Food and Chemical Toxicology 51 (2013) 370–378

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Rosmarinic acid ameliorates acute liver damage and fibrogenesis in carbon tetrachloride-intoxicated mice

Robert Domitrović [a,*], Marko Škoda [b], Vanja Vasiljev Marchesi [c], Olga Cvijanović [d], Ester Pernjak Pugel [e], Maja Bival Štefan [f]

[a] Department of Chemistry and Biochemistry, School of Medicine, University of Rijeka, Rijeka, Croatia
[b] Department of Physiology and Immunology, School of Medicine, University of Rijeka, Rijeka, Croatia
[c] Department of Social Medicine and Epidemiology, School of Medicine, University of Rijeka, Rijeka, Croatia
[d] Department of Anatomy, School of Medicine, University of Rijeka, Rijeka, Croatia
[e] Department of Histology and Embriology, School of Medicine, University of Rijeka, Rijeka, Croatia
[f] Department of Pharmacognosy, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia

ARTICLE INFO

Article history:
Received 16 August 2012
Accepted 19 October 2012
Available online 29 October 2012

Keywords:
Rosmarinic acid
Hepatotoxicity
Oxidative/nitrosative stress
Inflammation
Apoptosis
Liver fibrosis

ABSTRACT

The aim of this study was to investigate the therapeutic potential of rosmarinic acid (RA), a natural phenolic, in the treatment of acute liver toxicity. RA at 10, 25 and 50 mg/kg was administered by gavage once daily for 2 consecutive days, 6 h after CCl_4 intoxication. CCl_4 intoxication caused hepatic necrosis and increased serum ALT activity. In the livers, oxidative/nitrosative stress was evidenced by increased 3-nitrotyrosine (3-NT) and thiobarbituric acid reactive substances (TBARS) formation and a significant decrease in Cu/Zn superoxide dismutase (SOD) activity. CCl_4 administration triggered inflammatory response in mice livers by activating nuclear factor-kappaB (NF-κB), which coincided with the induction of tumor necrosis factor-alpha (TNF-α) and cyclooxygenase-2 (COX-2). RA improved histological and serum markers of liver damage and significantly ameliorated oxidative/nitrosative stress and inflammatory response in liver tissue. Additionally, RA prevented transforming growth factor-beta1 (TGF-β1) and alpha-smooth muscle actin (α-SMA) expression, suggesting suppression of profibrotic response. Furthermore, RA significantly inhibited the CCl_4-induced apoptosis, which was evident from decreased cleavage of caspase-3. The hepatoprotective activity of RA coincided with enhanced NF-E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) expression. The results of this study indicates that RA possesses antioxidant, anti-inflammatory, antiapoptotic and antifibrotic activity against acute liver toxicity.

1. Introduction

Liver injury is one of the major worldwide health problems due to non-adequate nutrition, alcohol and drug abuse, viral infections and incidental poisoning. Carbon tetrachloride (CCl_4) intoxication is a frequently used model of liver injury (Weber et al., 2003). CCl_4 is activated by hepatic microsomal cytochromes to form the trichloromethyl radical, CCl_3 This radical induces oxidative stress by binding to cellular molecules, thus impairing numerous cellular processes that lead to necrotic cell damage, inflammation and apoptosis (Sun et al., 2001a). Nonlethal intoxication triggers liver tissue remodeling and healing through the activation of hepatic stellate cells (HSCs), leading to liver fibrosis (Friedman, 2000). Natural phenolics have been shown to ameliorate oxidative stress-mediated liver injury and prevent hepatic failure.

Rosmarinic acid (Fig. 1) is a phenolic compound commonly found in various plants from the *Lamiaceae* (the mint) family, such as *Rosmarinus officinalis* (rosemary), *Origanum vulgare* (oregano), *Thymus vulgaris* (thyme), *Mentha spicata* (spearmint), *Perilla frutescens* (perilla), *Ocimum basilicum* (sweet basil) and several other medicinal plants, herbs and spices (Shetty, 2005). It has been reported that rosmarinic acid exerts different biological activities, such as antioxidant (Tepea et al., 2007), anti-inflammatory (Chu et al., 2012), antiallergic (Osakabe et al., 2004), anticancer (Moon et al., 2010), antimicrobial (Moreno et al., 2006) and neuroprotective (Ono et al., 2012) activity.

Previously, we showed that natural compounds, including isoquinoline alkaloid berberine (Domitrović et al., 2011), flavone luteolin (Domitrović et al., 2009), secoiridoid oleuropein (Domitrović et al., 2012) and anthocyanidin delphinidin (Domitrović and Jakovac, 2010) exhibit the hepatoprotective activity in a dose range depending on the particular compound. The most promising one,

* Corresponding author. Address: Department of Chemistry and Biochemistry, School of Medicine, University of Rijeka, B. Branchetta 20, 51000 Rijeka, Croatia. Tel.: +385 51 651 135; fax: +385 51 678 895.
E-mail address: robertd@medri.uniri.hr (R. Domitrović).

0278-6915/$ - see front matter © 2012 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.fct.2012.10.021

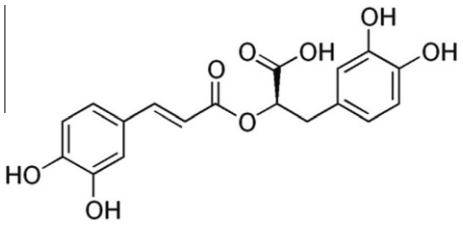

Fig. 1. The chemical structure of rosmarinic acid.

berberine, showed high clinical efficacy in patients with liver disease (Zhao et al., 2008). Silymarin and *N*-acetyl-L-cysteine, compounds already used in a clinical treatment of liver injury, exhibit a potent hepatoprotective activity, but with certain restraints (Squires et al., 2012; Fried et al., 2012). This indicates that there is still the need for finding highly effective and reliable drug for the treatment of liver damage and the prevention of acute liver failure. In this study, we used a mouse model of acute liver injury induced by CCl_4 to examine the therapeutic effects of rosmarinic acid and the mechanisms of its hepatoprotective activity.

2. Materials and methods

2.1. Chemicals and antibodies

Rosmarinic acid (96% pure), bovine serum albumin (BSA), bovine Cu/Zn superoxide dismutase (SOD), xanthine, xanthine oxidase, cytochrome *c*, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), thiobarbituric acid, trichloroacetic acid (TCA), 1,1,3,3-tetramethoxypropane (TMP) and Entelan were purchased from Sigma–Aldrich (St. Louis, MO, USA). Carbon tetrachloride (CCl_4) and *n*-butanol were obtained from Kemika (Zagreb, Croatia). Acetic acid and pyridine were from Merck (Darmstadt, Germany). Mouse monoclonal antibodies to tumor necrosis factor-alpha (TNF-α) (ab1793), 3-nitrotyrosine (3-NT) (ab78163), and alpha-smooth muscle actin (α-SMA) (ab18460) and rabbit polyclonal antibodies to nuclear factor-kappaB (NF-κB), cyclooxygenase-2 (COX-2) (ab15191), NF-E2-related factor 2 (Nrf2) (ab31163), HO-1 (ab13243) and transforming growth factor-beta1 (TGF-β1), were purchased from Abcam (Cambridge, UK). Rabbit polyclonal antibody to cleaved caspase-3 (Asp 175) (#9661) was from Cell Signaling Technology, Danvers, MA, USA. Glass slides for immunohistochemistry and DAKO EnVision + System were from DAKO Corporation (Carpinteria, CA, USA). Radioimmunoprecipitation assay (RIPA) buffer (sc-24948) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinylidene fluoride (PVDF) membrane was purchased from Roche Diagnostics GmbH (Mannheim, Germany), milk blocking reagent from Santa Cruz Biotechnology (Santa Cruz, CA, USA), peroxidase-labeled goat anti-mouse F(ab′)2 and Amersham ECL Prime from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals were of the highest grade available.

2.2. Animals

Male BALB/cN mice, 2 months old, weight 20–26 g, were obtained from the breeding colony of the School of Medicine, University of Rijeka. The animals were housed in standard environmental conditions and had free access to tap water and a standard rodent diet (pellet, type 4RF21 GLP, Mucedola, Italy). All experimental procedures were conducted in compliance with the Declaration of Helsinki principles and approved by the Ethical Committee of the Medical Faculty, University of Rijeka.

2.3. Experimental procedure

Mice were divided into 6 groups of 5 animals each. The control group (group I) received vehicle (saline with 5% DMSO (v/v)) and group II received rosmarinic acid at 50 mg/kg, by gavage, once daily for 2 consecutive days. The addition of DMSO markedly increased drug solubility (Fichera et al., 2007). Group III was administered CCl_4 dissolved in corn oil (2 mL/kg, 10% v/v), intraperitoneally. Rosmarinic acid, dissolved in saline with 5% DMSO (v/v), was given by gavage at doses of 10, 25 and 50 mg/kg (groups IV, V and VI) once daily for 2 consecutive days, 6 h after CCl_4 intoxication. The doses used were selected on the basis of our preliminary studies (data not shown). Twenty-four hours after the last dose of rosmarinic acid or saline mice were sacrificed by cervical dislocation under ether anesthesia. Previously, blood was collected from the orbital sinus and the serum was separated for determination of ALT activity. The livers were removed, washed with saline, blotted dry and divided into samples. Tissue specimens were frozen in liquid nitrogen and stored at −80 °C if not used for analysis immediately. Liver samples were used to assess the antioxidant status, protein content and for Western blot. Additionally, liver samples were preserved in a 4% paraformaldehyde solution for histology and immunohistochemistry.

2.4. Serum ALT activity

Serum activity of ALT was measured spectrophotometrically using diagnostic kit for ALT (Dijagnostika, Sisak, Croatia; IFCC method, 2006) on a Bio-Tek EL808 Ultra Microplate Reader (BioTek Instruments, Winooski, VT, USA) following the manufacturer's instructions.

2.5. Liver antioxidant status

The livers were homogenized in 50 mM phosphate buffered saline (PBS), pH 7.4, using a Polytron homogenizer (Kinematica, Lucerne, Switzerland). The supernatants were separated using Beckman L7-65 Ultracentrifuge (Beckman, Fullerton, USA) at 15000*g* for 20 min, at 4 °C. The level of lipid peroxidation in liver homogenates was quantified by thiobarbituric acid reactive substances (TBARS) assay as described previously (Domitrović et al., 2011). Samples were incubated with 8.1% SDS and 20% acetic acid solution. Later, supernatants were mixed with 0.8% TCA and heated at 95 °C for 1 h. The resulting chromogen was extracted with 1-butanol/pyridine solution (15:1, v/v) and absorbance was measured spectrophotometrically at 532 nm. TBARS were calculated as nmol malondialdehyde equivalent per milligram of protein according to the standard curve prepared from TMP. The supernatants were used for determination of Cu/Zn SOD activity. The Cu/Zn SOD activity was measured at 550 nm by the decrease in cytochrome *c* reduction by superoxide radicals generated in the xanthine/xanthine oxidase system, as described previously (Domitrović et al., 2011). The protein content in liver homogenates was estimated by Bradford's method (Bradford, 1976).

2.6. Histopathology

Liver tissues were placed in plastic cassettes and immersed in 4% paraformaldehyde for 48 h. The fixed tissues were processed as described previously (Domitrović et al., 2012). The degree of hepatocellular damage was evaluated by measuring the area of necrosis in liver sections stained with hematoxylin and eosin (H and E). For this purpose, we used light microscopy (Olympus BX51, Tokyo, Japan). The necrotic zones were manually selected and the percentage of necrotic area was determined using the Cell F v3.1 software, Olympus Soft Imaging Solutions (Münster, Germany).

2.7. Immunohistochemical analysis

For immunohistochemistry, 4 μm thick deparaffinized liver tissue sections were used, as described elsewhere (Domitrović et al., 2012). Briefly, deparaffinized liver slices were incubated overnight with the antibodies against NF-κB, Nrf2, α-SMA and cleaved caspase-3. Endogenous peroxidase activity was blocked by incubation in 0.075% hydrogen peroxide in PBS. For antibody detection DAKO EnVision + System, Peroxidase/DAB kit was employed. The sections were then counterstained with hematoxylin, dehydrated using graded alcohols and xylene, and mounted with Entelan. The immunostaining intensity was analyzed by light microscopy (Olympus BX51). The Cell F v3.1 software, Olympus Soft Imaging Solutions (Münster, Germany), was used to quantify immunohistochemical staining across 10 high-power fields (400×).

2.8. Western blot analysis

Western blot analysis with monoclonal antibodies against TNF-α, COX-2, HO-1, 3-NT, TGF-β1 and cleaved caspase-3 was performed as described elsewhere (Domitrović et al., 2012). Briefly, liver samples were lysed in RIPA buffer containing 50 mM Tris–HCl (pH = 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 2 μg/ml of each aprotinin, leupeptin and pepstatin. Volumes equivalent to 50 μg of proteins were transferred to 12% polyacrylamide gel. After electrophoresis, gels were blotted onto polyvinylidene fluoride membrane. After protein transfer, membranes were blocked overnight by milk blocking reagent at 4 °C. The proteins were visualized by addition of the respective antibodies, followed by peroxidase-labeled goat anti-mouse F(ab′)2. The β-actin was used as a control of protein load. Membranes were washed three times with TBST buffer, incubated with Amersham ECL Prime (GE Healthcare, Uppsala, Sweden) and scanned (Kodak Image Station 440CF, Kodak, New Haven, CT, USA). The intensity of the bands was quantified using ImageJ software (http://rsb.info.nih.gov/ij/).

2.9. Statistical analysis

Normality of data distribution was assessed by the Kolmogorov–Smirnov normality test. The distribution did not qualify the normality test so non-parametric test of Kruskal–Wallis was applied and post hoc comparisons were carried out with Dunn's multiple comparison test. Results of multiple comparisons tests were