Increased nitric oxide excretion in patients with severe acute pancreatitis: evidence of an endotoxin mediated inflammatory response?

S H Rahman, B J Ammori, M Larvin, M J McMahon

Background and aims: Nitric oxide represents a potential key mediator of the local and systemic manifestations of acute pancreatitis (AP) in experimental models but its role in human disease is uncertain. We therefore sought to assess if systemic nitric oxide (NO) production is elevated in severe AP and determine whether this is a reflection of biochemical severity or endotoxin exposure.

Patients and methods: Patients were recruited within 72 hours of pain onset. NO derived nitrite excretion determined from a 24 hour sterile urine collection was correlated with intestinal macromolecular permeability (polyethylene glycol excretion ratio), markers of systemic endotoxin exposure [lgG:lgM endotoxin core antibody (EndoCAb) ratio], disease severity, and the magnitude of systemic inflammation [peak C reactive protein (CRP) and Acute Physiology and Chronic Health Evaluation score II (APACHE-II)].

Results: In patients with a severe attack (n=20), nitrite excretion was increased significantly compared with patients with a mild attack [n=45, 20.6 µg v 15.65 µg; p<0.00] and the latter with healthy controls [n=20, p=0.004]. Nitrite excretion correlated strongly with both intestinal permeability [r=0.7, p<0.006] and EndoCAb ratio [r=0.7, p=0.01] but not with CRP or APACHE-II scores [p>0.1].

Conclusions: Total urinary nitrite excretion is increased in patients with severe AP, and may not be simply a reflection of systemic inflammation, but potentially a consequence of endotoxin mediated upregulation of inducible NO synthase activity.
The aims of the present study were: (1) to correlate 24 hour urinary nitrite excretion with the severity of AP; and (2) to examine the relationship between TUN excretion and empirical evidence of BT.

**PATIENTS AND METHODS**

Local research ethics committee approval was obtained from the four study institutions that participated in the study: the General Infirmary at Leeds, Bradford Royal Infirmary; Huddersfield Royal Infirmary, and Pontefract District General Hospitals. Adults admitted with AP and hyperamylasaemia (serum levels greater than three times the upper limit of normal) were considered for inclusion if their symptoms were of less than 48 hours’ duration. Patients with evidence of coexistent infection or inflammatory disease, chronic organ failure, or previous intestinal surgery were excluded. All patients had received at least 24 hours of aggressive fluid rehydration and were excluded if there was evidence of renal failure that did not respond to fluid therapy (urine output persistently below 0.4 ml/kg/h or plasma creatinine greater than 180 g/dl). Attacks were classified as mild or severe according to the Atlanta criteria of 1992, which are based on clinical outcome. A total of 74 patients with AP were recruited of which 65 satisfied the selection criteria. Of the patients excluded, two had active rheumatoid arthritis requiring immunosuppressants, three had a history of malignant mal) were considered for inclusion if their symptoms were of less than 48 hours’ duration. Patients with evidence of coexistent infection or inflammatory disease, chronic organ failure, or previous intestinal surgery were excluded. All patients had received at least 24 hours of aggressive fluid rehydration and were excluded if there was evidence of renal failure that did not respond to fluid therapy (urine output persistently below 0.4 ml/kg/h or plasma creatinine greater than 180 g/dl). Attacks were classified as mild or severe according to the Atlanta criteria of 1992, which are based on clinical outcome. A total of 74 patients with AP were recruited of which 65 satisfied the selection criteria. Of the patients excluded, two had active rheumatoid arthritis requiring immunosuppressants, three had a history of malignant

**Measurement of urinary nitrite excretion**

An aliquot of total urine collected over the 24 hour period after enteral administration of PEG solution was assayed for nitrite concentration using the Greiss reaction method, as previously described. Urinary nitrite excretion was subsequently calculated based on the volume of urine collected. A nitrite standard reference curve was generated using urine from healthy human volunteers.

**Measurement of intestinal permeability**

Differential urinary excretion of the two PEG molecules (PEG 3350/400 ratio) over 24 hours, measured using high flow liquid chromatography as previously described, was calculated to provide an index of intestinal permeability.

**Measurement of antiendotoxin core antibody levels**

Endogenous immunoglobulin IgG and IgM antiendotoxin core antibody (EndoCAb) levels to core glycolipid antigens were measured by a direct enzyme linked immunosorbent assay, as previously described. Plasma IgG EndoCAb:IgM EndoCAb ratio was used as a marker of systemic exposure to endotoxin.

**Measurement of C reactive protein levels**

CRP was measured using an enzyme linked immunosorbent assay (Dako, High Wycombe, UK). Normal CRP in serum is less than 10 mg/l.

**Statistical analysis**

Results are expressed as median (range). Comparison between groups was performed using the Mann-Whitney U test. Pearson correlation coefficient was calculated where indicated, and Spearman’s coefficient was used for non-Gaussian data. Significance was accepted at the 1% level.

**RESULTS**

In total, 65 patients with AP (mild 45, severe 20) and 20 control subjects were studied. The median interval between the onset of abdominal pain and admission to hospital was 24 hours (range 4–48 hours). Details of aetiology and demographics are outlined in table 1.

Groups were matched for age and sex. The aetiology of AP was identified in 64 of 65 patients (gall stones 39, alcohol abuse 13, endoscopic retrograde cholangiopancreatography six, hyperlipidaemia four, and drug related one). Clinical outcomes of patients with severe AP are shown in table 2. Pancreatic necrosis was demonstrated in nine patients using contrast enhanced computer tomography (>30%). In one patient, necrotic tissue became secondarily infected with

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**Table 1** Comparison of patients and control subjects

<table>
<thead>
<tr>
<th></th>
<th>Mild pancreatitis (n=45)</th>
<th>Severe pancreatitis (n=20)</th>
<th>Controls (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)†</td>
<td>60.0 (43–74.5)</td>
<td>63.0 (49–73)</td>
<td>55.7 (23–82)</td>
</tr>
<tr>
<td>Sex ratio [M/F]</td>
<td>20/25</td>
<td>13/7</td>
<td></td>
</tr>
<tr>
<td>Aetiology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gall stones</td>
<td>29</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Alcohol</td>
<td>9</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>ERCP</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Drug induced</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Idiopathic</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>APACHE II scores at 24 h*t</td>
<td>7.0 (5–27)</td>
<td>11.5 (8.3–15.5)</td>
<td></td>
</tr>
<tr>
<td>Prophylactic antibiotics</td>
<td>18 (38%)</td>
<td>14 (70%)</td>
<td></td>
</tr>
<tr>
<td>Intravenous nutrition</td>
<td>1 (2.2%)</td>
<td>10 (50%)</td>
<td></td>
</tr>
</tbody>
</table>

†Median (range).

ERCP, endoscopic retrograde cholangiopancreatography.

*p<0.001, Mann-Whitney U test.
enteric Gram negative organisms (>50% necrosis). Eight patients developed MOSF among which two had pancreatic necrosis. In all, five patients died (one with sterile pancreatic necrosis and MOSF, one with sterile necrosis and single organ failure (adult respiratory distress syndrome), one with infected necrosis and MOSF, and one with MOSF alone. Pancreatic necrosis was established using contrast enhanced computer tomography criteria within 1–5 days of onset of severe abdominal pain.

Urinary nitrite excretion and clinical severity of AP
Urinary nitrite excretion was significantly increased in patients with severe attacks (median 20.61 µg (interquartile range (IQR) 13.20–42.94)) compared with patients with mild attacks (median 15.65 µg (IQR 11.50–23.53); p=0.003) (fig 1).

Furthermore, patients with mild attacks showed significantly higher nitrite excretion compared with healthy controls (p=0.004).

Urinary nitrite excretion and PEG retrieval
Gut macromolecular permeability (PEG retrieval ratio) was increased in patients with severe attacks compared with mild attacks (0.06 (0.01–0.19) and 0.008 (0.005–0.013) respectively, p<0.001) (fig 2). A positive and significant correlation was demonstrated between nitrite excretion and both the PEG retrieval ratio and PEG 3350 percentage retrieval in patients with a severe attack of acute pancreatitis (r=0.7, p<0.01).

Urinary nitrite excretion, antiendotoxin core antibody (EndoCAb) ratio, and PEG retrieval
The immune response to endotoxaemia, IgG:IgM EndoCAb ratio, demonstrated a strong positive relationship with nitrite excretion in patients with severe AP (r=0.7, p<0.01) (fig 4). In addition, among this group, PEG 3350 retrieval correlated strongly with the IgG:IgM EndoCAb ratio (r=0.7, p<0.01) (fig 5).

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No (%)</th>
<th>Died (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudocyst</td>
<td>4 (20)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Pancreatic necrosis</td>
<td>9 (45)</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Septicaemia</td>
<td>3 (15)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Single organ failure</td>
<td>7 (35)</td>
<td>2 (10)</td>
</tr>
<tr>
<td>MOSF</td>
<td>8 (40)</td>
<td>4 (20)</td>
</tr>
</tbody>
</table>

MOSF, multiorgan system failure.
Urinary nitric oxide and severe acute pancreatitis

As expected, patients with severe AP demonstrated significantly higher 48 hour APACHE II scores (median 12 (range 5–27)) and 0–72 hour peak CRP levels (median 274 g/dl (range 108–384)) compared with those with mild disease (median 7 (range 2–16), p=0.002, and median 90 g/dl (range 1–278) p<0.001, respectively). In all patients with pancreatitis, urinary nitric oxide excretion failed to significantly correlate with either CRP level (48 hours and 72 hours; p>0.1) or 48 hour APACHE-II scores (N5, Spearman’s rank correlation).

DISCUSSION

This is the first study to investigate the relationship between NO derived urinary nitric oxide excretion and the severity of AP as well as its relationship to altered intestinal permeability in patients with AP Total urine nitrate excretion was shown to be significantly greater in patients with severe attacks compared with mild attacks, and in the latter compared with controls. In patients with severe attacks, alterations in intestinal macro-molecular permeability correlated strongly with urinary nitric oxide excretion and systemic exposure to endotoxin. An increase in intestinal permeability to large toxic molecules, such as endotoxin, and possibly bacteria, is a derangement in gut barrier function that is central to the hypothesis implicating the gut in the development of sepsis and MOSF. Previous experimental and clinical studies demonstrated an increase in intestinal permeability to macromolecules and identified the gut as an important source of infection during AP.

A number of studies have also demonstrated increased exposure to endotoxins in patients with severe AP. It is unclear if this is simply a reflection of the intensity of systemic endotoxaemia may in turn act through a positive feedback mechanism, either directly or through release of cytokines, to further increase intestinal permeability, impair host immunity, and promote BT from the gut, resulting in a vicious circle.

Abnormalities in immune function such as a reduction in circulating levels of CD4 positive (T helper) lymphocytes, a decrease in delayed-type skin hypersensitivity, impaired cell mediated immunity, and systemic phagocytic function have all been described in experimental pancreatitis.

Although we have demonstrated an association between nitric oxide excretion, severity of AP and empirical evidence of BT (altered gut permeability and systemic exposure to endotoxin), it is unclear if this is simply a reflection of the intensity of a non-specific inflammatory illness or a consequence of altered gut macro-molecular permeability.

The observed increase in NO derived nitrite in patients with severe attacks may be mediated by a subpopulation of neutrophils or monocytes activated either local to the pancreatic inflammation, systemically, or via the gut. Evidence supporting a role for NO producing enzymes in mediating increased gut permeability comes from a number of experimental studies using specific iNOS inhibitors. Decreased levels of NO metabolites occurred in mice pretreated with N-w-nitro-L-arginine prior to an intraperitoneal injection of LPS. In rats, administration of oral live, but not heat inactivated, Salmonella enteritidis LPS was followed by an increase in urinary NO derived metabolites in addition to positive faecal quantification, and mesenteric lymph node culture. Hence endotoxin induced mucosal injury and BT are likely to be associated with increased iNOS activity and therefore increased NO production. Furthermore, a dose dependent induction of NO by LPS in vitro has been demonstrated in two in vitro studies. Bogle et al found a nearly linear relationship between LPS concentration and nitrite formation in culture medium. Keller et al described a sigmoid-like relation between LPS and nitrite production, in agreement with the findings of Oudenhoven and colleagues. Unlike observations of mesenteric lymph node and gut mucosal tissue, urinary nitric oxide excretion reflects systemic pathogen load of the host and thus an estimate of the severity of infection.

Support for a specific relationship between nitric oxide excretion and gut permeability observed in this study is (1) the strong positive correlates with altered gut permeability and systemic exposure to endotoxin, and (2) lack of significant correlation with either CRP or APACHE-II scores. The latter therefore suggests that our observations of increased nitric oxide excretion are unlikely to be secondary to the non-specific systemic inflammation.

CONCLUSION

The observed associations of increased NO metabolites in patients with severe AP and its correlation with empirical markers of BT further implicates endotoxaemia as a central mechanism in the pathogenesis of MOSF and septic complications of this disease. Identification of the prime source(s) of NO release in early AP may merit the introduction of selective iNOS inhibitors either directly into the intestinal lumen to ameliorate the changes in intestinal permeability or systemically in order to reduce morbidity from sepsis.

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Authors’ affiliations
S H Rahman, M Larvin, J M McMahon, Academic Unit of Surgery, the General Infirmary, Leeds, UK
B J Ammori, Manchester Royal Infirmary, Manchester, UK

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