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Original article

Chlorogenic acid protects against liver fibrosis in vivo and in vitro through inhibition of oxidative stress

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SUMMARY

Liver fibrosis is a scaring process related to chronic liver injury of all causes and as yet no truly effective treatment is available. Chlorogenic acid (CGA) is a phenolic compound and exerts anti-inflammatory and anti-oxidant activities. Our former studies suggested that CGA could prevent CCl_4-induced liver fibrosis through inhibition of inflammatory signaling pathway in rats. However, whether the anti-oxidant activity is involved in the anti-fibrotic effect of CGA on liver fibrosis is not yet fully understood. This study examined whether CGA may prevent CCl_4-induced liver fibrosis by improving anti-oxidant capacity via activation of Nrf2 pathway and suppressing the PDGF-induced profibrotic action via inhibition of NOX/ROS/MAPK pathway. The studies in vivo showed that the liver fibrosis degree, hydroxyproline content and expression of α-SMA, Collagen I, Collagen III and TIMP-1 were increased in CCl_4-injected rats and which were alleviated markedly by CGA. Furthermore, CGA significantly decreased CYP2E1 expression and increased the expression of nuclear Nrf2 and Nrf2-regulated anti-oxidant genes (HO-1, GCLC and NQO1). CGA decreased MDA level and increased GSH, SOD and CAT levels in liver tissues. In vitro studies PDGF could induce NOX subunits (p47phox and gp91phox) expression, ROS production, p38 and ERK1/2 phosphorylation, HSCs proliferation and profibrotic genes expression in HSCs, all of which were reduced by CGA treatment. In conclusion, the results suggest that CGA protects against CCl_4-induced liver fibrosis, at least in part, through the suppression of oxidative stress in liver and hepatic stellate cells.

1. Introduction

Liver fibrosis, characterized by a pathological exposition of extracellular matrix (ECM), is an early stage of cirrhosis. The various chronic liver injuries, including viral hepatitis viruses, alcoholic, drug-related, metabolic or autoimmune diseases, could result in fibrosis and cirrhosis [1]. Hepatic stellate cells (HSCs) are recognized as a major player in the development of liver fibrosis. Following a fibrogenic stimulus, the hepatic stellate cells (HSCs) are activated and acquire a myofibroblast-like phenotype that is proliferative and fibrogenic [2,3]. The activated HSCs are primary source of ECM, including collagen I and III and also produce high levels of the tissue inhibitor of metalloproteinase 1 (TIMP-1), which modulate the apoptosis of HSCs and the degradation of ECM [4]. In present times, the management of liver fibrosis and cirrhosis is still unsolved and more researches are needed [2].

Several researches indicated that oxidative stress plays a considerable role in the development of liver fibrosis. Reactive oxygen species (ROS) are believed to induce HSCs proliferation, migration and collagen accumulation [5]. The main sources of ROS in liver include NADPH oxidase (NOX), the mitochondria uncoupling, cytochrome P450 enzymes, nitric oxide syntheses and xanthine oxidase [6]. NOX, which is a multiprotein complex, consists of membrane-integrated proteins (p22phox and gp91phox) and cytosolic proteins (p40phox, p47phox, p67phox, and Rac). The accumulating evidences indicate that NOX mediates the development of liver fibrosis [7,8]. The phagocytic form of NOX existed in Kupffer cells has been demonstrated to induce HSCs activation. While the non-phagocytic form of NOX existing in HSCs,

Abbreviations: CGA, chlorogenic acid; HSCs, hepatic stellate cells; CCl_4, carbon tetrachloride; α-SMA, α-smooth muscle actin; ECM, extracellular matrix; ROS, reactive oxygen species; TIMP-1, tissue inhibitor of metalloproteinase 1; NOX, NADPH oxidase; PDGF, platelet derived growth factor; CYP2E1, cytochrome P450 2E1; Nrf2, Nuclear factor erythroid-2-related factor 2; HO-1, heme oxygenase-1; GCLC, glutamate-cysteine ligase Catalytic Subunit; NQO1, NAD(P)H:quinone oxidoreductase-1; AREs, antioxidant response elements; MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase.

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endothelial cells and fibroblasts is necessary for the activation of cell signaling pathways [9]. NOX expressed in HSCs is able to mediate HSC activation and proliferation in response to profibrotic cytokines such as platelet derived growth factor (PDGF) [10], angiotensin II [11], leptin [12], apoptotic body [13] and advanced glycation end products [14]. NOX may therefore become an exciting therapeutic target for liver fibrosis. PDGF is an important mediator of HSCs proliferation during liver fibrosis [15]. Previous studies showed that NOX-derived ROS activated p38 MAPK and ERK1/2 and played a key role in PDGF-induced HSCs proliferation and collagen synthesis in vitro [10]. Therefore, in this study we used PDGF as a stimulus to promote NOX activation and ROS production in HSCs. CCl_4 is a well known hepatoxin, which is widely used to induce liver fibrosis in experimental animal models [16]. It has been reported that cytochrome P450 2E1 (CYP2E1) in rat liver activates CCl_4 to produce a trichloromethyl free radical ($CCl3\cdot$) and a proxy trichloromethyl ($CCl3OO\cdot$) and increase ROS production in Kupffer cells, such as $\cdot O2$-, H2O2 and $\cdot OH$, which damage the liver [17,18]. Nuclear factor erythroid-2-related factor 2 (Nrf2) was considered as an important transcription factors that activates the transcription of phase II detoxifying anti-oxidant genes such as heme oxygenase-1 (HO-1), glutamate-cysteine ligase (GCL) and NAD(P)H:quinone oxidoreductase-1 (NQO1) through binding to antioxidant response elements (AREs) [19,20]. The studies showed that animals deficient in Nrf2 are subjected to more oxidant injury due to decreased anti-oxidant protection [21]. Hence, Nrf2 serve as a major regulator of cell defense. In conclusion, antioxidants are potentially therapeutic compounds in the treatment of liver disease.

Chlorogenic acid (CGA) is a phenolic compound (Fig. 1) found in coffee, fruits, and vegetables, has been reported to exert anti-oxidant [22,23], anti-inflammatory [24,25], anti-carcinogenic [26], anti-obesity [27], anti-microbial [28], anti-diabetic and anti-lipidemic activity [29]. The studies in vivo and in vitro suggested that CGA could scavenge free radical and stimulate anti-oxidant enzymatic activities [22,30]. One recent study found CGA could inhibit vascular NADPH oxidase activity and mitigate hypertension in spontaneously hypertensive rats [31]. Our previous studies showed CGA could significantly inhibit CCl_4-induced liver fibrosis and the mechanisms might be related with pro-apoptotic and anti-inflammatory effects [32–34]. However, the role of anti-oxidant effect in anti-fibrotic action of CGA against liver fibrosis remains unclear. The present study is to explore the effect of CGA on oxidative stress in liver and HSCs, especially the Nrf2 and NOX/ROS/MAPK signaling pathway.

2. Materials and methods

2.1. Reagents

PDGF was purchased from Peprotech (Rocky Hill, NJ, USA). Chlorogenic acid [CGA, #C3878, ≥95% (titration)], DMSO, MTT, Diphenyleneiodonium (DPI), N-acetylcysteine (NAC) and CCl_4 were purchased from Sigma (St. Louis, USA). Polyclonal antibodies against p47phox, gp91phox, α-SMA, Nrf2, CYP2E1, Lamin B and β-actin were purchased from Proteintech group (Chicago, IL, USA). Monoclonal antibodies against p38, p-p38, ERK1/2, p-ERK1/2, JNK and p-JNK, SB203580 and PD98059 were purchased from CST (Boston, MA, USA). The secondary antibodies were purchased from Rockland Immunochemicals (Gilbertsville, PA, USA). PVDF membrane was purchased from Millipore Corp. (Billerica, MA, USA). SuperSignal™ West Pico Chemiluminescent Substrate kit was purchased from Pierce Biotechnology Inc (Rockford, IL, USA). The RevertAidTM cDNA Synthesis Kit was purchased from Fermentas (Vilnius, Lithuania). SYBR Green Supermix was purchased from Takara Bio (Otsu, Shiga, Japan). Trizol and 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) were purchased from Invitrogen (Eugene, OR).

2.2. Animals and experimental design

Thirty-two male Sprague–Dawley rats (220–250 g) had free access to food and drinking water and maintained under a controlled environment. This experiment was approved by the ethics committee of the second affiliated hospital of Medical School of Xi'an Jiao Tong University, Xi'an, China. These rats were randomly divided into four groups: (1) Control group (n = 8): treated with vehicle only (distilled water and olive oil); (2) CGA group (n = 8): treated by the intragastric route with CGA at a dose of 60 mg/kg (dissolved in distilled water) once daily and same volume of olive oil as CCl_4 group; (3) CCl_4 group (n = 8): injected by the intraperitoneal route with CCl_4 at a dose of 3 mL/kg [mixed with olive oil (40%, V/V)] twice a week and same volume of distilled water as CGA group; (4) CCl_4 + CGA group (n = 8): treated with same volume of CCl_4 and CGA as CGA group and CCl_4 group. The doses of CCl_4 and CGA were chosen according our previous studies [32,33]. The rats were sacrificed after 8 weeks. The blood and liver tissues were collected for later analysis.

2.3. Histological examinations

Liver tissues fixed in 10% formalin solution were embedded in paraffin and cut into 5 μm-thick section. Then the section was stained with hematoxyline-eosin (HE) and Masson (a trichrome stain) and photographed using an Olympus CKX 41 microscope. The liver fibrosis degree was evaluated by the percentage of collagen, which was decided through calculating the ratio of collagen to the total liver area using an image analyzer to analyze randomly 10 fields per slide.

2.4. Measurement of malondialdehyde, glutathione peroxidase, superoxide dismutase, catalase, and hydroxyproline in liver tissues

Liver tissues were homogenized with Tris–HCl. Then centrifuge the homogenate at 4 °C and 10,000 g for 10 min, collected the supernatant and analyze malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and hydroxyproline according to the protocol provided by the manufacturer (Nanjing Jiancheng Bioengineering Institute, China). The results were showed as mmol/mg protein (MDA and GSH), U/mg protein (SOD and CAT) or μg/mg in wet liver (Hydroxyproline).

2.5. Cell culture and treatment

The HSC-T6, an rat HSCs line [35], was provided by Dr SL Friedman (Mount Sinai School of Medicine, NY). The cells were cultured in high glucose DMEM medium containing 10% FBS in a humidified atmosphere of 5% CO_2 at 37 °C. At subconfluence, cells were serum starved for 24 h. Then HSCs were treated with PDGF (10 ng/ml) without or with CGA (50 μg/ml, 25 μg/ml, 12.5 μg/ml) to investigate ROS production, cell viability and the expression of profibrotic genes and NADPH oxidase components. The doses of CGA were chosen according our previous study [34].

2.6. MTT cell viability assay

5×10^4 HSC-T6 cells were seeded into 96-well plates and exposed to PDGF with or without CGA and inhibitors for 24 h before MTT (100 μl, 5 mg/ml) was added. Then incubated cells for 4 h at 37 °C and added DMSO (150 μl) to dissolve the formazan. Optical density (OD) was detected with an EL × 800 universal Microplate

Reader (BIO-TEK, INC) at 570 nm. Cell viability = [($OD_{treatment\ group}$−$OD_{control\ group}$)/$OD_{control\ group}$] × 100%. The inhibitors used in MTT assay included DPI (a NOX inhibitor), NAC (a scavenger of ROS), SB203580 (a p38 inhibitor) and PD98059 (a ERK1/2 inhibitor).

2.7. Measurement of ROS level

ROS level was monitored by the conversion of non-fluorescent 2, 7-dichlorofluorescein diacetate (DCF-DA). HSCs were pre-treated with CGA (50 μg/ml) or vehicle for 2 h and exposed to PDGF (10 ng/ml) for 30 min. Then cells were incubated in 10 μM DCFH-DA for 30 min. Finally, the fluorescence was examined by a flow cytometer.

2.8. Western blot analysis

The cytosolic and nuclear protein was prepared as described in previous study [33]. Samples of 50 μg protein were loaded into polyacrylamide gels and Electrophoresis was performed. Then the protein was transferred to PVDF membrane after blocking with 1 × TBST containing 5% non-fat milk for 2 h at room temperature, the membrane was incubated with primary antibodies against rat p47phox, gp91phox, α-SMA, Nrf2, CYP2E1, p38, p-p38, ERK1/2, p-ERK1/2, JNK, p-JNK, Lamin B and β-actin overnight at 4 °C. Subsequently, the membrane was washed with TBST and incubated with secondary antibody for 2 h. The specific blots were detected using a SuperSignal™ West Pico Chemiluminescent Substrate kit.

2.9. Quantitative real-time PCR analysis

Total RNA was isolated using Trizol reagent. 1 μg of total RNA was then converted into cDNA according to instructions from a RevertAid™ cDNA Synthesis Kit. The primers are listed in Table 1. The volume of real-time PCR reactions were 25 μl, containing 1 μl of the cDNA, 12.5 μl of SYBR Green Supermix, 9.5 μl of ddH_2O and 1 μl of the forward and reverse primer each. The conditions used were as follows: preliminary denaturation at 94 °C for 3 min, followed by 40 cycles were performed at 95 °C for 20 s and 60 °C for 20 s. The threshold cycle (CT) value was recorded. The genes expression was calculated with $2^{-\Delta\Delta CT}$ method [36].

2.10. Statistics

The experimental data were recorded as the mean ± S.D and analyzed with One-way ANOVA followed by SNK test. Values of $P < 0.05$ were considered to be statistically significant.

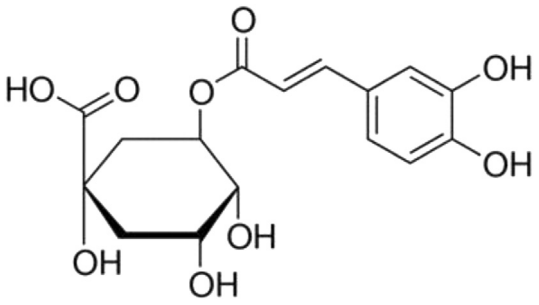

Fig. 1. Structure of chlorogenic acid.

3. Results

3.1. Effect of CGA on histopathological changes and hydroxyproline content in liver tissues

Examinations of the HE and Masson's trichrome-stained sections indicated that no evidence of fibrosis was observed in control and CGA group. The livers showed an integrity lobular architecture with normal central veins, without necrosis or excess collagen deposition. On the other hand, CCl_4 injection resulted in the development of marked liver fibrosis, including hepatocyte necrosis, excess collagen deposition and marked pseudo-lobules. However, CGA treatment significantly attenuated the degree of liver fibrogenesis in comparison to the CCl_4 group (Fig. 2A). The percentage of fibrosis was 17.5% in the livers of CCl_4-injected rats, whereas a decrease (5.9%) was found in the rats treated with CGA (Fig. 2B). The similar finding was also observed in the measurements of the hydroxyproline content in livers. The content of hydroxyproline were increased in CCl_4-injected rats ($P < 0.05$), which were attenuated by CGA treatment ($P < 0.05$) (Fig. 2C).

3.2. Effects of CGA on the expression of fibrogenic genes in liver tissues

α-SMA was considered as a marker of HSCs activation; Collagen I and III were the main content of ECM; TIMP-1 plays a key role in matrix remodeling during liver fibrosis. To explore the inhibition effect of CGA on liver fibrosis at molecular level, we detected the expression of these genes. Compared to the control group, the expression of α-SMA, Collagen I, Collagen III and TIMP-1 in liver were significantly increased in the CCl_4-injected group ($P < 0.05$), and these changes were suppressed by CGA ($P < 0.05$) (Fig. 2D and E).

Table 1
Primer sequences for Real-time PCR.

Target genes		Sequences	Accession no.
β-actin	Forward	5′- CTATCGGCAATGAGCGGTTCC -3′	NM_031144.2
	Reverse	5′-TGTGTTGGCATAGAGGTCTTTACG -3′	
α-SMA	Forward	5′-GGCTTCTCTATCTACCTTCC-3′	NM_031004.2
	Reverse	5′-ACATTCACAGTTGTGTGCTA-3′	
Collagen I	Forward	5′-ACAGGCGAACAAGGTGACAGAG-3′	NM_053304.1
	Reverse	5′-GCCAGGAGAACCAGCAGAGC-3′	
Collagen III	Forward	5′-AGATGCTGGTGCTGAGAAGAAAC-3′	NM_032085.1
	Reverse	5′-GCTGGAAAGAAGTCTGAGGAAGG-3′	
TIMP-1	Forward	5′-ACAGGTTTCCGGTTCGCCTAC-3′	NM_053819.1
	Reverse	5′-CTGCAGGCAGTGATGTGCAA -3′	
HO-1	Forward	5′-CTGGAAGAGGAGATAGAGC-3′	NM_012580.2
	Reverse	5′-CTGGTGTGTAAGGGATGG-3′	
GCLC	Forward	5′-AGAGGACAAACCCCAAC-3′	NM_012815.2
	Reverse	5′-CTAGCCTGGGAAATGAAG-3′	
NQO1	Forward	5′-AACGACATCACAGGGGAG-3′	NM_017000.3
	Reverse	5′-GCACCCCAAACCAATACA-3′	

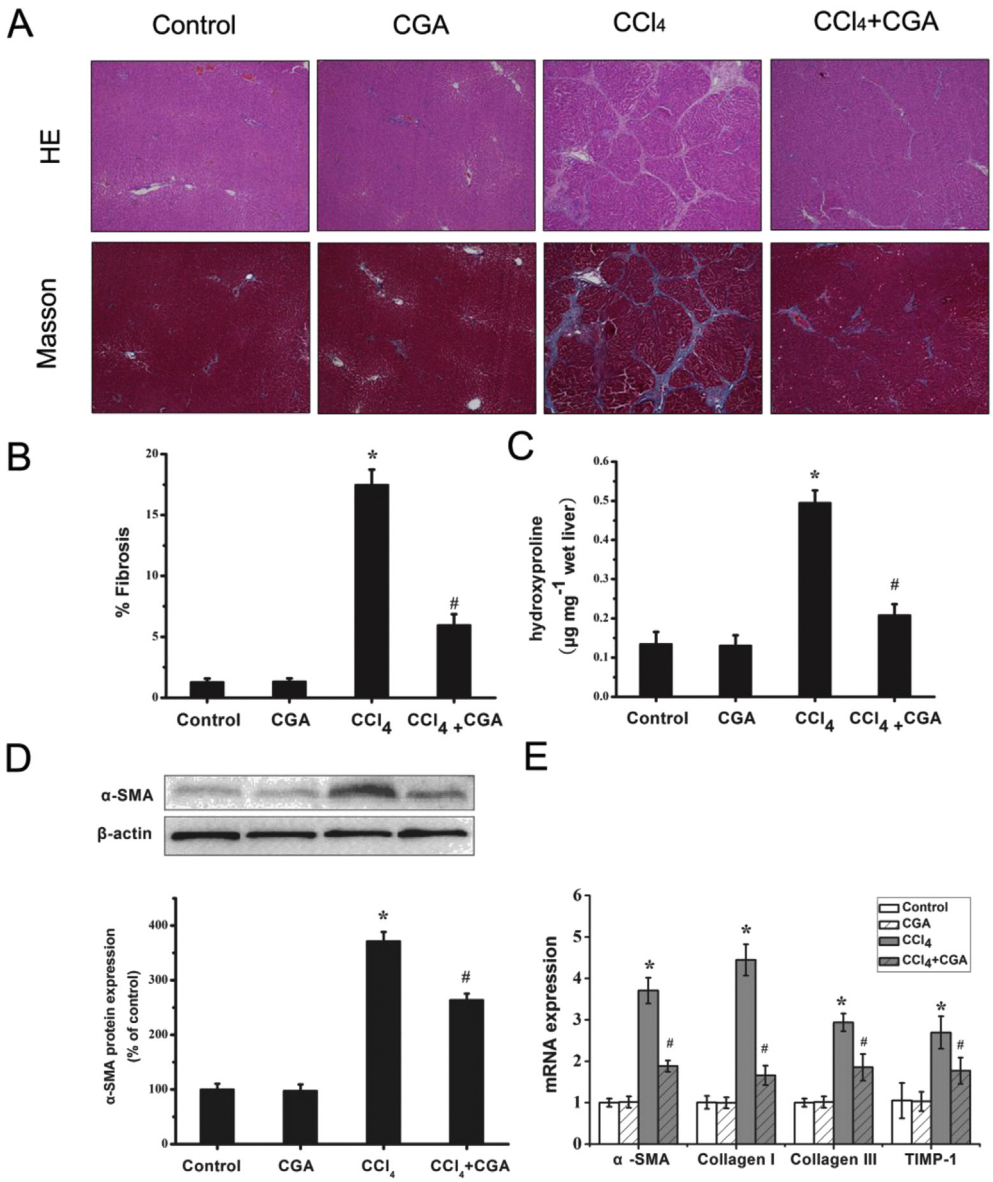

Fig. 2. Effect of CGA on the histological changes, hydroxyproline content and fibrogenic genes expression in the livers. (A) Histological changes were analyzed by HE and Masson. (B) The percentage of fibrosis in the liver tissues was determined using a computer-assisted automated image analyzer to analyze 10 random fields per slide and calculating the ratio of connective tissue to the total liver area. (C) Hydroxyproline content in liver tissues was measured with chromatometry. (D) The protein expression of α-SMA was analyzed by Western blot, β-actin served as control. (E) The mRNA expression of α-SMA, Collagen I, Collagen III and TIMP-1 were analyzed by Real-time PCR, normalized by β-actin, and expressed as $2^{-\Delta\Delta CT}$. Data of three independent experiments are expressed as mean ± SD. *P < 0.05 as compared with the control group; #P < 0.05 as compared with the CCl_4 group.

3.3. Effects of CGA on MDA, GSH, SOD and CAT level in liver tissues

Increased oxidative stress in liver has been recognized in CCl_4-induced liver fibrosis. In the present study, CCl_4 injection markedly increased MDA level and decreased GSH, SOD and CAT levels ($P < 0.05$), whereas CGA significantly protected the liver against this effect. Meanwhile, the SOD and CAT levels in liver were increased by CGA alone ($P < 0.05$) (Fig. 3A and B).

3.4. Effect of CGA on the expression of CYP2E1 and Nrf2 induced anti-oxidant genes in liver tissues

The nuclear translocation of Nrf2 is essential for the activation of anti-oxidant genes. CYP2E1 is one of the key markers of oxidative stress upregulated in liver fibrosis. To identify whether the anti-fibrotic effect of CGA was related with the regulation of Nrf2 and CYP2E1 expression, we investigated the protein expression of nuclear Nrf2 and CYP2E1 by western blot. We also detected the mRNA expression of anti-oxidant genes (HO-1, GCLC and NQO1) by Real-time PCR. Compared to the control group, the protein expression of CYP2E1 in liver were increased in the CCl_4-injected group ($P < 0.05$) and suppressed by CGA treatment ($P < 0.05$). The levels of nuclear Nrf2 and Nrf2-regulated genes were higher following CCl_4 injection ($P < 0.05$) and CGA increased the Nrf2, HO-1, GCLC and NQO1 expression ($P < 0.05$). In addition, we found that CGA-treated only could also increase the expression of nuclear Nrf2 and Nrf2-related anti-oxidant genes ($P < 0.05$) (Fig. 3C and D).

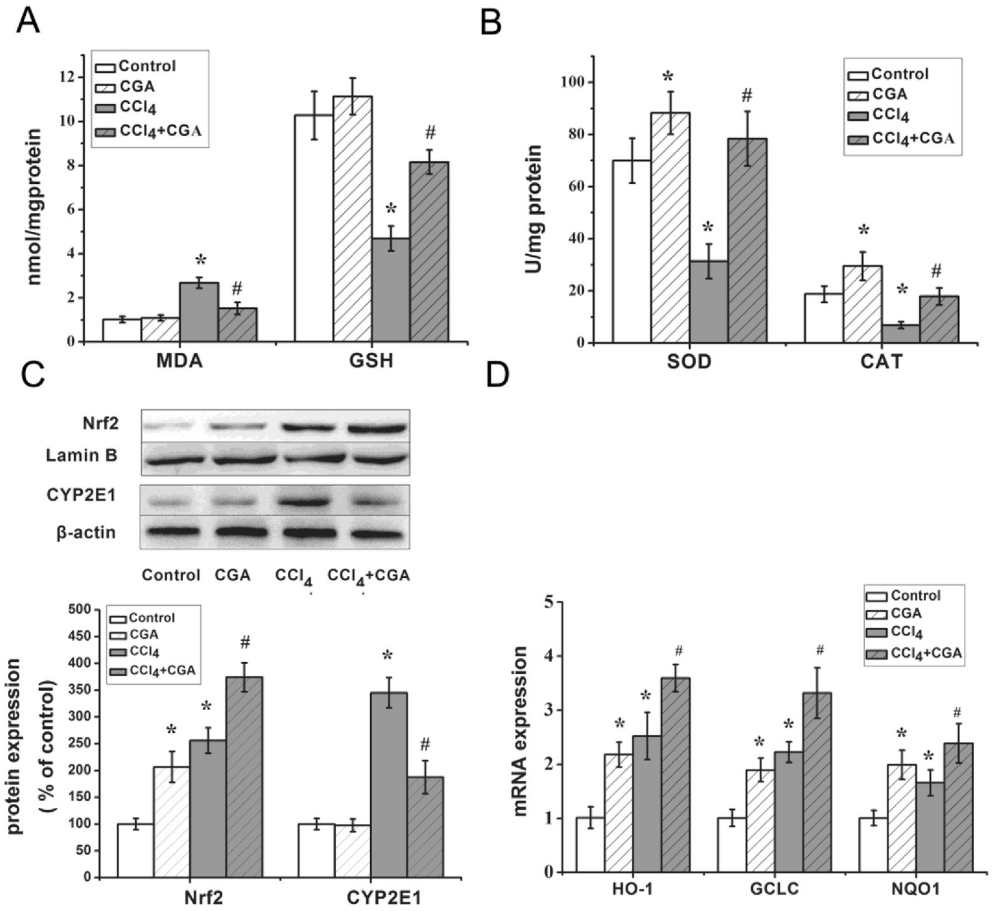

Fig. 3. Effect of CGA on oxidative stress and anti-oxidant defense in liver. (A), (B) The levels of MDA, GSH, SOD and CAT in liver tissues were detected with chromatometry. (C) The protein expression of Nrf2 and CYP2E1 were analyzed by Western blot. Lamin and β-actin served as control. (D) The mRNA expression of HO-1, GCLC and NQO1 were determined by Real-time PCR, normalized by β-actin, and expressed as $2^{-\Delta\Delta CT}$. Data of three independent experiments are expressed as mean ± SD. *P < 0.05 as compared with the control group; #P < 0.05 as compared with the CCl_4 group.

3.5. Effect of CGA on protein expression of gp91phox and p47phox in HSC-T6 cells

The relation of nonphagocytic form of NOX with liver fibrosis has also been verified by some studies demonstrating that either gp91phox or p47phox knockout leads to mitigation of liver fibrosis in mice. To evaluate the activity of NOX, we detected the protein expression of NOX subunit (gp91phox and p47phox) in HSCs. PDGF increased the expression of gp91phox and p47phox ($P < 0.01$), which were significantly decreased by CGA ($P < 0.05$) (Fig. 4A).

3.6. Effect of CGA on ROS level in HSC-T6 cells

Several investigators have demonstrated that PDGF-mediated profibrotic signaling is dependent on formation of ROS. So we investigate the effect of CGA on ROS production in HSCs. The result showed that 10 ng/ml PDGF induced 2-fold increase in ROS production ($P < 0.05$). However, 50 μg/ml CGA significantly reduced ROS level in HSC-T6 cells ($P < 0.05$) (Fig. 4B).

3.7. Effect of CGA on MAPK signaling pathway in HSC-T6 cells

Previous studies showed that NOX-derived ROS activated MAPK signaling pathway and played a key role in PDGF-mediated HSC proliferation and collagen synthesis in vitro. The present study showed that the phosphorylation of p38 and ERK1/2 were increased by PDGF ($P < 0.05$) and decreased by pretreatment with CGA, DPI or NAC ($P < 0.05$). However, PDGF, CGA, DPI or NAC had no significant effect on phosphorylation of JNK (Fig. 4C and D).

3.8. Effect of CGA on cell viability of HSC-T6

To assess whether CGA could inhibit PDGF-stimulated cell proliferation, HSCs were pretreated with CGA for 2 h and exposed to PDGF for 24 h. DPI, NAC, SB203580 and PD98059 exerted as positive controls. HSCs proliferation was detected by MTT assay. PDGF led to a significant increase of HSCs proliferation ($P < 0.05$). Treatment with CGA, DPI, NAC, SB203580 and PD98059 significantly inhibited HSCs proliferation in a dose-dependent manner ($P < 0.05$). However, CGA-treatment only had no significant effect on HSCs proliferation (Fig. 5A).

3.9. Effect of CGA on mRNA expression of Collagen I, Collagen III and TIMP-1 in HSC-T6 cells

PDGF could up-regulate mRNA expression of Collagen I, Collagen III and TIMP-1 in HSC-T6 cells ($P < 0.05$), which was markedly inhibited by CGA ($P < 0.05$). This result suggested that CGA could inhibit PDGF-induced collagen deposition and increased degradation of collagen (Fig. 5B, C and D).

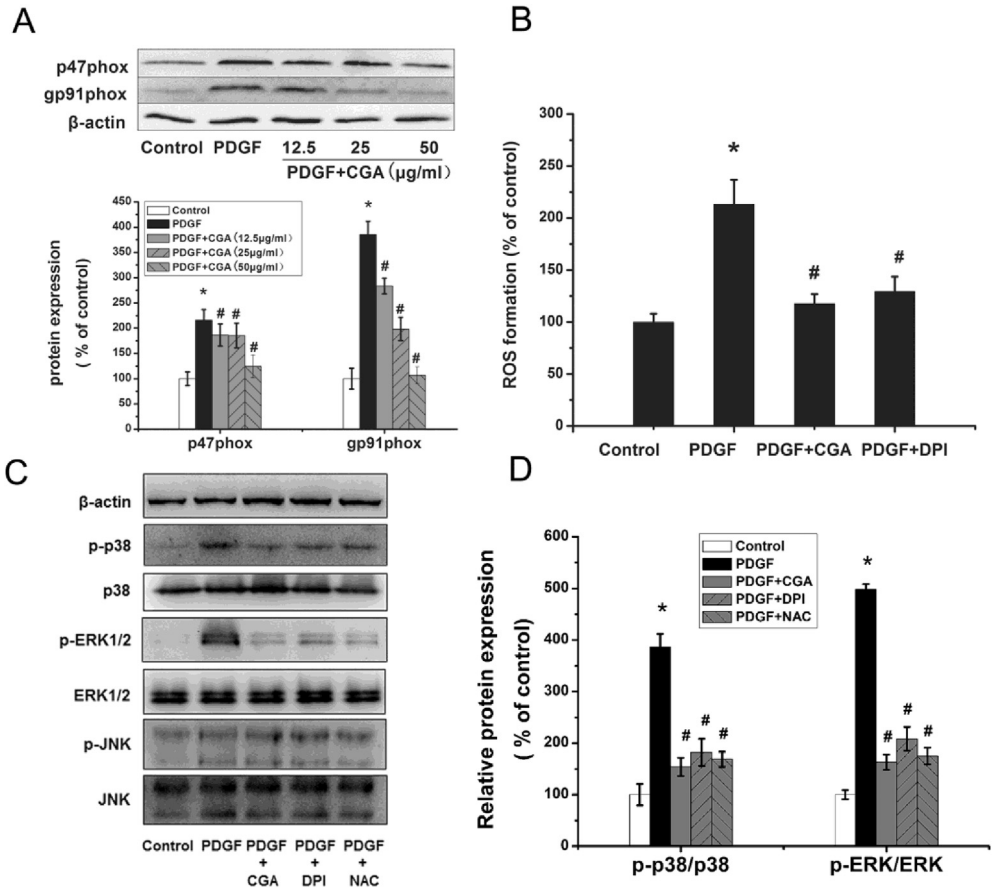

Fig. 4. Effect of CGA on PDGF-induced NOX/ROS/MAPK signaling pathway. (A) The protein levels of NOX subunit (p47phox and gp91phox) were analyzed by Western blot, β-actin served as control. (B) ROS production in HSCs was determined with flow cytometer. (C) p-p38, p-ERK1/2, p-JNK protein expression were analyzed by Western blot, β-actin, total p38, total ERK1/2 and total JNK served as controls. (D) The band intensity of p-p38 and p-ERK1/2 were quantified by densitometry and normalized to total p38 and total ERK1/2. Data of three independent experiments are expressed as mean ± SD. *P < 0.05 as compared with the control; #P < 0.05 as compared with PDGF alone.

4. Discuss

Our in vivo study found that CGA (a) decreased the degree of fibrosis and hydroxyproline content, (b) attenuated the expression of α-SMA, Collagen I, Collagen III and TIMP-1, (c) increased the levels of GSH, SOD and CAT, (d) reduced MDA level, (e) decreased CYP2E1 expression, (f) increased expression of nuclear Nrf2 and downstream anti-oxidant genes in the liver of CCl_4 rats. Meanwhile, in vitro study, we observed that CGA alleviated PDGF stimulated (a) gp91phox and p47phox protein expression, (b) ROS production, (c) p38 and ERK1/2 phosphorylation, (d) HSCs proliferation, (e) the expression of Collagen I, Collagen III and TIMP-1 in HSC-T6 cells. These findings indicated that CGA protected against liver fibrosis in vivo and in vitro through inhibition of oxidative stress.

Although the protective effects of CGA against liver fibrosis have already been studied by us, the present study is the first to demonstrate the role of anti-oxidant activity in anti-fibrotic effects of CGA and highlight the involvement of NOX and Nrf2 signaling pathway.

Indeed, increasing bulks of scientific data uncover the critical role of oxidative stress in the development of liver fibrosis. ROS, which are generated during cellular responses to various growth factors and cytokines, may act as one of the second messenger molecules within the cell [37,38]. PDGF and transforming growth factor-β (TGF-β), two pivotal profibrotic cytokines, are reported to transfer signal through ROS in liver fibrogenesis [10,39]. Oxidative stress-related molecules induce cell proliferation, collagen synthesize, and cell migration in HSCs [40]. ROS can induce lipid peroxidation and MDA usually reflects the level of lipid peroxidation [41]. It has been shown that GSH, SOD and CAT play key roles in combating oxidative stress. Accordingly, antioxidants have been proposed as effective strategies to inhibit the progress of liver fibrosis [42]. CYP2E1, the most important cytochrome P450 in liver, metabolizes CCl_4 and produces free radicals [43]. Therefore, oxidative stress is one cause of CCl_4-induced liver injury and fibrosis. This study showed that CGA markedly reduced MDA level and increased GSH, CAT and SOD levels in liver, which indicated that CGA could inhibit CCl_4-induced oxidative stress in liver.

Nrf2 is considered to be anti-oxidant enzyme inducer. Upon stimulation, Nrf2 translocates from cytosol to nucleus, binds to ARE, induces the expression of anti-oxidant genes and protects against oxidative damage [21]. Nrf2 is consider to be a significant target for the treatment of disorders related to oxidative stress, such as inflammation, cancer, fibrosis and obesity [44]. Nrf2 activation is observed in nonparenchymal cells including HSCs and Kupffer cells as well as in parenchymal hepatocytes. Nrf2 plays complex roles in liver inflammation, fibrosis, cancer, and regeneration [45]. Xu W reported that liver injury was strongly aggravated in the Nrf2-knockout mice after CCl_4 treatment and Nrf2 deficiency enhanced inflammatory and profibrogenic responses [46]. Therefore, Nrf2 is considered to be a significant target to treat various liver diseases. Our study showed CGA could induce nuclear translocation of Nrf2 and increase the expression of HO-1, NQO1, and GCLC.

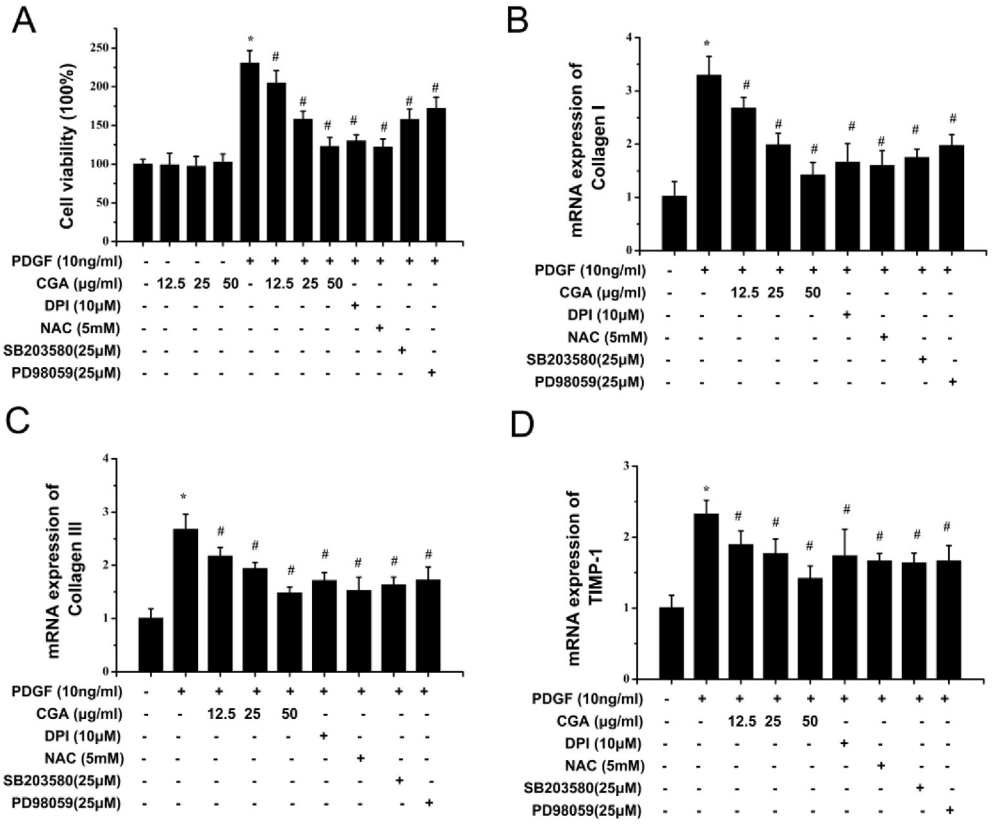

Fig. 5. Effect of CGA and specific inhibitors on PDGF-induced proliferation and production of fibrogenic genes in HSCs. (A) cell viability was determined with MTT assay. (B), (C), (D) The mRNA expression of Collagen I, Collagen III and TIMP-1 were determined by Real-time PCR, normalized by β-actin, and expressed as $2^{-\Delta\Delta CT}$. Data of three independent experiments are expressed as mean ± SD. *P < 0.05 as compared with the control; #P < 0.05 as compared with PDGF alone.

In the liver, the NOX expressed in HSCs is constitutively active and can response to several stimulation such as PDGF, leptin, angiotensin II and apoptotic bodies. Tohru Adachi [10] showed that NOX-derived ROS in response to PDGF induced HSCs proliferation through MAPK pathway. Samuele DM [12] found that NOX was an important mediator of proliferative and fibrogenic actions of leptin. Ramón Bataller [11] reported NOX mediated the actions of angiotensin II on HSCs and played a critical role in liver fibrogenesis. Shan-Shan Zhan [13] showed that the phagocytosis of apoptotic bodies by HSCs increased intracellular ROS production through activation of NOX. In vitro studies, PDGF was used to be an inducer of oxidative stress. The results showed CGA could reduce the PDGF-stimulated NOX expression, ROS production, p38 and ERK1/2 signaling pathway in HSCs.

In summary (Fig. 6), we demonstrated a new mechanism for CGA-mediated protection against CCl_4-induced liver fibrosis. The results showed CGA ameliorated liver fibrosis in CCl_4-injected rats through the inhibition of oxidative stress and increased antioxidant defense through Nrf2 pathway and exerted actions against PDGF-induced NOX/ROS/MAPK signaling pathway in HSCs. According to our current results, CGA may be a promising new treatment of liver fibrosis in a variety of chronic liver diseases.

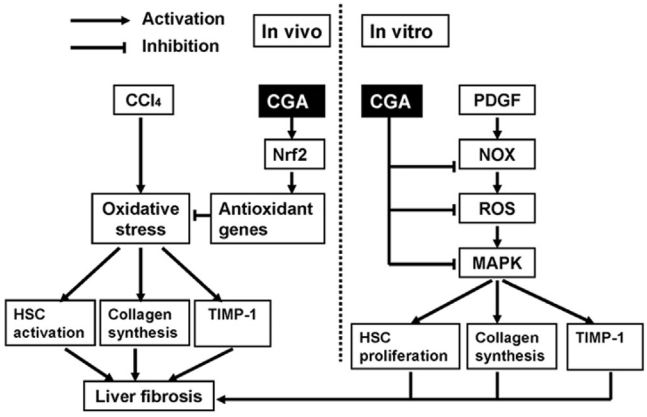

Fig. 6. CGA protected against liver fibrosis in vivo and in vitro through inhibition of oxidative stress. In vivo CGA increased anti-oxidant defense through regulating Nrf2 pathway and thus combated oxidative stress in liver of CCl_4-injected rats. CGA also exerted actions against PDGF-induced NOX/ROS/MAPK signaling pathway in HSCs, which finally inhibited HSCs proliferation, collagen synthesis and TIMP-1 expression. As a result, CGA protected against liver fibrosis.

Conflict of interest

The authors declare no conflict of interest.

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