International Immunopharmacology 54 (2018) 125–130

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Activation of Nrf2 pathway and inhibition of NLRP3 inflammasome activation contribute to the protective effect of chlorogenic acid on acute liver injury

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ARTICLE INFO

Keywords:
Chlorogenic acid
Carbon tetrachloride
Liver injury
Nrf2
NLRP3 inflammasome

ABSTRACT

Chlorogenic acid (CGA), a kind of polyphenol found in coffee, fruits and vegetables, has potent anti-oxidant and anti-inflammatory properties. Our previous studies showed CGA could efficiently alleviate liver fibrosis in rats. However, whether CGA regulates nuclear factor erythroid-2-related factor 2 (Nrf2) anti-oxidant pathway and NLRP3 inflammasome activation and protects against carbon tetrachloride (CCl_4)-induced acute liver injury are unknown. We found that CGA could increase Nrf2 activation and expression of Nrf2-related anti-oxidant genes, including HO-1, NQO1 and GCLC. Pretreatment with CGA could reduce CCl_4-induced elevation of serum transaminases and alleviate liver pathological abnormalities. CGA also reversed CCl_4-induced increase in MDA level and decrease in the levels of GSH, SOD and CAT in liver tissues. Meanwhile, CGA inhibited NLRP3 inflammasome activation, as indicated by the reduced protein expression of NLRP3, Pro-Caspase-1, Caspase-1, Pro-IL-1β and IL-1β. Moreover, CGA reduced serum levels and liver mRNA expression of TNF-α, IL-6 and IL-1β. These results demonstrate that CGA protects against CCl_4-induced acute liver injury probably through enhancing Nrf2-mediated anti-oxidant pathway and inhibiting NLRP3 inflammasome activation.

Liver disease is one of the most common causes of death all over the world, because the liver takes part in metabolism and detoxification in the body. Liver injury can be induced by various factors including alcohol, drug abuse, viral infections, metabolic and autoimmune attack [1,2]. Acute liver injury can be easily caused by various toxicants. CCl_4 is a chemical hepatotoxin that induces hepatocellular damage through metabolic activation by cytochrome P450 2E1 (CYP2E1). CYP2E1 metabolized CCl_4 to form trichloromethyl radical (·CCl3) and proxy trichloromethyl radical (·OOCCl3), which can cause lipid peroxidation [3]. The lipid peroxidation products can induce liver cell apoptosis and necrosis [4]. Thus, chemicals or drugs with anti-oxidant activity may be used to alleviate CCl_4-induced liver injury.

Nuclear factor erythroid-2-related factor 2 (Nrf2), a member of the Cap'n'Collar family of basic leucine-zipper transcription factors, activates the transcription of phase II detoxifying anti-oxidant enzymes such as heme oxygenase-1 (HO-1), glutamate-cysteine ligase (GCL) and NAD(P)H:quinone oxidoreductase-1 (NQO1) through binding to the anti-oxidant response elements (AREs) [5,6]. Animals deficient in Nrf2, which has decreased anti-oxidant defense capacity, are highly susceptible to various injuries from oxidative stresses [6]. Therefore, Nrf2 is considered as an important endogenous regulator of oxidative stress [7]. A great deal of research has indicated that some herbals and dietary polyphenol could protect liver injury by activating Nrf2 signaling [8–10].

Recent findings indicated that the inflammasome can switch on the inflammatory response of tissues to a variety of stimuli. The nucleotide-binding domain- (NOD-) like receptor protein 3 (NLRP3) inflammasome, the most extensively investigated inflammasome identified so far, is composed of a sensor protein, NLRP3, the adaptor protein apoptosis-associated speck-like protein (ASC), and the pro-inflammatory Caspase-1. NLRP3 can trigger proteolytic cleavage of dormant Pro-Caspase-1 into active Caspase-1, which converts the cytokine precursors Pro-IL-1β and Pro-IL-18 into mature and biologically active IL-1β and IL-18, respectively [11]. These proinflammatory cytokines further exacerbate

Abbreviations: ALT, alanine transaminase; ASC, apoptosis-associated speck-like protein; AST, aspartate transaminase; AREs, antioxidant response elements; CAT, catalase; CGA, chlorogenic acid; CCl_4, carbon tetrachloride; CYP2E1, cytochrome P450 2E1; GCLC, glutamate-cysteine ligase catalytic subunit; GSH, glutathione; HO-1, heme oxygenase-1; MDA, malondialdehyde; NLRP3, nucleotide-binding domain- (NOD-) like receptor protein 3; Nrf2, nuclear factor erythroid-2-related factor 2; NQO1, NAD(P)H:quinone oxidoreductase-1; SOD, superoxide dismutase

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https://doi.org/10.1016/j.intimp.2017.11.007
Received 22 August 2017; Received in revised form 30 October 2017; Accepted 5 November 2017

the inflammatory process. NLRP3 inflammasome activation plays an important role in the development of many inflammatory diseases including acute liver injury and can serve as a therapeutic target [12,13].

Chlorogenic acid (CGA), the ester of caffeic acid and quinic acid, is a kind of polyphenol compound found in coffee, fruits and vegetables. CGA has potent anti-oxidant and anti-inflammatory properties. Ali [14] showed that CGA protected against methotrexate induced oxidative stress, inflammation and apoptosis in rat liver. Feng [15] showed that chemopreventive effect of CGA might be through its up-regulation of Nrf2-related cellular anti-oxidant enzymes. Ji [16] found that CGA reversed acetaminophen-induced reduced GSH levels, GCL and glutathione reductase activity in liver. Zheng [17] presented that CGA ameliorated experimental colitis in mice through suppressing the secretion of TNF-α and IL-6. Ye [18] showed that CGA attenuated LPS-induced acute kidney injury by inhibiting TNF-α, IL-6 and IL-1β production both in serum and kidney tissues. Our previous studies showed CGA could inhibit CCl_4-induced chronic liver injury in rats and the mechanisms might be related with anti-oxidant and anti-inflammatory [19–21]. However, the effect of CGA on CCl_4-induced acute liver injury and whether it is related with Nrf2 anti-oxidant pathway and NLRP3 inflammasome activation is unclear. This study was designed to explore the protective effect and mechanism of CGA on CCl_4-induced acute liver injury.

1. Materials and methods

1.1. Reagents

CCl_4, CGA (#C3878) and Dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, USA). CGA was resolved in DMSO and then sterile filtered before used. Polyclonal antibodies against Nrf2, HO-1, NQO1, GCL catalytic subunit (GCLC), Lamin B and β-actin were purchased from Proteintech group. Polyclonal antibodies against NLRP3, Pro-Caspase-1, Caspase-1, Pro-IL-1β and IL-1β were purchased from Santa Cruz Biotechnology. The secondary antibodies were purchased from Cell Signaling Technology.

1.2. Animals and experimental treatments

Thirty-two male Sprague-Dawley rats (220–250 g) were allowed free access to food and water. These rats were maintained under a controlled environment at 21 °C and 50% relative humidity with a 12-h dark/light cycle for 1 week. For determining the dose-dependent effect of CGA on Nrf2 activation, 18 rats were administered CGA dissolved in ddH_2O at 15 mg/kg, 30 mg/kg or 60 mg/kg (n = 6) once a day for 7 consecutive days by gavage. The control rats (n = 6) received ddH_2O. For determining the effect of CGA pretreatment on acute liver injury, another 18 male rats were divided into three groups (n = 6): (1) Control group: given vehicles (ddH_2O and olive oil). (2) CCl_4 group: given ddH_2O for 7 consecutive days firstly, and then given CCl_4 [3 mL/kg, dissolved in olive oil (40%, V/V) intraperitoneal injection] for 24 h; (3) CCl_4 + CGA group: given 60 mg/kg CGA for 7 consecutive days by gavage and same volume of CCl_4 as mentioned in CCl_4 group for 24 h. At the end of each experiment, rats were sacrificed. The blood and liver tissues were collected for later analysis. The study was approved by the ethics committee of the second affiliated hospital of Xi'an Jiao Tong University, Xi'an, China.

1.3. Liver function test

Alanine transaminase (ALT) and aspartate transaminase (AST) activities were analyzed by a biochemistry analyzer (Olympus AU2700, Japan).

1.4. Histological examinations

Liver tissues were fixed in 10% formalin and embedded in paraffin. Sections of 5 μm thick were placed on glass slides and then stained with hematoxylin-eosin (HE) according to standard procedure. Sections were examined under a Nikon Eclipse 50i microscope and images were recorded.

1.5. Measurement of malondialdehyde, glutathione, superoxide dismutase, and catalase in liver tissue

Liver tissues were weighed and homogenized with Tris–HCl. The total homogenate was centrifuged at 4 °C and 10,000*g* for 10 min and the supernatant was collected for analysis of malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT), following the protocol provided by the manufacturer (Nanjing Jiancheng Bioengineering Institute). Results are expressed as mmol/mg protein (MDA and GSH) or U/mg protein (SOD and CAT).

1.6. Western blot analysis

The cytosolic and nuclear proteins were performed as described in previous study [21]. Samples of 50 μg protein were loaded on polyacrylamide gels and electrophoresis was carried out and then the proteins transferred to PVDF membranes (Millipore Corp., Billerica, MA, USA). The PVDF membranes were blocked with 1 × Tris-buffered-saline-Tween (TBST) containing 5% non-fat milk for 2 h at room temperature and incubated overnight at 4 °C with primary antibodies against rat Nrf2 (1:800), HO-1 (1:500), NQO1 (1:500), GCLC (1:500), NLRP3 (1:500), Pro-Caspase-1 (1:500), Caspase-1 (1:500), Pro-IL-1β (1:500), IL-1β (1:500), Lamin B (1:1000) and β-actin (1:2000) in 1 × TBS containing 5% skimmed milk. After washing, they were incubated for 1 h at room temperature with anti-rabbit IgG at a 1:2000 dilution. After washing 4 times with TBST, they were incubated for 2 h at room temperature with secondary antibody at a 1:2000 dilution. Bands were visualized using a Super Signal Substrate Chemiluminescence Kit.

1.7. Quantitative real-time PCR

Total RNA were extracted using Trizol reagent (Invitrogen, USA) and then reverse-transcribed into cDNA according to manufacturer's instructions from a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA). The primers used in real-time PCR analysis are listed in Table 1. Quantitative real-time PCR analysis was carried out using the iCycler iQ system (Bio-Rad, USA). The volume of PCR reactions were 25 μl, containing 1 μl of the cDNA, 12.5 μl of 2 × iQ SYBR Green Supermix, 9.5 μl of ddH2O and 1 μl of the forward and reverse primer each. The conditions used were as follows: preliminary denaturation at

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′	
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′	
TNF-α	Forward	5′- CCACCACGCTCTTCTGTCTAC-3′	NM_012675.3
	Reverse	5′- GCTACGGGCTTGTCACTCG-3′	
IL-6	Forward	5′-CTTCCAGCCAGTTGCCTTCTTG-3′	NM_012589.1
	Reverse	5′-TGGTCTGTTGTGGGTGGTATCC-3′	
IL-1β	Forward	5′-AATCTCACAGCAGCATCTC-3′	NM_031512.2
	Reverse	5′-AGCAGGTCGTCATCATCC-3′	