Increased nitric oxide activity in a rat model of acute pancreatitis

R A Al-Mufti, R C N Williamson, R T Mathie

Abstract

Background—Overproduction of nitric oxide (NO) via induction of the inducible NO synthase (iNOS) is an important factor in the haemodynamic disturbances of several inflammatory states.

Aims—To identify the role of NO in a caerulein induced model of acute pancreatitis in the rat.

Methods—Arterial blood pressure and plasma NO metabolites were measured at zero and seven hours in adult male Wistar rats administered caerulein (n=10) or saline (n=10). Pancreatic activity of NOS (inducible and constitutive) was assayed biochemically. The pancreatic expression and cellular localisation of NOS and nitrotyrosine (a marker of peroxynitrite induced oxidative tissue damage) were characterised immunohistochemically.

Results—Compared with controls at seven hours, the pancreatitis group displayed raised plasma NO metabolites (mean (SEM) 70.2 (5.9) versus 22.7 (2.2) μmol/l, p<0.0001) and reduced mean arterial blood pressure (88.7 (4.6) versus 112.8 (4.1) mm Hg, p=0.008). There was notable iNOS activity in the pancreatitis group (3.1 (0.34) versus 0.1 (0.01) pmol/mg protein/min, p<0.0001) with reduced constitutive NOS activity (0.62 (0.12) versus 0.96 (0.08) pmol/mg protein/min, p=0.031). The increased expression of iNOS was mainly localised within vascular smooth muscle cells (p=0.003 versus controls), with positive perivascular staining for nitrotyrosine (p=0.0012 versus controls).

Conclusions—In this experimental model of acute pancreatitis, iNOS induction and oxidative tissue damage in the pancreas is associated with raised systemic NO and arterial hypotension. Excess production of NO arising from the inducible NO synthase may be an important factor in the systemic and local haemodynamic disturbances associated with acute pancreatitis.

Keywords: acute pancreatitis; nitric oxide; inducible nitric oxide synthase; peroxynitrite; caerulein induced pancreatitis

Evidence is accumulating that a very early event in the evolution of acute pancreatitis is the release of endogenous inflammatory mediators from the inflamed pancreas. Interleukin 1 (IL-1), IL-6, and tumour necrosis factor α (TNF-α) concentrations are all elevated in the serum of animals with acute pancreatitis within one hour of onset. These cytokines derive from the inflamed pancreas, and their serum concentrations correlate well with the degree of pancreatic inflammation. Likewise, serum IL-6 concentrations from patients with acute pancreatitis correlate with the severity of the disease. One quarter of patients with acute pancreatitis develop multiple organ dysfunction syndrome (MODS) characterised by a systemic inflammatory response syndrome (SIRS) akin to that of sepsis. Of the multitude of mediators operating in SIRS/sepsis, the most influential seem to be TNF-α, IL-1, and IL-6. These cytokines and other mediators including endotoxin can activate the production of the inducible nitric oxide (NO) synthase, resulting in overproduction of NO, which acts as a key final cellular and intercellular mediator.

NO, a highly reactive free radical, is produced from the amino acid l-arginine by the action of a family of isoenzymes, the nitric oxide synthases (NOS). Two broad groups can be identified: constitutive (cNOS) and inducible (iNOS). cNOS is present predominantly as a normal constituent of healthy endothelial cells (the endothelial isoform, eNOS) and synthesises NO in small amounts in response to physical or receptor stimulation. iNOS is not a normal cellular constituent, but can be expressed in a wide variety of cells and generates large amounts of NO in a sustained and largely uncontrolled manner. Excess production of NO causes vasodilatation and hypotension that is refractory to vasoconstriction, together with increased microvascular permeability and extravascular third spacing. The inability physiologically to correct these adverse responses results in end organ hypoperfusion, oedema, initiation of anaerobic metabolism, and end organ dysfunction. Moreover, the reaction of NO with superoxide leads to the formation of peroxynitrite, which is a powerful oxidant and cytotoxic agent and may play an important role in the cellular damage associated with the overproduction of NO. The spontaneous reaction of peroxynitrite with proteins results in the nitration of tyrosine residues to form nitrotyrosine, which is a specific nitration product of peroxynitrite and a marker for peroxynitrite induced oxidative tissue damage. Limited data from experimental models of acute pancreatitis have produced equivocal evidence in support of the role of NO in pancreatitis, with some showing a protective effect for NO donors, and others a beneficial effect for NO inhibitors. No previous
Nitric oxide activity in a rat model of acute pancreatitis

work has been carried out to characterise directly the activity of the enzyme NOS, nor have animal models adequately established the time course of altered expression of NO during the progress of the disease. Using a caerulein induced model of acute pancreatitis in the rat, the present study set out to: (1) measure the plasma concentrations of NO metabolites and correlate these data with arterial blood pressure changes associated with the development of acute pancreatitis; (2) assay biochemically the activity of NOS (both iNOS and eNOS isoforms) in the pancreatic tissue; and (3) characterise the pancreatic expression and cellular localisation of NOS and nitrotyrosine by immunohistochemistry.

**Methods**

**EXPERIMENTAL RAT MODEL**

Adult male Wistar rats weighing 295–458 g were randomised into two experimental groups. Acute oedematous pancreatitis was induced in rats (n=10) by hyperstimulation of the exocrine function of the pancreas with the cholecystokinin analogue, caerulein. Control animals (n=10) were age and weight matched with the experimental group. Each rat was anaesthetised by intraperitoneal administration of 2.7 ml/kg of a mixture of one part midazolam + one part Hypnorm + two parts sterile water. The carotid artery was exposed and cannulated using an intravenous cannula (Venflon, 20G/32 mm). The cannula was connected to a blood pressure monitor and recorder (Lectromed, UK). Blood samples (0.5 ml) were taken at the beginning of the procedure for the measurement of NO metabolites (NO₂ + NO₃) and serum amylase. The experimental group received an infusion of caerulein (Sigma, UK) of 10 µg/kg/hour for seven hours (caerulein was dissolved in sterile normal saline). The control group received an equivalent volume infusion of normal saline of 1 ml/hour for seven hours. The arterial blood pressure was monitored throughout the procedure in both groups. The temperature was monitored using a digital rectal thermometer and was maintained at 37°C (±1°C) using a heat blanket. After seven hours of caerulein/normal saline infusion, further arterial blood samples were obtained for the measurement of NO metabolites and serum amylase. The animals were then allowed to die by exsanguination. The pancreas was harvested and divided for biochemical assay of NOS activity, for immunohistochemical staining of pancreatic NOS and nitrotyrosine, for histological paraffin wax section examination, and for measurement of pancreatic wet: dry weight ratio as described below. All animal experiments were carried out in accordance with UK government regulations.

**BIOCHEMICAL ASSAY OF NITRIC OXIDE SYNTHASES**

This method, a modification of that described by Bredt and Snyder, measures the conversion of radiolabelled L-arginine to L-citrulline, the equimolar coproduct of NO in the reaction catalysed by NO synthase. Pancreatic tissues were stored at −80°C until assayed. Tissues were homogenised in the presence of a buffer (pH 7.4) containing 25 mM HEPES, 1 mM dithiothreitol, 50 mM sucrose, 5 µg/ml leupeptin, 5 µg/ml pepstatin A, 5 µg/ml bestatin, 5 µg/ml chymostatin, 10 µg/ml soybean trypsin inhibitor, and 10 µg/ml phenylmethylsulphonyl fluoride. The homogenates were then centrifuged at 15 000 g for three minutes and the supernatants were separated for assay. All the supernatants were assayed in duplicate. A 100 µl aliquot of the supernatant was added to 100 µl of a reaction buffer (at 0°C, pH 7.4) containing 25 mM HEPES, 1 mM dithiothreitol, 20 µM tetrahydrobiopterin, 10 µM flavin adenine dinucleotide, 10 µM flavin mononucleotide, 1 mM nicotinamide adenine dinucleotide phosphate reduced form, 2 µM L-arginine, 5 µCi/ml [³H]-L-arginine, 25 mM L-valine, 10 U/ml calmodulin, and 10 µM calcium chloride.

To characterise the activity of the calcium independent inducible isoform of NOS, calcium chloride and calmodulin were omitted and 2 mM EDTA was included in the buffer. The mixtures were then incubated at 37°C for 30 minutes, and the reaction terminated by adding 200 ml of a 0.2 M sodium acetate buffer (pH 5.2) containing 2 mM EDTA and 0.5 mM citrulline. All chemicals were obtained from Sigma, UK. The L-citrulline formed during the reaction was separated from unreacted L-arginine by thin layer chromatography and the radioactivity in the citrulline band counted by liquid scintillation counting. The percentage conversion of L-arginine to L-citrulline was calculated. The results obtained using the buffer containing calcium chloride represent the total NOS activity (includes both calcium dependent constitutive and calcium independent inducible isoforms) while the buffer containing EDTA represents the calcium independent inducible NOS activity. The calcium dependent constitutive NOS activity was determined by the difference in activities obtained in the two buffers. The protein content of each pancreatic tissue homogenate was measured to correct for any differences in enzymatic activities due to a difference in the protein content of tissues. Enzymatic activity was expressed in terms of pmol/mg protein/min.

**MEASUREMENT OF NITRIC OXIDE METABOLITES IN PLASMA**

Plasma concentrations of nitrite and nitrate were assayed using an NO chemiluminescence analyser (Model 270 B NOA, Sievers Instruments Inc., Colorado, USA). This is a highly sensitive method (< 2 pmole NO/s) for measuring NO and its metabolites in biological fluids. Reduction of nitrate and nitrite to NO was performed using 0.1M vanadium chloride in 1 M hydrochloric acid at 80–90°C in a purge vessel. All samples were assayed in triplicate. A standard curve was established with a set of serial dilutions (10⁻¹ to 10⁻⁷ mol/l) of sodium nitrate. The concentrations of NO metabolites in the samples were determined by comparison with the standard curve. All chemicals used in this assay procedure were obtained from Sigma, UK.
IMMUNOHISTOCHEMICAL STAINING

Pancreatic tissues were fixed in 1% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for four hours and then transferred into a storage buffer, PBS/sucrose buffer, at 4°C until the cryostat blocks were prepared. Tissue sections (4 µm) were cut on a cryostat and applied to slides. The avidin-biotin complex immunoperoxidase staining system was used. The primary antibodies, anti-iNOS polyclonal, anti-eNOS polyclonal, and antinitrotyrosine, were produced in rabbit and were obtained from Ayniti Research Products Ltd, Exeter, UK. The presence of positive staining with the specific antibody was indicated by the development of a reddish-brown stain in the section, as a consequence of using the 3,3'-diaminobenzidine substrate. The presence or absence of positive staining and its cellular localisation was noted in each section, using each type of primary antibody in both groups.

MEASUREMENT OF SERUM AMYLASE ACTIVITY

Sigma amylase reagent was used for the quantitative kinetic determination of α amylase activity in the serum (Sigma, UK). Amylase activity was expressed as U/l; the expected normal range of amylase activity was 0–72 U/l.

ASSAY FOR ENDOTOXIN

The Limulus amoebocyte lysate (LAL) test (E-TOXATE) (Sigma, UK) was used for the detection of endotoxin. The samples, taken after seven hours, were assayed in duplicate. A positive test was indicated by the formation of a hard gel.

MEASUREMENT OF PANCREATIC WET:DRY WEIGHT RATIO

Pancreatic tissue was blotted on paper and weighed. This represented the wet weight. The tissue was then placed in an oven at 60°C for five days and reweighed to obtain the dry weight. The pancreatic wet:dry weight ratio was calculated for both the control and the pancreatitis groups.

STATISTICAL ANALYSIS

The distribution of data from each group of observations was tested (Shapiro-Francia W’ test and Normal probability plots) to confirm Normality. A two sample t test was then performed to compare data between the two groups. Comparisons were done at zero hours (baseline) and at seven hours. The summary statistics are presented as means, 95% confidence intervals (CI), and SEM. Fisher’s exact test was used for the analysis of categorised outcome (immunohistochemistry and endotoxin assay). A “split plot repeated measures analysis of variance (ANOVA)” with Bonferroni correction was used for analysis of the results of mean arterial blood pressure (MABP) throughout the seven hour procedure.

Results

EXPERIMENTAL ANIMAL MODEL

Four criteria were used to confirm that the rats in the experimental group developed pancreatitis: macroscopic appearance of the pancreas,
Nitric oxide activity in a rat model of acute pancreatitis

Histological examination, increase in pancreatic wet:dry weight ratio, and raised serum amylase activities. All four criteria confirmed that the rats in the experimental group developed acute oedematous pancreatitis. The pancreas was swollen, enlarged, and embedded in an oedematous gel at the end of the procedure in the pancreatitis group. Figure 1 shows the typical difference in the microscopic appearances between the pancreatitis group and the control group. Pancreatic wet:dry weight ratio was increased in the pancreatitis group compared with controls (mean 3.94 (SEM 0.18) versus 2.32 (0.11), p<0.0001). There was also a fifty-fold increase in serum amylase activities in the pancreatitis group after seven hours of caerulein infusion (2021.0 (278.0) versus 39.0 (2.0) U/l, p<0.0001).

ARTERIAL BLOOD PRESSURE

Figure 2 compares the means and 95% CI for the MABP between the two groups. There was a significant reduction in MABP at six and seven hours after induction of pancreatitis compared with the control group, which maintained a stable blood pressure throughout the procedure. At the end of the seven hour procedure, MABP was lower in the pancreatitis group compared with controls (mean 88.7 (SEM 4.6) versus 112.8 (4.1) mm Hg, p=0.008) (fig 3A).

PLASMA NITRITE AND NITRATE

The concentrations of the NO metabolites, nitrite and nitrate, were notably higher at the end of the seven hour procedure in the pancreatitis group compared with controls (mean 70.2 (SEM 5.9) versus 22.7 (2.2) µmol/l, p<0.0001) (fig 3B).

BIOCHEMICAL CHARACTERISATION OF NITRIC OXIDE SYNTHASE ACTIVITY

The total activity of NOS, which includes the combined activity of iNOS and cNOS isoforms, was higher in the pancreatitis group compared with the control group (mean 3.72 (SEM 0.18) versus 2.32 (0.11), p<0.0001). There was also a fifty-fold increase in serum amylase activities in the pancreatitis group after seven hours of caerulein infusion (2021.0 (278.0) versus 39.0 (2.0) U/l, p<0.0001). There was also down regulation in the activity of cNOS, the constitutive Ca2+ dependent isoform, in the pancreatic tissue of the pancreatitis group compared with controls (0.62 (0.12) versus 0.96 (0.08) pmol/mg protein/min, p=0.031) (fig 4B).
PANCREATIC IMMUNOHISTOCHEMISTRY
There was positive staining with the anti-iNOS antibody in the pancreatitis group, which was mainly expressed in the vascular smooth muscle cells (fig 5A). Only tissues from the pancreatitis group expressed such positive staining for iNOS (seven versus zero, \( p = 0.003 \)). There was also some evidence of positive staining of endothelial cells with anti-iNOS antibody in the pancreatitis group (three versus zero, \( p = 0.210 \)) (fig 5B). The control group expressed negative staining with the anti-iNOS antibody in all sections (fig 5C).

Staining with the anti-eNOS antibody revealed pronounced and similar expression of eNOS within endothelial cells in both the pancreatitis and the control groups (\( p = 0.630 \)).

There was clear positive perivascular staining with the antinitrotyrosine antibody in six sections of the pancreatitis group (fig 6A) compared with zero staining in control tissues (fig 6B) (\( p = 0.0012 \)).

ENDOTOXIN ASSAY
Endotoxin was detected in the plasma of two control rats and one with pancreatitis (\( p = 0.789 \)).

Discussion
The study showed increased production of NO in acute pancreatitis. These changes were associated with a reduction in the mean arterial blood pressure at the end of the seven hour procedure, consistent with the peripheral vasodilatory effect of NO at a time of maximal enzymatic activity of iNOS.\(^{20-33}\) During the first five hours of the procedure, the arterial blood pressure remained stable and showed no difference from the control group.

Our study is the first to show induction of iNOS in the pancreas and notable overproduction of NO in an experimental animal model of acute pancreatitis, and is the first to document the activity of iNOS and cNOS in whole pancreatic tissue. The biochemical assay showed increased iNOS activity plus down regulation in eNOS activity, a pattern that has been noted in other inflammatory conditions. MacNaul and Hutchinson\(^{34}\) performed northern hybridisations to examine the differential expression of iNOS and eNOS mRNA in human aortic smooth muscle cells and endothelial cells cultured in the presence or absence of cytokines (IL-1\( \beta \) and TNF-\( \alpha \)) and lipopolysaccharide (LPS). iNOS mRNA was induced in smooth muscle cells, whereas eNOS mRNA expression was down regulated in endothelial cells, suggesting that under specific inflammatory conditions the generation of NO in vascular tissue switches from eNOS in the endothelium to iNOS in the smooth muscle. Likewise, Liu and coworkers\(^{35}\) showed that rats treated with LPS in vivo displayed down regulation of eNOS mRNA in the heart, lung, and aorta, with a parallel up regulation of iNOS mRNA. Many studies have reported cytokine induced stimulation of Ca\(^{2+} \) independent NOS in other tissues—for example, in aortic smooth muscle cells.\(^{36}\)

Our immunohistochemical findings broadly support the biochemical data and illustrate the cellular localisation of iNOS expression in the pancreas. Histochemical staining showed clear expression of iNOS in the vascular smooth muscle cells in pancreatitis. Such induction of iNOS has previously been reported in response to inflammatory stimuli.\(^{37}\) iNOS is not normally expressed in vascular smooth muscle cells but may be stimulated by endotoxin or by cytokines such as TNF-\( \alpha \) and IL-1.\(^{38}\) The induction of iNOS in vascular smooth muscle cells, with its associated overproduction of NO, may be an important mediator of the systemic effects.
vasodilatation and hypotension associated with sepsis.\textsuperscript{39} Our findings also indicate some evidence for iNOS expression in pancreatic endothelial cells, an interesting observation in the light of how seldom such effects have been noted in vivo in other inflammatory conditions.\textsuperscript{32 40}

In addition to the vasodilatory effect of NO, its overproduction has a direct cytotoxic effect. Oxygen derived free radicals have been incriminated in the pathogenesis of acute pancreatitis.\textsuperscript{41} If the production of free radical species exceeds cellular antioxidant defences, then oxidative stress leads to a disturbance in cellular homeostasis, including peroxidation of membrane lipids, and damage to nuclear DNA, intracellular lysosomes, and mitochondria.\textsuperscript{41-43} Lipid peroxidation ultimately causes increased membrane permeability and cell death. The production of large amounts of NO by iNOS in response to proinflammatory cytokines or endotoxin has been implicated in the pathophysiology of cell and organ damage associated with circulatory shock. Under such circumstances, the vascular smooth muscle is a key site of NO overproduction, leading to reduced responsiveness to vasoconstrictor agents and a progressive dilatation of the vasculature.\textsuperscript{20}

The immunohistochemical staining of the pancreatic sections from our experimental animal model showed positive staining with the antinitrotyrosine antibody in the pancreatitis group, mainly in the vicinity of blood vessels. The formation of nitrotyrosine represents a specific peroxynitrite mediated protein modification that is different from modifications mediated by other reactive oxygen species, and is a useful marker for peroxynitrite detection.\textsuperscript{21 22} Our study is the first to provide evidence for the production of peroxynitrite in the pancreas during acute pancreatitis. Szabó and colleagues\textsuperscript{39} showed that injection of endotoxin into rats leads to the expression of iNOS in the thoracic aorta at six hours and increased circulating concentrations of nitrite/nitrate. At the same time point, they observed a notable increase in the immunoreactivity of nitrotyrosine, suggesting that during endotoxin shock, part of the NO produced following the induction of iNOS is converted into peroxynitrite in the vicinity of large blood vessels.

For many years it has been known that endotoxaemia is a recognised feature of acute pancreatitis. Foulis and colleagues\textsuperscript{44} detected endotoxin in the serum of 13 of 26 patients with acute pancreatitis. Six of seven patients with systemic complications of the disease had endotoxaemia. Windsor and colleagues\textsuperscript{7} reported a significant fall in the serum concentrations of endogenous antienidotoxin core antibodies in 28 of 33 patients with acute pancreatitis, implying exposure to endotoxin, and in all those with severe pancreatitis. Exley and colleagues\textsuperscript{45} found endotoxaemia present in 51\% of patients with acute pancreatitis on day 1; it was more common in non-survivors than survivors (91\% versus 35\%). As noted above, endotoxin can result in the induction of iNOS and overproduction of NO.\textsuperscript{12 13} In our experimental model of oedematous pancreatitis, endotoxaemia was essentially absent in both the pancreatitis and the control groups, indicating that the observed induction of iNOS was associated with the inflammatory process and not with endotoxaemia.

In summary, we have shown in an experimental model of acute pancreatitis that there is substantial induction of iNOS with down regulation of eNOS in the pancreas, resulting in an increase in total pancreatic NOS activity together with the production of tissue peroxynitrite. Increased plasma NO was associated with a notable reduction in arterial blood pressure. We conclude that excess NO arising from the inducible NO synthase is an important factor underlying the systemic and local haemodynamic disturbances and oxidative tissue damage associated with acute pancreatitis. Whether overexpression of NO in other vascular beds also contributes to the systemic hypotension is the subject of work currently underway in our laboratory.

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Figure 6  (A) Pancreatic section from the pancreatitis group showing positive staining with the antinitrotyrosine antibody. (B) Section from the control group with negative staining for the antinitrotyrosine antibody (original magnification ×200).
Al-Mufti, Williamson, Mathie et al

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