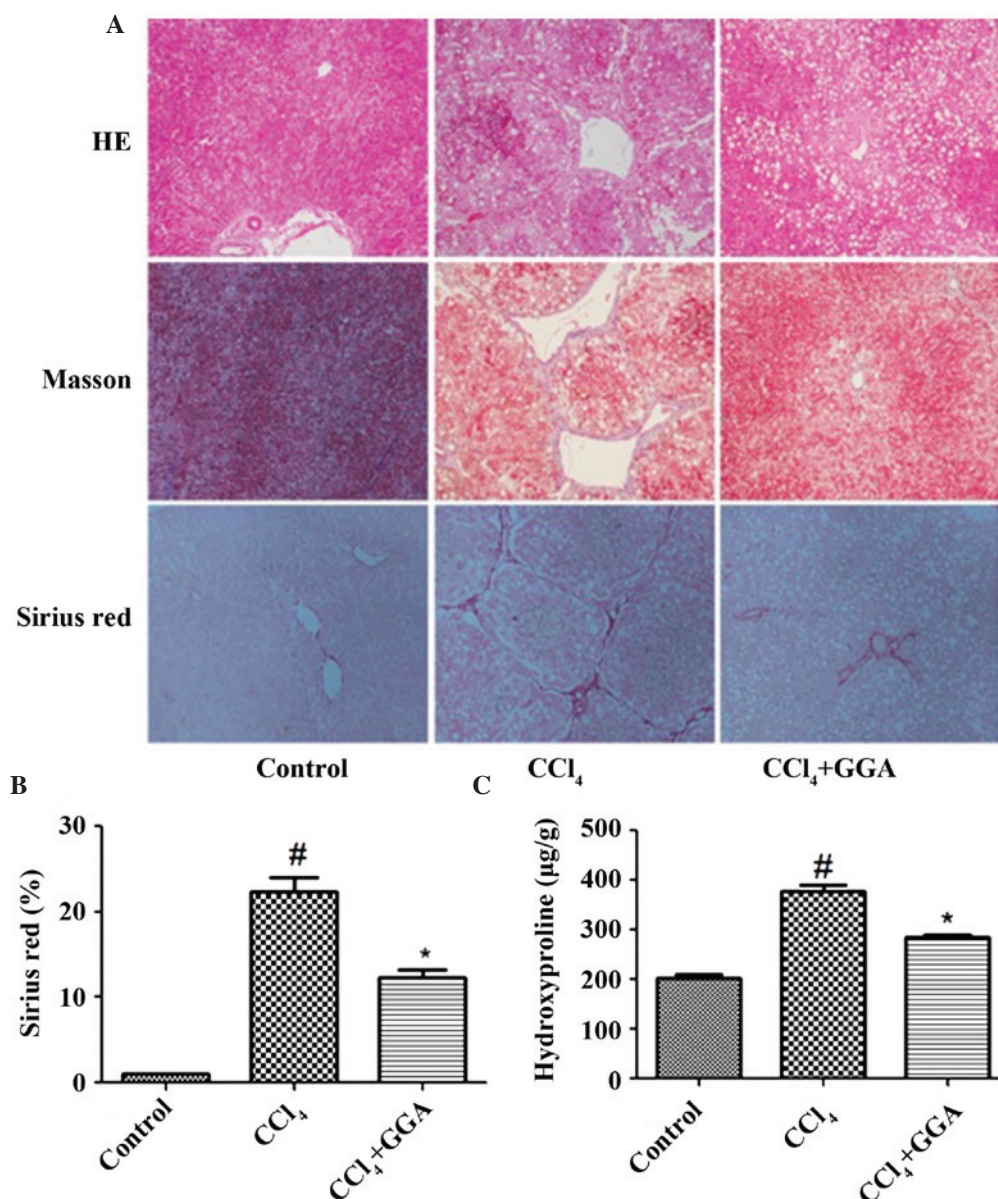Figure 1. GGA attenuated CCl_4-induced hepatic fibrosis in rats. (A) CCl_4 was used to construct a hepatic fibrosis model, to evaluate the therapeutic effect of GGA. Liver sections were stained with HE, Masson's reagent and Sirius Red for histological examination to determine hepatic morphology, structural changes and ECM content in the liver tissue of each group (magnification, x200). (B and C) Levels of ECM were determined by quantitative estimation of the red-stained areas in the Sirius Red-stained sections, and of hydroxyproline content. #$P<0.05$, compared with the control group; *$P<0.05$, compared with the CCl_4 group. GGA, geranylgeranylacetone; CCl_4, carbon tetrachloride; HE, hematoxylin and eosin; ECM, extracellular matrix.

Statistical analysis. Statistically significant differences between the control and treatment groups were detected using a simple analysis of variance, followed by Dunnett's multiple comparison tests. SPSS 13.0 was used for statistical analysis. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of GGA on hepatic fibrosis in CCl_4-induced rats. As shown in Fig. 1, the rats in the CCl_4-induced group exhibited extensive fibrotic deposition, which was demonstrated by HE, Masson's trichrome and Sirius red staining. In addition, quantitative estimation of hydroxyproline content indicated that the hydroxyproline content was significantly higher in the CCl_4-induced group, compared with the control group ($P<0.05$). However, treatment with GGA resulted in significant decreases in fibrotic deposition and the content of hydroxyproline, compared with the CCl_4-induced group ($P<0.05$).

Effects of GGA on liver function in CCl_4-induced rats. As shown in Fig. 2, the ALT, AST, and TB levels were markedly increased in the CCl_4-treated rats, compared with the control group. However, the GGA-treated CCl_4 rats exhibited marked decreases in ALT, AST and TB levels, compared with the CCl_4-treated rats.

Effects of GGA on the expression of HSP70 in CCl_4-induced rats. To determine the role of HSP70 in the protective effects of GGA against CCl_4-induced hepatic damage, the expression
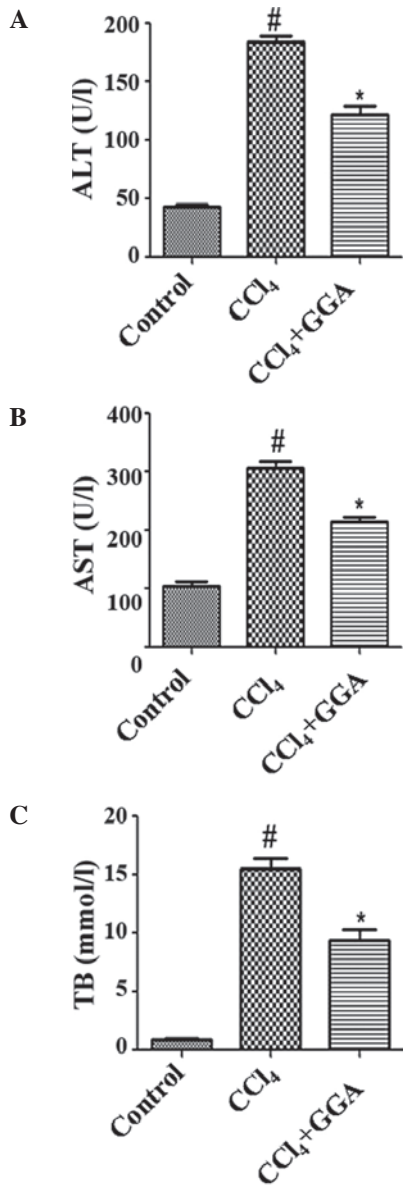
Figure 2. GGA improves liver function in CCl$_4$-induced rats. The levels of (A) ALT, (B) AST, and (C) TB were examined to assess hepatic function. $^#$P<0.05, compared with the control group; *P<0.05, compared with the CCl$_4$ group. GGA, geranylgeranylacetone; CCl$_4$, carbon tetrachloride; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TB, total bilirubin.
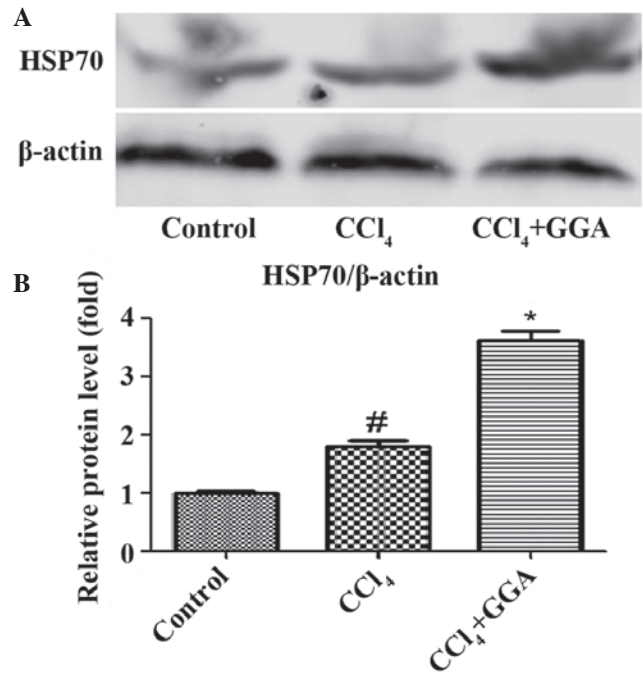

Figure 3. GGA further increases the expression of HSP70 in CCl$_4$-induced rats. (A) Protein samples (50 μg) were loaded for western blot analysis, in order to determine the expression levels of HSP70. Similar results were obtained from three repeated experiments. (B) Quantitative analysis was performed for HSP70 using a densitometer. Data are presented as the mean ± standard deviation. $^#$P<0.05, compared with the control group; *P<0.05, compared with the CCl$_4$ GGA group. GGA, geranylgeranylacetone; CCl$_4$, carbon tetrachloride; HSP70, heat shock protein 70.

levels of HSP70 were examined using western blotting. Treatment with CCl$_4$ resulted in a significant increase in the protein expression levels of HSP70 in the liver, compared with the control group. Treatment with GGA was found to further enhance CCl$_4$-induced expression of HSP70 in the liver (Fig. 3). These results indicated that GGA induced the expression of HSP70, which may be associated with the protective effects of GGA against CCl$_4$-induced hepatic fibrosis.

Effects of GGA on the expression levels of profibrogenic proteins in CCl$_4$-induced rats. Immunofluorescence staining and western blotting were used to detect the expression levels of α-SMA and TGF-β1 in the liver tissues of the rats. α-SMA and TGF-β1 have been reported to be involved with the development of hepatic fibrosis (23,24). The expression levels of α-SMA and TGF-β1 were low in the liver tissue of the control rats; however, a significant increase in the expression of α-SMA and TGF-β1 was observed with the development of CCl$_4$-induced hepatic fibrosis. Treatment with GGA was found to markedly reduce the expression levels of hepatic fibrosis-associated proteins (Fig. 4).

Discussion

In previous decades, there have been significant advances in understanding of the cellular and molecular mechanisms underlying liver fibrogenesis. Hepatic fibrosis is the common response to chronic liver injury, which may result in slow, progressive damage to liver function and disrupt the structure of liver tissue (1,2). Hepatic fibrosis is also a reversible wound-healing response to either acute or chronic cellular injury, which reflects the balance between liver repair and scar formation (25). Identifying the mechanisms underlying liver fibrogenesis is important for the development of targeted therapies to reverse the fibrotic response and improve the prognosis of patients with chronic liver disease. HSP70 is involved in several cellular processes and exerts a wide range of functions. HSP70 is involved in the prevention of protein aggregation through redirecting unfolded or misfolded proteins to the proteasomal degradation system (26). Previous studies have demonstrated that HSP70 has a protective role against fibrogenesis (25,27-29). However, further investigation is required to clearly determine its roles in hepatic fibrogenesis (30).
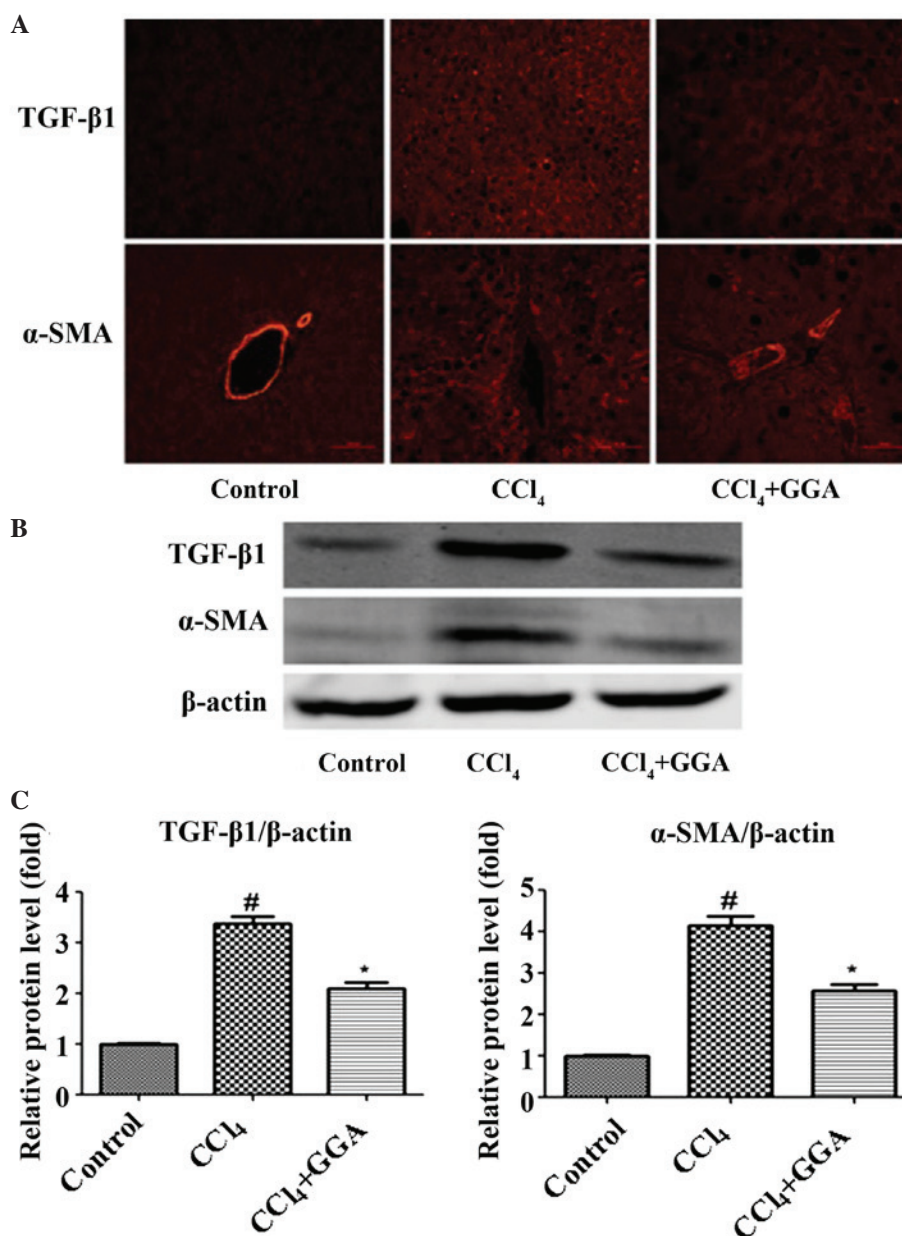
Figure 4. GGA attenuates profibrogenic protein expression in CCl$_4$-induced rats. (A) Immunofluorescence was used to examine the expression levels of TGF-β1 and α-SMA in the liver tissues (magnification, x200). (B) Protein samples (50 μg) were loaded for western blot analysis of TGF-β1 and α-SMA. Similar results were obtained from three repeated experiments. (C) Quantitative analysis was performed for TGF-β1 and α-SMA using a densitometer. Data are presented as the mean ± standard deviation. #P<0.05, compared with the control group; *P<0.05 compared with the CCl$_4$ group. GGA, geranylgeranylacetone; CCl$_4$, carbon tetrachloride; TGF-β1, transforming growth factor-β1, α-SMA, α-smooth muscle actin.

The results of the present study demonstrated that CCl$_4$ significantly increased the expression levels of HSP70 in the liver, compared with the control group. Treatment with GGA, an inducer of HSP70, further enhanced the CCl$_4$-induced expression of HSP70 in the liver, compared with the CCl$_4$-induced group. Notably, GGA administration attenuated CCl$_4$-induced hepatic fibrosis, as evidenced by the results of the HE, Masson's trichrome and Sirius red staining, and the quantitative estimation of hydroxyproline content. In addition, GGA improved the liver function, compared with the CCl$_4$-induced rats. As indicators of hepatic fibrosis, the expression levels of TGF-β1 and α-SMA in the GGA-treated CCl$_4$ group were found to be markedly lower than those in the CCl$_4$-induced rats. These results indicated that GGA attenuated hepatic fibrosis through increasing the expression of HSP70.

In conclusion, the identification of therapeutic applications to prevent the progression of liver fibrosis is clinically beneficial. The present study is the first, to the best of our knowledge, to demonstrate that daily oral administration of GGA may induce the expression of HSP70 in CCl$_4$-treated rat liver. Furthermore, HSP70 upregulation may be used as a novel therapeutic approach for the prevention of hepatic fibrosis.

Acknowledgements

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