Simvastatin treatment improves liver sinusoidal endothelial dysfunction in CCl₄ cirrhotic rats

Juan G. Abraldes, Aina Rodríguez-Vilarrupla, Mariona Graupera, Carmen Zafra, Héctor García-Calderó, Juan Carlos García-Pagán, Jaime Bosch*

Hepatic Hemodynamic Laboratory, Liver Unit, IMDIM, Hospital Clinic, Ciberedh and Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona, Villarroel 170, 08036 Barcelona, Spain

1. Introduction

Portal hypertension is a leading cause of morbidity and mortality in patients with cirrhosis of the liver [1]. In these patients a sufficient reduction in portal pressure is associated with protection from complications of portal hypertension [1,2], but currently available therapies are effective in a limited number of patients [1]. Advances in the knowledge of the pathophysiology of portal hypertension have opened new possibilities to explore novel therapies for the treatment of this syndrome. In particular, the recognition of a dynamic and, thus, reversible component in the increased hepatic resistance of cirrhotic livers [3] due to an increased hepatic vascular tone set the rationale for the use of vasodilators in portal hypertension [1]. Sinusoidal endothelial dysfunction with decreased nitric oxide (NO) production has been identified as a major contributor to the increase in the hepatic vascular tone in cirrhosis [4–7]. Increasing NO availability at the liver circulation is, therefore, a rational approach to the treatment of portal hypertension [8]. The use of NO donors has been hampered because they impair systemic hemodynamics with the
ensuing risk of renal failure [9]. The development of liver-selective NO donors may overcome this problem [10–12], but they have not been tested so far in patients with cirrhosis.

Sinusoidal endothelial dysfunction in the cirrhotic liver has been related to a decreased eNOS activity despite normal protein expression [4,5]. This has been associated with disturbances in the post-translational regulation of the activity of the enzyme, which include a decreased Akt-dependent eNOS phosphorylation [13,14]. HMG-CoA reductase inhibitors, commonly called statins, are widely used drugs that have been shown to improve endothelial function increasing NO production in endothelial cells from peripheral vasculature [15–20]. This effect is mediated, among other mechanisms, by an increase in Akt-dependent eNOS phosphorylation at serine 1176 (1177 in the human sequence) and ensuing increased eNOS activity. Statins, therefore, could be good candidates to improve endothelial function in liver vasculature. Very recently, we demonstrated that short-term administration of simvastatin improves flow-mediated hepatic vasodilatation and decreases hepatic resistance in patients with cirrhosis, and that this effect was probably mediated by an increase in hepatic NO release [21].

The current study was designed to further characterize the molecular and hemodynamic effects of statins on the liver circulation in rats with CCl4 induced liver cirrhosis.

2. Materials and methods

2.1. Induction of cirrhosis by CCl4

Male Wistar rats weighing 175–200 g underwent repeat inhalation exposure to CCl4. Phenobarbital (0.3 g/L) was added to the drinking water as previously described [22,23]. A high yield of micronodular cirrhosis is obtained, and after approximately 12–15 weeks of CCl4 inhalation the animals develop ascites. The animals were kept in environmentally controlled animal facilities at the IDIBAPS. All experiments were performed according to the criteria of the Committee for the Care and Use of Laboratory Animals in the Hospital Clinic and IDIBAPS.

2.2. Simvastatin treatment

After cirrhotic rats had developed ascites, CCl4 and phenobarbital administration were discontinued. Ascitic cirrhotic rats were then housed in pairs and received no drugs for 7 days. After this period, one rat of each pair was treated with simvastatin whereas its littermate was treated with vehicle. Simvastatin was suspended in distilled water and administered by gavage (25 mg/kg body weight) once a day for 3 days. This dose has been shown to inhibit the HMG-CoA activity in the liver as early as 30 min after oral administration, with a peak of inhibitory activity at one hour [24]. Experiments were performed 1 h after the last dose of the drug.

2.3. In vivo hemodynamic study

Rats were anaesthetized with Inactin (Thiobutabarbital sodium, 100 mg/kg body weight, Sigma chemical Co., St. Louis, MO) and fastened to a surgical board. Indwelling catheters were placed in the carotid artery and the superior mesenteric vein to monitor mean arterial pressure (MAP) and portal pressure (PP), respectively [25]. Both pressures were continuously measured by using two independent pressure transducers. These were shown to detect changes of the order of 0.1 mm Hg. Superior mesenteric artery blood flow (SMAFB, ml/min) was measured with a non-constrictive peri-vascular ultrasonic transit-time flow probe (IRB, 1 mm diameter. Transonic Systems Inc., Ithaca., NY, USA) placed around the vessel close to its aortic origin. The flow probe was connected to a small animal flowmeter (Transonic Systems Inc., Ithaca, NY, USA). After 20 min of stabilization, to explore the flow-induced relaxation of liver vasculature portal venous inflow was increased in a stepwise fashion by volume expansion with incremental doses (1–8 ml/kg) of hydroxethylstarch 6% (Hesteril® 6% 200/0.5, Fresenius Kabi, Barcelona, Spain) [7]. In an independent group of rats treated with simvastatin (n = 4) or placebo (n = 4) the effects of volume expansion were tested 15 min after nitric oxide synthase inhibition with l-NAME (15 mg/kg, iv.).

2.4. Isolated-perfused liver system

A flow-controlled perfusion system was employed in this study. The system has been described elsewhere [22]. After the cirrhotic rats had been anaesthetized with ketamine hydrochloride (100 mg/kg) plus midazolam (5 mg/kg), the abdomen was opened and the peritoneal cavity was explored for ascites. The rats were perfused via a PALL Ultipor filter (model SQ4OS by Pall, East Hills, NY) from the reservoir into an overflow chamber of 0.5 M EDTA pumped (MasterFlex, Cole-Parmer Instrument Co., Vernon Hills, IL) to monitor bile-flow. A ligature was passed around the inferior vena cava (IVC) above the renal veins, and the IVC was injected with 1400 U heparin/kg body weight. The portal vein was cannulated with a polyethylene tubing (PE-100; Portex, Kent, UK) and the liver was perfused immediately with Krebs’ solution containing 200 μmol of 0.5 M EDTA pumped (MasterFlex, Cole-Parmer Instrument Co., Vernon Hills, IL) and oxygenated via a Silastic tubing oxygenator with a mixture of 95% O2 and 5% CO2 at 37 ± 0.5°C. Abdominal aorta and inferior vena cava were cut allowing the exsanguination of the animal and the perfusate to escape. Thereafter, the thorax was opened and the right atrium was cannulated (PE-240; Portex, Kent, UK) advancing the catheter through the inferior vena cava up to the outlet of the hepatic vein. When the perfused effluent was clear the system was closed and the liver was perfused in a recirculating fashion with a reservoir volume of 100 ml at a constant flow rate of 35 ml/min. An ultrasonic flow probe (T201, Transonic System, Ithaca, NY) and a pressure transducer were placed on line, immediately ahead of the portal inlet cannula, to continuously monitor portal flow and perfusion pressure. Another pressure transducer was placed immediately after the thoracic vena cava outlet cannula for measurement of outflow pressure.

As in in vivo hemodynamic studies, the flow probe and the two pressure transducers were connected to a PowerLab (4SP) linked to a computer using the Chart v4.0.1 for Windows software (ADInstruments, Mountain View, LA). The average portal flow, inflow and outflow pressures were sampled and recorded every second and later exported to data management software to be analyzed.

The perfused rat liver preparation was allowed to stabilize for 30 min before the vasoactive substances were added. The criteria of liver viability included gross appearance of the liver, stable perfusion pressure, bile production over 0.4 ml/min/g liver and a stable buffer pH (7.4 ± 0.1) during the initial 30 min stabilization period [22]. If either viability criteria were not satisfied, the experiment was discarded. After stabilization, the pressure response to the α-adrenergic methoxamine (10−4 M) was evaluated by adding the drug to the reservoir. Endothelium-independent relaxation was evaluated after pre-constriction with methoxamine, by adding incremental doses of acetylcholine (ACh; 10−2–10−1 mol/L), following the methodology described by Gupta et al. [6]. In an independent group of rats (four treated with simvastatin and four treated with placebo) this experiment was conducted in the presence of the nitric oxide synthase inhibitor l-NNAME (10−3 M).
2.5. Western blotting of eNOS, P-eNOS, Akt, P-Akt and tissue cGMP concentration

After anaesthesia and laparotomy the liver was perfused through the portal vein with saline for exsanguination, and liver samples were immediately frozen in liquid nitrogen and kept at −70 °C until processing. The samples were crushed to powder while frozen and subsequently homogenized in Triton-lysis buffer containing Tris/HCl (pH 7.4, 20 mM), NaCl (150 mM), NaF (20 mM), Na2PO4 (10 mM), okadaic acid (10 nM), Na3VO4 (2 mM), antipain (2 µg/ml), aprotinin (2 µg/ml), chymostatin (2 µg/ml), leupeptin (2 µg/ml), pepstatin A (2 µg/ml), trypsin inhibitor (2 µg/ml), phenylmethylsulfonyl fluoride (40 µg/ml), and Triton X-100 (1% v/v), left on ice for 10 min, and then centrifuged at 10,000g/10 min. Protein concentration in supernatants was quantified using the Bradford assay. Then, aliquots from each sample containing equal amounts of protein (40–100 µg) were run on a 10% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. Equal loading was ensured by Ponceau staining. The blots were subsequently blocked for 1 h with Tris-buffered saline containing 0.05% (vol/vol) Tween 20 and probed with a mouse antibody recognizing eNOS (BD Transduction Laboratories, Lexington, KY), a rabbit anti-phosphorylated eNOS at Ser1176, a rabbit antibody recognizing Akt (Cell Signaling Technology, Beverly, MA) or a rabbit P-Akt at Ser473 (Cell Signaling Technology, Beverly, MA) for 16 h at 4 °C followed by an incubation with rabbit anti-mouse (1:10,000) or goat anti-rabbit (1:10,000) HRP-conjugated secondary antibody. After washing, blots were revealed by chemiluminescence. Protein expression was determined by densitometric analysis using the Science Lab 2001, Image Gauge (Fuji Photo Film Gmbh, Düsseldorf). After stripping, blots were assayed for GAPDH used for comparisons of two means. ANOVA test for repeated measures with SPSS 12.0 statistical package (SPSS, Chicago, IL). Unpaired t-test or Mann–Whitney test was used for comparisons of two means. ANOVA test for repeated measures was used when appropriate. Results are expressed as mean ± SEM. Significance was established at the 0.05 level.

2.6. Statistics

All analyses were performed with SPSS 12.0 statistical package (SPSS, Chicago, IL). Unpaired t-test or Mann–Whitney test was used for comparisons of two means. ANOVA test for repeated measures was used when appropriate. Results are expressed as means ± SEM. Significance was established at the 0.05 level.

3. Results

All rats had macroscopic cirrhosis and signs of portal hypertension as shown by the presence of ascites and collateral circulation.

3.1. In vivo studies

Treatment with simvastatin did not modify baseline systemic and splanchnic hemodynamics in cirrhotic rats: MAP, SMABF and PP were not significantly different in rats treated with vehicle (n = 9) or simvastatin (n = 8) (Table 1). After baseline measurements rats underwent volume expansion with incremental doses (1–8 ml/kg) of hydroxyethylstarch 6%. Volume expansion induced similar, dose-dependent increases in MAP and SMABF in both groups of rats. However, the increase in PP was significantly attenuated in rats treated with simvastatin in comparison to those treated with vehicle (Fig. 1). In contrast, in rats pre-treated with l-NAME there were no significant differences in volume expansion induced increase in PP in both group of rats (0.4 ± 0.3 vs 0.6 ± 0.2 mm Hg in placebo and simvastatin after 1 ml/kg, 0.6 ± 0.4 vs 0.8 ± 0.2 mm Hg after 2 ml/kg, 1.3 ± 0.8 vs 1.7 ± 0.2 mm Hg after 4 ml/kg and 2.7 ± 0.8 vs 2.5 ± 0.3 mm Hg after 8 ml/kg; ns), with similar increases in MAP and SMABF. This suggests that pre-treatment with simvastatin improved flow-stimulated relaxation of liver vasculature through an NO-dependent mechanism.

3.2. Liver perfusion studies

To further characterize the effects of simvastatin on liver vasculature, livers from rats treated with vehicle (n = 8) or with simvastatin (n = 7) were isolated and perfused. There were no significant differences in baseline perfusion pressure between the two groups (8.5 ± 0.5 mm Hg in vehicle vs 8.9 ± 0.5 in simvastatin; ns).

Methoxamine (10−4 M) significantly increased portal perfusion pressure in both groups of rats but this response was significantly blunted in the simvastatin group (Fig. 2a). This was not observed in the presence of l-NNA (Fig. 2b).

Dose–response curves to ACh showed that cirrhotic livers treated with vehicle showed endothelial dysfunction, since there was slight vasorelaxation in response to ACh at the 10−7 dose, no vasodilation at the 10−6 doses, and paradoxical vasoconstriction at the 10−5 dose of ACh. Simvastatin pre-treatment abolished paradoxi-

Table 1

Baseline hemodynamics in rats treated with simvastatin or vehicle (SMABF, superior mesenteric artery blood flow)

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Simvastatin</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>510 ± 17</td>
<td>482 ± 20</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>12.9 ± 0.7</td>
<td>11.8 ± 0.5</td>
</tr>
<tr>
<td>Mean arterial pressure (mm Hg)</td>
<td>110 ± 6</td>
<td>113 ± 6</td>
</tr>
<tr>
<td>Portal pressure (mm Hg)</td>
<td>12.2 ± 0.5</td>
<td>11.3 ± 0.7</td>
</tr>
<tr>
<td>SMABF (ml/min/100 g bw)</td>
<td>2.6 ± 0.3</td>
<td>2.3 ± 0.1</td>
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ocal vasoconstriction induced by ACh (Fig. 3a), an effect not observed in the presence of L-NNA (Fig. 3b).

Taken together, these experiments show that simvastatin improves the manifestations of liver sinusoidal endothelial dysfunction in cirrhosis, and that this occurs through a NO-dependent mechanism.

3.3. Effects on eNOS regulation and NO production

To further characterize the mechanisms involved in the simvastatin mediated improvement in flow-mediated endothelial-dependent dilation of liver vasculature, we harvested livers from cirrhotic rats treated with simvastatin (n = 4) or vehicle (n = 4) for 3 days. iNOS expression was not detected in livers from either group of rats (Fig. 4). Simvastatin significantly enhanced liver eNOS protein expression in cirrhotic rats (Fig. 5a). Further, simvastatin enhanced eNOS phosphorylation at Ser 1176 in cirrhotic rats (Fig. 5a). Since Ser 1176 phosphorylation is associated with an increased activity of eNOS, these findings suggest that simvastatin increased both eNOS expression and activity. eNOS phosphorylation at Ser 1176 is Akt-dependent. Therefore we investigated whether simvastatin also increased Akt activity. Simvastatin did not modify Akt expression, but significantly activated Akt.

This was shown by the increased Akt phosphorylation at Ser 473, demonstrated by the significant increase in the P-Akt/Akt ratio (Fig. 5b). These findings were associated with an increase in liver tissue cGMP content in rats treated with simvastatin (14.15 ± 1.13 pmol/ml) as compared with those treated with vehicle (9.90 ± 1.93; p = 0.035), indicating an increased liver NO production and utilization in rats treated with simvastatin. Altogether, these results suggest that in cirrhosis with portal hypertension, simvastatin is able to enhance liver NO bioavailability by increasing eNOS expression and by inducing an Akt-dependent eNOS activation.

4. Discussion

In a previous study we have shown that acute simvastatin administration to cirrhotic patients with portal hypertension decreases the estimated hepatic resistance and attenuates the portal pressure increase to a test meal [21]. The observation that these hemodynamic effects were associated with an increased hepatosplanchnic output of NO suggested that simvastatin improved flow-mediated hepatic relaxation (and thereby, hepatic endothelial function) by enhancing the availability of

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Fig. 1. Hemodynamic changes after volume expansion in rats pre-treated with vehicle (white bars) or simvastatin (black bars). Simvastatin treatment did not modify the increase in mean arterial pressure or SMABF induced by volume expansion, but significantly attenuated the increase in portal pressure (MAP, mean arterial pressure; SMABF, superior mesenteric artery blood flow; PP, portal pressure).

Fig. 2. (a) Absolute change in portal perfusion pressure (PPP) after the addition of the α-adrenergic agonist methoxamine in livers from cirrhotic rats treated with vehicle (white bars) or simvastatin (black bars). Simvastatin treatment significantly attenuated the vasoconstrictive response to methoxamine (a), an effect prevented by pre-treatment with L-NNA (b).

Fig. 3. Response to ACh in livers pre-constricted with methoxamine from cirrhotic rats treated with vehicle (white circles) or simvastatin (black circles). Simvastatin treatment significantly attenuated the paradoxical constriction induced by ACh in cirrhotic livers (a), an effect that was no longer observed after pre-treatment with L-NNA (b). (PPP, portal perfusion pressure; ACh, acetylcholine).
NO at the intrahepatic circulation. However, the molecular mechanisms linking the administration of statins to improved hepatic hemodynamics cannot be adequately assessed in clinical studies. Furthermore, measurement of hepatic vascular resistance in vivo has limitations due to the dual liver perfusion and to the presence of collaterals. Because of this, all studies assessing hepatic circulation use the \textit{in situ} liver perfusion technique [8].

In the present study, in an experimental model of liver cirrhosis (which allows \textit{ex vivo} evaluation of vascular responses of the liver circulation) we provide solid data showing that simvastatin enhances eNOS-dependent liver NO production. This was associated with an improvement in the manifestations of liver sinusoidal endothelial dysfunction, namely hyper-response to vasoconstrictors, paradoxical vasoconstrictive response to an endothelium-dependent vasodilator and impaired flow-mediated relaxation of the liver vasculature [5,6]. Additionally, by measuring SMABF, a surrogate of portal blood inflow, we were able to show that simvastatin does not enhance splanchnic vasodilatation of cirrhosis. This, together with the lack of changes in MAP, suggests that simvastatin effects on NO production were liver-selective.

A number of \textit{in vitro} studies have shown that statins are able to increase eNOS-dependent NO production in the endothelial cell. This occurs both at the translational and at the post-translational level. The most immediate effect of statins is an increase in eNOS phosphorylation at Ser 1176, with subsequent increased eNOS activity [17]. This is mediated by the activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway [17] that leads to an increase in Akt phosphorylation with ensuing eNOS phosphorylation [26]. Statins have also been shown to upregulate eNOS expression in the endothelial cell. This occurs at the translational level, by increasing eNOS mRNA stability [27]. In the present study, we were able to demonstrate, in a complex \textit{in vivo} system, that these molecular events also occur in the cirrhotic liver circulation. We show here that oral administration of simvastatin increases liver Akt phosphorylation, Akt-dependent eNOS phosphorylation at Ser 1176 and liver eNOS expression. This was associated with an increase

![Fig. 4. Western blotting showing iNOS expression in livers from cirrhotic rats treated with simvastatin or vehicle. iNOS expression was not detected in livers from either group of rats.](image)

![Fig. 5. Representative blots and densitometry readings of (a) eNOS, P-eNOS/eNOS ratio and (b) Akt and P-Akt/Akt ratio in cirrhotic livers treated with vehicle (white bars) or simvastatin (black bars). Simvastatin treatment significantly increased eNOS expression, eNOS phosphorylation at Ser 1176, and Akt phosphorylation at Ser 473 (AU, arbitrary units).](image)
in liver cGMP, the second messenger of NO. Altogether these data suggest that simvastatin increased eNOS-dependent NO release.

Insufficient NO production by the liver endothelium has been shown to play a major role in the increased hepatic vascular tone observed in cirrhosis [4,6,7]. This insufficient endothelial NO production manifests itself as a hyper-response to vasoconstrictors and by an impaired endothelium-dependent vasodilation, demonstrated by a paradoxical vasoconstriction in response to ACh [6]. In the present study we show that simvastatin improves these disturbances, since it reduces by circa 50% the response to the α-adrenergic vasoconstrictor methoxamine, and attenuates the paradoxical constriction induced by ACh. The results from our in vivo studies also reinforce these findings. One of the manifestations of liver endothelial dysfunction in cirrhosis is the inability of the liver circulation to properly dilate in response to flow [5]. This leads to an abnormal increase in portal pressure under situations associated with an increase in splanchnic blood flow, such as post-prandial hyperemia [28]. In this study, to test the flow-mediated dilation of the liver circulation, we induced an increase in portal blood inflow by a stepwise volume expansion. This methodology (using blood from a rat donor instead of a plasma expander) has been previously used by others to explore flow-mediated dilation of liver vasculature [12]. Here, we show that simvastatin attenuated the increase in portal pressure induced by volume expansion by approximately 50%, while the increase in SMABF, a surrogate of portal blood inflow, was similar in vehicle and simvastatin groups. These data suggest that simvastatin improved the vasodilatory capacity of the liver circulation upon a flow challenge, such as the one that occurs physiologically after meals and which results in significant increases of portal pressure in patients with cirrhosis [21,28]. The vascular effects of simvastatin were abolished when tested in the presence of nitric oxide synthase inhibitors. This, together with our molecular data, indicates that they were mediated by eNOS upregulation.

Another important finding of the present study is that simvastatin does not modify systemic hemodynamics in rats with cirrhosis. The use of NO donors (and of any vasodilator) in liver cirrhosis has been limited by the risk of exacerbation of the vasodilation and hypotension associated with the hyperdynamic syndrome of portal hypertension [1]. Therefore, in liver cirrhosis therapies aimed at increasing liver NO bioavailability must be liver-selective [1]. Simvastatin seems to fulfill this requirement, since our results suggest that this drug targets the dysfunctioning hepatic endothelium, while it does not increase further the already enhanced NO production in the systemic circulation. This selectivity of statins for the dysfunctioning endothelium has been shown in other settings. For example, statins are able to decrease arterial pressure in spontaneously hypertensive rats, which show diffuse endothelial dysfunction, but do not modify arterial pressure in normal animals [29]. Also, a recent study shows that statins improve endothelium-dependent vasodilation in smokers, but not in normal subjects [30]. Altogether, our data support that statins might constitute a new and feasible way of selectively increasing liver NO without deleterious adverse effects. Therefore, our results add to the current corpus of data showing that interventions that selectively increase NO bioavailability are able to improve hepatic hemodynamics in cirrhosis. In previous works, transfection of the liver with eNOS, nNOS or a constitutively active form of Akt proved effective in decreasing hepatic resistance [13,31,32]. Oral administration of NCX-1000, a hepatic-selective NO donor obtained by adding a NO-releasing moiety to UDCA, also decreased hepatic resistance in the CCl4 and biliary models of cirrhosis [11,12]. Statins could offer clear advantages over these approaches, since a number of statins are already approved for use in patients. Though these drugs seem safe in patients with mild liver disease [33–36], potential hepatotoxicity in patients with cirrhosis needs to be carefully evaluated.

In conclusion, our data suggest that the administration of simvastatin might constitute a new way of selectively increasing NO availability in the cirrhotic liver circulation and, therefore, to improve the vascular disturbances that contribute to portal hypertension in liver cirrhosis.

Acknowledgements

We are indebted to Jordi Gracia for his expertise in eGMP assays, Marcos Pasarin for his expertise in Western blotting and to Maria Montano for expert secretarial assistance. This study was supported by grants from Plan Nacional de I + D + I (FIS 04/0655, 05/1285 and 06/0623 to J.B., CM04/00031 and FIS 05/0519 to J.G.A.).

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