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Ginsenoside Rg1 protects against acetaminophen-induced liver injury via activating Nrf2 signaling pathway *in vivo* and *in vitro*

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ABSTRACT

Acetaminophen (APAP) is a worldwide used drug for treating fever and pain. However, APAP overdose is the leading cause of drug-induced liver injury. The purpose of the current study is to evaluate the hepatoprotective effect of ginsenoside Rg1 (Rg1), the main pharmacologically active compounds of *Panax ginseng*, against APAP-induced acute liver injury, and further to elucidate the involvement of Nrf2 signaling pathway by *in vivo* and *in vitro* experiments. Male C57BL/6 mice were treated with Rg1 for 3 days before injection of APAP. Serum and liver tissue samples were collected 6 h later. The results indicated that Rg1 significantly attenuated APAP-induced hepatotoxicity and oxidative stress in a dose-dependent manner. Rg1 effectively enhanced antioxidant and detoxification capacity, which is largely dependent on up-regulating Nrf2 nuclear translocation, reducing Keap1 protein expression and up-regulating Nrf2 target genes including GCLC, GCLM, HO-1, NQO1, *Ugt1a1*, *Ugt1a6*, *Ugt2b1*, *Sult2a1*, Mrp2, Mrp3 and Mrp4. Furthermore, Rg1 repressed the activities of *Cyp2e1*, *Cyp3a11*, *Cyp1a2*, which are important enzymes in the formation of APAP toxic metabolite N-acetyl-p-benzoquinone imine. However, the changes in transporters and enzymes, as well as ameliorative liver histology induced by Rg1 were abrogated by Nrf2 antagonist all-transretinoic acid *in vivo* and Nrf2 siRNA *in vitro*. In conclusion, Rg1 produced hepatoprotective effects against APAP-induced acute liver injury via Nrf2 signaling pathway. Rg1 might be an effective approach for the prevention against acute liver injury.

1. Introduction

Acetaminophen (APAP), a widely-used analgesic and antipyretic drug, is relatively safe at therapeutic doses. However, an acute or cumulative overdose of APAP can result in severe liver injury, which is the most common cause of drug-induced acute liver failure in the United States and Great Britain (Larson et al., 2005; Lee, 2004). The mechanism of APAP toxicity is initiated by the accumulation of the toxic reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), which is generated by cytochrome P450 enzymes (Cyps) including Cyp2e1, Cyp3a11 and Cyp1a2 (Slitt et al., 2005). NAPQI subsequently triggers hepatic toxicity through covalently binding to cellular macromolecules and induces cellular oxidant stress and DNA damage, leading to severe centrilobular hepatotoxicity and acute liver failure (Saito et al., 2010). Several signaling pathways have been demonstrated to play crucial roles in antioxidant defense system.

Nuclear factor erythroid 2-related factor 2 (Nrf2) which is a master regulator of the antioxidant defense system, mediates a cell survival response (Bataille and Manautou, 2012). Under basal conditions, Nrf2 interacts with Kelch-like ECH-associated protein 1 (Keap1), which is an

Abbreviations: ALT, alanine transaminase; ARE, antioxidant element; AST, aspartate transaminase; CAT, catalase; GCL, glutamate-cysteine ligase; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; GSH, reduced glutathione; HO-1, hemeoxygenase-1; Keap1, kelchlike 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; CYP450, cytochrome P450; Rg1, ginsenoside Rg1; SFN, sulforaphane; SOD, superoxide dismutase; Ugt1a1, UDP-glucuronosyltransferase 1a1

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inhibitor for Nrf2 and regulates the degradation of Nrf2 in the cytoplasm (Kaspar et al., 2009; Wang et al., 2016). In response to oxidative/electrophilic stress, Nrf2 is released from Keap1 and translocates into the nucleus, and subsequently activates antioxidant response element (ARE)-responsive gene expression (Sykiotis and Bohmann, 2010). Through binding to ARE, Nrf2 regulates the expression of a variety of genes encoding intracellular detoxifying enzymes and antioxidant proteins, including heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), glutamate-cysteine ligase catalytic and modifier subunits (GCLC and GCLM) (Jaiswal, 2004; Nguyen et al., 2009; Enomoto et al., 2001). Nrf2 has also been reported to regulate efflux transporters such as multi-drug resistance-associated proteins Mrp2, Mrp3 and Mrp4, and Cyp enzymes including Cyp2e1, Cyp3a11 and Cyp1a2 (Pang et al., 2016; Jiang et al., 2016). Therefore, targeting Nrf2 signaling pathway can serve as a novel therapeutic approach to treat APAP-induced hepatotoxicity.

Natural products, one of the most important resources in drug discovery, have been widely investigated worldwide (Ilyas et al., 2016). Due to the advantages of high efficacy and low toxicity, more and more researchers have focused on these chemicals, and many natural chemicals including silymarin, liquiritigenin and arjunolic acid are considered to be potential chemicals for the treatment of liver injury (Wagoner et al., 2010; Kim et al., 2006; Manna et al., 2007). Ginsenosides Rg1 (Rg1, shown in Fig. 1A) is one of the major pharmacological bioactive constituents of ginsenosides, which is a perennial herbaceous herb of Araliaceae family and a highly valued medicinal plant renowned for its diverse pharmacological actions (Lee et al., 1997; Helms, 2004; Attele et al., 1999). It has been reported that Rg1 has excellent clinical effects of preventing cancer, enhancing physical functions and neuroprotective activities (Kiefer and Pantuso, 2003; Nah, 2014; Xu et al., 2016). Rg1 has also been demonstrated to have favorable efficacy on hepatoprotective properties against acute liver injury (Gao et al., 2017a, 2017b). However, the role of Rg1 on the expression of transporters and detoxifying enzymes via Nrf2 signaling pathway to reduce APAP-induced acute liver disease has not been reported in detail. In the present study, we aimed to investigate the hepatoprotective effects of Rg1 against APAP-induced acute liver injury by *in vivo* and *in vitro* experiments and further explore the underlying mechanism.

2. Materials and methods

2.1. Chemicals and materials

Rg1 (purity > 98%) was purchased from Chengdu Must Bio-technology Co., Ltd. (Chengdu, China). APAP and sulforaphane (SFN) were obtained from Dalian Meilun Medical Science and Technology Company Ltd. (Dalian, China). All-transretinoic acid (ATRA, purity > 98%) which is the inhibitor of Nrf2/ARE signaling pathway, was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit antibodies against Keap1, Nrf2, GCLC, GCLM, NQO1, HO-1, Cyp2e1, Mrp2, Mrp3, Mrp4, β-Actin and Lamin B were purchased from Proteintech Biotechnology (Wuhan, China). All biochemical indicator kits and other chemicals were commercially available.

2.2. Animals and treatments

Male C57BL/6 mice (8–10 weeks, weight 20–25 g) were housed in laboratory animal facilities under a standard 12-h light/dark cycle. All animals received human care and treatment protocols were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Dalian Medical University, Dalian, China. For prophylactic group, mice were randomly divided into nine groups: (1) control group, (2) Rg1 (60 mg/kg)-treated group, (3) APAP-treated group, (4) Rg1 (15 mg/kg)/APAP-treated group, (5) Rg1 (30 mg/kg)/APAP-treated group, (6) Rg1 (60 mg/kg)/APAP-treated group, (7) SFN (50 mg/kg)/APAP-treated group. (8) ATRA (10 mg/kg)/APAP-treated group, (9) ATRA (10 mg/kg)/Rg1 (60 mg/kg)/APAP-treated group. Rg1 (15, 30 or 60 mg/kg) was dissolved in saline solution and administered to mice by oral gavage seven times with an interval of 12 h for 3 consecutive days. The control group and APAP group were administered by saline solution. To induce APAP hepatotoxicity, APAP was dissolved in saline solution that kept in warm boiling water bath (37 °C) and administered by intraperitoneal (i.p.) injection at a single dose of 400 mg/kg fifteen minutes later after the last dose of Rg1, all animals were fasted overnight before APAP administration. The control group and Rg1 alone group were injected i. p. with saline solution. The mice were injected intraperitoneally with 10 mg/kg of ATRA 4 h before administration of Rg1 every time. All mice were sacrificed under anesthesia (pentobarbital sodium, 65 mg/kg, intraperitoneal injection) 6 h after APAP treatment. For therapeutic group, mice were injected intraperitoneally with 400 mg/kg APAP. At 0, 2, 4 h after APAP administration, Rg1 (15, 30, 60 mg/kg), SFN (50 mg/kg) or saline solution was administered to mice. Six hours after APAP administration mice were sacrificed under anesthesia. Serum samples and liver tissues were harvested, a portion of the liver was immediately fixed in 10% buffered formalin for histology, and the remaining tissues were flash frozen in liquid nitrogen and stored at -80 °C for further use.

2.3. Serum biochemical analysis

The plasma was collected from suborbital veins into heparinized tubes and the alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LDH) enzymatic activity were determined using commercially available kits according to the manufacturer's instructions (Nanjing Jiancheng Institute of Biotechnology, Nanjing, China).

2.4. Parameter measurements for oxidative stress in the liver

The frozen liver tissues were homogenized in phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide. The activities of malondialdehyde (MDA), glutathione transferase (GSH), catalase (CAT) and superoxide dismutase (SOD) were measured using commercially available kits according to the manufacturer's instructions (Nanjing Jiancheng Institute of Biotechnology, Nanjing, China).

2.5. Histopathlogical examination

To detect histopathological changes, small pieces from different lobes of the livers were collected and fixed in 10% neutral buffered formaldehyde solution for at least 24 h. Then the tissues were washed with phosphate-buffered saline (PBS), embedded in paraffin wax and stained with hematoxylin and eosin (H&E) in 5 μm sections with a standard protocol. Finally, the histopathological changes of the liver tissues were examined under a light microscopy. The histological changes were evaluated by a point-counting method for severity of hepatic injury using an ordinal scales in accordance with the methods as previous described (Camargo et al., 1997). The stained sections were graded as a four-point scale from 0 to 3 as follows: 0, 1, 2, and 3 represent no damage, mild damage, moderate damage and severe damage, respectively.

2.6. Isolation and culture of mouse primary hepatocytes

Hepatocytes from male C57BL/6 mice (8–9 weeks) were isolated by the two-step collagenase digestion method as described previously (Klaunig et al., 1981). The obtained hepatocytes were cultured with the William's E medium, supplemented with 0.1 μmol/L dexamethasone (Sigma-Aldrich, St Louis, MO, USA), 10% heat-inactivated fetal bovine