Chloroquine Improved Carbon Tetrachloride-Induced Liver Fibrosis through Its Inhibition of the Activation of Hepatic Stellate Cells: Role of Autophagy

Wei He, a,b Bin Wang, h,i Jing Yang, a Yun Zhuang, a Liangzhi Wang, a Xiaodan Huang, c and Jianping Chen*.a

Department of Gastroenterology, the Third Affiliated Hospital of Soochow University; Changzhou 213003, China:
Division of Hepatology, Huashan Hospital and Institute of Hepatology, Fudan University; 12 Wulumuqi Middle
Road, Xuhui, Shanghai 200040, P. R. China: and Institute of Digestive Endoscopy and Medical Center for Digestive
Diseases, Second Affiliated Hospital of Nanjing Medical University: Nanjing 210011, China.

Received April 11, 2014; accepted June 18, 2014

September 2014 1505


Autophagy is involved in the activation of hepatic stellate cells (HSCs), which is the key process of liver fibrosis. We reasoned that chloroquine, based on its ability to inhibit autophagy, might exert beneficial effects in liver fibrosis. Liver fibrosis in rats was induced by carbon tetrachloride (CCL4). Rats were divided into three groups, a normal control group (N group), model group (M group), and chloroquine group (CQ group). Liver fibrosis in the rats was evaluated by hematoxyline–eosin (H&E) and Masson staining. The activities of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TB) were determined using an automated biochemistry analyzer. Total hepatic hydroxyproline levels were determined with a kit. The expressions of α-smooth muscle actin (α-SMA) and transforming growth factor-β1 (TGF-β1) were detected by immunofluorescence staining, and the expressions of LC3-II and p62 were determined by Western blotting. Compared with N group, M group showed impaired liver function, liver fibrosis, increased hydroxyproline content, up-regulated expressions of α-SMA and TGF-β1, which have been reported to be pro-fibrogenic genes in vivo, and increased autophagy flux as indicated by the accumulation of LC3-II and degradation of p62. These changes were attenuated by chloroquine treatment. Chloroquine exerts beneficial effects in CCL4-induced liver fibrosis. The mechanism of action includes inhibition of autophagy pathways and inhibition of activation of HSCs.

Key words chloroquine; liver fibrosis; autophagy; hepatic stellate cell

Hepatic fibrosis, characterized by excessive scar formation due to overproduction and deposition of the extracellular matrix, is the common response to chronic liver injury, ultimately leading to cirrhosis. This process usually occurs over a long period of time and can lead to organ dysfunction and death. Activation of hepatic stellate cells (HSCs) is considered to play a major role in the occurrence and development of hepatic fibrosis. Insights into mechanisms regulating HSC activation are considered as key targets for the treatment of hepatic fibrosis.

Autophagy, a highly evolutionarily energy-dependent process implicated as a cell death mechanism, degrades and recycles subcellular organelles. During autophagy, the cytoplasmic form LC3-I is processed and recruited to phagophores, where LC3-II is generated by site-specific proteolysis and lipidation at the C-terminus. Thus this characteristic conversion from endogenous LC3-I to LC3-II can be used to monitor autophagic activity. Recent studies suggest that autophagic flux is increased during HSC activation, and in vitro study have showed that treatment with an autophagy inhibitor partly inhibited HSC activation. Hence, treatment with an autophagy inhibitor could be a potential new therapeutic strategy for hepatic fibrosis.

Chloroquine (CQ), an autophagy inhibitor, is a commonly used therapeutic agent in skin disorders. Growing evidence also suggest that CQ could be useful in fibroblastic diseases of the skin and suppresses Idiopathic pulmonary fibrosis. However, it has not been elucidated whether CQ could protect against carbon tetrachloride (CCL4)-induced liver fibrosis.

In this study, we examined the biological function of CQ in CCL4-induced fibrosis in rats. We also determined whether CQ could attenuate CCL4-induced fibrosis partially via inhibiting autophagy and the subsequent activation of HSCs.

MATERIALS AND METHODS

Materials: Reagents were obtained from the following sources: Chloroquine and α-SMA antibody were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). LC3 and p62 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, U.S.A.). Transforming growth factor-β1 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Carbon tetrachloride (CCL4) was purchased from Merck (Darmstadt, Germany).

Animal Models: The animal experimental protocols were approved by the Animal Care and Use Committee of the Soochow University. Twenty four male Sprague-Dawley rats (180 to 220 g) were housed under standard animal laboratory conditions in the specific-pathogen-free-grade animal room at the Experimental Animal Center of Soochow University. All the rats were fasted for two days before the experiment. They were randomly divided into three groups: normal controls (N group, n=8), model (M group, n=8), and CQ group (CQ group, n=8). The control group was administered with vehicle only. For the model group, liver fibrosis was induced in rats by subcutaneous injections of 400 mL/L CCL4 salad oil solution with a single dose of 3 µg/g/rat twice a week. For the CCL4...
model plus CQ group, after 4 weeks of modeling as previously mentioned, a total of 50 mg/kg/d CQ was given by intraperitoneal injections. All the rats were killed 9 weeks later. Blood samples and liver tissues were obtained.

Serological Examination Blood samples aspirated from the caudal vein of the rats were analyzed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TB) using an automated Sincron-7 analyzer in order to compare the differences of liver function between each group.

Histological Examination and Immunofluorescence Staining All paraffin-embedded liver tissues were HE stained for histopathological examination. Masson’s trichrome staining was used to assess collagen levels. Immunofluorescence examinations were used to detect the expression of α-smooth muscle actin (α-SMA) and TGF-β1.

Measurement of Hepatic Hydroxyproline Content Total hepatic hydroxyproline levels were determined in the hydrolysates of liver samples as described previously. One hundred milligrams of wet liver samples were subjected to acid hydrolysis to determine the amount of hydroxyproline according to the protocol outlined in the Hydroxyproline Testing Kit (A030-2, Jiancheng, Nanjing, China).

Western Blotting Analysis Total proteins were extracted using RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined using the BCA method (Beyotime Institute of Biotechnology, Haimen, China). Equal amounts of protein were loaded onto each lane, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After being blocked with 5% skimmed milk in Tris-buffered saline (TBS) (pH 7.6) at room temperature, the membranes were incubated overnight at 4°C with primary antibodies for LC3 (1:1000), p62 (1:1000), and β-actin (1:500). After being incubated with the respective secondary antibody, immune complexes were detected using ECL Western blotting reagents. The detected proteins were normalized to β-actin or the respective total protein as appropriate.

Statistical Analysis The statistical significance of a difference between the control and treatment groups was determined by simple ANOVA followed by Dunnett’s multiple comparison tests. All values are expressed as mean±S.D. and statistical significance was defined as p<0.05.

RESULTS

Effects of CQ on Hepatic Fibrosis Induced by CCl4 in Rats We assessed the effects of CQ in a rat model of CCl4-induced hepatic fibrosis. As shown in Fig. 1A, rats in M group showed extensive haemorrhagic necrosis and disruption of tissue architecture as demonstrated by hematoxyline–eosin

![Fig. 1. CQ Attenuates Hepatic Fibrosis Induced by CCl4 in Rats](image)

- (A) CCl4 was used to construct a hepatic fibrosis model to evaluate the therapeutic effects of CQ. Liver sections were stained with HE and Masson’s reagent for histological examination to determine hepatic morphology and architecture changes and the amount of ECM in the liver tissue of each groups.
- (B) The amount of ECM was quantitated by quantitative estimation of hydroxyproline content. *p<0.05 compared with healthy group. **p<0.05 compared with CCl4 without CQ group. (n=3).
(H&E) staining and increased fibrosis deposition as demonstrated by Masson’s trichrome staining relative to N group. These alterations were remarkably reduced in the liver sections of CQ-treated rats.

In addition, the result of quantitative estimation of hydroxyproline content indicated that the hydroxyproline content in M group rats was 203.875 µg/g, which was significant higher than that in N group (377.25 µg/g, \( p < 0.05 \)), however, the hydroxyproline content in CQ-treated rats was 262.125 µg/g, which was lower than that in the M group (\( p < 0.05 \)) (Fig. 1B).

**Effects of CQ on Liver Function in Fibrotic Rats**

As shown in Fig. 2, compared with N group, serum concentrations of AST, ALT, and TB were examined to assess hepatic function. \( \# p < 0.05 \) compared with healthy group. **\( p < 0.05 \) compared with CCl4 without CQ group. \( n=3 \).

![Fig. 2](image)

The serum of the rats in each group was collected for assessment of liver function. TB, AST, and ALT were examined to assess hepatic function. \( \# p < 0.05 \) compared with healthy group. **\( p < 0.05 \) compared with CCl4 without CQ group. \( n=3 \).

**Effects of CQ on Profibrogenic Gene Expression in Vivo**

Immunofluorescence was used to examine \( \alpha \)-SMA and TGF-\( \beta \)1 expression in liver tissues \( n=3 \).
tions of ALT, AST, and TB were significantly increased in M group (p<0.05). Treatment with CQ for 5 weeks significantly attenuated the increase of ALT, AST, and TB compared with M group (p<0.05).

**Effect of CQ on Profibrogenic Gene Expression in Vivo**

Immunofluorescence staining assays (IF) were used to detect the expression of α-SMA (an indicator of activated HSCs), and TGF-β1 (a major profibrogenic gene) in the liver tissues. The results demonstrated that CCl₄-treated rats exhibited an increase in both α-SMA and TGF-β1 content compared with the control group (Fig. 3). However, CQ significantly inhibited the increase of α-SMA and TGF-β1 content compared with M group.

**Chloroquine Inhibited Autophagy Flux in Vivo**

The expression of the autophagy markers, LC3-II/LC3-I, were determined by Western blot analysis in liver samples from rats of various groups. In normal livers, LC3-II/LC3-I was present at a low level but increased markedly in the livers of CCl₄-induced rats (Figs. 4A, B). As expected, chloroquine increased further the expression of LC3-II because chloroquine, as a lysosomal inhibitor, blocked the lysosomal degradation of LC3-II in autophagosomes, leading to a marked accumulation of LC3-II, consistent with inhibition of autophagy (Figs. 4A, B). We also examined the effects of chloroquine on p62, a protein known to be uniquely degraded by autophagy pathways. CCl₄ stimulation markedly reduced p62 levels, which were partly restored by chloroquine treatment (Figs. 4A, B).

**DISCUSSION**

In this study we investigated the effect of CQ on liver fibrosis. Our in vivo study was inspired by the fact that CQ attenuated the liver fibrosis, improved the hepatic function, inhibited autophagy, and prevented the activation of HSCs in CCl₄ induced liver fibrosis rats.

Fibrogenesis is a common feature of many diseases where there is severe insult to the liver. The hepatic stellate cells trans-differentiation into myofibroblasts has been identified as an important event in liver fibrogenesis and has been well investigated over the last few years in a number of liver diseases. Following chronic liver injury, the appearance of HSCs changed, including the missing of lipid droplets, and the abnormal proliferation of HSCs, then they can differentiate to myofibroblasts, secrete a huge mass of cytokine and extracellular matrix, eternally result in hepatic fibrosis. This process is termed “activation,” a remarkably pleiotropic yet tightly programmed response occurring in a reproducible sequence. Thus, Inhibiting the activation of HSCs is the key in reversing hepatic fibrosis.

Autophagy, a process by which cells degrade and metabolize own constituents is an evolutionary conserved fundamental cellular process. In addition to its well known role in energy regulation, autophagy plays a role in several pathological conditions, such as cancer, inflammation, and tissue fibrosis. Recent studies indicated that autophagy was implicated in major liver pathologies, such as HCV infection and hepatocarcinoma and HSC activation, is followed by an increased autophagic flux and that its inhibition can partially inhibit the HSC myofibroblastic transition. These results point to autophagy as a possible target in the prevention of HSC activation.

Chloroquine has exerted its anti-fibrogenesis effect in many
fibrosis diseases. However, to the best of our knowledge, the effect and precise mechanism of CQ on liver fibrosis have not yet been addressed. The results in our study indicated treatment with CQ showed an improved hepatic function and a relative moderate liver fibrosis as evidenced by liver function test and histopathology test (Figs. 1, 2).

We further explored the possible molecular mechanism involved in the protective effect of CQ in CCl4-induced liver fibrosis rats. The post-sequestration steps in the autophagic mechanism were the fusion of autophagosomes with lysosomes, resulting in autolysosomes, and, finally, the degradation of the ingested biological materials. Expectedly, CQ, an autophagy inhibitor, inhibited autophagy in the livers of CCl4-induced rats, as evidenced by the partial restoration of p62 and further increased LC3II/LC3I expression in the liver tissues of these animals (Fig. 4). Additionally, CQ was also found to downregulate the expression of α-SMA and TGF-β1 (Fig. 3), which have been reported to be profibrogenic genes involved in the protective effect of CQ in CCl4-induced liver fibrosis (Figs. 1, 2).

In conclusion, our study provides first evidence that CQ potently protect CCl4-induced liver fibrosis through inhibiting the autophagy process and the activation of HSCs. Our study may provide a rationale for in vivo evaluations of CQ in therapies for liver fibrosis.

REFERENCES