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Rosmarinic acid attenuates hepatic ischemia and reperfusion injury in rats

Leandra Naira Z. Ramalho [a], Ângelo Augusto C. Pasta [a], Vânia Aparecida Terra [b], Marlei Josiele Augusto [a], Sheila Cristina Sanches [a], Fernando P. Souza-Neto [c], Rubens Cecchini [c], Francine Gulin [a], Fernando Silva Ramalho [a,*]

[a] Department of Pathology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil
[b] Pontifical Catholic University of Paraná, Londrina, PR, Brazil
[c] Department of Pathological Sciences, State University of Londrina, Londrina, PR, Brazil

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ABSTRACT

Rosmarinic acid (RosmA) demonstrates antioxidant and anti-inflammatory properties. We investigated the effect of RosmA on liver ischemia/reperfusion injury. Rats were submitted to 60 min of ischemia plus saline or RosmA treatment (150 mg/kg BW intraperitoneally) followed by 6 h of reperfusion. Hepatocellular injury was evaluated according to aminotransferase activity and histological damage. Hepatic neutrophil accumulation was also evaluated. Oxidative/nitrosative stress was estimated by measuring the reduced glutathione, lipid hydroperoxide and nitrotyrosine levels. Endothelial and inducible nitric oxide synthase (eNOS/iNOS) and nitric oxide (NO) were assessed with immunoblotting and chemiluminescence assays. Hepatic tumor necrosis factor-alpha (TNF-α) and interleukin-1beta mRNA were assessed using real-time PCR, and nuclear factor-kappaB (NF-κB) activation was estimated by immunostaining. RosmA treatment reduced hepatocellular damage, neutrophil infiltration and all oxidative/nitrosative stress parameters. RosmA decreased the liver content of eNOS/iNOS and NO, attenuated NF-κB activation, and down-regulated TNF-α and interleukin-1beta gene expression. These data indicate that RosmA exerts anti-inflammatory and antioxidant effects in the ischemic liver, thereby protecting hepatocytes against ischemia/reperfusion injury. The mechanisms underlying these effects may be related to the inhibitory potential of RosmA on the NF-κB signaling pathway and the reduction of iNOS and eNOS expressions and NO levels, in addition to its natural antioxidant capability.

1. Introduction

Liver damage following ischemia and reperfusion (I/R) represents an inevitable outcome for a number of surgical procedures involving the liver, such as the resection of large intrahepatic tumors, extensive hepatic trauma surgery and liver transplantation. I/R injury is a biphasic phenomenon whereby cellular damage due to hypoxia is accentuated upon restoration of the blood supply and oxygen delivery. A complex network of hepatic and extrahepatic events is involved in the pathophysiology of liver I/R injury. Identified as a major mechanism of injury during the reperfusion phase, an excessive acute inflammatory response occurs within the liver parenchyma promptly after blood flow restoration. The activation of Kupffer cells (KC) and sinusoidal endothelial cells (SEC), CD4 + T lymphocyte and neutrophil recruitment to the hepatic interstitium, and the subsequent generation of reactive oxygen and nitrogen species (ROS/RNS) and cytokines have been identified as the most relevant events in the pathogenesis of this syndrome. In addition to directly inducing cellular destruction by massive membrane lipoperoxidation, ROS can affect the pathophysiology indirectly by supporting protease activity through the inactivation of antiproteases and by acting as second messengers in signal transduction pathways involved in the regulation of genes encoding adhesion molecules and proinflammatory mediators (Jaeschke, 2000, 2003; Peralta et al., 2013). Nuclear factor-kappaB (NF-κB), a member of the c-Rel oncogene family of transcription factors, has been recognized as a key transcription factor that is activated during reperfusion following an ischemic event in diverse organs. NF-κB is located in the cytoplasm and consists predominantly of a p50 and p65 heterodimer associated with regulatory proteins called inhibitors of κB (IκB). p50 serves mainly as a DNA-binding subunit, whereas p65 is the transcriptionally active member of the complex. Cellular or extracellular activating stimuli result in the phosphorylation and proteasomal degradation of IκB, leading to its dissociation from

* Corresponding author. Department of Pathology, School of Medicine of Ribeirão Preto, University of São Paulo, Av. Bandeirantes, 3900, Monte Alegre, 14049-900, Ribeirão Preto, SP, Brazil. Tel.: +55 16 3602 3122; fax: +55 16 3602 0224.
E-mail address: framalho@fmrp.usp.br (F.S. Ramalho).

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NF-κB. Free NF-κB translocates to the nucleus, binds to specific DNA sites in the promoter regions of numerous pro-inflammatory genes, and activates their transcription (Fan et al., 1999).

Rosmarinic acid (RosmA), an ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid, is a polyphenolic compound with potential use at the nutritional/pharmaceutical interface, which is commonly found in various plants from the Lamiaceae (the mint) family, such as *Perilla frutescens, Rosmarinus officinalis, Coleus blumei, Salvia officinalis, Prunella vulgaris, Ocimum basilicum* and several other medicinal plants, herbs and spices (Bulgakov et al., 2012; Petersen and Simmonds, 2003). RosmA possesses anti-inflammatory (Chu et al., 2012; Jiang et al., 2009; Ku et al., 2013), antioxidant (Domitrović et al., 2013; Gao et al., 2005; Zdarilová et al., 2009), anticancer (Moon et al., 2010; Scheckel et al., 2008; Xu et al., 2010), and antimicrobial (Jordán et al., 2012; Suriyarak et al., 2013) activities. Moreover, RosmA has been reported to have neuroprotective effects on the rat brain after acute ischemic damage. In an experimental model with diabetic rats subjected to cerebral I/R injury, RosmA attenuated blood–brain barrier breakdown, decreased the infarct volume, down-regulated high-mobility group box-1 expression and inhibited NF-κB activation in ischemic brain tissue (Luan et al., 2013). Thus, we hypothesized that RosmA could also protect the liver from I/R damage. To test this hypothesis, we investigated the effect of RosmA in a normothermic I/R model in the rat liver.

2. Materials and methods

2.1. Experimental animals

Male Wistar rats weighing 150 to 200 g were obtained from the Central Animal House of the University of São Paulo (Ribeirão Preto, Brazil). The rats were housed at 23 ± 2°C with a 12 h light/dark cycle and were fed *ad libitum* with standard chow. This experimental protocol was approved by the Animal Research Ethical Committee of the School of Medicine of Ribeirão Preto-USP (process n° 177/2009).

2.2. Surgical procedure

After anesthesia was induced with ketamine [80 mg/kg body weight (BW)] and xylazine (10 mg/kg BW), a median laparotomy was performed and the blood supply to the left lateral and median lobes of the liver was interrupted for 60 min by placing a microvascular clamp across the portal triad to these lobes. This model results in a partial (70%) hepatic ischemia and prevents mesenteric venous congestion during the clamping period by preserving the portal flow through the right lateral and caudate lobes. At the end of the ischemia period, reperfusion was initiated by releasing the clamp, and the abdominal wound was closed in layers with running 4-0 silk suture (Mendes-Braz et al., 2012). The rats were re-anesthetized with ketamine/xylazine 6 hours after reperfusion for sample harvesting.

2.3. Experimental design

RosmA (96% pure, Sigma Chemical Co., St. Louis, MO, U.S.A.) was diluted (10 mg/mL) in saline solution with 1% ethanol (v/v) and administered in doses of 5 mL/kg BW (Osakabe et al., 2002). A preliminary pilot study was performed in Wistar rats with different doses of RosmA to determine the RosmA dose with the maximum tolerability and efficacy.

The animals were randomly divided into three experimental groups (n = 6–8 per group) as follows: rats subjected to the surgical procedure described above and administered RosmA (50 mg/kg BW) intraperitoneally 30 min before ischemia, 30 min after ischemia and 2 hours after reperfusion (I/R RA group); rats subjected to the surgical procedure and administered a saline solution with 1% ethanol (v/v) (I/R group) in the same manner as the I/R RA group; and sham-operated rats (SHAM group).

At the time of sacrifice (6 h after reperfusion), venous blood samples were collected under anesthesia, and the serum was stored at 80C. Liver tissue specimens from the median and left lateral lobes were harvested and frozen at 80°C or fixed in 4% buffered formalin and embedded in paraffin blocks.

2.4. Measurement of serum aminotransferases

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), biochemical indicators of hepatocellular damage, were measured spectrophotometrically at an absorbance of 340 nm in serum samples using a commercial kit according to the manufacturer's instructions (Labtest, Lagoa Santa, Brazil). The results are expressed in units per liter (U/L).

2.5. Histological assessment of liver injury

Formalin-fixed and paraffin-embedded tissue sections were cut to a thickness of 5 μm and stained with hematoxylin and eosin for histological examination. Analysis of the degree of tissue damage was performed by a pathologist in a blinded manner on 30 randomly chosen high-power fields (HPFs; 400× magnification) on each slide. The severity of hepatic parenchymal injury, termed the necroinflammatory score, was graded as follows: grade 0, minimal or no evidence of injury; grade 1, mild injury consisting of cytoplasmic vacuolization and focal nuclear pyknosis; grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders; and grade 3, severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration (Serafin et al., 2002).

2.6. Histological evaluation of hepatic neutrophil infiltration

Infiltration of neutrophils into the liver was estimated using the naphthol AS-D chloroacetate esterase staining method, which identifies specific leukocyte esterases (Moloney et al., 1960). Briefly, the 5-μm paraffin sections were deparaffinized with xylene, rehydrated through an alcohol series, and then immersed in distilled water before being incubated in naphthol esterase solution at room temperature for 15 min. Naphthol esterase solution contains naphthol AS-D chloroacetate (Sigma Chemical Co.) in *N,N*-dimethyl formamide (2 mg/mL), 4% sodium nitrite, and 4% New Fuchsin in 2 N HCl combined in 0.1 M phosphate buffer (pH 7.6). Next, the sections were rinsed with tap water and counterstained with Gill's hematoxylin for 15 s. Red color was deposited only in neutrophils and mast cells. The identification of the stained neutrophils was made based on nuclear morphology and red small granular deposits scattered within the cytoplasm. Only polymorphonuclear cells (PMN) located within sinusoids or extravasated into the surrounding parenchyma and characterized by a multilobed nucleus and red granular deposits within the cytoplasm were counted. The number of esterase-positive PMN was counted in 30 HPFs (400× magnification) in each sample, and the mean values were calculated.

2.7. Assessment of neutrophil infiltration by hepatic myeloperoxidase activity

The extent of neutrophil accumulation in liver tissue was also measured using a myeloperoxidase (MPO) activity assay as previously described (Ivey et al., 1995). Briefly, liver samples (100 mg tissue per 2 mL buffer) were homogenized in buffer (100 mM NaCl, 20 mM NaH2PO4, 15 mM NaEDTA, pH 4.7) and centrifuged at 3000 rpm for 10 min. The pellet then underwent hypotonic lysis (5 mL 0.2% NaCl for 30 s followed by the addition of an equal volume of solution containing 1.6% NaCl and 5% glucose). After further centrifugation, the pellet was resuspended in 0.05 M NaH_2PO_4 buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB; Sigma Chemical Co.). One-milliliter aliquots of the supernatant were transferred into 1.5-mL tubes followed by three freezethaw cycles using liquid nitrogen. The samples were centrifuged for 12 min at 4000 rpm, and the supernatants were used for MPO measurements. MPO activity was assayed by measuring the change in optical density at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2, and the results are expressed in units per milligram of protein (U/mg protein).

2.8. Hepatic reduced glutathione measurements

To measure the levels of reduced glutathione (GSH), liver samples were homogenized in 1.1% KCl, and proteins were precipitated with 1 N perchloric acid. After centrifugation, the samples were neutralized with 10% K_2CO_3. GSH was measured using glutathione transferase and 1-chloro-2,4-dinitrobenzene. A 50-mL aliquot of the sample was mixed with 225 mL of 0.1 M potassium phosphate buffer, pH 7.0 and 10 mL of 10 mM 1-chloro-2,4-dinitrobenzene in ethanol. The reaction was started with 5 mL of glutathione transferase solution (12 U/L) and monitored at 340–400 nm, reaching the endpoint 5 min after enzyme addition (Brigelius et al., 1983). The results are expressed in micromoles per milligram of protein (μmol/mg protein).

2.9. Lipid peroxidation measurement by tert-butyl hydroperoxide-initiated chemiluminescence

Rat liver homogenates were obtained using an Ultraturrax homogenizer containing 10 mg/mL of tissue in 30 mM KH_2PO_4/K_2HPO_4 buffer and 120 mM KCl at pH 7.4. Reaction mixtures were placed in luminescence tubes containing 3% (w/v) of liver supernatant and 3 mM *tert*-butyl hydroperoxide, in a final volume of 1 mL. The *tert*-butyl hydroperoxide-initiated chemiluminescence reaction was measured in a GLOMAX TD/20 20 luminometer (Turner Designs, U.S.A.), with a response range of 300–650 nm. The tubes were kept in the dark until assayed at 28°C (Gonzalez-Flecha et al., 1991; Peres et al., 2011). For each rat liver, a 45-min curve, where each point represented the differential smoothing of 45 readings, was obtained by interpolation. Peak-height (PH) and area under the curve (AUC) were extracted by integral calculus from each rat curve and were used to determine the lipid hydroperoxides present in the sample. The results are expressed in relative light units per gram of liver tissue (RLU/g liver). Additionally, to confirm the assay sensitivity to lipid