Pharmacological Research 65 (2012) 451–464

Contents lists available at SciVerse ScienceDirect

Pharmacological Research

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

Preventive and therapeutic effects of oleuropein against carbon tetrachloride-induced liver damage in mice

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ARTICLE INFO

Article history:
Received 5 September 2011
Received in revised form
20 December 2011
Accepted 20 December 2011

Keywords:
Hepatotoxicity
Oleuropein
Oxidative stress
Apoptosis
Liver fibrosis
Inflammation

ABSTRACT

Olives and olive products, an inevitable part of the Mediterranean diet, possess various beneficial effects, such as a decreased risk of cardiovascular disease and cancer. Oleuropein is a non-toxic secoiridoid found in the leaves and fruits of olive (*Olea europaea* L.). In this study, we have investigated the hepatoprotective activity of oleuropein in carbon tetrachloride (CCl_4)-induced liver injury in male BALB/cN mice. Oleuropein in doses of 100 and 200 mg/kg was administered intraperitoneally (ip) once daily for 3 consecutive days, prior to CCl_4 administration (the preventive treatment), or once daily for 2 consecutive days 6 h after CCl_4 intoxication (the curative treatment). CCl_4 intoxication resulted in a massive hepatic necrosis and increased plasma transaminases. Liver injury was associated with oxidative/nitrosative stress evidenced by increased nitrotyrosine formation as well as a significant decrease in superoxide dismutase activity and glutathione levels. CCl_4 administration triggered inflammatory response in mice livers by inducing expression of nuclear factor-kappaB, which coincided with the induction of tumor necrosis factor-alpha, cyclooxygenase-2 and inducible nitric oxide synthase. In both treatment protocols, oleuropein significantly attenuated oxidative/nitrosative stress and inflammatory response and improved histological and plasma markers of liver damage. Additionally, in the curative regimen, oleuropein prevented tumor necrosis factor-beta1-mediated activation of hepatic stellate cells, as well as the activation of caspase-3. The hepatoprotective activity of oleuropein was, at least in part, achieved through the NF-E2-related factor 2-mediated induction of heme oxygenase-1. The present study demonstrates antioxidant, anti-inflammatory, antiapoptotic, and antifibrotic activity of oleuropein, with more pronounced therapeutic than prophylactic effects.

1. Introduction

In the traditional Mediterranean diet, olives are one of the main nutrients, with olive oil as the principle source of fat. The Mediterranean diet has been linked to the lower incidence of cardiovascular disease, cancer, and other chronic conditions [1–3]. Although many beneficial effects of olive oil have been attributed to a high proportion of monounsaturated fatty acids, the polar, minor components, mostly phenolics present in olive oil, also significantly contribute to its health-protective effects [4]. Oleuropein is one of the most abundant phenolics in olives, which constitutes up to 14% of the fruit's dry weight [5]. This non-toxic compound possesses a considerable antioxidant activity as well as a broad spectrum of other pharmacological effects, including anti-inflammatory, anti-cancer, antimicrobial, antiviral, anti-atherogenic, hypolipidemic and

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BSA, bovine serum albumin; CCl_4, carbon tetrachloride; COX-2, cyclooxygenase; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; GR, glutathione reductase; GSH, glutathione; HSCs, hepatic stellate cells; HO-1, heme oxygenase; iNOS, inducible nitric oxide synthase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-kappaB; Nrf2, NF-E2-related factor 2; 3-NT, 3-nitrotyrosine; PBS, phosphate buffer saline; RIPA, radioimmunoprecipitation assay; Cu/Zn SOD, Cu/Zn superoxide dismutase; TGF-β1, tumor growth factor-beta1; TNF-α, tumor necrosis factor-alpha.

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doi:10.1016/j.phrs.2011.12.005

hypoglycemic activity [6]. Recently, Kim et al. [7] and Park et al. [8] demonstrated hepatoprotective activity of oleuropein against non-alcoholic fatty liver disease and hepatic steatosis induced by a high fat diet. Oleuropein down-regulated the expression of numerous genes involved in hepatic lipogenesis, oxidative stress, and proinflammatory response. Both oleuropein and its main metabolite, hydroxytyrosol, showed the ability to inhibit lipid peroxidation in rat microsomes [9].

Carbon tetrachloride (CCl_4) is a strong hepatotoxin that induces excessive production of free radicals and oxidative stress [10]. Natural phenolics could ameliorate oxidative stress-mediated liver damage, thus preventing hepatic failure. Therefore, we used a mouse model of liver damage induced by CCl_4 to study the protective and therapeutic effects of the olive polyphenol oleuropein.

2. Materials and methods

2.1. Chemicals and antibodies

Oleuropein was purchased from Extrasynthese (Genay Cedex, France). Corn oil, bovine serum albumin (BSA), bovine Cu/Zn superoxide dismutase (Cu/Zn SOD), xanthine, xanthine oxidase, cytochrome c, ethylenediaminetetraacetic acid (EDTA), glutathione (GSH), glutathione reductase (GR), reduced nicotinamide adenine dinucleotide phosphate (NADPH), metaphosphoric acid, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and Entelan were purchased from Sigma–Aldrich (Taufkirchen, Germany). Carbon tetrachloride (CCl_4) was obtained from Kemika, Zagreb, Croatia. Diagnostic kits for the plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were from Dijagnostika (Sisak, Croatia). 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) p65 (ab7970), cyclooxygenase-2 (COX-2) (ab15191), iNOS (ab3523), NF-E2-related factor 2 (Nrf2) (ab31163), transforming growth factor-beta1 (TGF-β1), and HO-1 (ab13243) 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), and ortho CP-G Plus film from Agfa-Gevaert N.V. (Motsel, Belgium). All other chemicals were of the highest grade available.

2.2. Animals

Male BALB/cN mice, 3 months old, weight 23–25 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 approved by the Ethical Committee of the Medical Faculty, University of Rijeka.

3. Experimental design

Mice were divided into 8 groups of 5 animals each. Fig. 1 shows the experimental design scheme for both preventive and curative

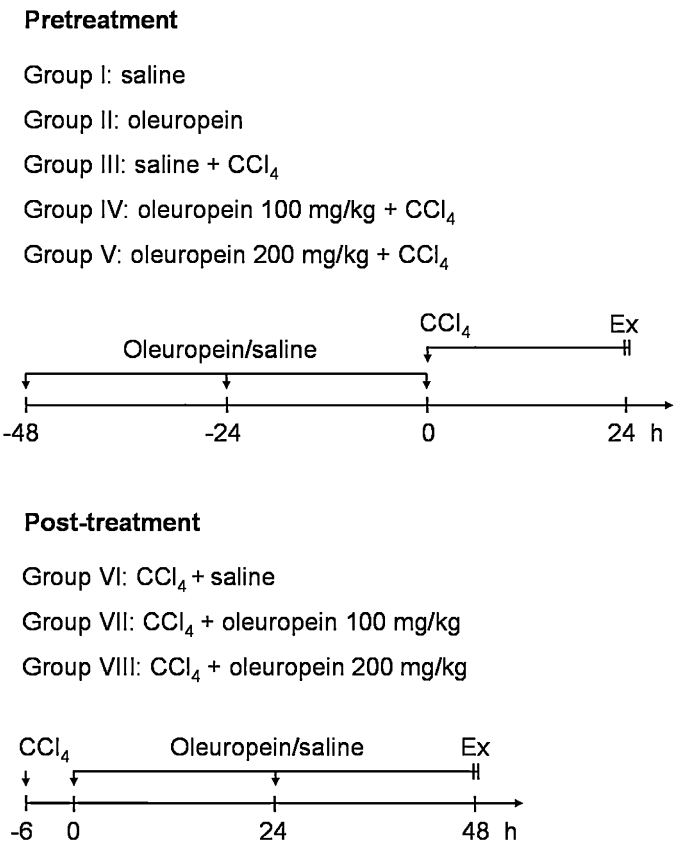

Fig. 1. The experimental design scheme.

treatments. The control group (group I) received saline and group II received oleuropein 200 mg/kg once daily for 3 days. Group III was administered CCl_4 dissolved in corn oil (2 ml/kg, 10% v/v), intraperitoneally (ip). Oleuropein, dissolved in saline and stabilized with DMSO (1% v/v), was given ip at doses of 100 and 200 mg/kg (groups IV and V, respectively) once daily for 3 consecutive days, with the last dose administered half an hour prior to CCl_4 administration (the preventive treatment). The doses used were selected on the basis of preliminary studies (data not shown). Groups I and II received the vehicle. In the curative treatment, group VI received CCl_4 dissolved in corn oil (2 ml/kg, 10% v/v). Two other groups received CCl_4 and then were treated with oleuropein 100 and 200 mg/kg, ip (groups VII and VIII, respectively), 6 h after CCl_4 intoxication, once daily for 2 consecutive days. Twenty-four hours after injection of CCl_4 or vehicle (the preventive treatment) or the last dose of oleuropein (the curative treatment), mice were sacrificed. Blood was collected by cardiac puncture and heparinized plasma was separated for determination of ALT and AST activities. 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% phosphate buffered formalin solution for histology and immunohistochemistry.

Additionally, we performed a time-course study in order to establish the relevance for HO-1 in oleuropein-mediated hepatoprotection. Mice were divided into 8 groups of 5 mice each and treated with CCl_4 alone or with CCl_4 and oleuropein 200 mg/kg, half an hour before intoxication, and sacrificed 3, 6, 12, and 24 h later. Blood and liver samples were collected for the measurement of the plasma AST and ALT activities and the liver expression of Nfr2, HO-1, and 3-NT.