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Tanshinone IIA protects against acetaminophen-induced hepatotoxicity *via* activating the Nrf2 pathway

Wenwen Wang[a,1], Cuiwen Guan[a,b,1], Xiaozhe Sun[a], Zhongxiang Zhao[c], Jia Li[a], Xinlu Fu[a], Yuwen Qiu[a], Min Huang[a], Jing Jin[a,*], Zhiying Huang[a,*]

[a] School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 510006, PR China
[b] The Third People's Hospital, Nanhai District, Foshan 528244, PR China
[c] School of Chinese MateriaMedica, Guangzhou University of Chinese Medicine, Guangzhou 510006, PR China

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ABSTRACT

Background: Tanshinone IIA (Tan), the main active component of *Salvia miltiorrhiza*, has been demonstrated to have antioxidant activity. Acetaminophen (APAP), a widely used antipyretic and analgesic, can cause severe hepatotoxicity and liver failure when taken overdose. Oxidative stress has been reported to be involved in APAP-induced liver failure.

Purpose: This study aimed to investigate the effect of Tan on APAP-induced hepatotoxicity and the underlying mechanisms involved.

Study Design: C57BL/6 J mice were divided into six groups: (1) control, (2) APAP group, (3) APAP + Tan (30 mg/kg) group, (4) Tan (30 mg/kg) group, (5) APAP + Tan (10 mg/kg) group, (6) Tan (10 mg/kg) group. Mice in group 3 and 5 were pre-treated with specified dose of Tan by gavage and subsequently injected with an overdose of APAP intraperitoneally (i.p., 300 mg/kg). The effect of Tan on Nrf2 pathway was investigated in HepG2 cells and mice.

Methods: Plasma aspartate transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH), liver glutathione (GSH), glutathione transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) levels were determined after mice were sacrificed. Lipid peroxidation and histological examination were performed. The effect of Tan on the Nrf2 pathway was detected by western blotting and qRT-PCR.

Results: Tan pretreatment reduced APAP-induced liver injury. Tan was able to activate Nrf2 and increase the expression levels of Nrf2 target genes, including glutamate-cysteine ligase catalytic subunit (GCLC), NAD(P)H:quinine oxidoreductase 1 (NQO1) and hemeoxygenase-1 (HO-1), in a dose-dependent manner in HepG2 cells. Consistent with our observations in HepG2 cells, Tan increased nuclear Nrf2 accumulation and upregulated mRNA and protein levels of the Nrf2 target genes GCLC, NQO1 and HO-1 in C57BL/6 J mice compared with mice treated with APAP alone.

Conclusions: Our results demonstrate that Tan pretreatment could protect the liver from APAP-induced hepatic injury by activating the Nrf2 pathway. Tan may provide a new strategy for the protection against APAP-induced liver injury.

Introduction

Acetaminophen (APAP), a widely used antipyretic and analgesic drug, can cause severe and fatal hepatotoxicity when an overdose is taken (Larson et al., 2005). At therapeutic doses, most APAP is metabolized by cytochrome P450 enzymes to form the highly reactive intermediate metabolite N-acetyl-p-benzoquinoneimine (NAPQI), which is readily detoxified *via* conjugation with glutathione (GSH) under normal conditions. However, when overdosed, NAPQI deplete hepatic glutathione and bind covalently to intracellular proteins, resulting in increased oxidative stress and

Abbreviations: ALT, alanine transaminase; APAP, acetaminophen; ARE, antioxidant element; AST, aspartate transaminase; CAT, catalase; Cry, cryptotanshinone; GCLC, glutamate-cysteine ligase catalytic subunit; GPx, glutathione peroxidase; GSH, glutathione; GST, glutathione transferase; HO-1, hemeoxygenase-1; Keap1, kelch-like ECH-associated protein 1; LDH, lactate dehydrogenase; MDA, malondialdehyde; NAPQI, N-acetyl-p-benzoquinoneimine; NQO1, NAD(P)H:quinine oxidoreductase 1; Nrf2, Nuclear factor erythroid-2-related factor 2; SFN, sulforaphane; SOD, superoxide dismutase; Tan, tanshinone IIA; TanI, tanshinone I.

* Corresponding authors. Tel.: +86 20 39943025; fax: +86 20 39943000.
E-mail addresses: jinjing@mail.sysu.edu.cn (J. Jin), hzhiying@mail.sysu.edu.cn (Z. Huang).

[1] These authors contribute equally to this study.

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hepatic necrosis (James et al., 2003). The oxidative stress caused by NAPQI has been shown to play a central role in the acute hepatic damage induced by APAP (Jaeschke and Bajt, 2006).

The nuclear factor erythroid-2-related factor 2 (Nrf2) mediated stress responses have been shown to be the important mechanism for cells to alleviate oxidative stress. Nrf2 is a transcription factor that regulates the expression of various cytoprotective enzymes by binding to the antioxidant element region (ARE) domain upstream of their promoter. Under normal conditions, kelch-like ECH-associated protein 1 (Keap1), a negative regulator of Nrf2, retains Nrf2 in the cytoplasm and mediates its proteasomal degradation (Itoh et al., 1999). Upon oxidative stress, Nrf2 dissociates from Keap1 and translocates into the nucleus, where it hetero-dimerizes with small Maf binding proteins and binds to ARE, promoting the transcription of its target genes (Kobayashi and Yamamoto, 2005; Li and Kong, 2009). Nrf2 target genes include glutamate-cysteine ligase catalytic subunit (GCLC), NAD(P)H:quinine oxidoreductase 1 (NQO1), and hemeoxygenase-1 (HO-1), among others. Nrf2 is assumed to be a potential target for the treatment of drug-induced toxicity.

Mice deficient of Nrf2 are highly susceptible to organ injury caused by toxic agents such as APAP, benzopyrene, hyperoxia, and diesel exhaust (Aoki et al., 2001; Cho et al., 2002; Enomoto et al., 2001; Ramos-Gomez et al., 2001) due to defects in generating antioxidants. In contrast, mice with a hepatocyte-specific deletion of Keap1 exhibit an increase in Nrf2 and its target genes and are resistant to APAP-induced hepatotoxicity (Okawa et al., 2006). Because the hepatotoxicity of APAP is primarily due to oxidative stress and the Nrf2 pathway plays an important role in protecting against oxidative stress, small molecules that could activate Nrf2 might be beneficial in protecting against APAP-induced liver injury.

Salvia miltiorrhiza Bunge (Lamiaceae) is a perennial plant that is highly valued for its roots and known as Danshen, which was widely used in cardiovascular diseases for centuries in traditional Chinese medicine (Cheng, 2007). Tanshinones, including tanshinone IIA (Tan), tanshinone I (Tan I) and cryptotanshinone (Cry), have been identified as the main active components in Danshen. Tan, one of the most active tanshinones, has been shown to have antioxidant, anti-inflammatory, anti-angiogenesis, anti-cancer and hepatoprotective activities (Fan et al., 2009; Fu et al., 2007; Zhu et al., 2010). Tan could activate the Nrf2 pathway in human aortic smooth muscle cells (Zhang and Wang, 2007). Further, pretreatment with Tan protects against carbon tetrachloride-induced liver injury (Liu et al., 2002). However, until recently, whether Tan can protect against APAP-induced liver damage has not been reported.

In the present study, we investigated the protective effects of Tan against APAP-induced hepatotoxicity. Here, we found that Tan could induce ARE-mediated expression of Nrf2 target genes in HepG2 cells. We also present evidence that Tan could protect against APAP-induced liver injury in C57BL/6 J mice *via* activating the Nrf2 pathway.

Materials and methods

Materials

Tan (>98% purity assayed by HPLC) (Supplementary Figs. 1–3) was a kind gift from Prof. Peiqing Liu ((Department of Pharmacology and Toxicology, School of Pharmaceutical Sciences, Sun Yat-sen University). Tan I (purity above 99%) and Cry (purity above 99%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China. Sulforaphane (SFN) was purchased from Enzo Life Sciences (Lausen, Switzerland). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from HyClone (Logan, UT, USA). Rabbit anti-Nrf2, anti-heme oxygenase-1 (HO-1) and anti-glutamate-cysteine ligase catalytic (GCLC) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit anti-NAD(P)H:quinone oxidoreductase (NQO1) antibody were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Rabbit anti-β-actin antibody and rabbit anti-goat IgG horseradish peroxidase (HRP)-linked whole antibody were purchased from Cell Signaling Technology Inc. (Beverly, MO, USA). Other chemicals were of analytical grade from commercial suppliers.

Cell culture

HepG2 cells were kindly provided by Prof. Jianmin Jiang (Department of Pharmacology and Toxicology, School of Pharmaceutical Sciences, SunYat-sen University). HepG2 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) at 37 °C in a humidified atmosphere with 5% CO_2.

Transient transfection and luciferase assays

The pGL3-ARE plasmid was a kind gift from Dr. Athanassios-Fragoulis (University Hospital Aachen). The expression vector for dominant positive Nrf2 (pEF-Nrf2) and the empty vector (pEF) were kindly provided by Dr. Shinya Ito (University of Toronto, Toronto, Canada). HepG2 cells at 80–90% confluence were transiently transfected with 0.1 μg of pGL3-ARE and 0.02 μg of phRL-TK (Promega, Mannheim, Germany) with or without cotransfection with 0.1 μg of Nrf2 expression vectors using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, CA, USA). Transfection was continued for 5 h, and then, the cells were recovered in complete medium overnight and treated with various compounds for 24 h. Firefly and Renilla luciferase activity was measured with the Dual Luciferase Assay system (Promega, Mannheim, Germany) according to the manufacturer's instructions. The activity of firefly luciferase was normalized to that of Renilla luciferase.

Animals and treatments

SPF male C57BL/6 J mice weighing 16–20 g were purchased from Laboratory Animal Center, Sun Yat-sen University. All mice were kept in a specific pathogen-free animal room under controlled conditions at 23 ± 3°C and 55 ± 15% humidity with a 12 h light–dark cycle. Mice were provided with water and standard chow ad libitum and had a 3-day acclimation period prior to the experiments. All animal experimental procedures were performed in accordance with the protocol approved by the Animal Ethical and Welfare Committee of Sun Yat-sen University.

Mice were randomly assigned into the following treatment groups (n=6 mice/group): (1) vehicle control group, (2) APAP group, (3) APAP + Tan (30 mg/kg) group, (4) Tan (30 mg/kg) group, (5) APAP + Tan (10 mg/kg) group, (6) Tan (10 mg/kg) group. Experimental design was shown in Table 1. Two different doses of Tan were selected. The mice in the control and APAP groups were pretreated only with 0.5% CMC-Na (20 ml/kg) orally; the other four groups were treated daily with indicated doses of Tan (20 ml/kg) orally for 4 consecutive days. Mice were fasted for 16 h prior to APAP administration. On day 4, mice in group 2 were injected intraperitoneally with a single dose of 300 mg/kg APAP (15 ml/kg in saline). Mice of group 3 and group 5 were injected the same dose of APAP 1 h after specified dose of Tan pretreatment, then were given with Tan orally again 6 h later. Compared with Group 3 and 5, mice in group 4 and group 6 were treated with saline instead of APAP. At 24 h after APAP administration, the mice were sacrificed. Blood and liver tissues were collected from each animal for biochemical and histopathological analyses.