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## Low NO bioavailability in CCl<sub>4</sub> cirrhotic rat livers might result from low NO synthesis combined with decreased superoxide dismutase activity allowing superoxide-mediated NO breakdown: A comparison of two portal hypertensive rat models with healthy controls

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### Abstract

**Background:** In cirrhotic livers, the balance of vasoactive substances is in favour of vasoconstrictors with relatively insufficient nitric oxide. Endothelial dysfunction has been documented in cirrhotic rat livers leading to a lower activity of endothelial nitric oxide synthase but this might not be sufficient to explain the low nitric oxide presence. We compared the amount of all nitric oxide synthase isoforms and other factors that influence nitric oxide bioavailability in livers of two portal hypertensive rat models: prehepatic portal hypertension and carbon tetrachloride induced cirrhosis, in comparison with healthy controls.

**Results:** Endothelial nitric oxide synthase was the solely detected isoform by Western blotting in all livers. In cirrhotic livers, the amount of endothelial nitric oxide synthase protein was lower than in healthy controls, although an overlap existed. Levels of caveolin-1 messenger RNA were within the normal range but endothelin-1 messenger RNA levels were significantly higher in cirrhotic livers ( $p < 0.05$ ). A markedly lower superoxide dismutase activity was observed in cirrhotic livers as compared to healthy controls ( $p < 0.05$ ).

**Conclusions:** In contrast to prehepatic portal hypertension, cirrhotic livers had decreased endothelial nitric oxide synthase protein and enhanced endothelin-1 messenger RNA amount. We hypothesise that a vasodilator/vasoconstrictor imbalance may be further aggravated by the reduced activity of superoxide dismutase. Decreased activity allows enhanced superoxide action, which may lead to breakdown of nitric oxide in liver sinusoids.

### Background

The balance of vasoactive substances in cirrhotic livers is

in favour of vasoconstrictors [1–3]. This contrasts with splanchnic and systemic vasodilatation characteristically

seen in this condition [1,2]. Nitric oxide (NO), prostacyclin and carbon monoxide are known intrahepatic vasodilating substances, whereas endothelin-1, superoxide ( $O_2^-$ ), angiotensin-II, epinephrine and others act as vasoconstricting agents [1–6]. NO is produced by 3 different nitric oxide synthase (NOS) isoforms: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) [1]. The latter is in a normal liver clearly present in endothelial cells of portal venules, portal arterioles and central venules, as well as in sinusoidal endothelial cells [7,8]. Other liver cell types such as hepatic stellate [9,10], Kupffer cells [9] or hepatocytes [7,9] do not express eNOS. A diminished hepatic activity of eNOS by about 30–50 % was documented in carbon tetrachloride ( $CCl_4$ ) induced cirrhosis [7–9,11], in biliary fibrosis of the rat [9,12] and in advanced human cirrhosis [13]. This led to the concept that decreased hepatic NO bioavailability in case of cirrhosis is due to decreased NO synthesis [7,9,11–13]. The contribution of nNOS and iNOS to portal hypertension is not well studied [3]. In the present study, we wanted to know which NOS isoform was the most abundant in rat livers in normal conditions and in two different models of portal hypertension: prehepatic portal hypertension and  $CCl_4$  cirrhosis.

Furthermore, the reason of decreased hepatic NO bioavailability in case of cirrhosis is not yet elucidated. One of the inhibitors of eNOS catalytic activity is caveolin-1 [14], whereas endothelin-1 counteracts the vasodilating effect of NO via endothelin-A receptors [1,3,5]. Finally, NO can be scavenged by  $O_2^-$  [1,15] and superoxide dismutase (SOD) catalyses  $O_2^-$  breakdown [15,16]. Because SOD and NO compete for  $O_2^-$ , SOD can be regarded as a "NO sparing" enzyme [17,18] (Fig. 1). This finding is relevant not only in the context of oxidative stress in cirrhotic livers. It also concerns eNOS itself, because eNOS can synthesise both NO and  $O_2^-$  [18,19] (Fig. 1). Hence, a balanced hepatic production of NO and  $O_2^-$  has to exist under physiological circumstances [19]. In the present study, we measured hepatic levels of caveolin-1 mRNA, endothelin-1 mRNA and SOD activity to find whether differences exist between healthy controls and two portal hypertensive models.

## Results

### Western blots of eNOS, iNOS and nNOS

The eNOS was the only NOS isoform detected in livers of all groups. The amount of eNOS protein in liver homogenates was similar in normal and PPVL rats (Fig. 2A), but was lower in  $CCl_4$  cirrhotic livers (Fig. 2A), although some overlap existed with healthy controls. This is in accordance with the variable severity of the cirrhosis in this model. The iNOS protein content was below the limit of detection in livers of healthy controls and the two groups with portal hypertension (Fig. 2B). The nNOS protein was

not detected in any liver homogenate (Fig. 2C). In Western blots of iNOS and nNOS but not in those of eNOS, some atypical bands of smaller proteins were observed (data not shown).

### Hepatic mRNA levels of caveolin-1 and endothelin-1

A large variation of caveolin-1 mRNA values was present in all groups. Levels in the two portal hypertensive groups were not significantly different from healthy control values (Table 2).

Levels of endothelin-1 mRNA in the PPVL group were comparable to those of healthy control rats (Table 2), but values of  $CCl_4$  cirrhotic livers were significantly and approximately 40-fold higher ( $p < 0.05$  vs controls) (Table 2).

### Hepatic SOD activity

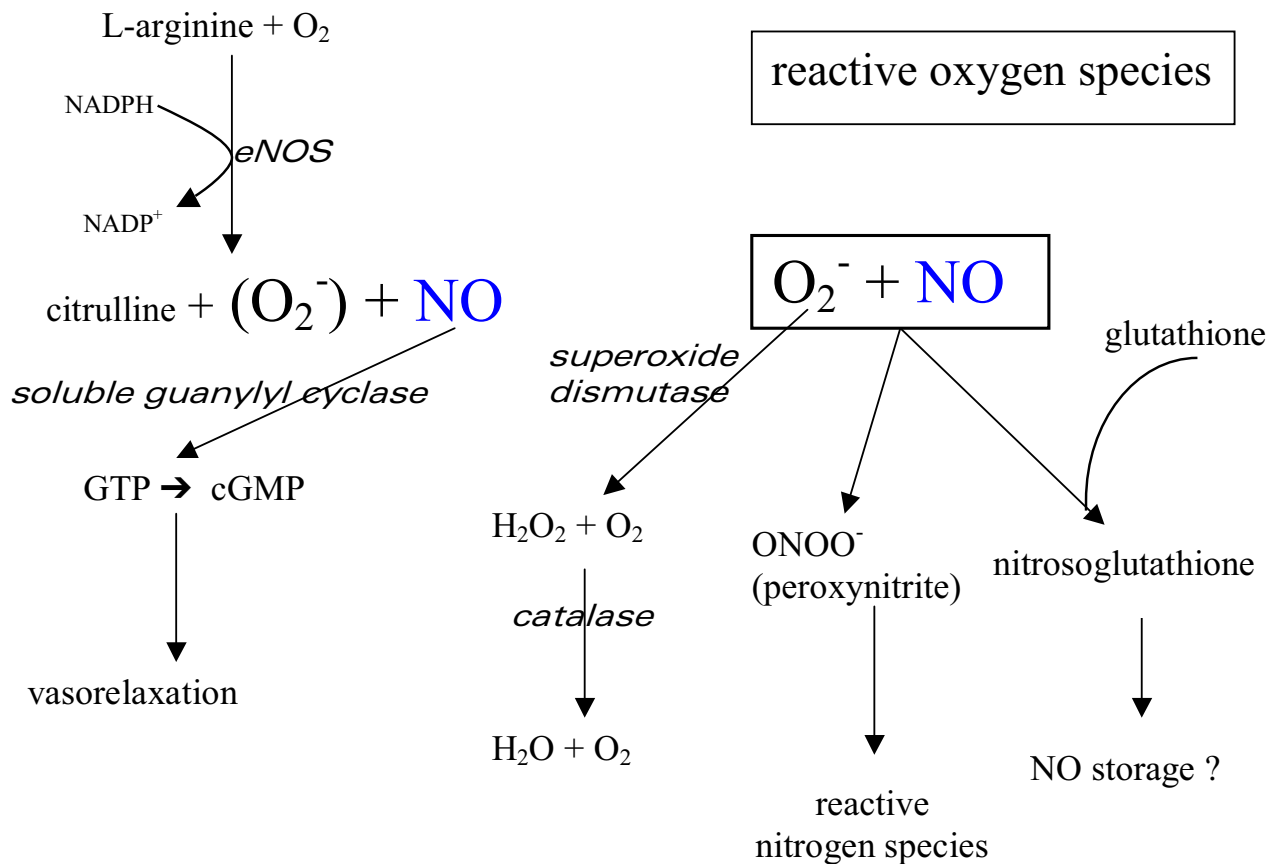
SOD activity in liver homogenates of healthy controls was 15 (7) U/mg protein and it was 14 (3) U/mg protein in PPVL rats (Table 2). In  $CCl_4$  cirrhotic livers, SOD activity was significantly reduced to 10 (3) U/mg protein ( $p < 0.05$  vs normal livers) (Table 2).

### Hepatic malondialdehyde levels

Malondialdehyde, a marker of lipid peroxidation, ranged in normal livers from 2 to 20 pmol/mg liver (median 15) and similar values were measured in PPVL rats. In  $CCl_4$  cirrhotic livers, malondialdehyde levels were significantly elevated, with a median of 26 pmol/mg liver (range 6 to 130) ( $p < 0.05$  vs normal livers) (Table 2).

## Discussion

Endothelial cells are the only liver cell type that expresses eNOS [7–10] in normal and pathological conditions. In cirrhotic livers, endothelial dysfunction results in reduced eNOS activity in rat [7,9,11,12] and man [13]. A decreased bioavailability of the vasodilator NO favours vasoconstriction of liver sinusoids, especially in the presence of enhanced endothelin-1, a strong vasoconstrictor [1–3],[20–22]. NO can be produced by 3 NOS isoforms [18]. Furthermore, NO might be consumed by reactive oxygen species before it exerts vasorelaxation, as has been documented in extrahepatic vessels [17–19,23,24] (Fig. 1). In the present study, eNOS protein was the solely detected NOS isoform in liver of normal rats and of PPVL and  $CCl_4$  cirrhotic rats (Fig. 2). The eNOS is derived from endothelial cells in various vascular structures inside the liver [7,9,11]. In our search for other NOS isoforms, we could not demonstrate hepatic iNOS in any of the 3 groups (Fig. 2B), which is in agreement with other studies in normal [25,26] and  $CCl_4$  cirrhotic livers [7,9,12]. Following LPS injection [27,28], hepatic iNOS could be detected (Fig. 2B). It can thus be concluded that iNOS is not contributing to portal hypertension in these two rat models. Al-

**Figure 1**

Proposed scheme of nitric oxide (NO) and superoxide signaling. Adapted from references [18], [24] and [34]. NO is a potent vasodilator acting through activation of soluble guanylyl cyclase in vasoactive effector cells. Superoxide is able to react with NO to form reactive nitrogen species, which could not have vasodilatory effects. Superoxide dismutase competes with NO to react with superoxide. Superoxide dismutase activity leads to breakdown of superoxide and may be regarded as a "NO sparing enzyme". Glutathione and NO may lead to possible storage of NO-derivatives.

though the nNOS protein content fell below the detection limit of Western blotting in all our rats, nNOS immunostaining by others showed a dense expression around the hepatic artery and bile duct branches in the hilum of rat liver [29]. With progressive ramifications of the hepatic artery, the number of nNOS positive fibres decreases [29]. This could render nNOS undetectable (Fig. 2C) or weak [28] in parenchyma at a distance of the hilum. The issue that unknown small-size proteins sometimes stain with commercially available NOS antibodies (not shown in Figs. 2B, 2C) is discussed in detail in reference [30].

The portal vein resistance in the PPVL rat model results from the mechanical stenosis laid around the extrahepatic part of the portal vein [31]. In PPVL rats, we could not document any change in hepatic eNOS protein, endothe-

lin-1 mRNA, caveolin-1 mRNA or SOD activity. Our findings suggest that the (atrophic) parenchyma in PPVL rats is not altering portal vein resistance importantly. In portal vein tissue below this stenosis, however, increased endothelin-1 levels have been documented and administration of endothelin-A receptor antagonists lowered the pressure in the prestenotic portal vein [32].

In the CCl<sub>4</sub> cirrhotic model, eNOS activity is subnormal as reported by different groups [7-9,11]. This could be due to several causes. In our CCl<sub>4</sub> cirrhotic rats, the amount of eNOS protein itself was subnormal (Fig. 2A), which we confirmed by immunohistochemistry [8]. Others did not find such a difference [7] but this might be related to differences in rat strains, degree of cirrhotic process or of the applied techniques, *e.g.*, since they used an immunopre-

Figure 2A. eNOS Western blot

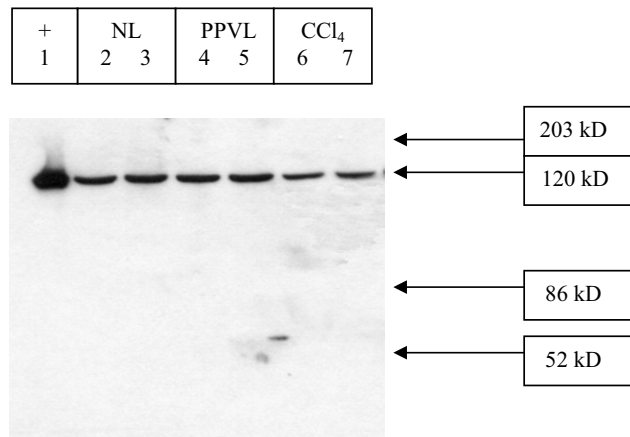


Figure 2B. iNOS Western blot

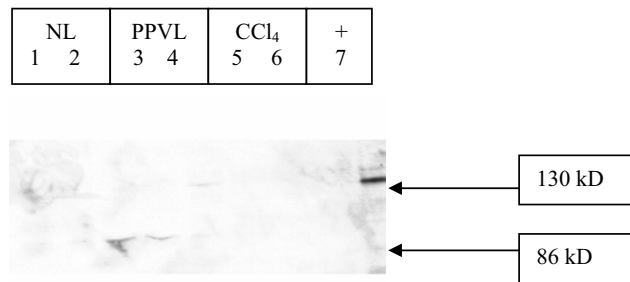
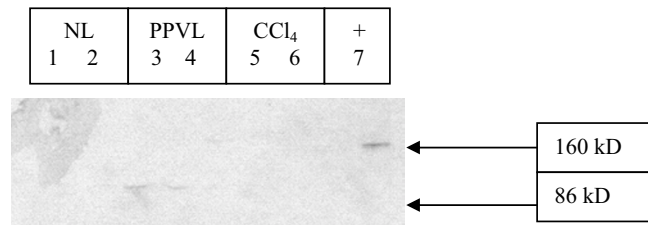


Figure 2C. nNOS Western blot



**Figure 2**

Western blots of NOS isoforms. Liver homogenates of rats were used in Western blots; see Methods section. Normal rats (NL) were compared with prehepatic portal hypertensive rats, achieved by partial portal vein ligation (PPVL) and rats with carbon tetrachloride/phenobarbital induced (CCl<sub>4</sub>) cirrhosis. **(A) Western blot of eNOS, representative of eight blots.** Lane 1, marked with +: Human endothelial cells were used as positive control. Lanes 2–3: two different NL livers. Lanes 4–5: two different PPVL livers. Lanes 6–7: two different CCl<sub>4</sub> cirrhotic livers. Prestained markers indicated the presence of 203, 120, 86, 52 kilodalton (kD) sized proteins. **(B) Western blot of iNOS, representative of two blots.** Lanes 1–2: two different NL livers. Lanes 3–4: two different PPVL livers. Lanes 5–6: two different CCl<sub>4</sub> cirrhotic livers. Lane 7, marked with +: liver from a rat previously treated with lipopolysaccharide was used as positive control for iNOS (see Methods). Prestained markers indicated the presence of 130 and 86 kilodalton (kD) sized proteins. **(C) Western blot of nNOS, representative of two blots.** Lanes 1–2: two different NL livers. Lanes 3–4: two different PPVL livers. Lanes 5–6: two different CCl<sub>4</sub> cirrhotic livers. Lane 7, marked with +: rat brain homogenate was used as positive control for nNOS (see Methods). Prestained markers indicated the presence of 130 and 86 kilodalton (kD) sized proteins.

cipitation step before protein blotting [7]. When eNOS spots on Western blots are very dense, densities do not correlate anymore with loaded protein amounts (own personal observation).

More important than eNOS protein amounts, is the understanding of eNOS enzymatic activity [18]. The eNOS activity is inhibited by protein-protein interaction of caveolin-1 with eNOS in hepatic [7,12] and extrahepatic vessels [14,18]. Others cast doubt on the very localisation of caveolin-1 in hepatic endothelial cells [33]. Caveolin-1 expression has been observed in hepatocytes, Kupffer cells and stellate cells as well [33]. We admit that in the present study we could not clarify the cellular localisation of caveolin-1 nor caveolin-1/eNOS interaction with mRNA measurements. We documented a small but not significant increase of caveolin-1 mRNA in CCl<sub>4</sub> cirrhotic livers (Table 2). If caveolin-1/eNOS interaction takes place in liver endothelial cells, our findings show that a clear-cut upregulation of caveolin-1 (as seen for endothelin-1 in Table 2) was not the case in CCl<sub>4</sub> cirrhotic liver tissue. The significant increase of endothelin-1 mRNA in this model (Table 2) is compatible with reports from other groups [20,21], where stellate cells [34] and hepatocytes [21] were identified as important endothelin-1 synthesising cells. An increased endothelin-1 synthesis together with changes in endothelin-A and B receptor density may be implicated in haemodynamic deteriorations [5,34]. A (relatively) insufficient NO production will thus allow vasoconstrictor effects.

SOD activity enhances NO bioavailability by removing O<sub>2</sub><sup>-</sup>, which otherwise could rapidly convert NO into peroxynitrite and other reactive nitrogen species [17–20,23,24,35], as is given schematically in Fig 1. The observed decrease of SOD activity might allow higher intrahepatic O<sub>2</sub><sup>-</sup> action. In the CCl<sub>4</sub> cirrhotic rat liver, we hypothesise that enhanced intrahepatic O<sub>2</sub><sup>-</sup> will further reduce the already low NO and this will further amplify vasoconstrictor supremacy [24]. The observation that exogenously administered superoxide doubled portal pressure in the isolated perfused liver of a normal rat [6] supports this hypothesis. The antioxidative defence enzyme SOD is present in different isoforms in all liver cell types [15]. Admittedly, we did not study SOD activity in particular liver cell types or in the vascular lumen (the latter regards the extracellular SOD isoform). SOD can easily interfere with NO and O<sub>2</sub><sup>-</sup> released by endothelial cells [29,36]. It is also known that activities of various SOD isoforms cannot easily be discriminated in rat liver tissue [36,37].

Presumed vasoconstrictive properties of reactive oxygen species may have consequences in chronic liver diseases with regard to the study of superoxide dismutase mimetics

as treatment for portal hypertension. A recent report showed that gene transfer of the extracellular SOD isoform was beneficial in rats with endothelial dysfunction related to arterial hypertension [38].

## Conclusions

In conclusion, we found that eNOS was the major if not the sole NOS isoform in livers of normal, PPVL and CCl<sub>4</sub> cirrhotic rats. In contrast to prehepatic portal hypertension, CCl<sub>4</sub> cirrhotic livers had decreased eNOS protein and enhanced mRNA levels of endothelin-1 but not of caveolin-1. This vasodilator/vasoconstrictor imbalance might be further aggravated by a reduced SOD activity, which could lead to enhanced superoxide-mediated inactivation of NO in liver sinusoids. The resulting low NO is unable to counteract the enhanced endothelin-1 and this results in a strong vasoconstricting effect in CCl<sub>4</sub> cirrhotic livers.

## Methods

### Animal models

Male Sprague-Dawley rats (Charles River Wiga, Germany) were used either as healthy controls (n = 14), for prehepatic portal hypertension (n = 6) or for CCl<sub>4</sub> induced cirrhosis (n = 11) (Table 1). In later experiments, male inbred Wistar rats (Animal House Leuven, Belgium) were used similarly as healthy controls (n = 9), for prehepatic portal hypertension (n = 5) or CCl<sub>4</sub> induced cirrhosis (n = 9) (Table 2). Prehepatic portal hypertension was achieved by partial portal vein ligation (PPVL) [31] and haemodynamic measurements were carried out 2 weeks later. CCl<sub>4</sub> induced cirrhosis was obtained by 12 weekly inhalations (Table 1) or ingestion (Table 2) of the hepatotoxin CCl<sub>4</sub>, together with phenobarbital 350 mg/l in the drinking water [39]. Rats were studied 2 weeks after the last CCl<sub>4</sub> administration. Under pentobarbital anaesthesia (50 mg/kg intraperitoneally), portal venous pressure was measured in all rats, the liver was removed and 2 g of liver tissue were homogenised in 8 ml ice-cold buffer I consisting of 250 mM sucrose, 5 mM MgCl<sub>2</sub>·6H<sub>2</sub>O and 50 mM Tris/HCl pH 7.4. Homogenates were divided into aliquots and stored at -20°C until further processing. A small slice of liver tissue was put in guanidinium buffer on ice for 30 minutes, snap frozen in liquid nitrogen and stored at -80°C until further processing. Additionally, a small liver sample was fixed and used for haematoxylin-eosin stained paraffin-embedded sections; only those CCl<sub>4</sub> rats with micronodular cirrhosis were maintained for analysis.

### Western blotting for nNOS, iNOS and eNOS

SDS/PAGE 7.5 % gel electrophoresis was run with diluted homogenates containing 30 µg of protein and with marker proteins (Sigma, St. Louis, USA) including a 120 kD protein, *E. coli* β-galactosidase. All protein concentrations were measured using the Bradford method (Bio-Rad Labs,

**Table 1: Livers used for NOS Western blots. Characteristics of normal and two types of portal hypertensive rats whose livers were used for the detection of NOS isoforms by Western blot in Fig. 2. Data are expressed as mean (SD).**

	Normal (n = 14)	PPVL (n = 6)	CCl <sub>4</sub> cirrhosis (n = 11)
Body weight (g)	478 (82)	360 (27)**	520 (70)
Liver weight (g)	15.4 (3.4)	10.3 (2.0)**	15.8 (6.0)
PVP (mm Hg)	8.1 (1.9)	11.3 (1.7)*	14.5 (2.7)**

\* p < 0.05 and \*\* p < 0.01 as compared to normal group. PPVL: partial portal vein ligation (= model of prehepatic portal hypertension). PVP: portal venous pressure. CCl<sub>4</sub> cirrhosis: carbon tetrachloride induced cirrhosis.

**Table 2: eNOS related parameters in rat livers: caveolin-1, endothelin-1, as well as SOD total activity and malondialdehyde levels.**

	Normal (n = 9)	PPVL (n = 5)	CCl <sub>4</sub> cirrhosis (n = 9)
Body weight (g)	272 (10)	280 (13)	355 (58)*
Liver weight (g)	10.4 (0.8)	9.7 (0.9)	14.8 (5.6)
Portal venous pressure (mm Hg)	5.0 (1.1)	9.9 (1.5)*	11.0 (2.9)*
cDNA dilutions still detecting caveolin-1 by RT-PCR	128 [4–512]	32 [8–256]	384 [128–2048]
cDNA dilutions still detecting endothelin-1 by RT-PCR	8 [0–64]	13 [2–144]	310 [16–512]*
SOD total activity (U/mg protein)	15 (7)	14 (3)	10 (3)*
Malondialdehyde (pmol/mg liver)	15 [2–20]	8 [4–16]	26 [6–130]*

\* p < 0.05 as compared with normal group. PPVL: partial portal vein ligation (= model of prehepatic portal hypertension). CCl<sub>4</sub> cirrhosis: carbon tetrachloride induced cirrhosis. Livers of normal and two types of portal hypertensive rats used for the study of eNOS related parameters: caveolin-1 and endothelin-1 as well as superoxide dismutase (SOD) activity and malondialdehyde levels. For cDNA dilutions see Methods. Data are expressed as mean (SD) for normally distributed data or median [range] for not normally distributed data.

Hemel Hempstead, UK) and with bovine serum albumin as standards. As positive controls were taken: a lysate of human aorta endothelial cells (Transduction Labs, Lexington, USA) for eNOS; a homogenate of rat brains for nNOS; and a liver of a rat given LPS 800 µg/kg IV (Sigma, St. Louis, USA) 6 hours before harvesting for iNOS. Samples of both portal hypertensive conditions and healthy controls were run simultaneously on the same gel. Two liver homogenate samples per liver were run. After blotting on a nitrocellulose membrane, blots were blocked overnight at 4°C. Blots were incubated for 2 hours with a mouse monoclonal antibody respectively against eNOS, nNOS or iNOS (Transduction Laboratories, Lexington, USA), dissolved at 1:1000 in buffer II (10 mM Tris-HCl pH 7.6, 0.1 M NaCl, Tween-20 at 0.1 %). Subsequently, blots were incubated with sheep anti-mouse IgG, horseradish peroxidase-labelled (Amersham, Bucks, UK), at 1:3000 diluted in 5 % skimmed milk powder blocking solution for one hour. After washing, detection reagents (ECL Western blotting system, Amersham, Bucks, UK) were added and blots were shortly exposed to an autoradiography film (Nen Life Science Products, Boston, USA) (Figs. 2-4) [8]. To check for adequate protein loading and

blotting, all blots were stained afterwards with Ponceau S red dye (Sigma, St. Louis, USA).

#### **Hepatic mRNA levels of caveolin-1 and endothelin-1 with RT-PCR**

Hepatic caveolin-1 or endothelin-1 mRNA levels were assessed semi-quantitatively with RT-PCR, using serial dilutions of cDNA as a measure for the amount of specific mRNA in the different livers. Briefly, total RNA was extracted in a single step procedure [40]. The precipitated RNA was dissolved in 20-µl DEPC-treated water and the concentration was measured using the Ribogreen RNA quantitation kit (Molecular Probes, Eugene, USA), with ribosomal RNA as standard. One µg of this RNA was used for cDNA synthesis with M-MLV reverse transcriptase (GibcoBRL, Life Technologies, Merelbeke, Belgium) and random primers (Amersham Pharmacia Biotech, Little Chalfont, UK) in a volume of 20 µl, 1 hour at 37°C. The reaction was stopped by heating in boiling water for 1 min.

The PCR primer set used for the detection of rat caveolin-1 mRNA (access number Z 46614) was:

P18: 5'-CCG.GGA.ACA.GGG.CAA.CAT.CTA.CAA.GCC-3'  
positions 82–108;

M28: 5'-GCC.GTC.RAA.ACT.GTG.TGT.CCC.TTC.TGG-3'  
positions 251–277, resulting in a fragment of 195 bp.  
Note that R stands for [A,G].

The PCR primer set used for the detection of rat endothelin-1 mRNA (preproendothelin-1) (access number NM 612548) was:

P1: 5'-CAG.GTC.CAA.GCG.TTG.CTC.CTG.CTC.CTC.C-3'  
positions 328–355;

M2: 5'-CAC.CAC.GGG.GCT.CTG.TAG.TCA.ATG.TGC.TCG-3'  
positions 782–811, resulting in a fragment of 483 bp.

PCR determinations were performed on a dilution series of each sample. The first sample contained cDNA equivalent to 0.25 µg total RNA; each following sample was diluted to half the concentration of the previous one. The PCR mixture contained 5 µl of cDNA solution, 6.25 pmol of each primer, 0.2 µM of each dNTP, 1 U of Taq DNA polymerase adjusted by PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl<sub>2</sub> and 0.01 % gelatin) in a final volume of 50 µl. Samples were overlaid with 100 µl mineral oil. PCR conditions were identical for both primer sets: denaturing 5 min at 95°C; 45 cycles of 1 min at 95°C, 45 sec at 58°C and 45 sec at 72°C; and a final step for 5 min at 72°C, after which the samples were stored at 4°C. Samples were analysed on a 2 % agarose gel. Samples of both portal hypertensive conditions and healthy controls were separated simultaneously on the same gel. The majority of the samples were amplified and analysed at least in duplicate. Results are given as the highest dilution that gave a positive signal on the gel (Table 2).

### Hepatic superoxide dismutase (SOD) activity

SOD activity in liver homogenates in buffer I was diluted 400 times with buffer I and measured with a RANSOD kit (Randox Laboratories, Crumlin, UK) according to the manufacturer's instructions. In brief, xanthine oxidase generates O<sub>2</sub><sup>-</sup>, which reacts with a chromogen to form a red formazan dye that is photometrically quantified. One SOD unit was defined as 55 % inhibition of dye formation. SOD activity was expressed as U/mg protein (Table 2).

### Hepatic malondialdehyde levels

Determination of malondialdehyde was performed as published before [41]. Briefly, liver homogenates in buffer I were run together with 1,1,3,3-tetraethoxypropane as standard and buffer I as blanks. After the addition of phosphoric acid and thiobarbituric acid, samples were heated

at 80°C for 15 min. Longer and more intense heating created too much interference of sucrose [42] (own personal observation). Ice cooled reaction products were further separated by high performance liquid chromatography-reverse phase technique [41]. The latter step is necessary to eliminate other substances that had reacted with thiobarbituric acid [43]. Results are expressed as pmol malondialdehyde/mg liver wet weight (Table 2).

### Statistical analysis

Data are given as mean (SD) or as median [range] for respectively normally and non-normally distributed data. We made comparisons of the 3 groups (normal, PPVL, CCl<sub>4</sub> cirrhosis) by one-way-analysis of variance in case of normally distributed data with equal variances. If other cases, we used analysis of variance-on-ranks, where the sum of ranks of each group was compared. When significant differences between groups means were found, the Scheffe's post hoc test was performed to identify the groups. Significance level was always taken at  $\alpha = 0.05$ . Statistical analyses were carried out with Sigma STAT 2.0 (Jandel Corporation, San Rafael, USA).

### Ethical committee

Written approval for the present experiments was obtained from the Ethical Committees for Animal Research of the Catholic University of Leuven, Belgium, and of the University of Berne, Switzerland.

### Authors' contributions

MV and JV carried out this study together with the statistical analysis. FN participated in the design of the study. JF and JR designed and co-ordinated the study.

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