ELSEVIER

Journal of Hepatology 44 (2006) 886–893

Journal of Hepatology

www.elsevier.com/locate/jhep

Increased phosphodiesterase-5 expression is involved in the decreased vasodilator response to nitric oxide in cirrhotic rat livers

Mauricio R. Loureiro-Silva[1,2], Yasuko Iwakiri[1,2,3], Juan G. Abraldes[1,2], Omar Haq[1,2], Roberto J. Groszmann[1,2,*]

[1]*Hepatic Hemodynamic Laboratory, VA Medical Center, West Haven, CT, USA*
[2]*Digestive Diseases Section, Yale University School of Medicine, New Haven, CT, USA*
[3]*Department of Pharmacology, Yale University School of Medicine, West Haven, CT, USA*

***Background/Aims*: Cirrhotic livers have a deficient vasodilator response to nitric oxide (NO). The vasodilator effect of NO is normally limited by the degradation of its second messenger cyclic guanosine 3′, 5′ monophosphate by phosphodiesterases. We investigated (1) the phosphodiesterase-5 (PDE-5) expression in normal and cirrhotic rat livers, (2) the location of the deficient response to NO in cirrhotic livers, and (3) the effect of the PDE-5 inhibitor Sildenafil citrate on this deficient response.**

***Methods*: Normal and ascitic cirrhotic rats were subjected to liver perfusion with continuous measurement of both perfusion and sinusoidal (wedge hepatic) pressures. After incubation with *N*-monomethyl-L-arginine and pre-constriction with Methoxamine, concentration–response curves to the spontaneous NO donor *S*-nitroso-*N*-acetylpenicillamine were obtained in the absence or presence of Sildenafil (10^{-8} M).**

***Results*: PDE-5 expression (Western blot) in cirrhotic livers was higher than in normal livers ($P=0.042$). Compared to normal livers, cirrhotic livers showed a decreased response to *S*-nitroso-*N*-acetylpenicillamine in the pre-sinusoidal area ($P=0.003$) but not in the sinusoidal/post-sinusoidal area ($P=0.433$). In the presence of Sildenafil, normal and cirrhotic livers showed similar pre-sinusoidal ($P=0.419$) and sinusoidal/post-sinusoidal ($P=0.875$) responses to *S*-nitroso-*N*-acetylpenicillamine.**

***Conclusions*: Increased PDE-5 expression is involved in the decreased vascular response to NO in cirrhotic livers.**

***Keywords*: Microcirculation; Liver perfusion; Soluble guanylyl cyclase; *S*-nitroso-*N*-acetylpenicillamine**

1. Introduction

Portal hypertension is the most common and lethal complication of cirrhosis. Although anatomical abnormalities are the main cause of the increased vascular resistance to portal blood flow through the cirrhotic liver, an enhanced intrahepatic vascular tone has also been demonstrated in cirrhotic patients [1–3] and cirrhotic rats [4]. Increased production of vasoconstrictors [5,6] and decreased production of the vasodilator nitric oxide (NO) [7,8] are major intrinsic mechanisms that cause a hemodynamic imbalance leading to the development of this increased vascular tone in cirrhotic livers [9]. We have previously observed that, in addition to a decreased production of NO, the cirrhotic liver has a deficient vascular response to NO [10].

NO promotes vasodilation mainly through activation of soluble guanylyl cyclase (sGC) in contractile cells [11]. Under normal conditions, activated sGC synthesizes cyclic guanosine 3′,5′ monophosphate (cGMP) initiating the cGMP cascade [12–15], a sequence of events that culminates with cell relaxation and vasodilation. The vasodilator effect of NO is normally limited by phosphodiesterases (PDEs) that break down cGMP to its inactive form GMP [11,16].

Received 30 June 2005; received in revised form 15 December 2005; accepted 10 January 2006; available online 28 February 2006

* Corresponding author. Address: Digestive Diseases Section/111H, VA Healthcare System, 950 Campbell Avenue, West Haven, CT 06516, USA. Tel.: +1 203 932 5711x5090; fax: +1 203 933 3665.

E-mail address: roberto.groszmann@yale.edu (R.J. Groszmann).

0168-8278/$32.00
doi:10.1016/j.jhep.2006.01.032

Increased PDE expression and/or activity have been associated with vascular dysfunction in different clinical conditions such as diabetes [17–19], subarachnoid hemorrhage, [20,21] and pulmonary hypertension [22,23]. Phosphodiesterase-5 (PDE-5) inhibitors have been shown to potentiate the vasodilator effect of both endogenous and exogenous NO improving vascular function in patients with erectile dysfunction [24], type two diabetes [25], and pulmonary arterial hypertension [26,27]. Therefore, we hypothesized that an increased PDE-5 expression may be involved in the development of vascular dysfunction in cirrhotic livers.

In the present study, we demonstrate that PDE-5 modulates the vascular tone in normal livers, and that its over-expression is involved in the decreased response to NO in cirrhotic livers.

2. Materials and methods

Male Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN) were used in accordance to the National Research Council Guide for the Care and Use of Laboratory Animals. After anesthesia with ketamine hydrochloride (Ketaset®, 100 mg/kg body weight, Fort Dodge Animal Health, Fort Dodge, IO) and xylazine (Rompum®, 40 mg/animal Bayer, Shawnee Mission, KA), ascitic cirrhotic rats and age-matched normal rats were submitted to either tissue collection or liver perfusion. It has been previously shown that intra-hepatic endothelial dysfunction in ascitic cirrhotic rats is more severe than in non-ascitic cirrhotic rats [7].

2.1. Induction of cirrhosis by carbon tetrachloride

Rats underwent exposure to carbon tetrachloride (CCl_4) by inhalation three times a week and phenobarbital (0.35 g/l) was added to their drinking water [28]. This technique induces micronodular cirrhosis in approximately 10–12 weeks of treatment. In this study we used rats with moderate to severe degree of cirrhosis; all of them had ascites, enlargement of portal vein, and collateral circulation.

2.2. Western blotting

PDE-5 and sGC expressions were determined in tissue samples of liver and aorta collected from normal and ascitic cirrhotic rats. After exsanguinations with Krebs–Henseleit solution, liver and abdominal aorta samples were collected, frozen in liquid nitrogen, and stored at −70 °C. Samples were homogenized in an appropriate lysis buffer containing 50 mM Tris–HCl, 1 mmol 4-(2-aminoethyl)-benzenesulfonyl fluoride, protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany), and 1% (v/v) Nonidet $P-40$, pH 7.5. Protein content in the supernatants was quantified using the Lowry method with bovine serum albumin as standard. The supernatants were subjected to the SDS-PAGE gel electrophoresis of protein (20 μg), and Western blotting was performed using antibodies that recognized PDE-5 (1:500, BD Transduction Laboratories), sGC (1:500; β_1-subunit; PAB, Calbiochem, San Diego, CA), and β-actin Mab (1:1000, Sigma, St. Louis, MO). Enhanced chemiluminescence was used for protein detection. Intensity of the bands corresponding to the protein of interest was measured using densitometry.

2.3. PDE-5 Immunofluorescent staining

Liver tissue was collected from normal and ascitic cirrhotic rats after a short exsanguination with Krebs–Henseleit solution followed by perfusion with 100 ml of 4%-paraformaldehyde/PBS. Samples were fixed overnight in 4%-paraformaldehyde/PBS at 4 °C dehydrated in 15%-sucrose/PBS and prepared for frozen section. Sections were processed for immunofluorescence using a protocol consisting of three major steps. First, non-specific antigenic sites were blocked in a blocking buffer containing 5% goat serum, 0.2%-BSA, and 0.5%-Triton X-100 in PBS. Second, samples were incubated with a primary antibody (PDE-5 antibody, 1:100) at 4 °C overnight. And third, samples were incubated with fluorescent secondary antibodies. After mounting with anti-fade mounting media, staining was visualized by a confocal microscope, Ziess LSM 510 Confocal Imaging System equipped with a krypton/argon mixed gas laser captured images. To ensure specificity of staining, images were obtained using confocal machine settings (i.e. aperture, gain, and black level) at which no fluorescence was detectable in another negative control samples treated with secondary antibody alone.

2.4. In situ rat liver perfusion

Rats were submitted to in situ liver perfusion as we previously described [29]. Briefly, the abdominal cavity was open and the portal vein was cannulated with a 14-gauge Teflon catheter. Liver exsanguination was initiated immediately by infusion (40 ml/min) of oxygenated (carbogen gas, 95% O_2/ 5% CO_2) Krebs–Henseleit solution containing dextrose (11 mM). The posterior vena cava was cut and cannulated with a second 14-gauge Teflon catheter (outflow catheter).

In order to measure the sinusoidal pressure, a PE-60 catheter was passed through the right atrium and the thoracic segment of the posterior vena cava, and then wedged into a hepatic vein in the left lobe. This procedure did not impair the drainage of perfusate through the abdominal segment of the posterior vena cava. The preparation was transferred to a temperature-controlled (37 °C) chamber and the perfusion system was changed to a recirculating mode. After exsanguination, all livers were perfused with 100 ml of recirculating Krebs–Henseleit solution at a constant flow rate of 40 ml/min using similar tubing.

Perfusion and sinusoidal pressures were measured continuously by using two independent strain-gauge transducers (P23XL, Spectramed, Oxnard, CA) attached to the perfusion cannula and to the wedged catheter, respectively. Before each experiment, both pressure measurement systems were calibrated with the zero point at the level of the hepatic hilum. Perfusion and sinusoidal pressures were continuously recorded by Chart 3.6 program using MacLab/4e hardware (AD Instruments).

Since the perfusion flow was kept constant, this technique also provided continuous recording of changes on vascular resistance. The perfusion pressure increase (or decrease) induced by a vasoactive agent was determined by the sum of the pre-sinusoidal, sinusoidal, and post-sinusoidal vascular resistance increases (or decreases) induced by this agent. The sinusoidal pressure increase (or decrease), however, was determined by the sum of the sinusoidal and post-sinusoidal vascular resistance increases (or decreases). Consequently, changes in pre-sinusoidal vascular resistance increase could be estimated as the difference between the change in total vascular resistance (obtained from perfusion pressure measurement) and the change in sinusoidal/post-sinusoidal vascular resistance (obtained from sinusoidal pressure measurement) [29].

During the experiment, the perfusate was oxygenated by means of a Silastic tubing lung [30]. Liver global viability was assessed by gross appearance of the liver, perfusion pressure curve pattern, and bile production (>1.0 μl/min/g liver in normal livers) [31,32].

2.4.1. Effect of sildenafil citrate on the pre-constricted intra-hepatic circulation

Concentration–response curves induced by sildenafil (10^{-7}–10^{-5} M; three doses; 1.5 min interval between doses) or its vehicle (saline) were obtained using normal livers after pre-constriction (5 min) of the intra-hepatic circulation with the α_1-adrenergic agonist Methoxamine (10^{-4} M). Sildenafil vasodilator effect was estimated as the ratio between the pressure decrease induced by each concentration of this PDE-5 inhibitor and the pressure increase induced by Methoxamine [10].

2.4.2. Effect of Sildenafil citrate in the intra-hepatic response to exogenous NO

Concentration–response curves to the spontaneous NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP; 10^{-7}–3×10^{-5}M; six doses; 2 min interval between doses) were obtained in normal and cirrhotic livers after