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Chlorogenic acid reduces liver inflammation and fibrosis through inhibition of toll-like receptor 4 signaling pathway

Haitao Shi, Lei Dong*, Jiong Jiang, Juhui Zhao, Gang Zhao, Xiaoyan Dang, Xiaolan Lu, Miao Jia

Department of Gastroenterology, Second Affiliated Hospital of Xi'an Jiaotong University, No. 157 Xiwu Road, Xi'an, Shaanxi 710004, China

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ABSTRACT

Chlorogenic acid (CGA) is a type of polyphenol with anti-inflammatory, antioxidant activities. Our previous studies showed CGA could efficiently inhibit carbon tetrachloride (CCl_4)-induced liver fibrosis in rats. However, the specific underlying mechanism remains unclear. The aim of this study is to investigate the effects of CGA on liver inflammation and fibrosis induced by CCl_4 and whether they are related to inhibition of toll-like receptor 4 (TLR4) signaling pathway. Male Sprague-Dawley (SD) rats were administrated CCl_4 together with or without CGA for 8 weeks. Histopathological and biochemical analyses were carried out. The mRNA and protein expression levels of proinflammatory and profibrotic mediators were detected by RT-PCR and Western blot, respectively. The levels of serum proinflammatory cytokines were detected by ELISA. CGA significantly attenuated CCl_4-induced liver damage and symptoms of liver fibrosis, accompanied by reduced serum transaminase levels, collagen I and α-smooth muscle actin (α-SMA) expression. As compared with the CCl_4-treated group, the expression levels of TLR4, myeloid differentiation factor 88 (MyD88), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were reduced in the treatment group of CCl_4 and CGA, whereas bone morphogenetic protein and activin membrane-bound inhibitor (Bambi) expression was increased. CGA also suppressed CCl_4 induced nuclear factor-κB (NF-κB) activation. Moreover, the hepatic mRNA expression and serum levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) were significantly increased in CCl_4-treated rats and attenuated by co-treatment with CGA. Our data indicate that CGA can efficiently inhibit CCl_4-induced liver fibrosis in rats and the protective effect may be due to the inhibition of TLR4/MyD88/NF-κB signaling pathway.

Liver fibrosis is a wound-healing process in injured liver caused by various agents, including infection with hepatitis viruses, chronic alcohol abuse, metabolic or autoimmune diseases (Friedman, 2003; Kawada, 2011). Upon liver injury, quiescent hepatic stellate cells (HSCs) are activated and produce excessive extracellular matrix (Bataller and Brenner, 2005; Friedman, 2003, 2010). In chronic liver injury, the injured or damaged cells release a number of cytokines, which then stimulate the Kupffer cells to release more inflammatory mediators (Luckey and Petersen, 2001). Kupffer cells and activated HSCs have been implicated to secrete proinflammatory cytokines, chemokines and adhesion factors which play important roles in liver inflammation, accelerating the liver fibrosis (Pinzani and Macias-Barragan, 2010).

Lipopolysaccharides (LPS) is the major constituent of outer membrane in gram-negative bacteria. It activates a variety of the mammalian cell types including monocytes/macrophages and endothelial cells through the toll like receptor 4 (TLR4) (Zhu and Mohan, 2010). Previous study shows serum LPS levels are significantly elevated in patients with chronic liver disease owing to the increased intestinal mucosal permeability and bacterial translocation (Choi et al., 2011; Lin et al., 1995; Parlesak et al., 2000). Many liver cells including Kupffer cells, HSCs and liver endothelial cells (LECs) can express TLR4 and respond to LPS, which has an important role in liver fibrosis (Aoyama et al., 2010). It is known that LPS stimulates Kupffer cells to release a number of cytokines to activate HSCs and TLR4 signaling in LECs regulates angiogenesis, which are linked to the development of liver fibrosis (Seki and Brenner, 2008). LPS sensitizes quiescent HSCs to TGF-β and Kupffer cell–mediated activation (Seki et al., 2007), while for activated HSCs, it can promote them to develop an inflammatory phenotype and make a contribution to the inflammatory network in the liver during endotoxemia (Brun et al., 2005; Paik et al., 2003; Thirunavukkarasu et al.,

Abbreviations: CGA, chlorogenic acid; HSCs, hepatic stellate cells; LPS, lipopolysaccharides; TLR4, toll-like receptor 4; CCl_4, carbon tetrachloride; α-SMA, α-smooth muscle actin; MyD88, myeloid differentiation factor 88; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; Bambi, bone morphogenetic protein and activin membrane-bound inhibitor; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1β.

* Corresponding author. Tel.: +86 29 87679272; fax: +86 29 87678758.
E-mail addresses: shihaitao7@163.com (H. Shi), Dong556@126.com (L. Dong).

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2006). Besides Kupffer cell, HSC is another target for LPS-induced liver injury and provide a direct link between inflammatory and fibrotic liver injury (Seki et al., 2007).

Some epidemiological researches suggest that coffee consumption may reduce the risk of liver fibrosis (Ruhl and Everhart, 2005; Tverdal and Skurtveit, 2003). Our study, as well as one from others, shows that coffee can improve carbon tetrachloride (CCl_4)-induced liver fibrosis in rats (Shi et al., 2010; Shin et al., 2010). However, it is not clear which components of coffee are responsible for the protective effect. Other than caffeine, kahweol and cafestol, coffee drink is also rich in chlorogenic acid (CGA). Caffeine, kahweol and cafestol have been shown to have hepatoprotective and antioxidant effects (Gressner et al., 2008; Lee et al., 2007). CGA is a type of polyphenol with anti-inflammatory, antioxidant, antiobesity and antimicrobial activities (Feng et al., 2005; Kono et al., 1997; Xu et al., 2010; Zhang et al., 2010). A recent study showed that CGA can protect mice from LPS-induced hepatotoxicity (Xu et al., 2010). Another study showed that CGA can inhibit LPS-induced inflammatory cytokines release in RAW264.7 cells (Shan et al., 2009). And our previous study showed that CGA can efficiently inhibit CCl_4-induced liver fibrosis in rats (Shi et al., 2009). However, the specific underlying mechanism remains unclear, but it may be related to its anti-inflammatory activities. This study is to investigate the effects of CGA on liver inflammation and fibrosis induced by CCl_4 and whether they are related to inhibition of TLR4 signaling pathway.

1. Materials and methods

1.1. Reagents

CGA was purchased from Sigma–Aldrich (St. Louis, USA). Anti-α-SMA was from Epitomic (Burlingame, CA, USA). Anti-TLR4, Bambi and Lamin B were from ProteinTech Group (Chicago, IL, USA). Anti-MyD88, IκB-α, p-IκB-α, and β-actin were from Bioworld Technology (St. Louis Park, MN, USA). Anti-NF-κB was from Abcam (Cambridge, UK). Anti-COX-2, iNOS and horseradish-peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The SuperSignal Substrate Chemiluminescence Kit was from Pierce (Rockford, USA). All other chemicals used in the experiment were of analytical grade.

1.2. Animals and experimental treatments

Thirty-two SD rats weighing (220–250 g) were housed with free access to food and water throughout the experiments. The rats were maintained in a controlled environment at 21 ± 2 °C and 50 ± 5% relative humidity under a 12-h dark/light cycle and were acclimatized for at least 1 week prior to use. The rats were randomly divided into four groups (8 rats per group): (1) control group: given the appropriate vehicles (distilled water and olive oil); (2) CGA group: given CGA (60 mg/kg, dissolved in distilled water, intragastric administration once daily); (3) CCl_4 group: given CCl_4 [3 mL/kg, dissolved in olive oil (40%, V/V), intraperitoneal injection twice weekly]; (4) CCl_4 + CGA group: given CCl_4 along with CGA (dosages and treatments as mentioned for CGA group and CCl_4 group, respectively). No rats died during the experimental period. At the end of 8 weeks and 24 h after the last dose of CCl_4 the rats were anesthetized with chloral hydrate and blood was drawn from inferior caval vein prior to the excision of organ tissues. Serum was collected by centrifugation at 13,000 rpm for 15 min at 4 °C and stored at −70 °C for liver function test and ELISA analysis. Liver was rapidly excised and stored in liquid nitrogen for later analysis. The experiment was performed in accordance with the guidelines of the Animal Care Committee of Xi'an Jiaotong University.

1.3. Liver function test

Alanine transaminase (ALT), aspartate transaminase (AST) activities and albumin were measured using a biochemistry analyzer (Olympus AU2700, Japan).

1.4. Histological examinations

A portion of each liver was fixed in 10% formalin, processed by routine histological procedures, embedded in paraffin, and cut into 5-μm sections. The samples were stained with hematoxylin and eosin (H&E) for histopathological examination and with Masson's trichrome for assessment of fibrosis. Sections were examined in a blinded manner under a microscope (Leica TCS SP, German). Fibrosis was graded according to the method of (Ruwart et al., 1989) as follows: grade 0, normal liver and no fibrosis; grade 1, increase in collagen without septa formation; grade 2, formation of incomplete septa from the portal tract to the central vein (septa that do not interconnect with each other); grade 3, complete but thin septa interconnecting with each other so as to divide parenchyma into separate fragments; and grade 4, the same as grade 3, except for the presence of thick septa (complete cirrhosis). To avoid sampling errors, biopsies were obtained from equivalent lobes and the semi-quantitative grades were performed by independent histopathologists who were blinded to the treatment assignment. Each sample was observed at 100× magnification. The degree of liver damage was expressed as the mean of ten fields of view on each slide.

1.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from liver tissue samples by the TRIzol kit (Invitrogen) according to the manufacturer's protocol. The RNA was then subjected to reverse transcription using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). The mRNA expression levels of TLR4, myeloid differentiation factor 88 (MyD88), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), bone morphogenetic protein and activin membrane-bound inhibitor (Bambi), α-smooth muscle actin (α-SMA), collagen I were measured by RT-PCR. The primer sequences used in PCR are shown in Table 1. The annealing temperatures and the thermal cycles for each target gene were as follows: TLR4 (56 °C, 30 cycles), MyD88 (54 °C, 30cycles), iNOS (56 °C, 30 cycles), COX-2 (54 °C, 30 cycles), TNF-α (56 °C, 34 cycles), IL-6 (56 °C, 30 cycles), IL-1β (53 °C, 30 cycles), Bambi (57 °C, 30 cycles), α-SMA (53 °C, 30 cycles) and collagen-I (53 °C, 30 cycles), β-actin (56 °C, 30 cycles). PCR products were run on a 2% agarose gel stained with ethidium bromide recorded on polaroid film, and the bands quantified by densitometry. The expression levels of all the transcripts were normalized to that of β-actin mRNA in the same tissue samples.

1.6. Western blot

The cytosolic and nuclear protein extracts from rat liver were performed as described previously (Choi et al., 2009). Samples of 50 μg of protein were mixed with gelloading buffer, boiled for 5 min, and loaded on 8% or 10% polyacrylamide gels. Electrophoresis was then carried out and the proteins transferred to nitrocellulose membranes. Non-specific antibody binding was blocked by preincubation of the membranes in 1× Tris-buffered saline (TBS) containing 5% skimmed milk for 2 h at room temperature. The membranes were incubated overnight at 4 °C with primary antibodies against rat TLR4 (1:500), MyD88 (1:500), NF-κB (1:400), IκB–α (1:500), p-IκB–α (1:500), iNOS (1:500), COX-2 (1:500), Bambi (1:800), α-SMA (1:1000), Lamin B (1:1000), and β-actin (1:1000) in 1× TBS containing 5% skimmed milk. After washing, they were incubated for 2 h at room temperature with anti-rabbit IgG at a 1:4000 dilution. Bands were visualized using a SuperSignal Substrate Chemiluminescence Kit.

1.7. Determination of cytokine levels.

Specific and sensitive enzymelinked immunoassays (ELISA) obtained from R&D Systems (Minneapolis, MN, U.S.A.) were used to determine concentrations of TNF-α, IL-6, and IL-1β in serum.

1.8. Statistical analysis

The experimental results were expressed as the mean ± SD. Statistical significance among the groups was analyzed by one-way analysis of variance with the Student–Newman–Keuls test. Histopathological data from the liver were analyzed by the Kruskal–Wallis non-parametric test, followed by the Mann–Whitney *U*-test. $P < 0.05$ was considered significant.

2. Results

2.1. Effects of CGA on general features and liver function

There was no difference in body weight and liver index [Liver-weight (g)/body-weight (100 g)] between control group and CGA control group. CCl_4-treated rats showed significantly decreased body weight ($P < 0.01$) and increased liver index ($P < 0.01$). However, CCl_4 and CGA co-treatment have no effect on body weight and liver index compared with CCl_4 treatment alone. For assessments of hepatocellular damage, the serum transaminase (AST and ALT) activities and albumin level were measured. There was no difference in serum transaminase activities and albumin level between control group and CGA control group. After administration for 8 weeks, a dramatic increase in liver enzyme activities and decrease in albumin level were observed in CCl_4-treated rats compared with controls ($P < 0.01$). CCl_4 and CGA co-treatment significantly decreased ALT and AST activities ($P < 0.01$) and increased