J Korean Med Sci 2003; 18: 520-6
ISSN 1011-8934

The Role of Nitric Oxide in Experimental Cerulein Induced Pancreatitis

An enhanced formation of nitric oxide (NO), due to the induction of inducible nitric oxide synthase (iNOS), has been implicated in the pathogenesis of shock and inflammation, but its role in acute pancreatitis still remains controversial. To clarify the role of NO in acute pancreatitis, the present experiment investigated the expression of iNOS and the effect of NOS inhibition on cerulein-induced pancreatitis in rats. Group I received intraperitoneal (ip) injection of normal saline. Group II received two ip injections of cerulein (20 μg/kg). Group III received injections of N^G-nitro-L-arginine methyl ester (L-NAME) (30 mg/kg) with cerulein. Group IV received L-arginine (250 mg/kg) with cerulein and L-NAME. The expression of iNOS in the pancreas was examined by western blot analysis. The plasma concentration of NO metabolites was measured. The severity of pancreatitis was assessed by measuring serum amylase, pancreas water content and histopathological examination. Compared with controls, the cerulein group displayed significantly increased expression of iNOS and raised plasma NO metabolites. Treatment with L-NAME significantly decreased hyperamylasemia, plasma NO level, and the extent of pancreatic injury. Treatment with L-arginine reversed the effects of L-NAME. These findings suggest that an enhanced formation of NO by iNOS plays an important role in the development of acute pancreatitis, and inhibition of NO production has the beneficial effects in reducing pancreas injury.

Key Words : *Pancreatitis, Acute Necrotizing; Inducible Nitric-Oxide Synthase; Nitric Oxide; Caerulein*

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Received : 3 February 2003
Accepted : 20 March 2003

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*This study was supported by a special research grant of Korea University, 1999.

INTRODUCTION

Acute pancreatitis is an inflammatory disease resulting from the autoactivation of digestive enzymes in the pancreatic acinar cell. Inflammatory cell recruitment, proinflammatory cytokines, and oxygen-derived free radicals have been reported to play an important role in determining the severity of acute pancreatitis (1-4). Exposure of many tissues and cells to proinflammatory cytokines results in the expression of the inducible isoform nitric oxide synthase (iNOS) (5). An enhanced formation of nitric oxide (NO) due to the induction of iNOS has been implicated in the pathogenesis of shock and inflammation (6). Al-Mufti et al. (7) reported that excess NO arising from the iNOS is an important factor underlying the systemic and local hemodynamic disturbances and oxidative tissue damage associated with acute pancreatitis. Furthermore, iNOS-deficient mice have been shown to exhibit resistance to the acute pancreatitis induced by cerulein (8). Therefore, production of NO by iNOS has been proposed as a pathogenic factor in acute pancreatitis.

NO is an inorganic, gaseous free radical that is involved in the physiology of circulation and in pathophysiologic conditions such as inflammation. NO is synthesized from the amino acid L-arginine by a family of enzymes, the NOS (9, 10). To date three isoforms of NOS have been identified: two of them are termed neuronal constitutive (nNOS or NOS-1) and endothelial constitutive (eNOS or NOS-3), which are constantly present and synthesize small amounts of NO in response to physical or receptor stimulation. The inducible NOS (iNOS or NOS-2), the third NO isoform, is found only after stimuli such as endotoxin or proinflammatory cytokines and generates large amounts of NO in a sustained and largely uncontrolled manner (5, 10-12).

Excess production of NO causes vasodilatation and hypotension that is refractory to vasoconstriction, together with increased microvascular permeability and extravascular third spacing (9). Moreover, NO rapidly reacts with the superoxide anion, which results in the formation of peroxynitrate and hydroxyl radicals, both powerful oxidants and cytotoxic agents (13). These findings suggest that the overproduction of NO by iNOS plays an important role in the hemodynamic disturbances and cellular damage of several inflammatory states. Several investigators have examined the role of NO in various animal models of acute pancreatitis (14-25). However, conflicting results have been reported. There are several lines of evidence showing that NO seems to have a protective effect on local pancreatic cellular damage, mainly by inhibition of neutrophil accumulation and improvement of microcircula-

tion (14-19). In contrast, several reports suggest that inhibition of NOS or iNOS has a beneficial effect in experimental pancreatitis (20-25).

To clarify the role of NO in acute pancreatitis, we examined the expression of iNOS and the effects of NOS inhibition on cerulein-induced pancreatitis in rats.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (200-250 g) were used. All rats were fed standard laboratory chow diet (Cheil-jedang Co., Seoul, Korea) and housed in 12 hr dark-light rooms under controlled temperature (23±1℃) and humidity (65%). All experiments were performed according to protocols approved by the Korea University Hospital Animal Research Committee, and maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals of Korea University.

Experimental Protocol

Forty rats were randomly divided into four groups of 10 animals each, and subjected to the treatment regimens list below. Group I (control) received four intraperitoneal injection of normal saline at 1 hr intervals. Group II (cerulein) received two intraperitoneal injection of cerulein (Research Plus, Bayonne, NJ, U.S.A.) at a dose of 20 μg/kg body weight at 1 hr intervals. Group III (cerulein+L-NAME) received two intraperitoneal injections of cerulein at 1 hr intervals and four intraperitoneal injections of N^G-nitro-L-arginine methyl ester (L-NAME, Sigma Chemical Co., St. Louis, MO, U.S.A.) at a dose of 30 mg/kg body weight at 1 hr intervals. First injection of L-NAME started with the first cerulein injection. Group IV (cerulein+L-NAME+L-arginine) received cerulein and L-NAME, as in group III and received two intravenous injection of L-arginine (Sigma Chemical Co.) at a dose of 250 mg/kg body weight as a bolus into the tail vein at 1 hr intervals simultaneously with cerulein and L-NAME.

Five hours after initiation of the cerulein or physiologic saline injection, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (Sigma Chemical Co.), a dose of 40 mg/kg body weight. Before killing the rats, the blood was collected from the abdominal aorta to assay serum amylase, plasma NOx (NO_2 plus NO_3). All blood samples were centrifuged at 1,800 rpm for 10 min at 4℃. The plasma was sampled using sterile pipettes. These samples were then immediately stored at -70℃ until assayed. After killing the rats, the pancreas was quickly removed and small pieces of the pancreas were excised for routine histological examination. The remainder of each pancreas was stored at -70℃ for western blot analysis of iNOS.

Amylase and NOx Assay

Serum amylase was measured by an enzymatic assay, using the Abbott spectrum analyzer (Abbott, Chicago, IL, U.S.A.). Measurements of plasma NOx concentration were determined by Griess reaction (26) using nitrate/nitrite colorimetric assay kit (Cayman, Ann Arbor, MI, U.S.A.)

Western Blot Analysis of iNOS

Pancreas samples were homogenized with ice-cold 25 mM Tris-HCl buffer (pH 7.5) using Ultra Turrax homogenizer (IKA, Wilmington, NC, U.S.A.) for 4 min. The homogenates were centrifugated at 14,000 rpm for 20 min. The supernatants were collected and the protein concentrations were mearsured by the method of Lowry et al. (27). Protein extracts (20 μg/lane) were diluted in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer in a ratio of 1:1 and heated at 100℃ for 5 min. Samples were electrophoresed on 10% Tris-Glycine polyacrylamide gel in 1× Tris-glycine SDS buffer at room temperature. After electrophoresis, the gel was transferred to 0.45 μm Hybond-P membrane for 2 hr at 4℃. The membrane was blocked in 5% blocking solution (5% skim milk, 1× PBS [pH 7.4], and 0.1% Tween20) for 1 hr and incubated for 1 hr with IgG polyclonal rabbit antibody against iNOS (anti-iNOS 1:2,000; Affinity, Golden, CO, U.S.A.). Blots were washed four times with washing solution (1× PBS [pH7.4], and 0.1% Tween20) and incubated for 1 hr with secondary antibody (1:1,500 dilution), peroxidased, labeled anti-rabbit immunoglobulin G. Labeled proteins were visualized by ECL kit (Ammersham Life Science, Buckinghamshire, U.K.) by following the manufacture's suggested protocol, followed by autoradiography.

Tissue Water Content

Fragments of pancreas were blotted dry and weighed to determine tissue wet weight. This tissue was then dessicated by overnight incubation at 80℃ and reweighed to determine tissue dry weight. Pancreas water content was calculated as the difference between wet and dry weights and expressed as a percentage of wet weight.

Histologic Grading

Pancreatic tissue was fixed in 20% formalin, embedded in paraffin wax, and sectioned into 5 μm slices. The tissue was stained with hematoxylin and eosin. Histologic assessment was performed on all tissue specimens of all parts. Morphological changes were evaluated in a blind manner by two independent pathologists. The histologic grading of inflammatory cell infiltration was carried out with reference to a scale ranging from 0 to 3 (minimal to maximal, respective-

ly). By contrast, the grading of necrosis and vacuolization was based on the percentage contained in the examined area. The samples were graded as follows: absence of lesion=0; involvement of 1-10%=1; 11-25%=2; 26-50%=3; >50%=4.

Data Analysis

All values were expressed as means±SEM. Differences between groups were evaluated by one-way ANOVA and Mann-Whitney rank-sum test. Statistical significance was assumed for p value <0.05.

RESULTS

iNOS expression

Western blot analysis showed expression of iNOS in pancreata from the pancreatitis group. In contrast, no expression of iNOS was detected in the control group (Fig. 1).

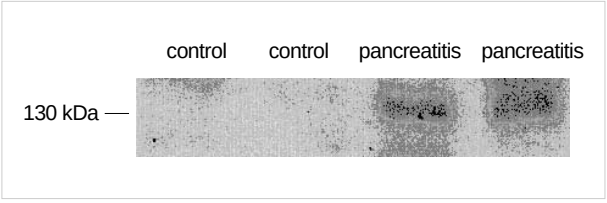

Fig. 1. The expression of iNOS. Western blot analysis showed expression of iNOS in pancreata from the pancreatitis group.

Effect of NOS Inhibitor or Substrate on Serum Amylase and Plasma NOx Activity

The serum amylase concentration was significantly increased in group II, which received cerulein, as compared with the control group. In the group III, treated with L-NAME, level of amylase was significantly reduced to 3,900 ±420 U/L, 50% of that in the cerulein group (p<0.01). In group IV, treated with coinfusion of L-NAME and L-arginine, serum amylase level did not change significantly as compared with the cerulein group (Fig. 2).

As shown in Fig. 3, the plasma NOx concentration in the cerulein group was significantly increased compared to that in the control group, from 5.3±2.3 to 41.2±3.8 μmol/L. On the other hand, a significant decrease in the plasma NOx level was observed in group III compared with that of group II (p<0.01). However, the combined administration of L-NAME and L-arginine (group IV) reversed the effect of L-NAME on plasma NOx level.

Effect of NOS Inhibition or Substrate on Pancreas Water Content

The pancreatic water content of rats receiving cerulein alone (group II) was significantly greater than that of the control group (86.6±2.3 to 73.9±2.1%, p<0.05). The pancreatic water content in group III (79.4±3.9%) was significantly less than that of the cerulein group (p<0.05). However, no significant changes were observed in group IV (84.7±1.8%).

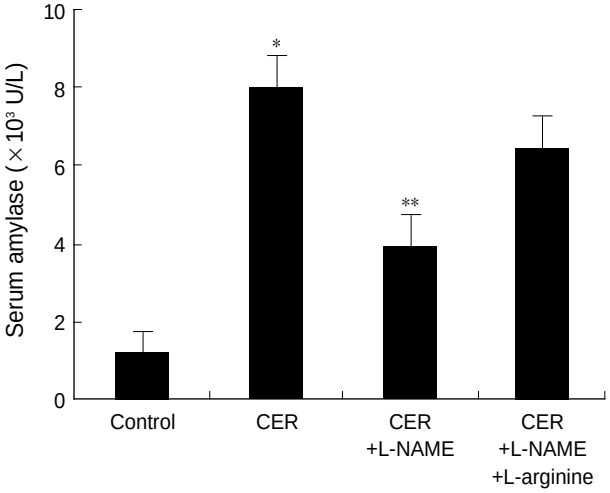

Fig. 2. Effect of NOS inhibitor or substrate on serum amylase activities in cerulein (CER)-induced pancreatitis. Serum amylase level was significantly decreased in group treated with L-NAME. Combined administration of L-NAME and L-arginine reversed the effect of L-NAME on serum amylase. Data represent the mean ±SEM. *p<0.001 vs. control; **p<0.01 vs. Cerulein group receiving injection cerulein.

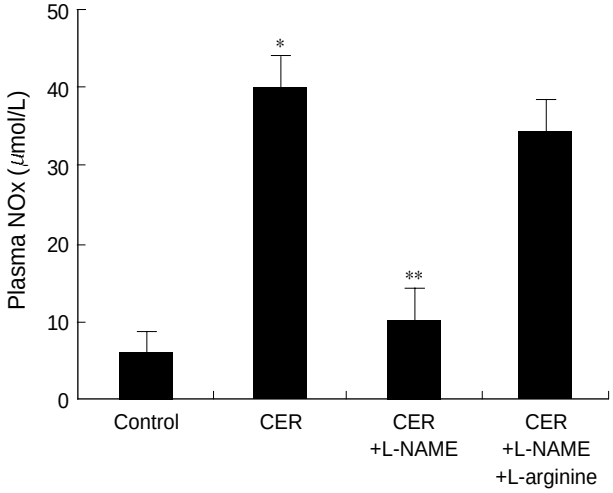

Fig. 3. Effect of NOS inhibitor or substrate on plasma NOx level in cerulein (CER)-induced pancreatitis. Plasma NOx activities was significantly decreased in group treated with L-NAME. Combined administration of L-NAME and L-arginine reversed the effect of L-NAME on plasma NOx level. Data represent the mean ±SEM. *p<0.001 vs. control; **p<0.01 vs. Cerulein group receiving injection of cerulein.

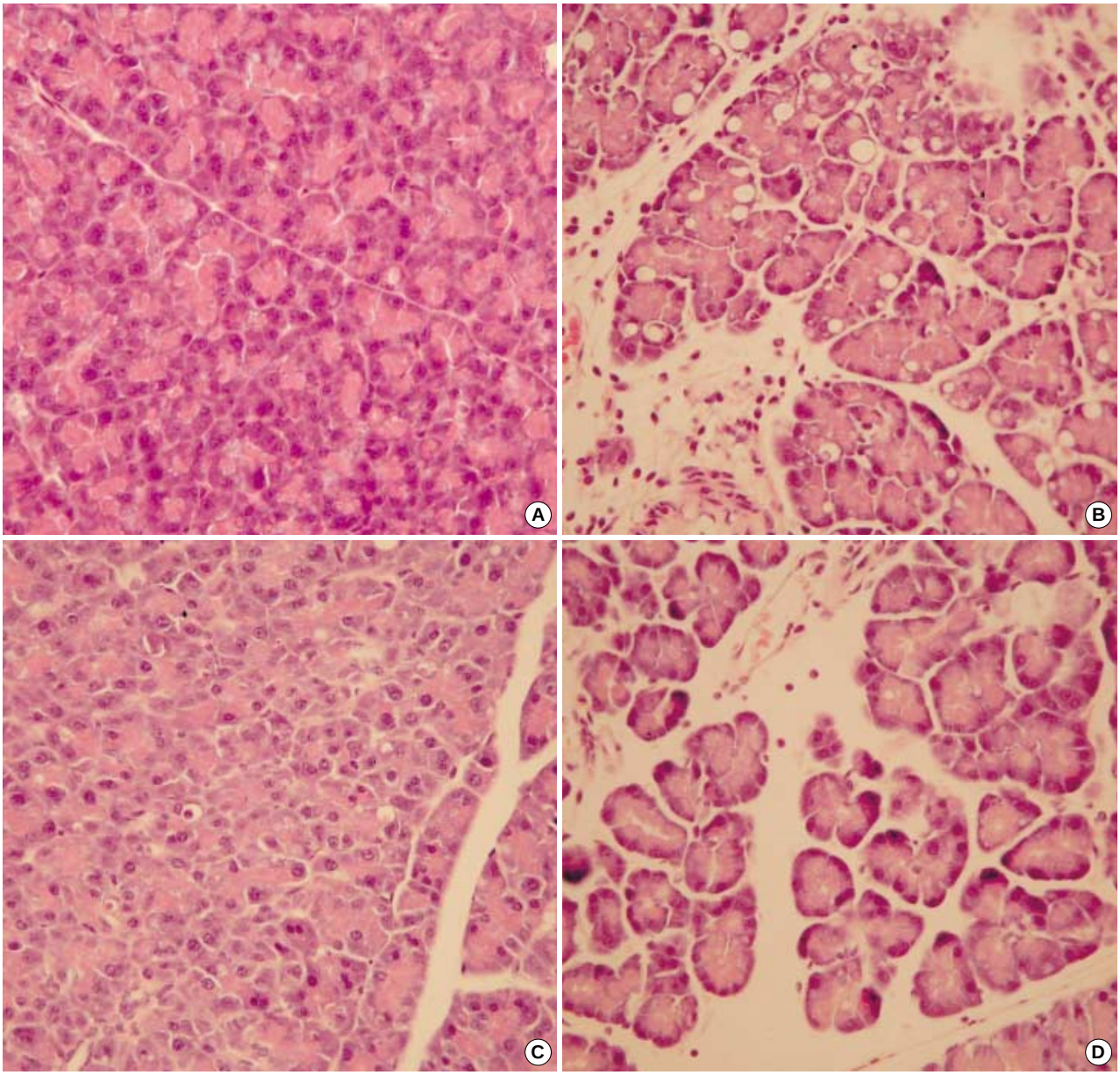

Fig. 4. Effect NOS inhibitor or substrate on pancreatitis in rats. (A) Pancreas from control rat. (B) Pancreas from rat injected with cerulein alone, showing extensive acinar cell vacuolization, interstitial edema and inflammatory cell infiltration. (C) Pancreas from rat treated with L-NAME showing marked reduction in acinar cell vacuolization, edema and inflammatory cell infiltration. (D) Pancreas from rat treated with L-NAME and L-arginine showing similar finding as B (H&E stain, × 400).

Effect of NOS Inhibitor or Substrate on Histological Changes of Pancreas

The histological change of the pancreas is presented in the Fig. 4 and 5. This histological examination of pancreata from group II showed changes compatible with an edematous form of acute pancreatitis such as interstitial edema, inflammatory cell infiltration, acinar cell vacuolization and minimal evidence of cell necrosis. Group III, which received L-NAME, showed a significant reduction of inflammatory cell infiltrations such as neutrophil compared to group II ($p<0.01$). Vacuolization of acinar cells or necrosis were also significantly decreased in group III ($p<0.01$). However, group IV, treated with both L-NAME and L-arginine, showed no significant histological changes when compared with group II.

DISCUSSION

It is generally believed that a very early event in the evo-

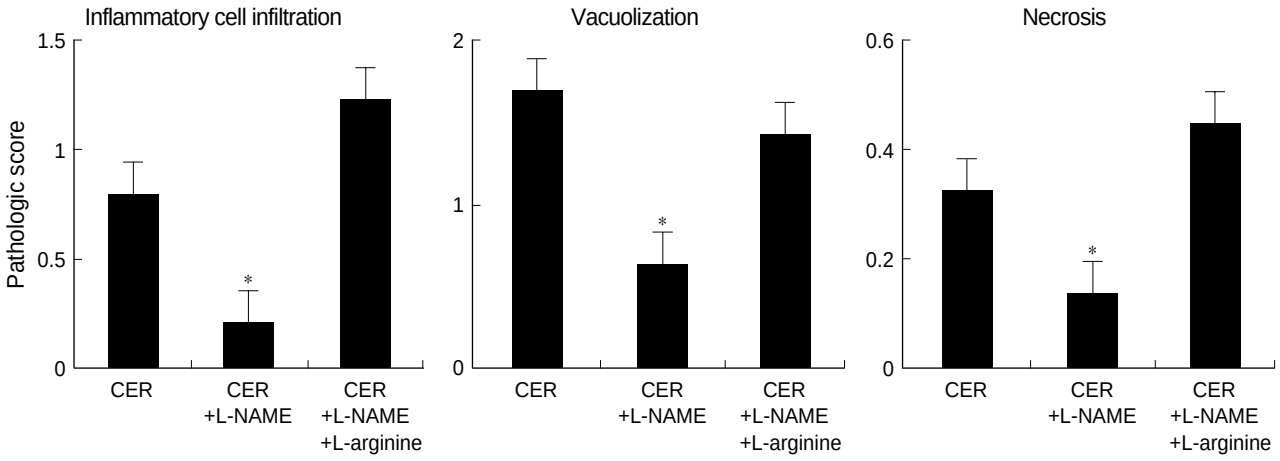

Fig. 5. Effect of NOS inhibitor or substrate on histological changes in cerulein-induced pancreatitis. In group treated with L-NAME shows significant reduction of inflammatory cell infiltration, vacuolization and necrosis. In group treated with concomitant administration of both L-NAME and L-arginine fail to show any effect on pathologic score. Data represent the mean±SEM. *p<0.01 vs. Cerulein group receiving injection of cerulein.

lution of acute pancreatitis is the release of proinflammatory cytokines including tumor necrosis factor-α, interleukin (IL)-6 and IL-8 from the inflamed pancreas. Levels of these cytokines from patients with acute pancreatits correlate with the severity of the disease (2-4). The proinflammatory cytokines activate the production of the iNOS, resulting in overproduction of NO, which acts as a key final cellular and intercellular mediator (5, 9-11). Recently, Al-Mufti et al. (7) have shown in an experimental acute pancreatitis that there is substantial induction of iNOS with down-regulation of constitutive NOS (cNOS) in the pancreas, resulting in an increase in total pancreatic NOS activity together with the production of tissue peroxynitrate. At the same time point, they observed that the increased expression of iNOS was mainly localized within vascular smooth muscle cells, with positive perivascular staining for nitrotyrosine, marker for peroxynitrite induced oxidative tissue damage. Taken together, the above findings suggest that induction of iNOS and the subsequent production of NO contribute to the pathophysiology of acute pancreatitis.

To investigate the pathophysiologic role of NO, a direct modulation of the NO pathway by application of either endogenous NOS inhibitors or NO donors was chosen in animal models of acute pancreatitis (14-25). However, controversial results were obtained as to whether the effects of enhanced NO generation are positive, negative, or negligible with respect to the development of local and systemic complications in the course of the disease. These differing results could result from different models of pancreatitis, or may be attributable to different drug regimens that achieve partial or complete inhibition of NOS. Recently, special iNOS gene deletion "knockout" mice have been used to characterize the impact of NO form iNOS during acute pancreatitis (8, 28). However, these animal studies also have yielded ambiguous evidence in support of the role of NO in pancreatitis.

In this study, we have shown that the administration of supramaximal dose of cerulein induces iNOS expression in pancreatic tissue, and significantly increased plasma concentration of NO metabolites. Also, our observations confirmed that overproduction of NO arising from the iNOS was associated with the development of acute pancreatitis as shown by marked hyperamylasemia, increased pancreas water content, and histologically extensive acinar cell vacuolization. Our data confirm previous observations of enhanced iNOS expression and increased NO metabolites during acute pancreatitis (7, 21, 24, 29, 30). Because the amount of NO produced by cNOS is too small to be reflected in the changes of NO levels, the induction of iNOS, presumably in macrophages and vascular smooth cells, may be responsible for the large release of NO in acute pancreatitis (7). Although the pathologic roles of iNOS and enhanced NO production in acute pancreatitis still remain controversial, many studies provide compelling evidence that iNOS is, in part, responsible for the activation of inflammatory cascade as well as pancreatic damage (5, 7, 10-12). Excessive production of NO contributes to the delayed vascular decompensation and to the hyporeactivity of the vasculature to vasoconstrictor agents observed in several experimental models of circulatory shock (10, 31). There is evidence that some of the cytotoxic effects of NO are due to the formation of peroxynitrate, a reactive oxidant formed by the rapid reaction of NO with superoxide anions (32). Accumulation data suggest that peroxynitrite may contribute to the tissue injury in a number of pathophysiologic conditions associated with inflammation and/or oxidant stress (33). Furthermore, Almufti et al. (7) showed that iNOS induction and oxidative tissue damage in the pancreas were associated with an increase in systemic NO and arterial hypotension. Strong evidence

suggests that iNOS-deficient mice exhibit resistance to the acute pancreatitis induced by cerulein (8). These notions may in part explain how cytotoxicity from NO may participate in the pancreatic damage associated with acute pancreatitis in this study.

We showed that the administration of universal NOS inhibitor, L-NAME reduced each of the parameters used to quantify the severity of cerulein-induced pancreatitis (i.e., serum amylase, pancreas water content, and histologic parameter), whereas concomitant administration of both L-NAME and NOS substrate, L-arginine fail to show any effect on injury parameters. Further, our study has shown that plasma concentration of NO metabolites after the administration of L-NAME significantly decreased. This effect was reversed by combined administration of both L-NAME and L-arginine. These results indicate that NO, and/or its reaction products, damage pancreas tissue and that L-NAME could confer some protection against the development of acute experimental pancreatitis. These results are similar to those generated other studies: administration of N^G-nitro-L-arginine (L-NNA), NOS inhibitor inhibited the elevation of serum NOx in cerulein-induced pancreatitis in mice pretreated with lipopolysacharide (21). L-NAME, competitive NOS inhibitor, has been shown to prevent lung injury in acute pancreatitis model (24). Further, animal studies also have documented that the administration of a selective iNOS inhibitor reduces bacterial translocation in acute pancreatitis and appears to have ameliorated the course of disease (25). Therefore, inhibition of NOS had favorable effects on pancreatitis which is complicated by the condition in which iNOS is expressed to produce excessive amounts of NO.

On the contrary, several investigations of acute experimental pancreatitis have shown that L-arginine reduced pancreatic edema formation and intrapancreatic trypsinogen activation, whereas L-NNA or L-NAME exacerbated the severity of pancreatitis (14-19). These studies have supported the view that general blockade of NOS might affect cNOS and cNOS blockade eliminates the beneficial effects of NO such as vasodilatation to keep blood flow and reduction in leukocyte adhesion. General blockade of NOS by universal inhibitors such as L-NAME and L-NNA in vivo may exhibit contrasting effects in organ function, depending on the predominant pathway or cell function that is disrupted, and on the alternative activation of compensatory mechanism (10). The possible effects of NOS inhibitor such as vasoconstriction, increased blood pressure, protection against NO-mediated tissue damage, enhanced platelet aggregation and enhanced leukocyte accumulation, may be beneficial or detrimental on pancreas inflammation. In an experimental pancreatitis, increased iNOS activity plus down-regulation in cNOS activity and reduced mean arterial blood pressure have been reported (7). In our study, administration of NOS inhibitor significantly reduced plasma NO level and pancreas injury in this pancreatitis model. Therefore, these observations suggested that general blockade of NOS may eliminate the harmful effects of NO rather than diminishing its beneficial effects. Consequently, L-NAME might affect iNOS and inhibit excessive NO production by iNOS that might damage pancreas tissue. To further clarify the significance of iNOS in acute pancreatitis, additional experiments with selective iNOS inhibitor using the pancreatitis model, isolated perfused pancreas, should be considered.

In conclusion, we have shown that NO excessively produced via iNOS was involved in cerulein-induced pancreatitis. We have also demonstrated that L-NAME, universal NOS inhibitor, attenuated the severity of pancreatic damage associated with acute pancreatitis. The attenuation effect of L-NAME may be attributable, in part, to a reduction of plasma NO levels. We would conclude that an enhanced formation of NO by iNOS plays an important role in the development of acute pancreatitis, and inhibition of NO production has a beneficial effect in reducing pancreatic inflammation.

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