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Three-day tetrahydrobiopterin therapy increases in vivo hepatic NOS activity and reduces portal pressure in CCl_4 cirrhotic rats☆

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***Background/Aims*: Tetrahydrobiopterin is an essential cofactor for NOS enzymes to synthesize NO. It has been suggested that reduced intrahepatic tetrahydrobiopterin decreases intrahepatic NO and contributes to increase hepatic vascular resistance and portal pressure in cirrhosis. The main aim of the study was to evaluate the effect of tetrahydrobiopterin supplementation in portal pressure in CCl_4 cirrhotic rats.**

***Methods*: Cirrhotic rats received vehicle or tetrahydrobiopterin (10 mg/kg/day i.p.) for 3 days. Hepatic and systemic hemodynamics and hepatic tetrahydrobiopterin, NOS activity and cGMP levels were measured. In addition, hepatic and systemic hemodynamics were evaluated in normal rats in which tetrahydrobiopterin deficiency was induced by administrating 2,4-diamino-6-hydroxy-pyrimidine (DAHP) for 8 h.**

***Results*: In cirrhotic rats, tetrahydrobiopterin administration increased liver NOS activity and cGMP levels and markedly and significantly reduced portal pressure. Amelioration of portal hypertension was associated with a normalization of arterial pressure. In normal rats DAHP decreased hepatic tetrahydrobiopterin and NOS activity and increased hepatic vascular tone. These effects of DAHP administration were corrected by tetrahydrobiopterin supplementation.**

***Conclusions*: The present study shows that tetrahydrobiopterin markedly reduces portal hypertension and improves systemic hemodynamics in cirrhotic rats. These data support the concept that tetrahydrobiopterin supplementation may represent a new therapeutic strategy for portal hypertension.**

***Keywords*: Portal hypertension; NOS uncoupling; NO biodisponibility**

1. Introduction

Portal hypertension is a frequent clinical syndrome in patients with liver diseases characterized by a pathological increase in the portal venous pressure and represents a major cause of morbi-mortality in these patients [1].

The main pathophysiological mechanism that leads to the development of portal hypertension in cirrhosis is an

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Abbreviations: Ach, acetylcholine; B, biopterin; CH, cirrhotic; CT, control; DAHP, 2,4-diamino-6-hydroxy-pyrimidine; eNOS, endothelial nitric oxide synthase; GTPCHI, guanosine triphosphate-cyclohydrolase I; L-NNA, N^{ω}-nitro-L-arginine; PP, portal pressure; Mtx, methoxamine; BH_4, tetrahydrobiopterin.

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increased resistance to portal blood flow through the cirrhotic liver. This increased resistance is due both to the disruption of the liver vascular architecture caused by cirrhosis and to an increased hepatic vascular tone. This in turn is caused by reduced nitric oxide (NO) availability within the liver, along with an increase in cyclooxygenase-1-derived vasoconstrictive prostanoids. Different pathophysiological mechanisms explaining reduced NO bioavailability within cirrhotic livers have been identified [2–4], including the existence of post-translational alterations in endothelial NO synthase (eNOS), such as increased caveolin-eNOS interaction [5], decreased eNOS phosphorylation [6] or cofactor deficiencies [7].

Tetrahydrobiopterin (BH_4) is an essential cofactor for the adequate generation of NO by NOS enzymes [8–10]. If adequate quantities of BH_4 are not present, a situation known as NOS uncoupling takes place and the production of NO is decreased. Previous studies from our lab [7] have shown that in cirrhotic livers there is a deficiency of BH_4, secondary to a reduction in the expression and activity of GTP cyclohydrolase I (GTPCHI), the limiting enzyme in BH_4 synthesis, which is associated with decreased NOS activity and NO availability. This study further demonstrated that the acute administration of BH_4 increased NOS activity in liver homogenates of cirrhotic rats and improved the vasodilatory response to acetylcholine in isolated and perfused cirrhotic livers [7]. However, the possible effects of more prolonged BH_4 administration on portal pressure and on systemic hemodynamics in cirrhotic rats have not been evaluated so far.

The present study evaluates the effects of three-day BH_4 supplementation on portal pressure and on splanchnic and systemic hemodynamics in CCl_4 cirrhotic rats. In addition, to further define the role of BH_4 deficiency modulating hepatic vascular tone, hepatic and systemic hemodynamic were assessed in normal rats in which tetrahydrobiopterin deficiency was induced by administrating 2,4-diamino-6-hydroxy-pyrimidine (DAHP), a specific inhibitor of GTPCHI.

2. Materials and methods

2.1. Hepatic and systemic effects of three-day BH_4 supplementation to CCl_4 cirrhotic rats

2.1.1. Animals and induction of cirrhosis

Male Wistar rats weighing 175–200 g were induced to cirrhosis by three-weekly inhalation of CCl_4, adding phenobarbital (0.3 g/l) to the drinking water as previously described [11,12]. When the cirrhotic rats developed ascites (12–18 weeks of CCl_4 inhalation), administration of CCl_4 and phenobarbital was stopped and studies were performed one week later.

Cirrhotic rats were treated over 3 days with BH_4, 10 mg/kg/day i.p. every 12 h or its vehicle. In vivo systemic and portal hemodynamics were evaluated 2 h after the last dose. In addition, BH_4 levels and NOS activity were evaluated in homogenized livers.

In vivo systemic and hepatic hemodynamics. BH_4 ($n = 9$) or vehicle ($n = 10$) treated rats were anesthetized with ketamine (100 mg/kg, i.p., Imalgene, Barcelona, Spain) and midazolam (100 mg/kg, i.p., Laboratorio Reig Jofre, Barcelona, Spain) and maintained at constant temperature of 37 ± 0.5 °C (continuously monitored during the experiment). A tracheostomy and cannulation with a PE-240 catheter (Portex, Kent, UK) was performed in order to maintain adequate respiration during the anaesthesia. Thereafter, PE-50 catheters were introduced into the femoral artery and in the portal vein through the ileocolic vein, in order to measure arterial pressure (MAP; mmHg) and portal pressure (PP, mmHg), respectively. A perivascular ultrasonic flow probe (Transonic System, Ithaca, NY) placed around the portal vein measured the portal vein blood flow (PBF). Results were indexed to liver weight (ml min^{-1} g^{-1}). Hepatic vascular resistance (HVR) was calculated as: PP/PBF. Blood pressures and flows were registered on a multichannel computer based recorder (Powerlab 4SP, ADInstruments, Mountain View, LA)

2.1.2. BH_4 determination

BH_4 levels were determined as previously described [7] in homogenized livers of cirrhotic rats treated with BH_4 ($n = 9$) or vehicle ($n = 10$), using a high performance liquid chromatography (HPLC) system, coupled to a fluorescence detector (Waters 474, Barcelona, Spain) (350 nm excitation, 450 nm emission). Results were expressed as pmol/mg protein. The protein content of each sample was determined by the Bradford method with bovine serum albumin (BSA) as the standard.

2.1.3. Measurement of nitric oxide synthase activity

Nitric oxide synthase (NOS) activity was measured in homogenized livers from cirrhotic rats treated with BH_4 ($n = 9$) and vehicle ($n = 10$) by determining the conversion of ^{14}C-labeled L-arginine to ^{14}C-labeled L-citrulline, according to a previously reported method [7,13,14]. Enzymatic activity was expressed as pmol min^{-1} mg^{-1} protein.

2.1.4. Measurement of cGMP

Measurements of cGMP, a marker of NO bioavailability, were performed in liver homogenates from rats treated with vehicle ($n = 9$) or BH_4 ($n = 10$) using an enzymeimmunoassay (Cayman Chemical Co., Ann Arbor, MI) as previously described [15,16]. Results were expressed as pmol/mg tissue.

2.1.5. Western blot analysis of caveolin-1

Caveolin-1 protein expression was assessed by Western blot in livers from cirrhotic rats treated with vehicle ($n = 4$) or with BH_4 ($n = 4$) as previously described [16]. Aliquots from each sample containing equal amounts of protein (100 μg) were run on a 12% SDS–polyacrylamide gel, and transferred to a nitrocellulose membrane. After the transfer, the blots were subsequently blocked for 1 h with Tris-buffered saline containing 0.1% (v/v) Tween 20 and 5% (wt/vol) nonfat dry milk, and probed with rabbit anti-caveolin antibody (BD Biosciences Pharmingen) overnight at 4 °C followed by incubation with goat anti-rabbit HRP-conjugated secondary antibody (1:10,000 in the same solution; Stressgen) for 1 h at room temperature. Blots were revealed by chemiluminescence. Protein expression was determined by densitometric analysis using the Science Lab, Image Gauge (Fuji Photo Film GMBH, Düsseldorf). After stripping, blots were assayed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotec., Santa Cruz, CA) expression as standardization of sample loading. Quantitative densitometric values of caveolin were normalized to GAPDH.

2.2. Induction of BH_4 deficiency in normal rats

In male Wistar rats weighting 330–380 g, BH_4 deficiency was induced by the administration of the selective GTPCHI inhibitor 2,4-diamino-6-hydroxy-pyrimidine (DAHP) at a dose of 1 g/kg i.p. twice at 4 h intervals. Controls rats were treated with PBS. Four hours after the second dose in vivo portal hemodynamics, hepatic BH_4 levels and NOS activity in homogenized livers were evaluated as described above.