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Nitric Oxide in Chronic Liver Disease

Abstract: The formation of nitric oxide (NO), a free radical, increases in liver disease, where the L-arginine-nitric oxide (L-arg-NO) pathway is activated by the increased levels of cytokines and endotoxins. In this study, we investigated the relationship between the L-arg-NO pathway and chronic active hepatitis and cirrhosis, which are classified as chronic liver disease. We determined the serum levels of nitrite and nitrate, the end products of the L-arg-NO pathway, in patients with chronic liver disease and compared them with the control group levels.

Total nitrite produced by the reduction of nitrate to nitrite in the presence of nitrate reductase was determined. The measurement of nitrite concentrations was based on the Griess reaction. The nitrate concentrations were obtained by subtracting the nitrite concentrations from the total nitrite+nitrate concentration.

The study group consisted of 21 patients with chronic active hepatitis and 19 patients with cirrhosis. In the cirrhosis group, serum levels of nitrite+nitrate, nitrate and nitrite were 84.8±9.43 μmol/L, 74.75±9.49 μmol/L and 10.03±1.59 μmol/L, respectively. In the chronic active hepatitis group, the values were 72.79±6.9 μmol/L, 59.9±7.09 μmol/L and 12.88±1.65 μmol/L, respectively. The control group values obtained from 10 normal persons were 29.0±4.6 μmol/L, 19.4±3.16 μmol/L and 9.59±1.60 μmol/L, respectively.

The statistical evaluation showed a significant difference between the control and study groups (p <0.0001). We found higher nitrite+nitrate and nitrate levels in the study group than in the control group. Nitrite levels did not show any significant difference between the two groups. No significant differences were found between the chronic active hepatitis and cirrhosis groups (p>0.5).

Our results show that serum nitrite and nitrate concentrations as an index of NO generation may be elevated in hepatic failure seen in chronic liver disease.

Key Words: Nitric oxide, chronic liver disease, nitrate and nitrite
cerebellum (10,11) and myocardium (12). In the case of chronic hepatitis, circulating levels of cytokines, such as TNFα and IL6, increase during disease (13). In many studies enhanced hepatic expression of a variety of different cytokines has been demonstrated in patients with hepatic inflammation. Many studies demonstrated that hepatocytes express iNOS following exposure to various cytokines such as tumor necrosis factor (TNF), interleukin 1 (IL1), interferon gamma (IFNγ), and interleukin six (IL6) (14,15,16). Furthermore, it was demonstrated that hepatocytes also produce NO in vivo during chronic hepatic inflammation (17,18) and in vitro in response to conditioned Kupffer cell supernatant (19) or to a mixture of lipopolysaccharide (LPS) (20) and the cytokines TNFα, IL1, and IFNγ (21). Human hepatocytes were also stimulated to produce NO by the same combination of endotoxin and cytokines as rat hepatocytes (5,6,22). Increased NO production and plasma nitrite/nitrate levels are also found during chronic hepatic inflammation, suggesting a role for NO in the hepatic response to inflammatory stimuli (23). The reason for the vasodilation observed in endotoxemia and the production of NO might be the induction of an NO synthase; thus, NO appears to be an important mediator of the vascular actions of endotoxin and cytokines. Since then, the role of NO in the pathogenesis of the hyperdynamic circulation has been evaluated by several groups of investigators in various animal models of chronic liver disease. Most data support an important role for NO in the development and maintenance as systemic and splanchnic hemodynamic complications of chronic liver disease (24).

In this study, we investigated the probable variability of serum nitrite and nitrate levels in patients with chronic hepatitis and cirrhosis.

Materials and Methods

Patients and samples

Forty hospitalized patients were included in this study, of which 21 had chronic active hepatitis (mean age, 46±13). The etiology was hepatitis B in eight patients, and hepatitis C in thirteen patients and nineteen patients had cirrhosis (mean age, 51±8). The diagnosis of chronic active hepatitis and cirrhosis was established on the basis of clinical, analytical and ultrasonographic findings in all patients observed by the department of gastroenterology. No patient had received any previous treatment. Ten healthy subjects with normal blood pressure were used as controls after we ruled out liver, kidney and heart disease. On the day of the study, all patients were fasting and resting in bed. Blood samples were collected at 8 AM in acid washed tubes. Following coagulation, all samples were centrifuged at 4000xg for 10 minutes and sera were stored at -70°C until experiments.

Nitrite determination

The concentration of nitrite was determined with the Griess reaction as described previously (25). All samples were diluted fourfold with distilled water and deproteinized by adding 1/20th volume of zinc sulfate (300g/L) to give a final concentration of 15 g/L. After centrifugation at 9500xg for 5 minutes at room temperature, 150 µl of supernatant was applied to a clean tube and 450µl of Griess reagent (1% sulfonilamide in 1N HCl, 15% N-1-naphtyletylenediamine dichloride) was added. After mixing well, all tubes were left in a dark place for 30 minutes at room temperature. At the end of the reaction time, the absorbances were measured on a spectrophotometer (Jasco-500 /Japan) at a wavelength of 550nm. A blank was prepared in the same way but 150µl potassium phosphate buffer (50mM) was used instead of serum. Calibration curves were made with sodium nitrite and potassium nitrate in distilled water (linear range 0 – 100µmol/L).

Nitrate determination

Nitrate was measured as nitrite after enzymatic conversion by nitrate reductase (EC1.6.6.2) as described by Moshage (26). This procedure is based on the reduction of all nitrate to nitrite and measured the sum of nitrite concentration. Then the nitrate concentration was calculated by subtracting the measured nitrite value from the total nitrite concentration. 40 µl nitrate reductase (20mU), 50 µl FAD (5mM), 10µl NADPH (0.6mM) and 250µl phosphate buffer (50mM) were added to 100µl deproteinized samples prepared by mixing with 1/5 zinc sulfate solution before the assay. The mixtures were incubated for 1 hour at 37°C, then 150µl of the mixture was added to 450µl of Griess reagent and incubated again for 30 min at room temperature. The absorbances were measured on the spectrophotometer at a wavelength of 550nm. To test the recovery of nitrite and
nitrate in plasma, we used nitrite and nitrate calibration curves made with normal plasma samples and with hyperbilirubinemic and hemolytic plasma samples.

The results obtained from the patient and control groups were analyzed statistically with the Mann-Whitney U test.

Results

The mean serum nitrite+nitrate, nitrate and nitrite concentrations in cirrhosis, chronic active hepatitis and control subjects were 84.8±9.43 μmol/L, 72.79±6.9 μmol/L and 29±4.6 μmol/L; 74.75±9.49 μmol/L, 59.9±7.09 μmol/L and 19.41±3.16 μmol/L, 10.03±1.59 μmol/L, 12.88±1.65 μmol/L, and 9.59±1.60 μmol/L respectively. Statistical evaluation showed that nitrite+nitrate and nitrate levels were increased in both patient groups compared with the control group (p<0.0001) and a positive correlation was found between these two parameters. However, there was no significant difference in terms of nitrite levels between the patient and control groups. Also no significant difference was determined between nitrite+nitrate and nitrate levels of the chronic hepatitis and cirrhotic groups (p>0.5) (Figure).

Discussion

This study demonstrates increased serum levels of nitrite and nitrate, which are metabolites of nitric oxide, in patients with cirrhosis. The initial cause of overproduction of nitric oxide is not known, whereas portal venous hypertension, which increases shear stress and up-regulates endothelial nitric oxide synthase, no doubt contributes to nitric oxide overproduction (27,28). The concentrations of nitric oxide are higher in portal venous plasma than in peripheral venous plasma in patients with cirrhosis, suggesting increased splanchic production of nitric oxide (29). The activity of nitric oxide synthase in polymorphonuclear cells and monocytes was increased in patients who had cirrhosis. These cells mainly contain the inducible type of nitric oxide synthase, a finding that provides evidence that this type of the enzyme may have a role in peripheral vasodilatation in patients with cirrhosis (30). Nitric oxide production also seems to be increased in patients with cirrhosis, but the role of nitric oxide in the pathogenesis of arterial vasodilatation and sodium and water retention in these patients remains unclear (30,31).

We also found high nitrite and nitrate concentrations in patients with chronic hepatitis. There is a discrepancy between our results and reported studies, which concluded that nitric oxide production was reduced or unchanged in chronic hepatitis (32). However, interestingly, these authors found even higher values of nitrite and nitrate levels in patients with hepatocellular carcinoma based on chronic hepatitis than in patients with carcinoma accompanied by elevated plasma nitrite and nitrate levels. These findings may suggest that some chronic hepatitis patients have elevated serum nitrite and nitrate concentrations, while some do not, and the reason for this remains unclear (32). A possible explanation that differences in the severity of inflammation and fibrosis may cause varying serum nitrite and nitrate levels. Also the etiology of chronic hepatitis may explain the discrepancy between some previously reported studies and our results.

In conclusion, serum nitrite and nitrate concentrations may be elevated in patients with chronic hepatitis. This increase may be due to enhanced cytokine expression, as mentioned by Simpson et al. (33). Nitrite and nitrate production also seems to be increased in patients with cirrhosis, but the role of nitric oxide in the pathogenesis of hemodynamic and renal abnormalities in patients with cirrhosis remains unclear (34). If the experimental data are confirmed, modulation of nitric oxide synthesis could represent a new approach to management of circulatory and renal dysfunction in patients with cirrhosis.
References


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