Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Resveratrol improves intrahepatic endothelial dysfunction and reduces hepatic fibrosis and portal pressure in cirrhotic rats

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Background & Aims: Resveratrol, a polyphenol found in a variety of fruits, exerts a wide range of beneficial effects on the endothelium, regulates multiple vasoactive substances and decreases oxidative stress, factors involved in the pathophysiology of portal hypertension. Our study aimed at evaluating the effects of resveratrol on hepatic and systemic hemodynamics, hepatic endothelial dysfunction, and hepatic fibrosis in CCl_4 cirrhotic rats.

Methods: Resveratrol (10 and 20 mg/kg/day) or its vehicle was administered to cirrhotic rats for two weeks and hepatic and systemic hemodynamics were measured. Moreover, we evaluated endothelial function by dose-relaxation curves to acetylcholine, hepatic NO bioavailability and TXA_2 production. We also evaluated liver fibrosis by Sirius Red staining of liver sections, collagen-1, *NFκB*, *TGFβ* mRNA expression, and desmin and α-smooth muscle actin (α-SMA) protein expression, as a surrogate of hepatic stellate cell activation.

Results: Resveratrol administration significantly decreased portal pressure compared to vehicle (12.1 ± 0.9 *vs.* 14.3 ± 2.2 mmHg; $p < 0.05$) without significant changes in systemic hemodynamics. Reduction in portal pressure was associated with an improved vasodilatory response to acetylcholine, with decreased TXA_2 production, increased endothelial NO, and with a significant reduction in liver fibrosis. The decrease in hepatic fibrosis was associated with a reduced collagen-1, *TGFβ*, *NFκB* mRNA expression and desmin and α-SMA protein expression.

Conclusions: Resveratrol administration reduces portal pressure, hepatic stellate cell activation and liver fibrosis, and improves hepatic endothelial dysfunction in cirrhotic rats, suggesting it may be a useful dietary supplement in the treatment of portal hypertension in patients with cirrhosis.

Introduction

In cirrhosis, the initial factor determining the onset of portal hypertension is the increase in intrahepatic vascular resistance. This is not only due to morphological changes resulting from chronic liver inflammation and fibrosis, but also to reversible functional alterations, including an exaggerated response of the porto-hepatic vascular bed to vasoconstrictors and a deficient response to vasodilators [1]. A decreased nitric oxide (NO) availability and an increase in cyclo-oxygenase-1 (COX-1)-derived prostanoids within the liver play a major role in the pathogenesis of these dynamic alterations [2–5].

Reduced NO availability has been shown to be in part due to an increase scavenging by superoxide (O_2^-) and different strategies aimed at reducing O_2^- levels [6,7], such as superoxide dismutase (*SOD*) gene transfer, are able to reduce portal pressure in experimental models of cirrhosis in the rat.

Resveratrol (3,5,4′-trihydroxystilbene) is a natural polyphenolic flavonoid found in a large amount of plant species, including grapes and their derivatives, berries and nuts. It has been suggested to have important health benefits attributed to its demonstrated anti-oxidant, anti-neoplastic, anti-inflammatory and anti-platelet aggregation activities [8–11]. Specifically, in different experimental models, resveratrol improves vascular dysfunction, an effect that is attributed to its ability to reduce oxidative stress, to upregulate endothelial nitric oxide synthase (eNOS) expression and activity, and to inhibit COX-1 activity [12–15].

Resveratrol has been shown to exert anti-oxidant effects in experimental models of liver injury induced by ischemia/reperfusion and ethanol by inducing the enzymatic activity of SOD and catalase [16,17], and to attenuate fibrosis development when co-administered with CCl_4 to rats [18]. Additionally, resveratrol

Keywords: Oxidative stress; Endothelial dysfunction; Nitric oxide; Thromboxane.
Received 28 June 2012; received in revised form 29 November 2012; accepted 4 December 2012; available online 20 December 2012

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Abbreviations: NO, nitric oxide; COX-1, cyclooxygenase-1; eNOS, endothelial nitric oxide synthase; CCl_4, carbon tetrachloride; MAP, mmHg, mean arterial pressure; PP, mmHg, portal pressure; PBF, ml min^{-1}, portal blood flow; SMABF, ml min^{-1}, superior mesenteric artery blood flow; Mtx, methoxamine; Ach, acetylcholine; TXB_2, thromboxane B_2; O_2^-, superoxide; DHE, dihydroethidium; cGMP, cyclic guanosine monophosphate; P-eNOS, phosphorylated eNOS; α-SMA, α smooth muscle actin.

reduces the hepatotoxicity induced by acetaminophen, ethanol and carbone tetrachloride (CCl_4), and prevents liver damage due to ischemia-reperfusion, irradiation, and high-fat diet [19]. Overall, we hypothesized that resveratrol may exert beneficial effects in the pathophysiological mechanisms involved in the development of portal hypertension in cirrhosis.

Therefore, the aim of the present study was to investigate the effects of chronic administration of resveratrol in CCl_4-cirrhotic rats with portal hypertension.

Materials and methods

Induction of cirrhosis by CCl_4 and resveratrol administration

In male Wistar rats (50–75 g), cirrhosis was induced by inhalation of CCl_4 three times a week, and phenobarbital (0.3 g/L) was added to the drinking water as previously described [6]. When cirrhotic rats had developed ascites, after approximately 12–15 weeks of CCl_4 inhalation, administration of CCl_4 and phenobarbital was discontinued. One week later, the animals were randomized to receive resveratrol (10 mg/kg body weight (bw); Sigma, Tres Cantos, Madrid, Spain) or its vehicle (carboximethylcellulose 0.7%) daily by gavage for two weeks. Resveratrol or its vehicle was prepared and administered by a third person and, therefore, the investigators performing the experiments were not aware of the treatment received by the rats. Experiments were initiated 1 h after the administration of the last dose of resveratrol or vehicle. The dose of 10 mg/kg bw/day of resveratrol has been shown to reduce liver oxidative damage after bile duct ligation [20] and to decrease acute liver damage induced by acute CCl_4 intoxication [21]. To evaluate a possible dose-dependent effect of resveratrol, an additional group of rats were treated with resveratrol at 20 mg/kg bw/day (n = 10) or its corresponding vehicle (n = 8). The animals were kept in environmentally controlled animal facilities at the Institut d'Investigacions Biomèdiques August Pi y Sunyer (IDIBAPS). All procedures were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance with European Community guidelines for the protection of animals used for experimental and other scientific purposes (EEC Directive 86/609).

In vivo hemodynamic studies

Rats were anesthetised with ketamine hydrochloride (100 mg/kg; Merial Laboratories, Barcelona, Spain) plus midazolam (5 mg/kg; Laboratorios Reig Jofré, Barcelona, Spain) intraperitoneally. A tracheostomy was performed and a polyethylene tube PE-240 was inserted into the trachea to ensure a patent airway. PE-50 catheters were introduced into the femoral artery to measure mean arterial pressure (MAP; mmHg) and into the ileocolic vein to measure portal pressure (PP, mmHg). Perivascular ultrasonic flow probes connected to a flow meter (Transonic Systems Inc., Ithaca, NY, USA) were placed around the portal vein, as close as possible to the liver to avoid portal-collateral blood flow, in order to measure portal blood flow (PBF; ml min^{-1}) going through the liver, and at the superior mesenteric artery to measure superior mesenteric artery blood flow (SMABF; ml min^{-1}). Blood pressures and flows were registered on a multichannel computer-based recorder (PowerLab; AD Instruments, Colorado Springs, CO). The temperature of the animals was maintained at 37 ± 0.5 °C and hemodynamic data were collected after a 20 min stabilization period.

Evaluation of endothelial function

After *in vivo* hemodynamic measurements, livers were quickly isolated and perfused by a flow-controlled perfusion system as previously described [22]. The perfused rat liver preparation was allowed to stabilize for 20 min before vasoactive substances were added. The intrahepatic microcirculation was preconstricted by adding the α_1-adrenergic agonist methoxamine (Mtx; 10^{-4} mol/L; Sigma) to the reservoir. After 5 min, concentration–response curves to cumulative doses of acetylcholine (Ach; 10^{-7}, 10^{-6}, and 10^{-5} mol/L; Sigma) were evaluated. The concentration of Ach was increased by 1log unit every 1.5 min interval. Responses to Ach were calculated as the percentage change in portal perfusion pressure [5]. The gross appearance of the liver, stable perfusion pressure, bile production over 0.4 µl/min/g of liver and a stable buffer pH (7.4 ± 0.3) were monitored during this period. If any viability or stability criteria were not satisfied, the experiment was discarded.

Effects of resveratrol on oxidative stress and hepatic SOD activity

Measurement of O_2^- content.
To evaluate if resveratrol is able to reduce intrahepatic O_2^- levels, livers from cirrhotic rats treated with resveratrol (n = 2) or vehicle (n = 2) were promptly removed after the hemodynamic measurements and *in situ* O_2^- content was evaluated in fresh liver cryosections (10 µm) stained with the oxidative fluorescent dye dihydroethidium (DHE) (Molecular Probes, Eugene, Oregon USA), as previously described [23,24].

SOD activity.
Total SOD activity was measured in liver homogenates obtained from CCl_4-cirrhotic rats treated with resveratrol or vehicle (n = 9 per group), using a commercially available immunoassay (Sigma). The assay is based on the competition reaction between the sample-containing SOD and the highly water-soluble tetrazolium salt (WST) that produces a water-soluble formazan dye upon reaction with O_2^-. Briefly, livers were homogenized in buffer containing 20 mM Hepes, 1 mM EDTA, 210 mM mannitol and 70 mM sucrose. After centrifugation at 1500g for 5 min at 4 °C, the supernatant was collected and protein concentration was quantified. SOD activity assay was performed according to manufacturer's instructions.

Evaluation of NO pathway

Nitric oxide bioavailability.
Measurements of cyclic guanosine monophosphate (cGMP), a marker of NO bioavailability, were performed in liver homogenates from cirrhotic rats treated with resveratrol (n = 8) or vehicle (n = 8) using an enzyme immunoassay (Cayman Chemical Company, Tallin, Estonia), as previously described [7,25].

eNOS and P-eNOS protein expression.
Total eNOS and P-eNOS protein expression was determined by Western blot in liver homogenates from cirrhotic rats treated with resveratrol (n = 4) or vehicle (n = 4) as previously described [7]. Antibodies against phosphorylated eNOS (Ser1176; Cell Signaling Technology, Beverly, MA) and total eNOS (BD Biosciences, San Jose, CA) were incubated for 16 h at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Stressgen, Victoria, BC, Canada) for 1 h at room temperature. Blots were revealed by chemiluminescence. Protein expression was determined by densitometric analysis using Science Lab 2001, Image Gauge (Fuji Photo Film Gmbh, Düsseldorf, Germany). Quantitative densitometry values of eNOS and P-eNOS were normalized to GAPDH.

Measurement of thromboxane A_2

In liver-perfusion experiments, samples of the perfusate were obtained before Mtx administration and after the dose–response to Ach. The samples were stored at −80 °C, and thromboxane B_2 (TXB_2), the end metabolite of thromboxane A_2 (TXA_2), was quantified in duplicate using a commercially available enzyme immunoassay (Cayman) [26,27]. TXB_2 production was expressed as absolute increment after dose–response curve to Ach over baseline before Mtx administration [28].

Effects of resveratrol on sinusoidal endothelial cells

Isolation of sinusoidal endothelial cells.
Sinusoidal endothelial cells were isolated from control and cirrhotic rats as previously described [24]. Briefly, after collagenase perfusion of the livers and isopycnic sedimentation of the resulting dispersed cells through a two-step density gradient of Percoll, pure monolayer cultures of SEC were established by selective attachment on a substrate of rat tail collagen type I. Afterwards, cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 and studies were performed on cells from the first passage, 12 h after their isolation, to preserve their typical phenotype.

Measurements of NO levels and TXA_2 production in SEC.
To determine whether resveratrol administration could increase NO bioavailability in SEC isolated from five cirrhotic rat livers (CH-SEC), CH-SEC were incubated for 24 h at 37 °C with resveratrol (50 µM) or with vehicle (ethanol). Then, nitric oxide levels were assessed with DAF-FM-DA as previously described [29]. In addition, nitrites/nitrates (NOx) production was assessed in aliquots of SEC supernatants using specific microelectrodes (Lazar Laboratories, Los Angeles, CA) according to manufacturer's instructions.