Plasma Nitrate/Nitrite and Endothelin-1 in Patients With Liver Cirrhosis

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The aims of this study were to examine the plasma nitrate/nitrite (NOx; two end products of nitric oxide metabolism) and endothelin-1 (ET-1) concentrations in patients with liver cirrhosis, and to investigate whether there is a relationship between these two vasoactive parameters and the course of disease. Twenty-eight patients with liver cirrhosis (11 HBV-related, four HCV-related, four alcohol-related, and nine with idiopathic etiology) and 25 healthy subjects (controls) were included in the study. The venous plasma concentrations of NOx and ET-1 were significantly higher ($P < 0.01$ and $P < 0.001$) in the patients with cirrhosis than in the controls. A significant increase in ET-1 was observed in the Child B subgroup vs. Child A ($P < 0.05$), and in the Child C subgroup vs. either subgroup A or B ($P < 0.05$). There were no statistical differences between study subgroups (Child A–C) in the mean of NOx values. Plasma NOx and ET-1 were significantly increased in

patients with ascites compared to those without ascites ($P < 0.05$ and $P < 0.01$). Increased nitric oxide synthesis may be a compensation mechanism against endothelial injury. The highest ET-1 levels in Child C and moderately increased ET-1 levels in Child B, and the lower increase of ET-1 levels in Child A patients suggest that plasma ET-1 increases with the progression of the disease. The fact that NOx and ET-1 levels were higher in patients with decompensated cirrhosis (patients with ascites) than in those with compensated cirrhosis (patients without ascites), and the presence of a strong correlation between ET-1, NOx, and the degree of varices, supports the suggestion that there is a relationship between NOx, ET-1, and portal hypertension. Our study demonstrates that increased ET and nitric oxide metabolism is associated with the hemodynamic alterations induced by portal hypertension. J. Clin. Lab. Anal. 19:177–181, 2005.

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INTRODUCTION

Portal hypertension is a common clinical syndrome associated with chronic liver disease. Because of the altered hemodynamic profile in portal hypertension, dramatic changes occur in the mechanical forces, which may play a pivotal role in controlling endothelial and vascular smooth muscle cell signaling, structure, and function in cirrhotics. Endothelin (ET) and nitric oxide are two of the most powerful vasoactive substances released from the endothelium in response to both humoral and mechanical stimuli that can profoundly affect both the function and structure of the vascular smooth muscle (1).

Nitric oxide is an ubiquitous messenger that is involved in a number of cellular processes, such as

neurotransmission, immune regulation, and vascular homeostasis (2). Nitric oxide causes vasodilatation, inhibits platelet aggregation, and suppresses smooth muscle proliferation (3). The continuous release of nitric oxide from the endothelium maintains basal vascular tone, and alterations in the release of nitric oxide allow the autoregulation of blood flow (4). Nitric oxide is synthesized from its substrate L-arginine by enzymes

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called nitric oxide synthases (NOS), which are either constitutive or inducible. Inducible NOS (iNOS) is produced in macrophages and other cell types (e.g., hepatocytes) after stimulation by cytokines, endotoxin, and other substances. The constitutive isoforms of NOS are localized mainly at neuronal cells (nNOS) and endothelial cells (eNOS) (5,6). Endothelium-derived nitric oxide has been reported to be an important modulator of vascular tone. It diffuses rapidly into the surrounding smooth muscle and causes relaxation by increasing the production of cGMP in these cells (7). Increased levels of plasma nitrate/nitrite (NOx; two end products of nitric oxide metabolism) in patients with cirrhosis have been reported (8).

ETs are members of a family of polypeptides that have potent and distinct vasoactive properties, and are synthesized in different tissues (9,10). ET-1 secretion is prompted by a variety of stimuli, including hypoxia (11), dehydration (12), endotoxin (13), shear stress (14), and baroreceptor activation (15). It is also influenced by several vasoactive substances, such as catecholamines, renin-angiotensin, atrial natriuretic peptide, arginine-vasopressin, nitric oxide, and prostacyclin (9,10). It has been hypothesized that altered activity of the ET system is involved in hemodynamic disturbances of cirrhosis (16–18). Although the relevant literature is rapidly increasing, the pathophysiology of the ET system in cirrhosis remains poorly defined. The data about the production of ET in cirrhosis are controversial. ET production has variously been shown to be reduced (19), normal (17,20,21), or elevated (18,22) compared to healthy subjects. Previous studies have reported increased nitric oxide and reduced, normal, or elevated ET levels in cirrhotic patients. Therefore, the purposes of this study were to estimate the levels of NOx and ET-1 in the plasma of patients with cirrhosis, and to investigate whether there is any relationship between these parameters, the stage of disease, the presence or absence of ascites, and the degree of portal hypertension.

MATERIALS AND METHODS

Twenty-eight patients with liver cirrhosis in the care of the Department of Internal Medicine of Istanbul Medical Faculty were enrolled in this study. The diagnosis of liver cirrhosis was established on the basis of clinical, biochemical, and ultrasonographic findings. Percutaneous liver biopsy was performed in patients without decompensated liver disease to make an accurate diagnosis of cirrhosis. All of the patients had undergone upper gastrointestinal system endoscopy to detect oesophageal varices. The control group consisted of 25 healthy subjects (15 males and 10 females, 47 ± 13

years old). The characteristics of the patients are summarized in Table 1.

Venous blood samples were collected in EDTA.K₃ tubes from the antecubital vein, 24 hr after the patients were prescribed a diet low in nitrate and nitrite (i.e., no spinach, beets, or cured meat—the most abundant sources of alimentary nitrite and nitrate) followed by an overnight fasting. The tubes were centrifuged immediately at 1,500 *g* (10 min) to remove the plasma. The plasma samples were kept at –80°C until they were used. For measurement of plasma NOx, Grisham's method was used (23). The plasma samples were thawed on ice. Plasma (100 µL) was added to 50 µL (0.5 M) N-2-hydroxyethylpiperazine-N-2ethanesulfonic acid (HEPES), 10 µL (5 mM) of the reduced form of nicotinamide adenine dinucleotidephosphate (NADPH), and 316 µL distilled water. Nitrate reductase (15 µL, 10 U/mL) was pipetted to the reaction mixture, followed by incubation at 37°C for 60 min. Then 10 µL (750 U/mL) lactate dehydrogenase (LDH), 50 µL (10 mM) 9 pyruvic acid, and 1,000 µL Griess reagent were added. After 10 min of incubation at room temperature, the absorbance were read at 543 nm against the blank. Sodium nitrate (200 µM) was used as a standard. The results were expressed in µmol/L. The intra-assay coefficient of variation (CV) was 5.2%, and the interassay CV was 10.2%. Plasma ET-1 concentrations were assigned by means of an ET (¹²⁵I) (Euro-Diagnostica AB, Ideon, Sweden) kit after extraction with Sep-Pac C18 columns (24). The measuring range of the assay was 0–125 pmol (0–250 pg/mL) and its sensitivity was 0.4 pmol/L. All measurements were performed singly. The intra-assay CV was 9.5%.

All results are expressed as the mean ± SD. We assessed the differences between the patient and control groups with Student's *t*-test. Pearson's correlation coefficient value was used as a correlation test.

RESULTS

The peripheral venous plasma concentrations of ET-1 and NOx were significantly higher ($P < 0.001$ and

TABLE 1. Clinical features of patients with liver cirrhosis

Features	Patients
Sex (male:female)	18:10
Age (years, mean ± SD)	52 ± 10
Etiology of cirrhosis (HBV:HCV:alcohol:idiopathic)	11:4:4:9
Albumin (g/dL, mean ± SD)	2.81 ± 0.55
AST (IU/L, mean ± SD)	96 ± 72
ALT (IU/L, mean ± SD)	46 ± 31
Child-Pugh (A:B:C)	10:12:6
Ascites (+):(–)	15:13
Bilirubin (mg/dL, mean ± SD)	1.93 ± 1.36
Degree of varices (1:2:3)	10:7:11

$P < 0.01$) in patients with cirrhosis compared to controls (Table 2). The reference ranges for normals were 34.3–89.9 $\mu\text{mol/L}$ for NOx, and 10.86–19.11 pmol/L for ET-1.

The cirrhotic patients were divided into Child-Pugh subgroups (Child A–C). Plasma ET-1 was 14.72 ± 3.23 pmol/L in Child A, 18.49 ± 4.31 pmol/L in Child B, and 26.42 ± 12.05 pmol/L in Child C. A significant increase in ET-1 was observed in Child B vs. Child A ($P < 0.05$), and in Child C vs. either subgroup A or B ($P < 0.05$) (Table 2). Plasma NOx levels were 70.00 ± 13.98 $\mu\text{mol/L}$ in Child A, 81.96 ± 24.34 $\mu\text{mol/L}$ in Child B, and 73.51 ± 23.94 $\mu\text{mol/L}$ in Child C. There were no significant differences in NOx levels among these subgroups.

When plasma ET-1 (22.51 ± 8.33 pmol/L) and NOx (83.74 ± 24.06 $\mu\text{mol/L}$) levels in cirrhotic patients with ascites were compared with ET-1 (14.61 ± 3.33 pmol/L) and NOx (66.81 ± 13.17 $\mu\text{mol/L}$) levels of cirrhotic patients without ascites, a significant increase was observed in the cirrhotic patients with ascites ($P < 0.01$ and $P < 0.05$) (Table 2). There was a strong correlation between ET-1 and degree of varices, and NOx and degree of varices ($P < 0.01$). There was a significant correlation between ET-1 and NOx concentrations in study group ($P < 0.01$). There was no correlation between ET-1 and aspartate aminotransferase/alanine aminotransferase (AST, ALT) or between NOx and AST, ALT levels.

TABLE 2. Levels of NOx ($\mu\text{mol/L}$) and ET-1 (pmol/L) in healthy controls and patients with liver cirrhosis (mean $\pm$ SD and P value)

	Nox	ET-1
Controls (n = 25)	50.75 ± 14.35	13.83 ± 2.39
Study group (n = 28)	75.88 ± 21.24^a	18.84 ± 7.55^a
Child-Pugh score		
A (n = 10)	70.00 ± 13.98	14.72 ± 3.23
B (n = 12)	81.96 ± 24.34	18.49 ± 4.31^b
C (n = 6)	73.51 ± 23.94	$26.42 \pm 12.05^{c,d}$
Ascites		
Patients with ascites (n = 15)	83.74 ± 24.06	22.51 ± 8.33
Patients without ascites (n = 13)	66.81 ± 13.17^e	14.61 ± 3.33^e

^a $P < 0.001$ for NOx and $P < 0.01$ for ET-1, when study group was compared with controls.

^b $P < 0.05$, when patients with Child-Pugh B were compared to those with Child-Pugh A.

^c $P < 0.05$, when patients with Child-Pugh C were compared to those with Child-Pugh A.

^d $P < 0.05$, when patients with Child-Pugh C were compared to those with Child-Pugh B.

^e $P < 0.05$ for NOx and $P < 0.01$ for ET-1, when patients without ascites were compared with patients with ascites.

DISCUSSION

Patients with cirrhosis and portal hypertension exhibit characteristic hemodynamic changes with a hyperkinetic systemic circulation and abnormal distribution of the blood volume. The primary disturbance appears to be widespread cell injury and disturbed endothelial cell function (1). Our findings revealed a significant increase in the plasma NOx concentrations in patients with cirrhosis vs. healthy controls. A few other reports demonstrated similar results for this phenomenon (8,25,26). Whether this reflects accentuated NO production in the liver is controversial. Guarner et al. (27) suggested that this phenomenon is the result of iNOS activation in the liver due to high lipopolysaccharide (LPS) concentration in the portal blood flow. Vallance and Moncada (28) and Jimenez et al. (29) concluded that overproduction of nitric oxide in the liver is closely linked to the hyperdynamic circulation that is often observed in patients with liver cirrhosis. However, Stumm et al. (30) suggested that in chronic portal hypertension, the main source of NO production is eNOS rather than iNOS activity in the liver. In addition to hepatocytes and endothelial cells, monocytes, macrophages, and neutrophils are other sources of nitric oxide in cirrhotic patients (29,31,32). In this study no significant correlation was recognized between the disease stage and plasma nitrite/nitrate concentration in cirrhotic patients. Therefore, no relationship between hepatocytes and nitric oxide was identified. Similarly to Stumm et al.'s (30) study, our study results suggest that the source of the nitric oxide may be the eNOS rather than the iNOS in the liver.

In addition to increased nitric oxide production, this study clearly indicates that the ET system is activated in patients with liver cirrhosis. It has been hypothesized that altered activity of the ET system is involved in hemodynamic disturbances in cirrhosis (16–18). However, the data regarding the production of ET-1 in cirrhosis are controversial. ET production has been shown to be reduced (19), normal (17,20,21), or elevated (18,22) compared to results from healthy subjects in different studies. These discrepancies could be due to either methodological differences or different etiologies and severities of cirrhosis in the different studies. Most studies that reported significantly higher levels of ET-1 in cirrhosis used radioimmunoassay (RIA) techniques, while most studies that used enzyme-linked immunosorbent assay (ELISA) reported only slightly increased or unchanged levels. Therefore, it is possible that RIA methodologies are more sensitive for measuring ET-1 concentrations. The source of the increased ET is under investigation. Endothelial cell damage due to cirrhotic changes may be responsible for the high concentrations

of plasma ET-1. An alternative mechanism for the increased ET concentration may be a defective clearance mechanism. Although the clearance mechanism of ETs by ET-converting enzyme in the liver has not been fully elucidated, it seems that it is impaired in cirrhotic liver, since the frequency and intensity of immunoreactivity for ET-1 and ET-3 in cirrhotic hepatocytes are higher than those in normal hepatocytes. In addition, a disturbance of ET excretion into the bile may also be responsible for ET storage, which is also related to decreased liver function. Disturbance of ET degradation and/or leakage of bile into the blood may cause elevated serum ET levels, since ET is excreted through the biliary system (33). On the other hand, the increased plasma ET-1 concentration in liver cirrhosis is probably due to an increased synthesis of ET-1. Several potential stimuli may be involved in this process. Tissue hypoxia and increased shear stress due to the increased blood flow and low systemic vascular resistance in patients with liver cirrhosis are thought to be involved in this process (34). Also, increased circulating endotoxin may contribute to the increased plasma ET-1 in cirrhosis as well (35).

In this study the highest plasma ET-1 concentrations were found in patients with advanced liver cirrhosis (Child C subgroup), moderate ET-1 increases were found in Child B patients, and lower ET-1 increases were observed in Child A patients. This suggests that plasma ET-1 increases with the progression of the disease. The same relationship between disease stage and plasma NOx was not found. However, when plasma NOx and ET-1 levels in patients with ascites were compared with NOx and ET-1 levels in patients without ascites, a significant increase was observed. The difference between patients with and without ascites may be the consequence of portal hypertension. The fact that there is a significant correlation between NOx and the degree of varices, and ET-1 and the degree of varices, supports this hypothesis. Arkenau et al. (8) also reported that the presence of ascites, and the progression of oesophageal varices were associated with higher circulating nitrate levels. On the other hand, the presence of a strong correlation between NOx and ET-1 suggests that the regulatory mechanisms of nitric oxide and ET-1 interact. Nitric oxide inhibits the production of ET-1 and modulates both the number and affinity of the ET_A receptors. Nitric oxide synthase is functionally coupled to the ET_B receptor (36,37). Because nitric oxide and ET-1 have opposite effects on vascular tone, platelet aggregation, and monocyte and macrophage activation, increased endothelial production of nitric oxide is probably a compensatory mechanism against the endothelial injury seen in liver cirrhosis. Therefore, increased nitric oxide production in cirrhosis may both

balance the increased concentrations of vasoconstrictor mediators (including ET-1) and further delay the endothelial damage. In our study, cirrhosis-related endothelial dysfunction was indicated by the increased ET-1 and nitric oxide levels. Increased nitric oxide synthesis may be a compensation against endothelial injury. The highest ET-1 levels were observed in patients with advanced cirrhosis (Child C), and moderate levels were found in Child B patients, which suggests that plasma ET-1 increases with the progression of the disease. On the other hand, the fact that patients with decompensated cirrhosis (patients with ascites) have higher NOx and ET-1 levels than patients with compensated cirrhosis (patients without ascites) supports the notion that there is a relationship between NOx, ET-1, and portal hypertension severity. Our study demonstrates that increased ET and nitric oxide metabolism are associated with the hemodynamic alterations induced by portal hypertension.

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