Clinics and Research in Hepatology and Gastroenterology (2015) 39, 188—197

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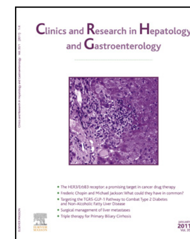

ORIGINAL ARTICLE

Protective effects of ursolic acid in an experimental model of liver fibrosis through Nrf2/ARE pathway

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Available online 29 October 2014

Summary
Aim: Liver fibrosis is a reversible wound-healing response that occurs following liver injury. In this study, we aimed to investigate the possible protective effects of ursolic acid in liver fibrosis induced by carbon tetrachloride (CCl_4).
Methods: ICR mice were randomly divided into six groups (Group 1: normal; Group 2: CCl_4-treated group; Group 3: CCl_4 plus ursolic acid 25 mg/kg group; Group 4: CCl_4 plus ursolic acid 50 mg/kg group; Group 5: CCl_4 plus colchicine 1 mg/kg group; Group 6: ursolic acid 50 mg/kg group). Mice were administered with CCl_4 (2 mL of CCl_4 in olive oil (1:1, v/v) per kg body weight twice weekly) by intraperitoneal injection and oral injection of colchicine (1 mg/kg) or ursolic acid (25, 50 mg/kg) daily. After six weeks, serum aminotransferase activity, hepatic reactive oxygen species (ROS) production, thiobarbituric acid reactive substances (TBARS), antioxidase (SOD, CAT, GPx) activity and histopathological analysis were performed. The levels of nuclear factor E2-related factor 2 (Nrf2), NAD(P)H: quinone oxidoreductase-1 (NQO1), glutathione S-transferase (GST) and heme oxygenase-1 (HO-1), tumor necrosis factor-alpha (TNF-α), prostaglandin E2 (PGE2) and inducible nitric oxide synthase (iNOS), Bcl-2 and caspase-3 were measured.
Results: Ursolic acid significantly prevented CCl_4-induced hepatotoxicity and fibrosis, indicated by both diagnostic indicators and histopathological analysis. CCl_4-induced profound elevations of oxidative stress, inflammation and apoptosis in liver were suppressed by ursolic acid.

Abbreviations: ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; ARE, Antioxidant response elements; CAT, Catalase; CCl_4, Carbon tetrachloride; GPx, Glutathione peroxidase; GSH, Reduced glutathione; GST, Glutathione S-transferase; HO-1, Heme oxygenase-1; iNOS, Inducible nitric oxide synthase; NQO1, NAD(P)H:quinone oxidoreductase-1; Nrf2, Nuclear factor E2-related factor 2; PGE2, Prostaglandin E2; α-SMA, α-smooth muscle actin; SOD, Superoxide dismutase; TBARS, Thiobarbituric acid reactive substances; TNF-α, Tumor necrosis factor-alpha; UA, Ursolic acid.

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http://dx.doi.org/10.1016/j.clinre.2014.09.007
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Conclusions: These results suggest that ursolic acid has the hepatoprotective actions. The inhibition of CCl_4-induced liver fibrosis, inflammation and apoptosis by ursolic acid is due at least in part to its ability to modulate the Nrf2/ARE signalling pathway.
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Introduction

Chronic liver disease is a major public health problem worldwide. All chronic liver diseases have in common the gradual progressive substitution of the functioning hepatic parenchyma by fibrotic tissue [1]. Hepatic fibrosis is a dynamic process characterized by excessive deposition of extracellular matrix (ECM) components, including collagen type I and α-smooth muscle actin (α-SMA), and can ultimately cause liver cirrhosis [2]. Hepatic stellate cells (HSCs) are the main matrix-producing cells involved in the hepatic fibrosis development, orchestrating the deposition of ECM in normal and fibrotic liver [3]. Activated HSCs is the major producers of ECM in response to liver injury. Because liver injury involves a complex cascade of events, the precise mechanism of hepatic fibrosis is not fully understood. However, many evidences had shown that oxidative stress-mediated inflammation initiate and then perpetuate the fibrosis [2,4—6].

Nuclear factor E2-related factor 2 (Nrf2) is referred to as the ''master regulator'' of the antioxidant response via the antioxidant response elements (ARE), modulating the expression of hundreds of genes, including not only the familiar antioxidant enzymes, but large numbers of genes that control seemingly disparate processes such as immune and inflammatory responses, tissue remodelling and fibrosis, carcinogenesis and metastasis, and even cognitive dysfunction and addictive behavior [7—9]. Many researches have demonstrated that Nrf2 prevents the liver from many hepatotoxicants. The Nrf2-mediated protection is accompanied by induction of antioxidant genes, suppression of inflammatory responses, inhibition of apoptosis and attenuation of oxidative stress [7,8,10].

Triterpenoids are ubiquitously distributed throughout the plant kingdom, and some are increasingly being used for medicinal purposes for a variety of clinical diseases in many Asian countries [11]. Ursolic acid (UA: 3β-hydroxy-urs-12-en-28-oic acid), a natural pentacyclic triterpenoid, has been found in various plants including apples, basil, cranberries, peppermint, rosemary, oregano and prunes and has been reported to possess many biological activities, including antioxidant, anti-inflammatory, trypanocidal, antirheumatic, antiviral and antitumoral properties [12,13]. Previous study had revealed that UA ameliorated hepatic fibrosis in the rat by specific induction of apoptosis in hepatic stellate cells [14]. However, the molecular mechanisms of CCl_4-induced liver injury and anti-fibrosis effects of UA are not yet completely understood. Therefore, we have used an experimental model of mice chronically treated with CCl_4 as a model of hepatic fibrosis. In the present study, we aimed to determine whether UA can protect mouse liver from CCl_4-induced fibrosis by the Nrf2/ARE pathway.

Materials and methods

Chemicals and reagents

UA and CCl_4 were obtained from Sigma Chemical Co. (St. Louis, MO, USA); anti-α-SMA antibody, anti-Bcl-2 antibody, anti-caspase-3 antibody, anti-TNF-α antibody, anti-PGE2 antibody, anti-iNOS antibody, anti-Nrf2 antibody, anti-HO-1 antibody, anti-NQO1 antibody and anti-GST antibody are from Santa Cruz Biotechnology (Santa Cruz, CA, USA); aminotransferase activities in serum assay kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) and Jingmei Biotech ltd. (Shenzhen, China); BCA assay kit from Pierce Biotechnology, Inc. (Rockford, IL, USA). All other reagents unless indicated were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and experimental design

Male ICR mice (20—25 g) were obtained from the Branch of National Breeder Center of Rodents (Beijing) and kept in an environmentally controlled room ($23 \pm 2\,^{\circ}C$, $55 \pm 10\%$ humidity) with a 12-h light/dark cycle and allowed free access to food and water. Hepatic fibrosis was induced by intraperitoneal (i.p.) injection of 2 mL of CCl_4 in olive oil (1:1, v/v) per kg body weight twice weekly for up to 6 weeks [15]. Sixty mice were randomly divided into six groups (10 mice/group). Mice in Group 1 were given twice weekly injections of olive oil (vehicle control); mice in Group 2 were injected with CCl_4 and received water containing 0.1% Tween 80 by oral gavage; mice in Group 3 were injected with CCl_4, as in group 2, and received UA in distilled water containing 0.1% Tween 80 at a dose of 25 mg/(kg day) by oral gavage; mice in Group 4 were injected with CCl_4, as in group 2, and received UA in distilled water containing 0.1% Tween 80 at a dose of 50 mg/(kg day) by oral gavage; mice in Group 5 were injected with CCl_4, as in group 2, and received colchicine 1 mg/kg (kg day) in distilled water by oral gavage; Group 6 were injected with olive oil, as in group 1, and received UA in distilled water containing 0.1% Tween 80 at a dose of 50 mg/(kg day) by oral gavage [16,17].

At the end of treatment, seven mice in each group were used for the biochemical analysis; the others were used for histological evaluations. Mice were sacrificed and about 2 mL of blood samples were drawn by cardiac puncture with heparined tubes. The plasma was collected after centrifugation at 5000 rpm for 10 min and stored at $-70\,^{\circ}C$ freezer for further analysis. The liver tissues were immediately collected for experiments and placed in ice-cold 0.9% NaCl solution, perfused with the physiological saline solution to remove blood cells, blotted on filter paper. And then, the removed

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