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Nephroprotective activities of rosmarinic acid against cisplatin-induced kidney injury in mice

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ABSTRACT

Rosmarinic acid (RA) is a natural phenolic compound with a broad range of applications, from food preservatives to cosmetics. Increasing amounts of evidence suggests its beneficial effects against various pathological conditions. The aim of this study was to investigate the therapeutic activity of rosmarinic acid (RA) against cisplatin (CP)-induced nephrotoxicity. RA was administered by oral gavage at doses of 1, 2 and 5 mg/kg for two successive days, 48 h after intraperitoneal CP injection (13 mg/kg). Twenty four hours later, mice were sacrificed. Treatment with RA significantly ameliorated histopathological changes and the increase in serum creatinine and blood urea nitrogen (BUN) induced by CP. Oxidative stress induced by CP, evidenced by increased renal 4-hydroxynonenal (4-HNE), cytochrome P450 2E1 (CYP2E1) and heme oxygenase (HO-1) expression, was significantly reduced by RA administration. Moreover, RA inhibited the expression of nuclear factor-kappaB (NF-κB) and tumor necrosis factor-α (TNF-α), indicating the inhibition of inflammation. Additionally, RA exhibited antiapoptotic activity through the reduction of p53, phosphorylated p53 and active caspase-3 expression in the kidneys. These findings show that RA ameliorates CP-induced oxidative stress, inflammation and apoptosis in the kidneys. The nephroprotective activity of RA could be, at least in part, attributed to reduced CYP2E1 expression.

1. Introduction

Cisplatin (cis-diamminedichloroplatinum(II), CP) is an antitumor drug commonly used in the treatment of testicular, ovarian, bladder, cervical, esophageal, head and neck and small cell lung cancer (Giaccone, 2009). Unfortunately, numerous side effects are related to CP therapy, including ototoxicity, gastrointestinal toxicity, myelosuppression, neurotoxicity and kidney injury (Miller et al., 2010; Hartmann and Lipp, 2003). Kidneys represent the main route of CP excretion, with proximal tubule cells as a primary site of CP accumulation (Yao et al., 2007). Thus, nephrotoxicity is one of the most serious dose-limiting side effects in CP chemotherapy. The major mechanisms of CP-induced nephrotoxicity include tubular necrosis, oxidative stress, inflammation and apoptosis (Miller et al., 2010).

Natural phenolics, such as hesperidin, rutin, silymarin and ganistein, were shown to ameliorate CP-mediated nephrotoxicity (Sahu et al., 2013; Kang et al., 2011; Ninsontia et al., 2011; Sung et al., 2008). Moreover, quercetin prevented the nephrotoxic activity of CP without affecting its anti-tumor activity (Sanchez-Gonzalez et al., 2011). These findings indicate that natural phenolic compounds could be utilized as a nephroprotective agents against CP-induced kidney injury.

Rosmarinic acid (RA), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, is a widely occurring natural product with a broad range of applications, from food preservatives to cosmetics (Petersen and Simmonds, 2003). It possesses numerous biological activities, including antioxidative (Zhang et al., 2010), anti-inflammatory (Chu et al., 2012), antiapoptotic (Lee et al., 2008), antitumor (Venkatachalam et al., 2013), antialergic (Costa et al., 2012), antibacterial (Moreno et al., 2006) and antiviral (Swarup et al., 2007). The antioxidant activity of RA may be attributed to its phenolic structure. Phenolic compounds can easily donate electrons or hydrogen atoms to neutralize free radicals, whereas resulting phenoxyl radicals could be enzymatically recycled to parent phenolic (Sakihama et al., 2002). The antioxidant capacity of phenolics seems greatly dependent on the number and even more the position of hydroxyl groups. Hydroxyl groups in the *ortho* position of the aromatic ring, such as in RA, can greatly enhance the

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antioxidant capacity (Sroka and Cisowski, 2003). RA and luteolin with four phenolic hydroxyl groups, including one catechol structure, showed similar lipid peroxidation inhibition and free radical scavenging activity *in vitro* (Özgen et al., 2011). Antioxidant activity of RA makes this compound a good drug candidate for treatment of oxidative stress-related pathological conditions. Previously, we showed that RA and luteolin ameliorated acute liver damage in mice through the suppression of oxidative stress, inflammation and fibrogenesis (Domitrović et al., 2009, 2013). Recently, Tavafi and Ahmadvand (2011) demonstrated that RA inhibits gentamicin-induced renal oxidative damage in rats. However, the nephroprotective activity of RA against CP-induced kidney injury has not been studied previously.

In the current study, we investigated the therapeutic activity of RA against oxidative stress, inflammation and apoptosis induced by administration of CP as a possible mechanisms of the nephroprotective activity of RA.

2. Materials and methods

2.1. Chemicals

Rosmarinic acid (96%), cis-diamineplatinum(II) dichloride and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (Steinheim, Germany). Diagnostic kits for blood urea nitrogen (BUN) and creatinine were from Dijagnostika (Sisak, Croatia). Radioimmunoprecipitation assay (RIPA) buffer (sc-24948) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinylidene difluoride (PVDF) membrane and blocking reagent were obtained from Roche Diagnostics GmbH (Mannheim, Germany). Mouse monoclonal antibodies to tumor necrosis factor-alpha (TNF-α) (ab1793) and p53 (ab26), and rabbit polyclonal antibodies to nuclear factor-kappa B (NF-κB) p65 (ab7970), heme oxygenase-1 (HO-1) (ab13243), 4-hydroxynonenal (4-HNE) (ab46545) and cytochrome P450 2E1 (CYP2E1) (ab19140), HRP-conjugated anti-mouse IgG (ab97023) and HRP-conjugated anti-rabbit IgG (ab97051) for Western blotting were purchased from Abcam (Cambridge, UK). Rabbit polyclonal antibodies to cleaved caspase-3 (Asp175, #9661) and phospho-p53 (p-p53) (Ser15, #9284) were from Cell Signaling Technology (Danvers, MA, USA). DAKO EnVision + System, Peroxidase/DAB kit with secondary antimouse/antirabbit antibodies (K500711) were from DAKO Corporation (Carpinteria, CA, USA). Enhanced chemiluminescence (ECL) substrate was purchased from Pierce Chemical Co. (Rockford, IL, USA). Anesthetic and analgesic (Narketan 10 and Xylapan, respectively) were purchased from Vetoquinol (Bern, Switzerland). All other chemicals were of the highest grade commercially available.

2.2. Animals

Male BALB/cN mice from our breeding colony, 14 week old, weighting 24-28 g, were divided into 6 groups with 5 animals per group. Mice were fed a standard rodent diet (pellet, type 4RF21 GLP, Mucedola, Italy), and water *ad libitum*. The animals were maintained at 12 h light/dark cycle, at constant temperature (20 ± 1 °C) and humidity (50 ± 5%). All experimental procedures were performed in compliance with the Declaration of Helsinki and approved by the Ethical Committee of the Medical Faculty, University of Rijeka.

2.3. Experimental design

Group I (control group) received DMSO diluted with saline (5% DMSO, v/v) by oral gavage. Group II was treated with RA (5 mg/kg) dissolved in the vehicle. Group III received CP (13 mg/kg) dissolved in 5% DMSO (v/v) immediately before its administration as a single intraperitoneal (*ip*) injection. Groups IV, V and VI were treated with RA solution orally by gavage at doses of 1, 2 and 5 mg/kg, respectively, for two consecutive days, two days after CP, whereas groups I, II and III received the vehicle only. We minimized a possible effect of DMSO on cisplatin by reducing its content. Additionally, controls received the same amount of DMSO as the CP- and CP + RA-treated animals. RA or vehicle were given to mice after *ip* administration of the combination of anesthetic and analgesic. We performed a preliminary investigation using RA in a dose range of 1–30 mg/kg with small groups of animals to establish the suitable doses for usage in the main study. Several studies (Brahmi et al., 2012; Sahu et al., 2011; Vijayan et al., 2007) demonstrated that pre-treatment of animals with natural antioxidants was similarly or less renoprotective than post-treatment. Since kidney damage develops gradually, we administered RA on day 2 and 3 following CP injection, targeting ongoing kidney injury. Twenty-four hours after the last dose of RA or vehicle, mice were sacrificed. Previously, blood was collected from retro-orbital sinus and serum was separated to determine serum creatinine and blood urea nitrogen (BUN) concentration. The abdomen was open and kidneys were removed. One kidney was frozen are later used for Western blotting and other was immersed in buffered 4% paraformaldehyde solution for histological sections.

2.4. Serum markers of kidney damage

The level of serum markers of kidney function, BUN and creatinine, was measured by using a Bio-Tek EL808 Ultra Microplate Reader (BioTek Instruments, Winooski, VT, USA) according to manufacturer's instructions.

2.5. Histopathology

Paraformaldehyde-fixed tissues were processed as described previously (Domitrović et al., 2012). Histopathological changes in the kidneys were evaluated in 4 μm thick deparaffinized sections stained by hematoxylin and eosin (HE). Tubular damage was assessed by scoring tubular dilatation, necrosis, apoptosis and cast formation in 10 different fields (Olympus BX51 microscope, Tokyo, Japan, 400× original magnification) in the corticomedullary junction of the kidneys (Leemans et al., 2005). Histopathological changes were blindly scored by a pathologist on a 5-point scale: 0 = no damage, 1 = 10% of the corticomedullary junction injured, 2 = 10–25%, 3 = 25–50%, 4 = 50–75%, 5 = more than 75%.

2.6. Immunohistochemistry

Immunohistochemical analysis on deparaffinized tissues sections was performed using the primary antibodies against 4-HNE (1:1000), NF-κB p65 (1:1000), and cleaved caspase-3 (1:200), the secondary antimouse/antirabbit antibodies and the DAKO EnVision kit, as described previously (Domitrović et al., 2011). Stained slides were analyzed by light microscopy (Olympus BX51, Tokyo, Japan).

2.7. Western blot

Kidneys were lysed in RIPA buffer as described previously (Domitrović et al., 2011). Volume equivalents of 50 μg of proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis for 2.5 h at 4 °C under reducing and denaturing conditions. Gels were blotted onto the PVDF membrane for 45 min at room temperature, incubated in 1% blocking reagent for 1 h at 37 °C and incubated with the primary antibodies against CYP2E1 (1:5000), HO-1 (1:2000), TNF-α (1:1000), p53 (1:1000) and p-p53 (1:1000) overnight at 4 °C. The membranes were washed in Tris-buffered saline plus Tween 20 (TBST) and incubated with the secondary antibodies for 1 h at 37 °C. Finally, the membranes were exposed to ECL substrate, bands were detected and scanned (Allianze 4.0, Cambridge, UK). The intensity of the bands was assayed by computer image analysis software (NIH Image J software, available at http://rsb.info.nih.gov/ij).

2.8. Statistical analysis

Data were analyzed using StatSoft STATISTICA version 12.0 software by Kruskal-Wallis test and post hoc comparisons were carried out with Dunn's multiple comparison test. Results of multiple comparisons tests were indicated by different letters. Means with letters in common are not significantly different from each other. Values in the text are means ± standard deviation (SD). Differences with $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Kidney weight and serum markers of kidney damage

Both body weight and relative kidney weight of control and RA-treated mice were similar (Table 1). CP-intoxication resulted in a significant body weight reduction and increased relative kidney weight. These changes were significantly ameliorated by RA. The serum creatinine and BUN levels were significantly higher in CP-treated animals when compared to control mice. Treatment with RA significantly decreased the creatinine and BUN levels in a dose-dependent manner.

3.2. Kidney histopathology

Normal tubular morphology was observed in cortical and medullary regions of kidney in control mice (Fig. 1A) and mice treated with RA (Fig. 1B). CP administration resulted in