NFkappaB Mediated Expression of iNOS in Pancreatic Acinar Cells Contributes to the Severity of Acute Pancreatitis and Associated Lung Injury

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Abstract
The role of nitric oxide (NO) has been increasingly implicated in pathophysiology of acute pancreatitis (AP). Studies have shown increased NO production in AP although not all are agreeable on whether NO is beneficial or detrimental in AP. This study aims to investigate the role of iNOS in AP. AP was induced in mice by administration of cerulein (50 µg/kg/h). For the NFkappaB inhibition study, mice were pretreated with Bay 11-7082 (10mg/kg), iNOS inhibition was achieved by administration of 1400w (2 µg/kg/h) and L-NAME (25mg/kg/h) was used to inhibit all NOS activity. Acinar cells were hyperstimulated with cerulein (10-7M) with or without Bay 11-7082 pretreatment. NO production was determined by measuring the total nitrite and nitrate (NOx) content while iNOS expression and phospho-IkappaB were measured by western blot. Pancreatic and acinar cell iNOS expression and NOx production increased sharply and was sustained in response to cerulein hyperstimulation. These increases were significantly attenuated following NFkappaB inhibition. Non-selective NOS and selective iNOS inhibition resulted in similar amelioration of AP. These data shows that NFkappaB mediated expression of iNOS is the main contributor of NO production in acinar cells during AP and the inhibition of iNOS results in an improvement in AP.

Keywords: pancreatitis, iNOS.

INTRODUCTION
Acute pancreatitis (AP) is a common clinical condition with about 40 cases of AP per 100,000 adults reported every year (Granger 2005). Most of the patients suffer mild pancreatitis, which is self-limiting and recover in few days while others may require intensive care treatment for haemorrhagic and necrotic lesions of the pancreas with a mortality rate of 40 %. High incidence of death is due to the systemic inflammatory response syndrome (SIRS) leading to multiple organ failure (Gomez 2002). The pathogenesis of AP involves a complex cascade of events involving the interplay of local and systemic immune responses. However, the detailed pathogenesis and etiology have not yet been fully understood.

Nitric oxide (NO) is involved in physiological events such as modulation of vascular tone and neurotransmission as well as pathological conditions such as inflammation (Cirino 2006, Salvemini 2003, Luss 1996). The role of nitric NO has been increasingly implicated in the pathophysiology of AP though studies are still divided on whether NO plays a beneficial (Lomis 1995, Dabrowski 1994, Cuzzocrea 2002, Sandstrom 2005) or detrimental role (Molero 1995, Werner 1998, Qui 2001). Of the three NOS isoforms, only iNOS is inducible and is capable of producing large amounts of NO in a Ca” independent manner (Stuehr 1997, Galea 1999). Pancreatic iNOS expression (Lomis 1995, Rau 2001, Vaquero 2001) and activity (al-Mufti 1998, Viola 2000) have been shown to increase
in AP and of the two iNOS-knockout in AP studies published, both were conflicting (Qui 2001, Cuzzocrea 2002).

To further clarify the role of iNOS in AP, we investigated the expression of iNOS in the pancreas and subsequent NO production in the progression of cerulein-induced AP. Similar experiments were conducted with acinar cells in response to cerulein hyperstimulation and the influence of NFκB activation on the expression of iNOS in the pancreas and acinar cells were also studied. Finally we compared the effect of non-selective NOS inhibition and selective iNOS inhibition in cerulein-induced AP to determine the role of NO derived from iNOS in AP.

MATERIALS AND METHODS

Cerulein-induced pancreatitis

All animal experiments were approved by the animal ethics committee of National University of Singapore and carried out in accordance with established International Guiding Principles for Animal Research. Swiss mice (20–25 g) were randomly assigned to control or experimental groups using eight or more animals for each group. For the NFκB inhibition study, mice were given a single dose of Bay 11-7082 (10mg/kg) (Calbiochem, San Diego, CA) intra-peritoneally (i.p.) 30 mins before cerulein (Bachem, Bubendorf, Switzerland) administration. Animals were then given hourly i.p. injections of normal saline or cerulein (50 µg/kg) in saline for 3, 6 and 10 h. For the NOS inhibition studies, L-NAME (25mg/kg) (Sigma, St. Louis, MO, USA) or 1400w (1µg/kg) (Sigma, St. Louis, MO, USA) were injected i.p. 30 mins before cerulein and every hour thereafter. Animals were then given hourly i.p. injections of normal saline or cerulein (50 µg/kg) in saline for 10 hours. One hour after the last cerulein injection animals were sacrificed by an i.p. injection of a lethal dose of pentobarbitone. Harvested heparinized blood was centrifuged, the plasma removed and stored at −80 °C for subsequent measurement of plasma amylase content. Samples of pancreas and lungs were weighed and stored at −80 °C for subsequent measurement of tissue myeloperoxidase (MPO) activity, NOx content and western blotting. Random segments of the pancreas and lungs were fixed in 10% neutral phosphate-buffered formalin then embedded in paraffin wax and subsequently stained with haematoxylin and eosin. Random segments of the pancreas were also collected to measure water content.

Pancreatic acini preparation and in vitro treatment with cerulein

Pancreatic acini were obtained from mouse pancreas by collagenase treatment as described previously (Bhatia 2002). Briefly, pancreas from three Swiss mice (20–25 g) were removed, infused with buffer A, containing (in mM) 140 NaCl, 4.7 KCl, 1.13 MgCl2, 1 CaCl2, 10 glucose, and 10 HEPES (pH 7.2) and 200 IU/ml collagenase and 0.5mg/ml soybean trypsin inhibitor, and incubated in a shaking water bath for 10 min at 37°C. The digested tissue was passed through 50 mg/ml BSA and washed twice with buffer A before further experiments. Cells were incubated with a supraphysiological dose of cerulein, 10−7M for 15, 30, 45 and 60 mins. For the NFκB inhibition study, cells were pre-incubated Bay 11-7082 (10 µM) 30 mins prior to addition of cerulein. At the end of each time point cells are pelleted down, the supernatant was assayed for NOx and the cell pellet was used for western blotting.

Amylase estimation

Amylase activity was measured using a kinetic spectrophotometric assay. Plasma samples were incubated with the substrate, 4,6-ethylidene (G7)-p-nitrophenyl (G1)-1-D-maltoheptoside (Sigma,St. Louis,MO, USA) for 2 min at 37 °C and absorbance measured every minute for the subsequent 2 min at 405 nm (Pierre 1976). The change in absorbance was used to calculate the amylase activity and expressed as U/L.

Myeloperoxidase estimation

Neutrophil sequestration in pancreas and lung was quantified by measuring tissue MPO activity (Bhatia 2000). Tissue samples were thawed, homogenized in 20 mM phosphate buffer (pH 7.4), centrifuged (10,000 ×g, 10 min, 4 °C) and the resulting pellet resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO). The suspension was subject to four cycles of freezing and thawing and further disrupted by sonication (40 s). The sample was then centrifuged (10,000 ×g, 5 min, 4 °C) and the supernatant used for the MPO assay. The
reaction mixture consisted of the supernatant, 1.6 mM tetramethylbenzidine (Sigma, St. Louis, MO), 80 mM sodium phosphate buffer (pH 5.4), and 0.3 mM hydrogen peroxide. This mixture was incubated at 37 °C for 110 s, the reaction terminated with 2 M H$_2$SO$_4$ and the absorbance measured at 450 nm. This absorbance was then corrected for the DNA content of the tissue sample (Labarca 1980) (fold increase over control).

**Preparation of tissue and cell lysates for Western blot analysis**

After treatment, pancreatic acinar cells or tissue samples were homogenized on ice in radioimmunoprecipitation assay (RIPA) buffer, supplemented with the protease inhibitor cocktail containing pepstatin, leupeptin, chymostatin, antipain, and aprotinin (Roche Diagnostics, Indianapolis, IN), and centrifuged at 4°C for 15 min at 13,000 rpm. The supernatants were collected and stored at -80°C. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

**Western blot analysis**

Cell and tissue lysates (50 μg) were separated on 4-12% SDS-polyacrylamide gel and electrophoretically transferred to PVDF membranes. Nonspecific binding was blocked by 1-h incubation of the membranes in 5% nonfat dry milk in 0.05% Tween 20 in PBS (PBST). The blots were then incubated overnight with the primary antibody iNOS, (Santa Cruz Biotechnology) and phospho-IκBα (Cell Signalling) at 1:1,000 dilutions in the buffer containing 2.5% nonfat dry milk in PBST, after which they were washed four times with PBST and finally incubated for 1 h with goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology) at 1:2,000 dilutions in the buffer containing 2.5% nonfat dry milk in PBST. The blots were developed for visualization using enhanced chemiluminescence detection kit (Pierce, Rockford, IL). Hypoxanthine-guanine phosphoribosyl transferase (HPRT) (Santa Cruz Biotechnology) was used as the housekeeping protein.

**NOx estimation**

Nitrite and nitrate content was estimated based on the colorimetric Griess Assay (Green 1982) with slight modifications. Tissues were homogenized in 20mM sodium phosphate buffer and pelleted down at 10,000g for 15 minutes. The tissue supernatants, plasma and acinar cell supernatants were then passed through centrifugal filter tubes (Millipore, Billerica, MA) with a pore size of $5k$Da to de-proteinize the influence of Bay pretreatment. Values are means±SE of 6 animals in each group. *P<0.05 compared with cerulein only treatment and control. #P<0.05 compared with cerulein with Bay pretreatment and cerulein only treatment.
samples. The samples were subsequently incubated with 20mU nitrate reductase (Sigma, St. Louis, MO) supplemented with 5μM FAD and 100μM β-NADPH (Sigma, St. Louis, MO) for 30 mins at 37°C in the dark to convert existing nitrate to nitrite. Finally, Griess reagent (1% sulphanilamide, 0.1% N-NED, 2.5% H3PO4, Sigma, St. Louis, MO) was added in equal volumes and incubated for 15 minutes in the dark. The absorbance is then measured at 540nm and the concentration was calculated using sodium nitrate standards. This absorbance was then corrected for the DNA content of the tissue sample (Labarca 1980) (fold increase over control).

Statistics

Data are expressed as the mean±standard error of the mean (SEM). The significance of changes was evaluated by using analysis of variance (ANOVA). If ANOVA indicated a significant difference, the data were analyzed by using Tukey’s method as a post hoc test for the difference between groups. A P value of <0.05 was considered to indicate a significant difference.

RESULTS

AP results in an NFκB dependent increase in pancreatic iNOS expression and NO production

Pretreatment with Bay 11-7082, an NFκB inhibitor, resulted with an expected reduction in phosphorylated pancreatic IκB as shown in Fig 1a. Induction of AP resulted in a significant increase of pancreatic iNOS expression at all time points of 3, 6 and 10 hours (Fig 1b). iNOS expression increased markedly as early as 3 hours and peaked at 6 hours with an increase of 500% as compared to the control. At 10 hours, iNOS levels decreased but still remained significantly higher by 300% over the control. NOx levels were also significantly increased in the pancreas at all time points with an initial increase of 130% that gradually decreased but still remained significantly higher as compared to the control (Fig 1c). The observed increase in pancreatic iNOS expression as well as NOx levels were significantly reduced following NFκB inhibition at all time points (Fig 1b and 1c).

Figure 2: (A) A representative immunoblot showing inhibition of IκB phosphorylation by Bay in acinar cells following cerulein hyperstimulation. (B) Histogram of densitometry of acinar cell iNOS expression as detected by western blot and representative immunoblot showing acinar cell iNOS expression following cerulein hyperstimulation and with pretreatment of Bay. (C) Effect of cerulein hyperstimulation on NO production in acinar cells as evidenced by NOx content and the influence of Bay pretreatment. Values are means±SE of 6 animals in each group. *P<0.05 compared with cerulein only treatment and control. #P<0.05 compared with cerulein with Bay pretreatment and cerulein only treatment.
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Figure 3: (A) Effect of L-NAME and 1400w treatment on NO production as evidenced by NOx content at 10 hours in the pancreas during AP. (B) Effect of L-NAME and 1400w treatment on NO production as evidenced by NOx content at 10 hours in the lungs during AP. Values are means±SE of 6 animals in each group. *P<0.05 compared with cerulein only treatment and control. #P<0.05 compared with cerulein with Bay pretreatment and cerulein only treatment.

Figure 4: (A) Effect of L-NAME and 1400w treatment on plasma amylase activity at 10 hours during AP. (B) Effect of L-NAME and 1400w treatment on pancreatic water content at 10 hours during AP. (C) Effect of L-NAME and 1400w treatment on pancreatic MPO activity at 10 hours during AP. (D) Effect of L-NAME and 1400w treatment on lung MPO activity at 10 hours during AP. Values are means±SE of 6 animals in each group. *P<0.05 compared with cerulein only treatment and control. #P<0.05 compared with cerulein with Bay pretreatment and cerulein only treatment.

In-vitro cerulein hyperstimulation results in an NFκB dependent increase in pancreatic acinar cells iNOS expression and NO release

Pretreatment with Bay 11-7082, an NFκB inhibitor, resulted with an expected reduction in phosphorylated pancreatic IkB as shown in Fig 2a. Cerulein hyperstimulation caused a marked 700% increase in acinar cell iNOS production as early as 15 minutes (Fig 2b). This increase was sustained up to 45 minutes with a peak of 10 fold at 30 minutes. This increase in iNOS expression was followed by an increase in NOx production by the acinar cells. Fig 2c shows a gradual increase in NOx at 15 minutes and peaked at 30 minutes in-line with the iNOS expression (Fig 2b), after which it plateaued till 60 minutes. The observed increase in acinar cell iNOS expression as well as NOx production was almost completely abolished following NFκB inhibition at all time points (Fig 2b and 2c).

Effect of L-NAME and 1400w on NOx production in cerulein induced pancreatitis

Treatment with L-NAME and 1400w resulted in a similar reduction in NOx levels in the pancreas and lungs following 10 hours of cerulein administration (Fig 3a and b). With either treatment, the NOx levels were reduced by about 60% in the pancreas to reach basal levels (Fig 3a). In the lung, both L-NAME and 1400w resulted in about 50% reduction in NOx levels to reach basal levels (Fig 3a), similar to that seen in the pancreas.
Figure 5: Representative H&E stains of the pancreas: (A) control, (B) AP, (C) AP with L-NAME treatment and (D) AP with 1400w treatment. (B) Shows marked edema (e) formation and increased neutrophil infiltration (arrows) while (C) and (D) shows significant improvement. Representative H&E stains of the lungs: (E) control, (F) AP, (G) AP with L-NAME treatment and (H) AP with 1400w treatment. (F) Shows thickening of the alveolar walls and marked neutrophil infiltration (arrows) while (G) and (H) shows significant improvement. Magnification is 200x.

Effect of L-NAME and 1400w on cerulein induced pancreatitis

Treatment with L-NAME and 1400w showed very similar significant improvements in AP. Plasma amylase activity was significantly reduced by approximately 2200U and 2000U in the L-NAME and 1400w groups respectively in comparison to the 15 fold increase observed in the cerulein only treated group (Fig 4c). L-NAME and 1400w treatment showed similar reduction in lung MPO activity, 1.6 and 1.8 fold respectively as compared with the cerulein only group of 2.5 fold as compared to the control (Fig 4d).

Histological examination of the pancreas and lungs by H&E showed similar protection against AP in the L-NAME and 1400w group. Fig 5b exhibits marked pancreatic edema formation and increased neutrophil infiltration and this was significantly reduced in the inhibitors treated groups (Fig 5c and d). The lungs show significant alveolar thickening and neutrophil infiltration in response to cerulein (Fig 5f) and this too was attenuated by treatment with the NO inhibitors (Fig 5g and h).

DISCUSSION

During inflammation, over-production of NO increases oxidative stress and cellular injury by increasing levels of COX-2 (von Knethen 1997) as well as activating it (Salvemini 1993, Vane 1994). Together with superoxide (O$_2^-$), NO readily forms peroxynitrite (Xia 1997) a powerful oxidant that is involved in lipid peroxidation, protein nitration, and DNA strand damage (Szabo 2003). High intracellular levels of NO are also capable of inhibiting mitochondrial respiration function by binding to NADH and succinate in an irreversible manner (Brown 1995). On the other hand, NO has been shown to decrease neutrophil recruitment by down-regulating expression of adhesion molecules in the blood vessels (Inagaki 1997, Secco 2006). This dual role of NO in inflammation is underscored by the conflicting results of previous studies on AP (Lomis 1995, Dabrowski 1994, Cuzzocrea 2002, Sandstrom 2005, Molero 1995, Werner 1998, Qui 2001).

Of the three NOS isoforms, only iNOS is capable of producing considerable amounts of NO as well as O$_2^-$ in varying amounts depending on the environmental conditions (Xia 1998). Coupled with the fact that iNOS expression is inducible especially under inflammatory conditions, our study focuses on its role in AP. In this study we have shown an increase in pancreatic iNOS expression in response to cerulein hyperstimulation. This corroborates previous findings that have shown similar increases in a variety of AP models by
immunohistology (al-mufti 1998, viola 2000, Ayub 2001), western blot (Rau 2001) and arginine to citrulline conversion in absence of Ca²⁺ (al-Mufti 1998, viola 2000). While the immunohistological and iNOS activity data showed upregulation at 7 hours post AP induction (al-mufti 1998, viola 2000, Ayub 2001), data from Rau et al and ours show similar early increase in iNOS expression by immunoblotting at 3 hours and this was sustained till 10 hours with a peak expression at 6 hours. This increased iNOS expression was accompanied with significant increase in NO production at the corresponding time points as evidenced by pancreatic NOx content. A similar observation was made in acinar cells that were exposed to supraphysiological doses of cerulein. We found a rapid and marked increase in iNOS expression as early as 15 minutes which was sustained for an hour. This increase in acinar cell iNOS expression was also accompanied with an increase in NO release as with the pancreas.

NFκB is among the three transcription factors identified in the expression of iNOS along with camp response element (CRE) and Ccaat-enhancer-binding proteins (C/EBP) (Galea 1999). The activation of NFκB in the pancreas during AP and in acinar cells in response to cerulien hyperstimulation (Steinle 1999, Han 1999) has been implicated in the progression of AP inflammatory response by activating the genes of pro-inflammatory molecules such as cytokines; TNF-α, IL-6 and IL-1, chemokine; MCP-1, IL-8 and RANTES; adhesion molecules; ICAM-1, VCAM and E-Selectin and enzymes; COX-2 (Grady 1997, Gukovskaya 1997, Christman 1998).

In this study we used Bay 11-7082 as an inhibitor of NFκB activation. Indirect evidence of the efficacy of this inhibitor was shown by means of semi-quantitative western blot in the reduction of phosphorylated IkB in the cerulein and Bay 11-7082 treated group as compared to the cerulein only group. With the use of this inhibitor, we observed a significant reduction in pancreatic iNOS expression and NO production following NFκB inhibition in AP by Bay 11-7082.

However, the reduction did not result in iNOS expression and NOx content falling to basal levels which could be due to other transcription factors involved in iNOS transcription such as cAMP response element (CRE) and Ccaat-enhancer-binding proteins (C/EBP) (Galea 1999) that may be activated in the progression of AP. A previous study using N-Acetylcysteine (NAC) as an NFκB inhibitor has also shown reduction in iNOS expression in a taurocholate-induced pancreatitis model (Vaquero 2001). However the use of NAC as an NFκB inhibitor in an inflammatory model may not be ideal as NAC also acts as a potent antioxidant. NAC has been shown to facilitate GSH biosynthesis and supplying GSH for GSH peroxidase-catalyzed reactions (Cotgrave 1997) that could contribute a reduction in cellular damage due to oxidative stress that is associated with AP and other inflammatory models. This protective effect of NAC itself may contribute towards the improvement of the inflammatory condition thus leading to a reduction of pro-inflammatory mediators in an NFκB independent manner. Bay 11-7082 on the other hand has not been shown to have anti-oxidative effects and has been shown to inhibit NFκB activation by irreversibly blocking the phosphorylation of IkBa (Pierce 1997).

Apart from the in-vivo model of AP, we have also shown that acinar cells over-express iNOS in response to cerulein hyperstimulation subsequently resulting in increased NO production. This rapid substantial increase of iNOS expression and NO release was almost completely abolished following NFκB inhibition by Bay 11-7082 as compared to the partial reduction observed in the in-vivo AP model. Therefore it is possible to speculate that this early production of NO by acinar cells as a result of NFκB activation may contribute to oxidative stress in the pancreas thus contributing to the probable initiation and progression of the pancreatic inflammatory response in cerulein-induced pancreatitis.

Through the use of iNOS selective inhibitor (1400w) and a non-selective NOS inhibitor (L-NAME), we have shown an identical complete reduction of NO production in the pancreas and lungs in AP. This once again affirms the central role that iNOS plays in the over-production of NO in AP. Our subsequent studies on the influence of selective and non-selective NO inhibition in AP and associated lung injury also showed almost identical reduction in AP parameters. Both L-NAME and 1400w showed similar significant improvement in AP as evidenced by reductions in plasma amylase, pancreatic water content and pancreatic as well
as lung MPO activity. This was supported by histological examination of the pancreas and lungs.

Though the role of NO in AP may be controversial with some studies showing a beneficial role (Lomis 1995, Dabrowski 1994, Cuzzocrea 2002, Sandstrom 2005) and others detrimental (Molero 1995, Werner 1998, Qui 2001), all of these studies show increased NOS activity and NO production in AP. In this study, we have shown iNOS to be the major contributor of NO in the pancreas during AP and that acinar cells too are capable of over-expressing iNOS in response to cerulein hyperstimulation. We have also shown that this increase in iNOS expression is dependent on NFκB activation and inhibition of iNOS activity improves the outcome of AP to the same extent as a non-selective inhibition of all NO activity. However an important factor in the activation of NFκB and expression of iNOS is the cyclical manner in which iNOS over-expression may lead to NFκB activation and translocation as well as the activation of NFκB leading to iNOS over-expression which will be the subject of further study. Taken together, we suggest that the NFκB dependent over-expression of iNOS plays a central role on the effect of NO in AP.

References


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