Nitric oxide synthase activity is increased in relation to the severity of liver dysfunction

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ABSTRACT

1. Nitric oxide is a potent vasodilator which plays a major role in the control of blood pressure. The hyperdynamic circulation of cirrhosis has been linked to nitric oxide.

2. We measured neutrophil nitric oxide synthase activity in relation to the level of hepatic dysfunction in patients with liver disease of varying aetiology and severity.

3. Neutrophils were isolated from 21 patients (7 Child-Pugh score A, 6 grade B and 8 grade C) aged 28–76 (median 49) years. Nitric oxide synthase activity was measured using the conversion of oxyhaemoglobin to methaemoglobin by nitric oxide and expressed in terms of cell protein. Blood pressure and biochemical indices were recorded. Data were assessed using Kruskal–Wallis one-way analysis of variance, Mann–Whitney U-test or Pearson correlation as appropriate.

4. Systolic, mean arterial and diastolic blood pressures decreased with increasing hepatic damage ($P = 0.031$, $P = 0.01$ and $P = 0.038$ respectively). Nitric oxide synthase activity increased with the degree of liver dysfunction ($P = 0.033$) and was highest in patients with Child-Pugh score C. Systolic blood pressure correlated with nitric oxide synthase activity in patients with Child-Pugh score C ($P = 0.029$).

5. Our results show that nitric oxide synthase activity increases with increasing Child-Pugh score and is associated with the development of systemic hypotension. These data may support the involvement of nitric oxide in the haemodynamic disturbances seen in liver disease.

INTRODUCTION

Nitric oxide is a vasodilator produced by the endothelium and other cells and is essential for the physiological control of blood pressure. It is produced from L-arginine by the action of the enzyme nitric oxide synthase (NOS), of which there are three distinct isoforms. The two constitutive forms, which are calcium and calmodulin dependent, are present primarily in endothelial cells and nerve cells. The inducible isoform is calcium independent and is synthesized in response to endotoxin and cytokines in many cells including macrophages, endothelial cells, vascular smooth muscle and hepatocytes. Human neutrophils also have NOS activity [1–4].

Portal hypertension is associated with a hyperdynamic circulation and peripheral vasodilatation. This vasodilatation is clinically important since it is thought to be involved in the pathogenesis of complications of portal hypertension such as ascites and the hepato-renal syndrome [5]. It has been hypothesized that excess nitric oxide (NO) production is responsible for the hyperdynamic circulation [6]. Increased urinary excretion [7]...
and circulating concentrations [8] of the metabolites of NO and increased exhaled NO [9,10] have been demonstrated in patients with cirrhosis. The increased NO production has been attributed to up-regulation of NOS in response to increased circulating concentrations of endotoxin [8]. In addition, increased production of NO by leucocytes from patients with cirrhosis correlated with cardiac index [3,11]. There have been no studies of NOS activity in groups of patients with different degrees of liver dysfunction. We measured NOS activity in relation to blood pressure in patients with liver disease of varying severity.

**MATERIALS AND METHODS**

The study was approved by the local ethics committee and written informed consent was obtained from each patient. Twenty-one patients attending the liver service were studied. Blood samples were obtained for isolation of polymorphonuclear leucocytes, and measurement of bilirubin, albumin and prothrombin time using standard techniques, for assessment of Child-Pugh score [12].

Blood pressure was taken using a mercury sphygmomanometer after subjects had been lying down for 5 min. Diastolic blood pressure was measured using the fifth Korotkoff sound. Since blood pressure changes in patients with liver failure are thought to be due to changes in peripheral vascular resistance, mean arterial pressure was calculated. Mean arterial pressure is a product of cardiac output and peripheral vascular resistance and can be calculated from systolic and diastolic pressures:

\[
\text{Mean arterial pressure} = \frac{\text{systolic BP} + (\text{diastolic BP} \times 2)}{3}
\]

Patients were assessed for the degree of ascites and encephalopathy present, to obtain a Child-Pugh score [12].

Polymorphonuclear neutrophils were isolated using a single-layer density gradient procedure [2]. Heparinized blood was layered on to Polymorphprep® separation medium (Nycomed UK Ltd, Birmingham, U.K.) at a rate of 5 ml of blood to 3.5 ml of medium. After centrifugation at 475 \(g\) for 30 min at 20 °C, the mononuclear cell band was discarded and neutrophils were retrieved and washed in Dulbecco’s modified Eagle’s medium (DMEM; ICN Biomedicals Ltd, Thame, Oxon, U.K.). Contaminating erythrocytes were removed by hypotonic shock and cells were washed three times in DMEM and resuspended in PBS (Dulbecco’s A, pH 7.4; Sigma-Aldrich Ltd, Poole, Dorset, U.K.). The cell separation technique results in a minimally activated cell population which is viable (> 95% by Trypan Blue exclusion) and comprises > 99% polymorphonuclear leucocytes [2].

Isolated cells were sonicated on ice for 1 min, and endogenous arginine was removed by incubating with washed Dowex-AG50W-X8 resin (Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, U.K.) for 5 min. After centrifugation for 5 min at 10000 \(g\), the clear supernatant was stored at −80 °C for NOS assay. Protein concentration of the lysate was measured using a technique modified from Lowry et al. (see [13]).

The assay for NOS is based on the quantitative conversion of oxyhaemoglobin to methaemoglobin by NO, which can be followed spectrophotometrically as a decrease in absorbance [14], and has been widely validated and used in a number of experimental systems [1,4]. The within- and between-assay coefficients of variation are 11.2% \((n = 20)\) and 22.1% \((n = 20)\) respectively. The enzyme activity is calculated from the reaction rate using the molar extinction coefficient for methaemoglobin. An incubation mixture containing 100 units/ml superoxide dismutase (from bovine erythrocytes, Sigma-Aldrich), 2000 units/ml catalase (from bovine liver, Sigma-Aldrich) and 1.29 \(\mu\)M human oxyhaemoglobin in PBS prepared as previously described [15], was pre-warmed to 37 °C. Incubation mixture (0.6 ml) was placed into 1-cm cuvettes with 0.2 ml of cell lysate. The cuvettes were then placed in a spectrophotometer (Lambda 14, with UV WinLab software package, Perkin Elmer, Beaconsfield, Bucks, U.K.) maintained at 37 °C, and the reaction was initiated by the addition of 0.1 ml of 2 \(\mu\)M \(\beta\)-NADPH (Sigma-Aldrich) and 0.1 ml of 0.3 M L-arginine (Sigma-Aldrich) in DMEM. The absorbance was recorded at 415 nm for 3 min, and the slope calculated, to give a reaction rate (change in absorbance units per minute). NOS activity was expressed as nmoles of NO released per minute per mg of cell protein.

**Statistical analysis**

Data are expressed as median (range) and were assessed using Kruskal–Wallis one-way analysis of variance with Mann–Whitney post-hoc testing as appropriate. Correlation between NOS activity and blood pressure was performed using Pearson’s correlation.

**RESULTS**

Twenty-one patients, median age 49 (28–76) years were studied. Seven patients were Child-Pugh score A (four with alcoholic liver disease, one with primary biliary cirrhosis, one with primary sclerosing cholangitis and one with hepatitis C). Six patients were Child-Pugh score B, all with alcoholic liver disease, and eight patients were Child-Pugh score C (six with alcoholic liver disease, and
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Table 1  Demographic data and blood pressure in patients with liver disease

Values are medians (range). BP, blood pressure.

<table>
<thead>
<tr>
<th>Child-Pugh score…</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Age (years)</td>
<td>59 (28–76)</td>
<td>49 (37–62)</td>
<td>49 (44–65)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>2/5</td>
<td>1/5</td>
<td>5/3</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>130 (110–160)</td>
<td>115 (100–150)</td>
<td>99 (70–145)</td>
</tr>
<tr>
<td>Mean arterial BP (mmHg)</td>
<td>89.9 (76.6–113.2)</td>
<td>84.9 (66.2–96.6)</td>
<td>71.3 (56.6–88.2)</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>70 (60–90)</td>
<td>65 (50–70)</td>
<td>60 (50–60)</td>
</tr>
</tbody>
</table>

Figure 1  Systolic and diastolic blood pressure in 21 patients with varying degrees of liver dysfunction (seven Child-Pugh score A, six score B and eight score C)

Box and whisker plots show median and 25th and 75th percentiles, with ranges as vertical lines. Data were assessed using Kruskal–Wallis one-way analysis of variance.

one each with primary biliary cirrhosis and chronic active hepatitis). None of the patients had a recent history of sepsis or gastrointestinal bleeding. The demographic data for each group are given in Table 1. Systolic, mean arterial and diastolic blood pressures decreased with increasing Child-Pugh score (P = 0.032, P = 0.013 and P = 0.038 respectively, Figure 1). Blood pressures for each group of patients are shown in Table 1.

NOS activity increased with increasing Child-Pugh score (P = 0.033, Figure 2). Activity was higher in patients with grade C Child-Pugh scores than we have found previously [14] in healthy subjects (P = 0.03) and also than in patients of grade A Child-Pugh score (P = 0.045). NOS activity correlated with systolic blood pressure (P = 0.029) and mean arterial pressure (P = 0.049) in those patients with grade C Child-Pugh score (Figure 3).

DISCUSSION

We have shown for the first time that NOS activity increases with worsening severity of liver damage and that this increased activity is reflected by decreases in both systolic and diastolic blood pressure. Patients with cirrhosis often develop portal hypertension and a hyperdynamic circulation characterized by systemic hypotension with a high cardiac output and low systemic vascular resistance [5]. These changes have been suggested as being due to excessive production of the profound vasodilator NO, resulting in peripheral vasodilatation [6]. The inducible isoform of NOS is activated on exposure to endotoxin and cytokines. Patients with cirrhosis have been found to have high circulating concentrations of endotoxin [8,16], which is thought to occur as a result of leakage of bacterial products into the peripheral circulation through the compromised liver [17]. Oral administration of colistin, a non-absorbable antibiotic with anti-endotoxin properties, for 7 days decreased serum nitrite and nitrate concentrations in patients with cirrhosis and ascites [8]. Concentrations of the pro-inflammatory cytokines tumour necrosis factor- \( \alpha \) and interleukin-1 \( \beta \) are also elevated in patients with chronic liver disease, and are higher in those patients with cirrhosis compared with non-cirrhotic patients [18], suggesting that increased endogenous cytokine levels are a consequence of liver dysfunction rather than inflammation. Our results also suggest that NOS activation is a phenomenon which occurs throughout the spectrum of liver dysfunction.
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Figure 2 Neutrophil NOS activity in 21 patients with varying degrees of liver dysfunction (seven Child-Pugh score A, six score B and eight score C)

Box and whisker plots show median and 25th and 75th percentiles, with ranges as vertical lines. The range for 10 healthy subjects is shown as a vertical bar [14]. Data were assessed for trends using Kruskal-Wallis one-way analysis of variance. Post-hoc testing was performed using Mann-Whitney U-test.

Figure 3 Correlation between systolic blood pressure and neutrophil NOS activity in eight patients with Child-Pugh score C liver dysfunction

Correlation was performed using Pearson correlation.

We found that neutrophil NOS activity increased as the Child-Pugh score increased, suggesting that the degree of activation of NOS increases as liver function deteriorates. This implies that circulating endotoxin concentrations increase with worsening liver function, resulting in widespread activation of inducible NOS within peripheral leukocytes and leading to increased NO production, as we have previously described in patients with septic shock [14]. Nitric oxide is rapidly converted to nitrite and nitrate in vivo and their measurement is used as an index of NO generation. Increased circulating concentrations [8] and urinary excretion [7] of nitrate and nitrite have been found in patients with cirrhosis, particularly in those with ascites, and plasma levels correlate with endotoxin concentrations [8]. Ex vivo NO production by leukocytes, measured both directly using conversion of radiolabelled arginine, the substrate for NOS [3], and functionally by the inhibition of platelet aggregation [3,11] or cyclic GMP generation [11], has also been shown to be elevated in patients with cirrhosis compared with healthy subjects. This finding is in agreement with the present study.

We have shown that NOS activity in neutrophils correlates with systolic blood pressure in patients with Child-Pugh score C, and ex vivo NO production by leukocytes from cirrhotic patients has previously been shown to correlate with cardiac index [3,11]. Several studies have shown that administration of the NO inhibitor N\textsuperscript{G}-monomethyl-L-arginine to cirrhotic rats with ascites increases mean arterial pressure in a dose-dependent fashion [19,20]. The poorer correlation of NOS activity with mean arterial blood pressure in the present study is not surprising and is likely to be due to the inaccurate measurement of diastolic blood pressure.

In summary, we have shown that NOS activity increases as the Child-Pugh score, and hence hepatic dysfunction, increases, and that this is associated with progressive decreases in systolic and diastolic blood pressure. These data provide further evidence for the possible involvement of NO in the pathophysiology of the vasodilatation and hyperdynamic circulation of liver disease. Since the haemodynamic disturbances in cirrhosis may themselves contribute to worsening hepatic and renal dysfunction, the finding that NOS activation and haemodynamic disturbances are associated may suggest a role for new prophylactic therapies in the early stages of chronic liver disease.

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REFERENCES

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