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Hepatic injury induced by thioacetamide causes aortic endothelial dysfunction by a cyclooxygenase-dependent mechanism

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ABSTRACT

Liver cirrhosis is associated with a wide range of cardiovascular abnormalities including hyperdynamic circulation and cirrhotic cardiomyopathy. The pathogenic mechanisms of these cardiovascular changes are multifactorial and include vascular dysregulations.

Aim: The present study tested the hypothesis that the systemic vascular hyporesponsiveness in thioacetamide (TAA)-induced liver injury model is dependent on nitric oxide (NO) and cyclooxygenase (COX) derivatives.

Main methods: Wistar rats were treated with TAA for eight weeks to induce liver injury.

Key findings: The maximal contractile response in concentration-effect curves to phenylephrine was decreased in aorta from TAA-treated rats, but no differences were found in aorta without endothelium, suggesting an endothelium-dependent mechanism in decreased contractile response. There was no difference in the contractile response with and without L-NAME (N(ω)-nitro-L-arginine methyl ester) in rats with liver injury, showing that the TAA treatment impairs NO synthesis. Pre-incubation of the aorta with indomethacin, a COX-inhibitor, normalized the reduced contractile response to phenylephrine in arteries from TAA group. Also, COX-2 and iNOS (inducible nitric oxide syntase) protein expression was increased in aorta from TAA group compared to control group. Animals submitted to TAA treatment had a reduction in systolic blood pressure. Our findings demonstrated that liver injury induced by TAA caused a decrease in aortic contractile response by a COX-dependent mechanism but not by NO release. Also, it was demonstrated an inflammatory process in the aorta of TAA-treated rats by increased expression of COX-2 and iNOS.

Significance: Therefore, there is an essential contribution of COX-2 activation in extra-hepatic vascular dysfunction and inflammation present in cirrhosis induced by TAA.

1. Introduction

Hepatic cirrhosis is considered a public health problem. The persistent hepatic injury is associated with the development of inflammation, progressive fibrosis, cirrhosis and, finally, cellular hepatocarcinoma [1]. Cirrhosis is a chronic liver disease considered a late stage of hepatic fibrosis. This complication is accompanied by the development of portal hypertension that causes an increase in hepatic vascular resistance to portal blood flow [2,3]. Hepatic cell damage can be caused by several factors, including: viral (hepatitis B and C virus), alcohol, alcoholic steatohepatitis, obesity, non-alcoholic fatty liver disease, and drugs [4].

Several studies have shown an association between hepatic injury and cardiovascular disease development, as reviewed by Fargion et al. [5]. The hepatic injury causes endothelial dysfunction in the hepatic arteries. The main mechanisms involved in this endothelial dysfunction are the lower bioavailability of nitric oxide (NO) and the increase of oxidative stress and thromboxane (TXA_2), which increase vascular contraction. NO is the primary vasodilator, responsible for vascular homeostasis, and its bioavailability is decreased in the cirrhotic frame [6].

Nevertheless, in the splanchnic and systemic circulation of animals and human with liver cirrhosis the opposite occurs, and this condition is associated with hyperdynamic circulation, characterized by increased

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blood flow and reduced vascular resistance [7]. Studies performed in cirrhotic patients and animals revealed that this vasodilation is associated with enhanced formation of vasodilators as well as vascular hyporesponsiveness to vasoconstrictors, vascular remodeling, and increased angiogenesis. The main mechanisms underlying this phenomenon are increased release of NO and dysfunctional prostaglandins secretion [8].

TXA_2 and prostacyclin (PGI_2) are COX (cyclooxygenase) derivatives that have gained relevance in the vascular response in cirrhosis. The administration of COX inhibitors to cirrhotic mice results in decreased hepatic TXA_2 production and increased intrahepatic NO synthesis, attenuating intrahepatic vascular resistance, endothelial dysfunction, and hyperreactivity to vasoconstrictors [9]. However, the effect of COX inhibitors on systemic circulation is unknown. Many studies performed with cirrhotic patients and cirrhotic animals with portal hypertension were aimed at revealing the mechanisms underlying the systemic vasodilation [7,10]. It is clear that systemic vasodilation cannot be mediated by a single mechanism, and these mechanisms could also vary depending on the animal model used to induce cirrhosis.

Liver injury induced by thioacetamide (TAA) is a model recognized for producing liver injuries, regenerative nodules, and fibrosis similar to those of human liver fibrosis. In view of this, the present study tested the hypothesis that the systemic vascular hyporesponsivity induced by liver injury in TAA model is dependent on NO and COX derivatives.

2. Material and methods

2.1. Animals

All animal procedures were performed following the regulations of the National Council on Animal Experimental Control (CONCEA, Brazil), which was approved by the Ethics Committee on the Use of Animals (CEUA, under protocol n° 23108.700353/14-0) of the Federal University of Mato Grosso. Rats were housed at constant room temperature and light cycle (12:12-h light-dark cycle) with free access to standard rat chow and tap water. The animals used were male Wistar rats weighing about 200 g, divided randomly into two groups: Control (C) and TAA-treated rats (TAA). The administered dose of TAA was 100 mg/kg intraperitoneally, twice weekly for 8 weeks [13]. Control rats received vehicle (saline) in the same volume and same administration protocol. After 8 weeks of the experimental period, the animals were euthanized under anesthesia with thiopental (50 mg/kg).

2.2. Systolic blood pressure

After 8 weeks of TAA treatment, the systolic blood pressure was assessed by using the non-invasive tail-cuff method with a Narco BioSystems® Electro-Sphygmomanometer (International Biomedical, Austin, TX, USA). The average of two pressure readings was recorded for each animal.

2.3. Biochemical analysis

Blood samples were collected in Falcon tubes, centrifuged (3000 rpm; 10 min; Eppendorf®Centrifuge 5804-R, Hamburg, Germany) and the serum was used for biochemical analysis. The levels of albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea and creatinine were analyzed using commercial kits (Labtest, Brazil).

2.4. Vascular reactivity of the aorta

Segments of the thoracic aorta (4 mm in length), free of fat and connective tissue, were mounted in an isolated tissue chamber containing Krebs-Henseleit solution (mmol/L: NaCl 113, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.1, KH_2PO_4 1.1, EDTA 0.03, glucose 5.5, pH 7.4), gassed with 95% O_2 and 5% CO_2, and maintained a resting tension of 1.5 g at 37 °C. Isometric tension was recorded using an isometric force transducer, connected to an acquisition system (ML T001, PowerLab/8S, ADInstruments, Ltd.). In some aortic rings the endothelium was kept intact, and in others, the endothelial layer was removed mechanically by friction using a thin rod wrapped with cotton soaked in Krebs Henseleit.

The stabilization procedure was performed by changing the nutrient solution three times at 15 min intervals while the tension was adjusted to 1.5 g. After stabilization, the vessels were stimulated twice with 75 mM KCl, to assess integrity and maximal contractility. Following the wash, vascular reactivity was investigated with cumulative concentration-response curves for the vasoconstrictor agent phenylephrine and the vasodilator acetylcholine (ACh). The effects of Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME – 100 mM), indomethacin (10 mM) and apocynin (30 μM) were investigated by their addition 30 min before phenylephrine curves in vessels with intact endothelium.

2.5. Histology

Tissue samples from the liver and aorta were fixed in 4% buffered formaldehyde for 24 h, and dehydrated in various concentrations of ethanol, then embedded in paraffin and stained with hematoxylin and eosin (HE). The aorta and liver tissue morphology were observed under light microscope. Aorta wall thickness was measured at four different places of aorta. HE-stained liver sections were evaluated for fibrosis (FS) and inflammation (IS) scores, both classified on a scale of 0–3 according to [11,12]. A mean liver injury score was calculated as follows: (FS + IS) / 2.

2.6. Western blotting

Proteins (30 μg) from aorta were extracted and separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered solution (10 mmol/l Tris,100 mmol/l NaCl, and 0.1% Tween 20) for 1 h at room temperature. Membranes were then incubated with primary antibodies overnight at 4 °C. Primary antibodies and the respective dilution used were: anti-COX 1 (1:750), anti-COX-2 (1:1.000), anti-iNOS (1:2.000) and anti-eNOS (endothelial nitric oxide synthase) (1:1500) (all antibodies from Cell Signaling Technology, Beverly, MA, USA). Membranes were washed with Tris-buffered solution and incubated for 1 h at room temperature with the appropriate secondary antibody. After incubation membranes were washed with Tris-buffered solution and signals were revealed with chemiluminescence and quantified using Alpha Imager software (Alpha Innotech, San Leandro, CA). The same membrane was stripped and used to determine β-actin protein expression using a monoclonal antibody against β-actin (1:15.000) (Cell Signaling Technology, Beverly, MA, USA), and its content was used to normalize protein expression in each sample. The intensity of the bands was quantified using the program ImageJ (NIH).

2.7. Statistical analysis

Results are shown as mean ± standard error deviation (SEM) and "n" represents the number of animals used in the experiments. The Student *t*-test was used to compare the results between the groups. Contractile responses are expressed in grams (g) as the maximal force to each agonist concentration. Concentration-response curves were fitted with nonlinear regression using an interactive fitting program (GraphPad Prism 4.0; GraphPad Software Inc.), and two pharmacological parameters were obtained: the maximal effect generated by the agonist (Emax) and pEC_{50} (negative logarithm of EC_{50} value). Statistical analyses of Emax and pEC_{50} values were performed using one-way ANOVA. The confidence level for the tests was 95%.