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Original Contribution

Caffeic acid prevents acetaminophen-induced liver injury by activating the Keap1-Nrf2 antioxidative defense system

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ABSTRACT

Acute liver failure induced by acetaminophen (APAP) overdose is the main cause of drug-induced liver injury (DILI). Caffeic acid (CA) is a phenolic compound from many natural products. This study aims to investigate the protective mechanism of CA in APAP-induced liver injury. The results of serum alanine/aspartate aminotransferases (ALT/AST), liver myeloperoxidase (MPO) activity, liver glutathione (GSH) and reactive oxygen species (ROS) levels demonstrated the protection of CA against APAP-induced liver injury. Liver histological observation provided further evidences of CA-induced protection. CA was found to reverse the APAP-induced decreased cell viability in human normal liver L-02 cells and HepG2 cells. CA also reduced the increased cellular ROS level induced by APAP in hepatocytes. The results of luciferase assay and Western-blot analysis showed that CA increased the transcriptional activation of nuclear factor erythroid 2-related factor 2 (Nrf2) in the presence of APAP. Nrf2 siRNA reduced the protection of CA against APAP-induced hepatotoxicity. CA also reversed the APAP-induced decreased mRNA and protein expression of heme oxygenase 1 (HO-1) and NAD(P)H: quinone oxidoreductase 1(NQO1). In addition, HO-1 inhibitor zinc protoporphyrin (ZnPP) and NQO1 inhibitor diminutol (Dim) reduced the protection of CA against APAP-induced hepatotoxicity. CA also decreased the expression of kelch-like ECH-associated protein-1(Keap1). Molecular docking indicated the potential interacting of CA with Nrf2 binding site in the Keap1 protein. CA had little effect on the enzymatic activity of cytochrome P450 (CYP) 3A4 and CYP2E1 *in vitro*. In conclusion, we demonstrated that CA prevented APAP-induced hepatotoxicity by decreasing Keap1 expression, inhibiting binding of Keap1 to Nrf2, and thus activating Nrf2 and leading to increased expression of antioxidative signals including HO-1 and NQO1.

1. Introduction

Caffeic acid (CA) is a natural polyphenolic compound derived from coffee, some fruits and traditional Chinese medicines [1–7]. CA and its analogs have shown a variety of pharmacological activities including anti-inflammation, anti-cancer and anti-virus [8]. In addition, this compound is easily absorbed through the gut barrier [9], so enhancing its mode of action. Previous reports have already shown that CA prevented liver reperfusion injury and griseofulvin-, nickel-, or doxorubicin-induced hepatotoxicity; furthermore its antioxidative capacity contributes to protecting liver against injury [10–13]. Previously, we demonstrated that CA, as a main compound in medicinal herb *Flos Lonicerae*, prevented acetaminophen (APAP)-induced hepatotoxicity in human normal liver L-02 cells *in vitro* [7]. CA has also been reported to inhibit APAP-induced liver injury in both mice and rats *in vivo* [14]. However, the precise mechanism involved in its protection

Abbreviations: APAP, acetaminophen; DILI, drug-induced liver injury; CA, Caffeic acid; ALT/AST, alanine/aspartate aminotransferases; MPO, myeloperoxidase; GSH, glutathione; ROS, reactive oxygen species; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase 1; NQO1, NAD(P)H: quinone oxidoreductase 1; ZnPP, zinc protoporphyrin; Dim, diminutol; Keap1, kelch-like ECH-associated protein-1; CYP, cytochrome P450; NAPQI, *N*-acetyl-*p*-benzoquinone imine; NAC, *N*-acetylcysteine; H_2DCFDA, 2′-7′-Dichlorodihydrofluorescein diacetate; FBS, fetal bovine serum; BSO, L-Buthionine-(S, R)-sulfoximine; H&E, haematoxylin and eosin; MTT, 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide; TRE, transcription response element; DDTC, sodium diethyldithiocarbamate trihydrate; GCL, glutamate-cysteine ligase; ARE, antioxidant-related elements; GR, glutathione reductase

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remains unclear.

Drug-induced liver injury (DILI) is a major clinical problem. Acute liver failure induced by APAP overdose is common, and is reported to be the main reason for DILI in the United States and the United Kingdom [15,16]. Despite great efforts made over the last 40 years, the mechanism of APAP-induced liver injury is still not completely elucidated. However, it is widely accepted that APAP can be metabolized by liver cytochrome P450 (CYP) into a reactive metabolite named *N*-acetyl-*p*-benzoquinone imine (NAPQI), which depleted cellular glutathione (GSH) and disturbed cellular redox balance, and thus led to oxidative stress-induced liver injury [17,18]. The commonly used antidote for APAP detoxification is *N*-acetylcysteine (NAC), which is a precursor for cellular GSH synthesis and also is a well-known antioxidant [19]. Although NAC attenuates APAP-induced hepatotoxicity, some patients may still develop liver injury despite administration of recommended dosage [20]. Therefore, there is a need to find a more effective and safe drug for APAP detoxification.

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) regulates the constitutive and inducible expression of a variety of genes involved in drug metabolism, detoxification, and antioxidative defenses [21]. Kelch-like ECH-associated protein 1 (Keap1), an adapter subunit of Cullin 3-based E3 ubiquitin ligase, regulates the degradation of Nrf2 [22]. The Keap1-Nrf2 system is thought to play a critical role in liver oxidative injury, and has been considered as a prospective target for liver disease [21,22]. Here, we investigated CA-induced protection against APAP-induced liver oxidative injury, and the involvement of the Keap1-Nrf2 signaling pathway.

2. Materials and methods

2.1. Chemical compounds and reagents

CA was purchased from Beijing Aoke Biological technology Co., Ltd (Beijing, China), and the purity is over 98.5%. Kits for analysis of alanine/aspartate aminotransferase (ALT/AST) and myeloperoxidase (MPO) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Cignal™ Reporter Assay kit for Nrf2&Nrf1, RNeasy® Plus Mini kit and Attractene were bought from Qiagen (Hilden, German). Duan-Glo® Luciferase Assay System was purchased from Promega (Madison, WI). Vivid® CYP2E1, Vivid® CYP1A2, and Vivid® CYP3A4 kits, Opti-MEM®, lipofectamine RNAiMAX, 2′-7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA), RPMI1640, and fetal bovine serum (FBS) were purchased from Life Technology (Carlsbad, CA). NE-PER® nuclear and cytoplasmic extraction reagents, and Pierce® BCA Protein Assay Kit were purchased from ThermoFisher Scientific (Waltham, MA). Whole cell protein extraction kit and enhanced chemiluminescence kit were all obtained from Millipore (Darmstadt, Germany). Antibodies for immunoblotting including anti-Actin, -Lamin B, and -Keap1 were all purchased from Cell Signaling Technology (Danvers, MA) (all 1:1000 dilutions). Antibodies for immunoblotting including anti-Nrf2, -HO-1, -GCLC, -GCLM, and -NQO1 were all bought from Santa Cruz (Santa Cruz, CA) (all 1:200 dilutions). Peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (H+L) and anti-mouse IgG (H+L) were purchased from Jackson ImmunoResearch (West Grove, PA). PrimeScript® RT Master Mix and SYBR® Premix Ex Taq™ were bought from Takara (Shiga, Japan). Control siRNA and Nrf2 siRNA were both purchased from Santa Cruz (Santa Cruz, CA). APAP, NAPQI, L-Buthionine-(S, R)-sulfoximine (BSO), ZnPP, Dim and other reagents unless indicated were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Animals and treatments

Specific pathogen-free male ICR mice (16–20 g body weight) were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Science (Shanghai, China). The animals were supplied with standard laboratory diet and water *ad libitum* at a temperature 22 ± 1 °C with a 12 h light–dark cycle (6:00–18:00) and 65 ± 5% humidity. All animals were received humane care in compliance with the institutional animal care guidelines approved by the Experimental Animal Ethical Committee, Shanghai University of Traditional Chinese Medicine.

Forty mice were randomly divided into 4 groups: (1) vehicle control, (2) APAP (400 mg/kg), (3) APAP (400 mg/kg)+CA (10 mg/kg), and (4) APAP (400 mg/kg)+CA (30 mg/kg). Mice were pre-administered orally with CA (10, 30 mg/kg per day) for 7 consecutive days. On the last day, mice were orally given a single dose of APAP (400 mg/kg) after administration of CA for 1 h. Animals were then killed 4 h after APAP intoxication, and plasma and liver tissue were collected.

For analyzing the effects of CA alone, 24 ICR mice were randomly divided into 3 groups: (1) vehicle control, (2) CA (10 mg/kg), and (3) CA (30 mg/kg). Mice were administered orally with CA (10, 30 mg/kg per day) for 7 consecutive days. Animals were then killed 5 h after the last administration of CA, and plasma and liver tissue were collected.

2.3. Analysis of serum ALT/AST activities

The blood samples obtained were kept at room temperature for 2 h. Serum was then collected after centrifugation at 840 × *g* for 15 min. Serum ALT and AST were measured with kits according to the manufacturer's instructions.

2.4. Analysis of liver GSH amount

Liver GSH amount was determined according to our previous reported method [23].

2.5. Analysis of liver MPO activity

Liver MPO enzymatic activity was determined according to the manufacturer's instruction. Protein concentration was detected by BCA kit and MPO activity was expressed as units/g protein.

2.6. Liver histological observation

Slices of mice livers were fixed in 10% phosphate buffered saline (PBS)–formalin for at least 24 h and then embedded in paraffin for histological assessment of tissue damage. Samples were subsequently sectioned (5 μm), stained with haematoxylin and eosin (H&E), and then observed under a light microscope (Olympus, Japan) to evaluate liver damage.

2.7. Cell culture

The L-02 cell line was derived from an adult human normal liver [24] (Cell Bank, Type Culture Collection of Chinese Academy of Sciences, Shanghai). Hepatoma-derived HepG2 cell line was obtained from the American Type Culture Collection (Manassas, VA). L-02 or HepG2 cells were cultured in RPMI1640 or MEM supplemented with 10% [v/v] fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.

2.8. Cell viability assay

Cells were seeded into 96-well plates. After attachment, cells