PANCREATIC DISEASE

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

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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 (IgG:IgM 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.

Acute pancreatitis (AP) is a common disease with a relatively high morbidity and mortality.¹⁻⁴ The reported incidence is approximately 30-40 per 100 000 population per year⁵ and 25% will develop severe or life threatening complications.¹⁻⁶ Although mortality has fallen over the last half century, it has remained at 10-15% for the last decade, despite improvements in intensive therapy.¹⁻⁹ Severe AP is now recognised as comprising an initial sterile systemic inflammatory response syndrome (SIRS) that may lead to multiple organ system failure (MOSF) within the first 72 hours.¹⁰⁻¹² There is an emerging consensus that SIRS and MOSF observed in severe pancreatitis arise as a result of bacterial translocation (BT) from the gut.¹³⁻¹⁴ Experimental and clinical studies have demonstrated increased intestinal permeability to macromolecules¹⁵⁻¹⁷ and identified the gut as an important source of infection during AP.¹⁸⁻²³ Exley *et al* detected endotoxaemia at presentation more commonly in non-survivors of AP (91% v 35%), and levels were significantly higher in severe and fatal attacks.²⁰ Similar findings were reported by Ammori *et al*, demonstrating a sevenfold rise in intestinal macromolecular permeability during severe attacks, which was strongly associated with increased antiendotoxin antibody levels indicating greater endotoxin entry into the systemic circulation.¹⁷ Although the route of migration of microorganisms from the intestinal lumen remains obscure,²⁴⁻²⁵ BT seems to be the most important route of bacterial infection,²⁴ and suggests an underlying failure in intestinal barrier function. Several mechanisms have been suggested whereby increased intestinal permeability may lead to translocation of endotoxin and enteric bacteria, including mucosal ischaemia,²⁶⁻²⁸ ischaemia-reperfusion injury,²⁹⁻³⁰ impaired immune defences,³¹⁻³² and changes in indigenous intestinal microbial ecology leading to bacterial overgrowth.³³⁻³⁴ Possible intracellular mechanisms include alterations in signal transduction, cellular signalling,

or expression of adhesion molecules on endothelial and epithelial cells.³⁵⁻³⁶

A potential mediator of the observed alterations in endothelia is nitric oxide (NO); a highly reactive and senescent molecule produced by a variety of cells, in particular endothelial cells, macrophages, and platelets. Endothelial cells possess multiple mechanisms for NO production via constitutive nitric oxide synthase (eNOS) and high output inducible NOS (iNOS) after inflammatory activation by cytokines or lipopolysaccharide (LPS). Induction of NO synthesis is a primary reaction of macrophages to bacteria, fungi, and protozoa, and additionally, has shown to be a key mediator of MOSF and sepsis.³⁷⁻⁴³

While NO is an unstable molecule, one means of investigating NO formation is to measure nitrite (NO₂⁻), which is one of two primary stable non-volatile breakdown products of NO. Evidence that NO generated during infections is oxidised to nitrite and nitrate and excreted in urine comes from studies in vivo using competitive inhibitors of NO synthase. Total urine nitrite excretion (TUN) over a 24 hour period has been shown to reflect NO synthesis⁴⁴⁻⁴⁶ and to correlate with the severity of septic diseases.⁴⁷ A dose dependent increase in nitrite has been demonstrated to occur when macrophages are activated with LPS both in vitro and in vivo.⁴⁸⁻⁵² It was therefore our hypothesis that endotoxin mediated increases in the NO metabolite nitrite in urine are related to the magnitude of intestinal macromolecular permeability and hence to AP.

Abbreviations: AP, acute pancreatitis; APACHE-II, Acute Physiology and Chronic Health Evaluation score II; BT, bacterial translocation; CRP, C reactive protein; EndoCAb, endotoxin core antibody; LPS, lipopolysaccharide; MOSF, multiorgan system failure; NO, nitric oxide; PEG, polyethylene glycol; SIRS, systemic inflammatory response syndrome; TUN, total urine nitrite; NOS, nitric oxide synthase.

Table 1 Comparison of patients and control subjects

	Mild pancreatitis (n=45)	Severe pancreatitis (n=20)	Controls (n=20)
Age (y)†	60.0 (43–74.5)	63.0 (49–73)	55.7 (23–82)
Sex ratio (M/F)	20/25	13/7	
Aetiology			
Gall stones	29	10	
Alcohol	9	4	
ERCP	3	3	
Hyperlipidaemia	3	1	
Drug induced	0	2	
Idiopathic	1	0	
APACHE II scores at 24 h†*	7.0 (5–27)	11.5 (9.3–15.5)	
Prophylactic antibiotics	18 (38%)	14 (70%)	
Intravenous nutrition	1 (2.2%)	10 (50%)	

†Median (range).

ERCP, endoscopic retrograde cholangiopancreatography.

* $p < 0.001$, Mann-Whitney U test.

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.³³ 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 tumours (breast, colon and bladder), two had positive urine bacterial cultures, and two patients developed acute renal failure within 24 hours of hospital admission (both requiring haemodialysis). A further 20 healthy volunteers matched for age and sex served as controls.

Methods

A 10 ml sample of whole blood was drawn from all patients for measurement of serum antiendotoxin core antibodies (IgG and IgM) within the first 48 hours of onset of abdominal pain. Serial 24 hourly measurements of C reactive protein (CRP) and Acute Physiology and Chronic Health Enquiry (APACHE) II scores were determined.³⁴ Polyethylene glycol (PEG) 3350 and 400, employed as permeability probes, were administered enterally at 48 hours after the onset of abdominal pain. Urine was subsequently collected in a sterile container over the following 24 hours and stored at -70°C for later analysis.

Urinary nitrite excretion was compared with:

- (1) intestinal permeability to PEG 3350;
- (2) serum antiendotoxin core antibody ratio (IgG:IgM ratio);
- (3) clinical severity of AP (Atlanta criteria); and
- (4) biochemical severity (72 hour peak CRP and 48 hour APACHE-II score).

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.⁴⁶ 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,^{38–55} 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.³⁶ 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

Table 2 Diagnosis and clinical outcome in 20 patients with severe acute pancreatitis

Diagnosis	No (%)	Died (%)
Pseudocyst	4 (20)	0 (0)
Pancreatic necrosis	9 (45)	3 (15)
Septicaemia	3 (15)	0 (0)
Single organ failure	7 (35)	2 (10)
MOSF	8 (40)	4 (20)

MOSF, multiorgan system failure.

Figure 1 Total urine nitrite excretion in the control, mild acute pancreatitis (AP), and severe AP groups.

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)

Figure 2 Polyethylene glycol (PEG) retrieval ratio (Peg 3350/400) in patients with acute pancreatitis (AP) stratified for severity of attack ($p<0.0001$). **Figure 3** Correlation of polyethylene glycol (PEG) 3350 percentage retrieval and total urine nitrite excretion in patients with a severe attack of acute pancreatitis ($r=0.7$, $p=0.006$). **Figure 4** Correlation of IgG:IgM endotoxin core antibody (EndoCAb) ratio and polyethylene glycol (PEG) 3350 percentage retrieval in patients with a severe attack of acute pancreatitis ($r=0.7$, $p<0.01$).

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, principally among patients with severe attacks ($r=0.7$, $p<0.01$, and $r=0.7$, $p=0.006$, respectively) (fig 3).

Urinary nitrite excretion, antiendotoxin core antibody 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).

Figure 5 Correlation of total urine nitrite excretion and IgG:IgM endotoxin core antibody (EndoCAb) ratio in patients with a severe attack of acute pancreatitis ($r=0.7$, $p<0.01$).

Urinary nitrite excretion and biochemical severity

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 nitrite excretion failed to significantly correlate with either CRP level (48 hours and 72 hours; $p>0.1$) or 48 hour APACHE-II scores (NS, Spearman's rank correlation).

DISCUSSION

This is the first study to investigate the relationship between NO derived urinary nitrite excretion and the severity of AP, as well as its relationship to altered intestinal permeability in patients with AP. Total urine nitrite 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 macromolecular permeability correlated strongly with urinary nitrite 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.²² Previous experimental and clinical studies demonstrated an increase in intestinal permeability to macromolecules^{15–17} and identified the gut as an important source of infection during AP.^{18,19}

A number of studies have also demonstrated increased exposure to endotoxins in patients with severe AP.^{17,21,22} IgM antiendotoxin antibodies fall in the presence of endotoxin within the circulation^{17,56,57} and although LPS is a T cell independent antigen, a switch from IgM to IgG antibody production has been observed.⁵⁸ Serum antibodies were measured at 48 hours after pain onset, and because of the slight variability in the length of time from recruitment and individual immune response, it was considered appropriate to use a ratio of the IgG and IgM EndoCAB response. Changes in intestinal permeability correlated strongly with this ratio in patients with severe attacks ($r=0.7$, $p<0.001$), similar to previous reports. The development of systemic endotoxaemia may in turn act through a positive feedback mechanism, either directly or through release of cytokines,⁵⁹ to further increase intestinal permeability,^{60,61} impair host immunity,⁶² and promote BT from the gut,^{61,63} thus resulting in a vicious circle. Abnormalities in immune function such as a reduction in circulating levels of CD4 positive (T helper) lymphocytes,⁶⁴ a decrease in delayed-type skin hypersensitivity,⁶⁵ impaired cell mediated immunity,⁶⁶ and systemic phagocytic function⁶⁷ have all been described in experimental pancreatitis.

Although we have demonstrated an association between nitrite 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 macromolecular permeability.

The observed increase in NO derived nitrite in patients with severe attacks may be mediated by a subpopulation of neutrophils or monocytes⁶⁸ 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.^{38–44} Decreased levels of NO metabolites occurred in mice pretreated with N^G -monomethyl-L-arginine prior to an intraperitoneal injection of LPS.^{45–47} 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.⁵² 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.^{49,50} Bogle *et al* found a nearly linear relationship between LPS concentration and nitrite formation in culture medium.⁴⁸ Keller *et al* described a sigmoid-like relation between LPS and nitrite production,⁴⁹ in agreement with the findings of Oudenhoven and colleagues.⁵² Unlike observations of mesenteric lymph node and gut mucosal tissue, urinary nitrite excretion reflects systemic pathogen load of the host and thus an estimate of the severity of infection.

Support for a specific relationship between nitrite 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 nitrite 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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