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Hyperdynamic circulation in patients with cirrhosis: direct measurement of nitric oxide levels in hepatic and portal veins

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Background/Aims: **Peripheral vasodilation represents the main vascular dysfunction associated with the hyperdynamic circulation of liver cirrhosis. This study was intended to measure directly regional and systemic levels of nitric oxide, a potent vasorelaxing mediator, in order to assess its role in the development of hemodynamic changes of cirrhosis.**

Methods: **We compared nitric oxide levels in the splanchnic and systemic circulation of 25 patients with cirrhosis undergoing transjugular intrahepatic portosystemic stent shunt and in the hepatic vein and peripheral blood of 10 patients without cirrhosis submitted to venous catheterization. Nitric oxide levels were measured through electron paramagnetic resonance spectroscopy as nitrosyl–hemoglobin complexes.**

Results: **Significantly higher nitric oxide levels were calculated in patients with cirrhosis with respect to controls, both in the peripheral and hepatic veins. In patients with cirrhosis, nitric oxide levels in the portal vein (3.44±2.17, expressed in arbitrary units) were higher than in the systemic circulation (1.89±1.15), but lower than in the hepatic vein (4.75±2.53; $p<0.001$ by variance analysis).**

Conclusions: **These data suggest that nitric oxide synthetic pathway activity as well as nitric oxide release are enhanced at the level of splanchnic vasculature and, more important, in the hepatic tissue, confirming evidence of the predominant role of nitric oxide in the pathogenesis of hemodynamic changes in patients with cirrhosis with portal hypertension.**

Key words: **Electron paramagnetic resonance spectroscopy; Liver cirrhosis; Nitric oxide; Portal hypertension; D-Sorbitol clearance; Transjugular intrahepatic portosystemic stent shunt.**

HYPERDYNAMIC circulation is a common feature in patients with cirrhosis, leading to related complications such as portal hypertension, ascites, hepatorenal and hepatopulmonary syndromes, congestive gastropathy and colopathy (1,2). This condition is characterized by increased cardiac output and heart rate, low arterial blood pressure, decreased systemic peripheral vascular resistances and hyporesponsiveness to endogenous vasoconstrictors (3,4). Several studies have demonstrated the multifactorial pathogenesis of the hyperkinetic circulatory state, suggesting that both humoral and local factors may contribute to the development and the maintenance of hemodynamic changes of liver cirrhosis (1,2,4).

Among humoral factors, nitric oxide (NO), a short-lived radical synthesized from L-arginine by NO-synthase, has been proposed to play a crucial role in the hemodynamic disturbances associated with cirrhosis (5,6). In 1991, Vallance & Moncada (7) hypothesized that the increased synthesis and release of NO induced by endotoxins directly, or indirectly by cytokines, could account for the peripheral cardiovascular complications of cirrhosis.

Thus far, however, a relationship between excess NO formation and the hyperkinetic circulation has

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TABLE 1
Clinical and laboratory findings of the studied patients

Case	Sex	Age, years	Diagnosis	Child-Pugh Class/score	Ascites	AST (IU/l)	ALT (IU/l)	GGT (IU/l)	PT %
1	M	62	Alcoholic	B/7	Severe	56	10	83	80
2	M	67	Post-viral	C/12	Severe	45	27	79	38
3	M	59	Post-viral	C/11	Severe	60	41	132	39
4	F	63	Post-viral	B/8	Light	41	24	42	44
5	M	57	Alcoholic	C/11	Severe	37	17	18	73
6	F	56	Post-viral	C/12	Severe	63	30	80	32
7	F	59	Alcoholic	C/12	Moderate	41	23	50	44
8	F	54	Post-viral	B/7	Moderate	18	12	40	85
9	M	69	Post-viral	B/8	Moderate	19	25	69	71
10	M	60	Post-viral	B/9	Severe	48	41	40	57
11	F	60	Post-viral	B/8	Severe	39	25	84	76
12	M	70	Post-viral	B/9	Absent	127	152	31	69
13	F	60	Post-viral	B/9	Moderate	36	17	28	70
14	M	55	Alcoholic	C/11	Severe	63	56	195	64
15	M	62	Alcoholic	B/7	Absent	32	21	122	75
16	M	57	Post-viral	C/11	Severe	135	85	79	35
17	M	47	Alcoholic	B/9	Absent	127	77	154	66
18	M	45	Alcoholic	C/11	Moderate	28	11	183	36
19	M	65	Post-viral	B/9	Absent	49	41	30	53
20	M	71	Post-viral	B/8	Light	42	25	51	63
21	M	56	Cryptogenetic	B/8	Absent	23	36	33	73
22	M	69	Alcoholic	B/8	Absent	47	28	93	68
23	F	64	Post-viral	C/13	Severe	31	81	20	44
24	F	61	Post-viral	B/9	Moderate	47	25	60	75
25	M	59	Alcoholic	B/9	Severe	19	37	141	59

not been adequately established, due to the difficulties encountered in the direct measurement of the NO amounts circulating in the blood and at the level of cellular sources (8). In view of the uncertainties still existing about NO involvement in the hyperdynamic circulation (9), we directly measured NO levels using electron paramagnetic resonance (EPR) spectroscopy in blood samples obtained from the portal vein, a hepatic vein and the systemic circulation of patients with cirrhosis undergoing transjugular intrahepatic portosystemic stent shunt (TIPS). Estimated NO levels were compared with those calculated in the hepatic vein and peripheral blood of patients without cirrhosis submitted to venous catheterization for varicocele sclerotherapy.

Materials and Methods

Patients

Twenty-five patients (17 males and 8 females; aged 45–71 years, mean age 57.8 years) with cirrhosis undergoing TIPS were studied. Fifteen patients had post-viral cirrhosis, nine alcoholic cirrhosis and one cryptogenetic cirrhosis. The diagnosis of liver cirrhosis was established on the basis of clinical, analytical and ultrasonographic findings in all the patients and confirmed with liver biopsy in 18 of them, while in the remaining cases this procedure was contraindicated. Baseline data and Child-Pugh scores of the patients studied are shown in Table 1. Indications for TIPS application included esophageal varices with failure of sclerotherapy and/or medical treatment in 11 cases (six with massive gastrointestinal bleeding), untreatable ascites in six cases, and both ascites and esophageal varices in eight cases. Hemodynamic parameters considered included blood pressure (mean systolic values 116±11.6 mmHg and mean diastolic values 66.8±8.4 mmHg, respectively) and heart rate (mean values 90.1±11.2 beats/min).

Ten normotensive male patients without cirrhosis (mean age 29.1, range 19–39 years old) undergoing venous catheterization for curative sclerotherapy of varicocele were considered as controls.

None of the studied subjects had clinical or analytical evidence of bacterial infection, or was an habit-

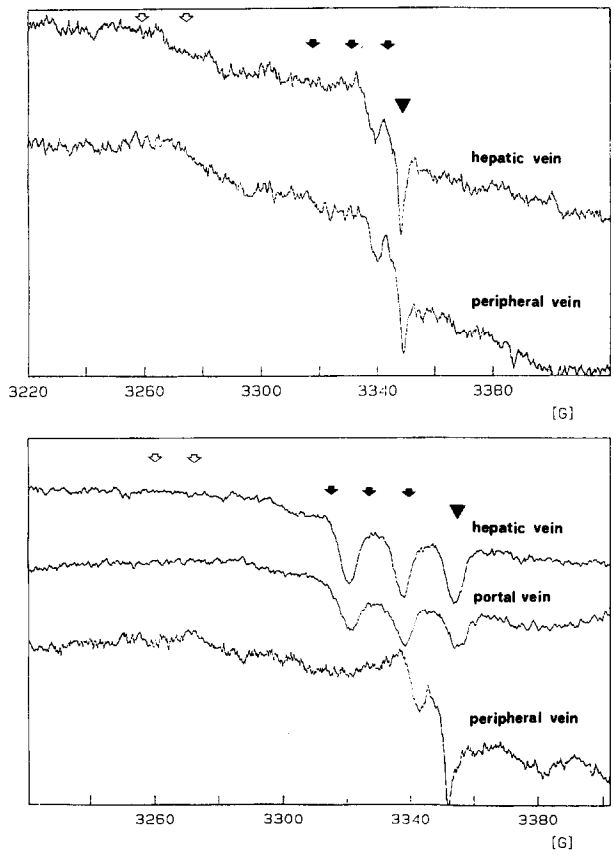

Fig. 1. EPR spectra of whole blood nitrosyl–hemoglobin complexes detected in a control subject (upper panel) and in a patient with cirrhosis (lower panel). Signals are characterized on the basis of triplet features centered at a spectroscopic splitting factor (g factor) value of 2.015 with 16.5 gauss line widths (arrows). Results were calculated by double integration of these spectra and expressed as arbitrary units. Two additional peaks at g=2.08 *and* g=2.07 *(open arrows) appeared in some samples, which referred to NO binding to heme iron in hexacoordinate state of hemoglobin tetramer. The excursion at* g=2.00 *(arrowhead) is due to the presence of nonspecific unpaired electrons in biological samples. EPR analysis was conducted at 77 K and instrument settings were as follows: 9.40 GHz microwave frequency, 10 mW microwave power, 1 gauss modulation amplitude, 163.84 ms time constant.*

ual smoker. All the patients gave their informed consent and the study was performed according to the 1975 Declaration of Helsinki ethical guidelines. In patients submitted to TIPS, 1–2 ml heparinized blood samples were drawn from the jugular vein, the hepatic vein and the portal vein during the procedure, performed as previously described (10). Briefly, after mild sedation and analgesic premedication, a 16 G needle was advanced transjugularly in a catheter into a hepatic vein, generally the right. Subsequently, an intrahepatic branch of the portal vein was punctured and the shunt was established by the implantation of Palmaz (Johnson and Johnson Interventional Systems, Warren, NJ) or Memotherm stent (Angiomed-Bard, Karlsruhe, Germany). Before stent application, caval and portal vein pressures were directly measured by the catheter.

In control subjects, blood specimens were drawn at the level of the jugular and hepatic veins through venous catheter.

EPR detection of nitrosyl–hemoglobin complexes

Blood samples were taken before stent opening on heparinized vacuum tubes and immediately placed in ice. After deoxygenation by bubbling through argon gas for 15–30 min, samples were transferred to 3-mm inner diameter quartz tubes sealed with a rubber septum and frozen in liquid nitrogen at 77K. Sampling tubes were stored at this temperature until EPR analysis was performed. An average time of 1–2 weeks elapsed between blood sampling and EPR processing. Preliminary experiments have shown the stability of EPR signals from samples stored in liquid nitrogen up to 6 weeks.

EPR observations were made at liquid nitrogen temperature on frozen probes and spectra were recorded on a Bruker ESP-300E spectrometer (X-band, 100 kHz field modulation). A computer system online with the EPR instrument allowed measurements of NO-hemoglobin levels, calculated as double integrals of first-derivative spectra and expressed as arbitrary units.

The technique of EPR spectroscopy allows measurement and quantification of molecules with unpaired electrons such as free radicals (11). In the EPR analysis, the sample is inserted in a magnetic field and is irradiated with electromagnetic energy typically in the microwave frequency range. Varying the magnetic field, the absorption of microwave energy is recorded as a function of magnetic field strength. For any given resonance absorption there is a characteristic g value, which is calculated using the ratio of the applied microwave frequency to the measured magnetic field strength at which resonance is observed. The position of absorption bands is indicated by this g factor, or spectroscopic splitting factor, which varies depending on the electronic configuration of the radical.

Due to the unpaired electron residing in a π–molecular orbital on the N-O triple bond, NO is paramagnetic, i.e. capable of interaction with a magnetic field. However, because of its extremely short half-life and strong reactivity, NO is detectable by EPR only if trapped and stabilized (11,12). Deoxyhemo-

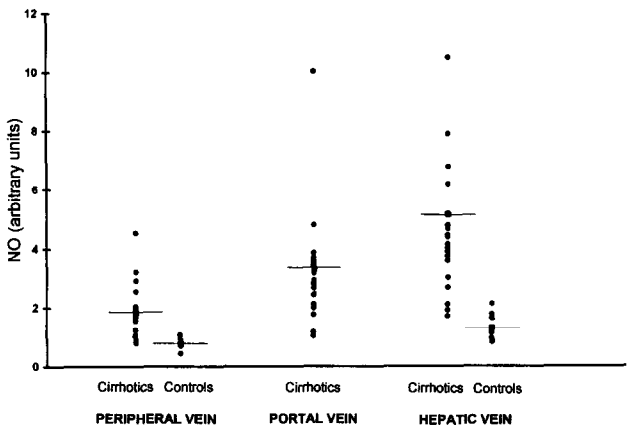

Fig. 2. Nitric oxide levels measured in a peripheral vein, in the portal vein and in a hepatic vein of 25 patients with cirrhosis submitted to TIPS application, as compared to those detected in a peripheral vein and in a hepatic vein of 10 control subjects undergoing venous catheterization for varicocele sclerotherapy. Individual and mean values are indicated. The levels were significantly higher in patients with cirrhosis than in the control group (p<0.05 and p<0.005 in the systemic vein and hepatic vein, respectively). Overall significance by analysis of variance of the difference among the three determinations in patients with cirrhosis was observed (p<0.001).

globin has been proposed as a naturally occurring trap for NO, leading to the relatively stable species nitrosyl–hemoglobin (13).

The paramagnetism of these complexes and their easy detection by conventional EPR spectroscopy in frozen solution at 77 K have prompted the use of this sophisticated technique for detecting and quantifying whole blood NO–hemoglobin derivatives, which have been extensively studied as a suitable marker for the generation of NO both in *in vivo* and *in vitro* biological models (14).

Typical EPR spectra attributable to NO-hemoglobin complexes detected in one patient with cirrhosis and in one control subject of our study population are shown in Fig. 1. The signal is characterized on the basis of a distinct triplet pattern centered at $g=2.013$–2.018 and provides the mean for the unequivocal identification of NO circulating bound to deoxyhemoglobin. NO derivatives of hemoglobin in venous blood arise from the kinetic heterogeneity of the NO binding to α and β subunits of hemoglobin (13). Under exposure to NO, the hemoglobin tetramer forms a mixture of nitrosylated valency hybrids including species that can be defined precisely by EPR spectroscopy (15). In particular, the three-line feature at $g=2.015$ shown in Fig. 1 results from NO–hemoglobin complexes in which NO binds to the heme iron of the α subunits in the ferrous and pentacoordinate state ("deoxy-like" or "low-affinity"). This three-line hyperfine structure of NO–hemoglobin has been recently shown to appear much more marked in venous blood than in arterial blood and to intensify linearly when O_2 saturation of hemoglobin decreases (15).

In each case, the magnitude of the EPR signals was considered to be proportional to the amount of NO-hemoglobin complexes. In addition, the hepatic clearance of D-sorbitol was measured in 15 patients with cirrhosis of our study population as an index of functional hepatic plasma flow, according to the previously described method (16).

The data were analyzed with the Student's paired *t*-test, the analysis of variance and linear regression. A value of $p<0.05$ was accepted for statistical significance. All results are expressed as mean±standard deviation.

Results

In patients with cirrhosis, greater amounts of NO–hemoglobin complexes were found in the hepatic vein than in the portal vein and systemic circulation (4.75±2.53 vs. 3.44±2.17 and 1.89±1.15, respectively; $p<0.001$). NO levels were found to be significantly higher in the portal vein than in the systemic circulation ($p=0.003$). Interestingly, the mean values of portal NO–hemoglobin were significantly lower than those calculated in the hepatic veins ($p<0.05$) (Fig. 2). There was no significant correlation between Child-Pugh scores and NO levels in any of the three venous specimens, nor were mean NO–hemoglobin concentrations in the peripheral and portal blood related to the values of portal vein pressure (31.6±5.7 mmHg) and portocaval gradient (24.4±4.5 mmHg), respectively. It is worth noting that hepatic clearance of D-sorbitol significantly correlated with systemic NO levels only in Child B patients ($p<0.05$; $r=-0.853$), whereas the statistical correlation was absent when Child C patients were analyzed as a group as well as when all patients with cirrhosis (Child B+Child C) were considered as a whole (Fig. 3). No significant correlation was otherwise found between nitrosyl–hemoglobin concentrations and parameters of liver cytolysis, synthesis and cholestasis. Furthermore, the presence and the degree of ascites did not influence NO levels.

In the systemic circulation of patients with cirrhosis the measured NO levels were higher than in control subjects (1.89±1.15 vs. 0.79±0.28; $p<0.05$) and significantly higher NO levels were detected in the hepatic vein of patients with cirrhosis with respect to controls (4.75±2.53 vs. 1.27±0.47; $p<0.005$).

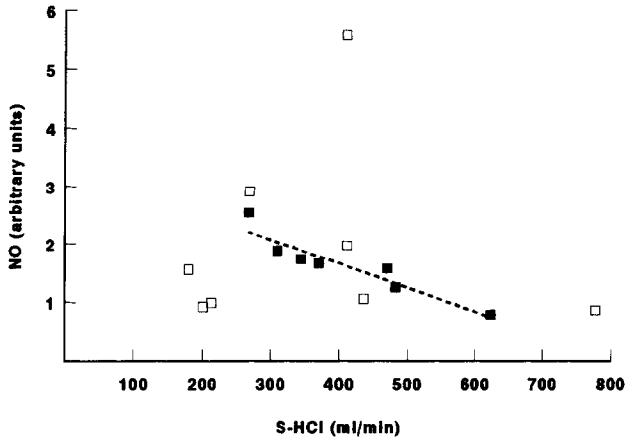

Fig. 3. Correlation between nitric oxide levels and hepatic clearance of D-sorbitol (S-HCl) in Child B patients with cirrhosis (n = 7), closed symbols, and Child C patients with cirhosis (n = 8), open symbols. Statistical significance was achieved only for Child B cases (dotted line, r = –0.853; p = 0.015)

We can exclude any interference due to different age of the two studied groups at least for results on peripheral blood, since our previous observations on a large cohort of normal healthy volunteers clearly demonstrated an age-independent behavior of NO levels (data not shown).

Discussion

Evidence currently available implicating the NO in the pathogenesis of hemodynamic disturbances of cirrhosis is indirect and derives from investigations using specific inhibitors of NO synthesis (17) or based on the measurement of NO metabolites (18). This study was intended to assess directly both regional and systemic NO levels in a group of patients with cirrhosis, taking advantage of EPR spectroscopy and the unique opportunity offered by TIPS application to collect blood samples at the level of different components of the liver vascular system. In fact, TIPS procedure provides the means for studying relative contributions of splanchnic as well as hepatic vasculature to the release and elimination of NO in cirrhotics.

Elevated NO levels detected in the portal blood of patients with cirrhosis may suggest that NO release occurs at a higher degree in the splanchnic circulation. At this level, upregulation of the L-arginine/nitric oxide pathway in endothelial as well as smooth muscle cells exposed to large amounts of endotoxin and/or exotoxins (19) is likely to induce excessive NO production (20). Our finding of a further increase in NO levels in the hepatic veins is attributable to a hyperfunctioning NO metabolic route in the hepatic tissue. Hepatocytes, which produce NO after *in vivo* exposure to endotoxin, are generally considered the major source of this mediator in the liver (21,22). However, Ito or perisinusoidal cells, Kupffer cells, infiltrating macrophages as well as other immune cells, and endothelial cells, increase in number during endotoxemia (20,22). All these cells may play a significant role in localized NO production in the liver during endotoxin-induced responses (23). Abnormal NO synthesis and release, in turn, may alter the activities and functions of liver parenchymal and non-parenchymal cell types, as a consequence of toxic properties of NO in excess, thereby leading to tissue damage and altering hepatic blood flow regulation (24).

From our findings, the possibility that, rather than a cause, activation of NO production was the consequence of the circulatory dysfunction and activation of other vasoactive systems in advanced liver disease cannot be excluded (25,26). The latter hypothesis is supported by the fact that stimulation of NO release is mediated also by shear stress and pulsatile flow, as well as by a series of hormonal substances, including prostanoids, endothelins, angiotensin II, vasopressin and norepinephrine (27). However, our experimental data are in agreement with the view that systemic and splanchnic vasodilation seen in cirrhosis with portal hypertension is due to increased NO synthetic pathway activity (7). It has been proposed that overproduction of NO may also account for vascular hyporeactivity to endogenous vasoconstrictors such as angiotensin II, vasopressin, norepinephrine, endothelin-1, with enhanced vascular response to NO-dependent vasodilators (4, 26).

Additional complexity in the study of NO formation in cirrhosis and portal hypertension arises from observations of the acute and chronic effects of nitrovasodilators on portal hemodynamics in cirrhosis (28). These drugs are known to act via release of NO and have been shown to reduce portal pressure by a variety of mechanisms. These include a decrease in portal venous inflow mediated by splanchnic vasoconstriction secondary to a reduction in cardiac preload or secondary to systemic hypotension (25). Alternatively, exogenous NO may act directly to reduce resistance at a portal-collateral or sinusoidal level (26). The occurrence of an upregulated NO synthetic activity and ongoing release of NO, as shown in our study through direct measurement of NO levels, both in the systemic and, even more important, in the portal and hepatic veins, might contribute to the imbalance between portal venous inflow and the compliance of portal collateral vasculature. The absence

of any statistical correlation between NO levels and considered hemodynamic parameters and liver functional tests according to our results clearly indicates that, in addition to NO, other mechanisms – functional, structural, or both – should be involved in the hemodynamic changes during liver diseases. Further information on the role of NO in portal hypertension of cirrhosis will derive from coupling NO direct measurement by EPR technique with the application of both dynamic and static liver functional assessment. Interestingly, in Child B patients of our study population the negative correlation existing between peripheral blood NO levels and hepatic clearance of D-sorbitol, a reliable index of functional hepatic plasma flow, supports the view that vasodilation could be an expression of both anatomical (portal blood escaping through portal systemic collaterals) and functional (liver cell necrosis, intrahepatic shunts) liver failure.

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