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Role of Nrf2 activation and NF-κB inhibition in valproic acid induced hepatotoxicity and in diammonium glycyrrhizinate induced protection in mice

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ABSTRACT

Diammonium glycyrrhizinate (DG), an active compound extracted and purified from liquorices root, has been reported to exhibit antioxidant and anti-inflammatory properties. The aim of this study was to investigate the effect and underlying mechanisms of DG on the hepatotoxicity induced by valproic acid (VPA). DG at the dose of 60 mg/kg was orally administered with VPA (100 mg/kg) to mice once daily for 14 consecutive days. DG treatment attenuated VPA-induced liver dysfunction, structural damage, glutathione depletion and decrease in antioxidant enzymes in BALB/C mice. DG prevented VPA-induced depletion of cytosolic nuclear factor E2-related factor 2 (Nrf2) and suppression of nuclear translocation of Nrf2, which, in turn, up-regulated phase II/antioxidant enzyme activities. The effects of VPA and DG on Nrf2 expression in HepG2 cells were in consistent with that of mice. Furthermore, an increase in the nuclear levels of nuclear factor-kappaB (NF-κB) was observed in the livers of VPA-treated mice that coincided with induction of inflammatory cytokines. In contrast, DG inhibited NF-κB translocation and that subsequently decreased inflammatory cytokines. Taken together, these results demonstrate that DG attenuates VPA-induced liver injury through increasing the expression of Nrf2 mediated phase II/antioxidant enzymes and simultaneously decreasing the expression of inflammatory mediators.

1. Introduction

Valproic acid (2-n-propylpentanoic acid, VPA) is a highly effective antiepileptic drug used for the treatment of various types of seizures and neurological disorders (Garcia-Morales et al., 2007; Haddad et al., 2009). However, the clinical use of VPA is often associated with hepatotoxicity (Fisher et al., 1991; Ghozzi et al., 2011; Sugimoto et al., 1987). The mechanism of VPA-induced hepatotoxicity is still not completely clear. *In vitro* and *in vivo* evidences have suggested that oxidative stress played a critical role in the pathogenesis of VPA-induced hepatotoxicity (Kiang et al., 2010; Tong et al., 2005a,b). It is indicated that excessive production of free radicals and depletion of antioxidants due to oxidative stress are implicated in VPA-induced liver dysfunction (Kiang et al., 2011).

The Nrf2–ARE pathway is regarded as one of the most important defense systems for the protection against oxidative damage. In response to oxidative stress, Nrf2 translocates to the nucleus where it transactivates a battery of genes encoding various phase II detoxifying and antioxidant enzymes by binding to antioxidant response elements (ARE). Typical Nrf2-target genes include phase II detoxification enzymes [e.g., glutathione-S-transferases (GST) and NAD(P)H quinine oxidoreductase 1 (NQO1)] and antioxidant-related enzymes [e.g., heme oxygenase-1 (HO-1) and glutamate cysteine ligase (GCL)] (Klaassen and Reisman, 2010; Kobayashi and Yamamoto, 2005). These enzymes play an important role in the counteraction against oxidative stress damage. The role of Nrf2 in the liver has been reported, as livers of Nrf2-null mice are more susceptible to various chemical- and oxidative stress-induced pathologies than wild-type mice. Several studies have revealed that Nrf2 activators could alleviate the liver injury induced by several compounds such as acetaminophen, carbon tetrachloride, and cadmium (Cho et al., 2011; Enomoto et al., 2001;

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Lee et al., 2014; Reisman et al., 2009; Wu et al., 2012). However, whether activation of Nrf2 can protect against VPA induced liver injury remains unclear.

Hepatic inflammation is a common finding during a variety of liver diseases including drug induced hepatotoxicity (Ramadori and Armbrust, 2001). Rats that develop liver injury displayed increased levels of inflammatory mediators such as nuclear factor-kappaB (NF-κB), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) (Diehl, 2000; Leiro et al., 2004; Liu et al., 2007). It was suggested that the release of inflammatory mediators from activated hepatic macrophages could potentiate drug induced hepatic injury. However, it has not been reported that whether these inflammatory mediators increased during VPA-induced hepatotoxicity.

Diammonium glycyrrhizinate (DG) (Fig. 1) is an active chemical component extracted and purified from liquorice root. DG possessed a wide range of pharmacological activities including anti-inflammation, resistance to biologic oxidation, membranous protection and immunomodulatory effect (Feng et al., 2007; Hou et al., 2012; Zhu et al., 2012). DG has been widely used in Asian countries for the treatment of several forms of liver disease including hepatitis and drug- and chemical-induced liver toxicity (Feng et al., 2007; Jin et al., 2005; Li et al., 2010). However, there is no previous experimental evidence describing the effect of DG on VPA-induced hepatotoxicity.

Taken the above into consideration, the present study has been undertaken to evaluate the protective effect of DG on the hepatotoxicity induced by VPA and to elucidate the underlying mechanisms. The data is helpful for the development of strategies to prevent or alleviate VPA-induced hepatotoxicity.

2. Materials and methods

2.1. Chemicals and reagents

Valproic acid (VPA) (>99% purity) was purchased from Hunan Xiangzhong Pharmaceutical Co., Ltd. (Shaoyang, Hunan, China). Diammonium glycyrrhizinate (DG) (>98% purity) was purchased from Chia Tai Tianqing Pharmaceutical Group Co., Ltd. (Nanjing, Jiangsu, China). HPLC and mass spectrometry were employed to check for interferences and confirm the chemical identity of DG. Anti-Nrf2 antibody, anti-GCLC antibody, anti-HO-1 antibody and anti-NF-κB p65 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-NQO1 antibody was obtained from Sigma–Aldrich (St. Louis, MO, USA). Anti-Histone H3 antibody and anti-β-actin antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Other chemicals were of analytical grade from commercial suppliers.

2.2. Animals and treatment

Male BALB/C mice (6 weeks) were purchased from the Laboratory Animals Center of Guangzhou University of Chinese Medicine, Guangzhou, China. All mice were kept in a specific pathogen-free animal facility under controlled conditions at the temperature (24 ± 2 °C), humidity (55 ± 15%), with a 12-h light–dark cycle. Food and water were provided ad libitum. All of the animal experimental procedures in this study were performed in accordance with the protocol approved by the Animal Ethical and Welfare Committee of Guangzhou University of Chinese Medicine, in accordance with National Institute of Health and Nutrition Guidelines for the Care and Use of Laboratory Animals. To investigate the protective activity of DG on VPA-induced hepatotoxicity, forty mice were randomly assigned to the following four groups: (1) control group; (2) VPA group (100 mg/kg); (3) DG group (60 mg/kg); (4) VPA (100 mg/kg) plus DG (60 mg/kg) group. The mice in the control group were given normal saline (NS) by intragastric administration for 14 days and the administration volume was 20 mL/kg. VPA and DG suspensions were prepared in normal saline (NS) and were given to the treatment group by intragastric administration (20 mL/kg) for 14 days. The dosages of VPA and DG were based on previous studies (Sokmen et al., 2012). In all treated groups, mice were anesthetized 24 h after the last administration and blood samples were collected for serum biochemical assays. Livers were removed and weighed immediately. For histopathological examination, one lobule of the liver was fixed in 10% formalin. The remaining parts of the liver were collected for biochemical analysis.

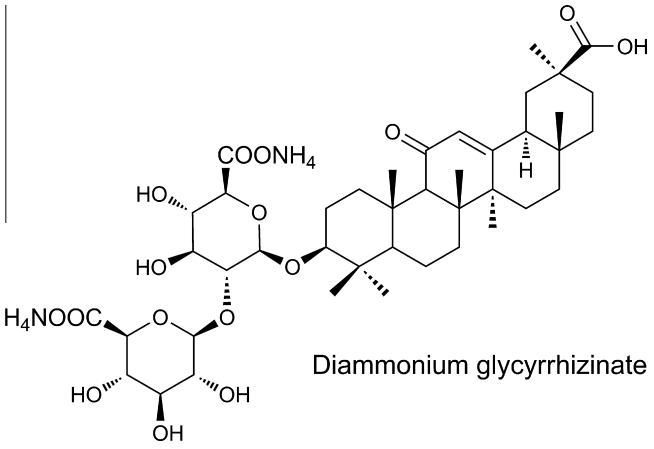

Fig. 1. Structure of diammonium glycyrrhizinate (DG).

2.3. Biochemical assays

Serum samples were assayed for ALT, AST, and LDH by using commercially available enzymatic assay kits (Leadman Group Co., Ltd., Beijing, China).

2.4. Histological studies

The specimens were immersed in a formaldehyde solution for 24 h. The recipe for formaldehyde solution is 10% of a 37–40% formaldehyde and 90% of a 0.01 mol/L pH 7.4 PBS. After fixation, the specimens were transferred to 70% ethanol and kept there until processed. The specimens were processed through graded alcohols, cleared in Van-clear (substitute for xylene) and embedded in paraffin. Sections of 3 μm were cut and stained with hematoxylin & eosin for overall morphological evaluation.

2.5. Measurement of oxidative stress marker

Activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) of homogenized liver were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). Contents of glutathione (GSH) and malondialdehyde (MDA) were also measured using GSH and MDA Detection Kit (Nanjing Jiancheng Bioengineering institute, Nanjing, Jiangsu, China) respectively, according to the manufacturer's instructions.

2.6. Cell culture

HepG2 cells were obtained from the American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, Utah, USA) and antibiotics (50 U/mL of penicillin and 50 μg/mL streptomycin). The cells were grown in a humidified incubator with 5% CO_2 at 37 °C.

2.7. ARE-luciferase assay

The plasmid pGL3-ARE was a kind gift from Dr. Athanassios Fragoulis (University Hospital Aachen) (Boesch-Saadatmandi et al., 2009). NRK-52E cells at 50% confluence were transfected with plasmids using Lipofectamine 2000 (Invitrogen Corporation, USA). Briefly, cells were incubated with the transfection complex containing 0.2 μg ARE-luciferase plasmid, 0.02 μg pRLtk control plasmid (Promega, Mannheim, Germany), and 0.5 μl Lipofectamine 2000 in serum- and antibiotic-free OptiMEM (Invitrogen Corporation, USA). Transfection was continued for 5 h; the cells then recovered in complete medium over night and treated with VPA (20, 40, 80 and 160 nM) for 24 h. After that, luciferase activities from firefly and Renilla luciferases in total cell lysates were determined using a Dual-Glo Luciferase Assay kit (Promega, Mannheim, Germany) with Flex Station 3 (Moleculardevices, USA).

2.8. Western blot analysis

Cytoplasmic and nuclear extracts were extracted using a Nuclear Extract kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's recommendations. Equivalent amounts of protein were separated by 12% SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore Co., Billerica, MA, USA). After being blocked in 5% non-fat milk in TBST [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% Tween 20] for 2 h at room temperature, the membranes were incubated with the appropriate primary antibodies at 4 °C overnight. The immunoblots were then incubated with a secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. The membranes were developed using an electrochemiluminescence (ECL) kit (Thermo Scientific/Pierce, Rockford, IL, USA) according to the manufacturer's protocol. The signals were