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Involvement of Nitric Oxide in Mesenteric Vascular Reactivity following Intraperitoneal Pancreatic Juice in Rats

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Abstract

The purposes of this study were to examine the protein expressions of endothelial and inducible nitric oxide synthase (eNOS and iNOS) of the rat intestinal smooth muscle, and to elucidate the role of nitric oxide (NO) in the reactivity of the superior mesenteric artery (SMA) to vasoconstrictors following intraperitoneal (i.p.) injection of pancreatic juice. Immunohistochemistry was used to observe the protein expressions of eNOS and iNOS in the intestinal tissues 15 h after i.p. injection of pancreatic juice (1 ml/100 g body weight). To test the vascular reactiveness, SMA was isolated and perfused with Tyrode's solution at a constant flow rate of 5 ml/min. The changes in perfusion pressure as the measure of contractile responses to phenylephrine (PE) were monitored. I.P. injection of pancreatic juice induced increases of plasma levels of tumor necrosis factor α (TNFα) ($P < 0.001$; N = 7) and NO ($P < 0.001$; N = 7). N^{ω}-nitro-L-arginine methyl ester (L-NAME) reduced the release of TNFα and NO. There were 8.3 ± 1.2-fold and 11.4 ± 2.8-fold increases in the protein expressions of eNOS and iNOS, respectively, in the intestinal tissue after pancreatic juice injection. PE (10^{-8} ~ 10^{-4} M) produced a dose-dependent vasoconstrictive effects on the SMA bed. Contractile responses to PE were attenuated in pancreatic juice-treated group. Addition of L-NAME (10^{-4} M) resulted in full recovery of the responses to phenylephrine in SMA bed, while aminoguanidine (AG, 10^{-4} M) caused only partial recovery. Our results indicate that i.p. injection of pancreatic juice results in a decrease in vascular reactivity of mesenteric vessels that is dependent on both eNOS and iNOS expressions in the intestinal vascular bed. Overproduction of NO elicits intestinal low vascular reactivity.

Key Words: pancreatic juice, intestinal vascular bed reactivity, nitric oxide synthase, nitric oxide

Introduction

During acute or chronic pancreatitis, pancreatic enzymes are activated within the pancreas resulting in digestion of pancreatic tissues (30). In the face of blocked pancreatic drainage such as stones in the common bile duct or alcoholism, hypersecretion of pancreatic juice causes acini to rupture and release active enzymes which digest pancreatic tissue and induce leakage of juice into peritoneal space. Furthermore, interstitial edematous pancreatitis, necrotizing pancreatitis, pancreatic pseudocyst and pancreatic

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abscess are potential to induce leakage of biologically active compounds into peritoneal space to induce ascites (21), lung injury (31-34), liver injury (21) and kidney injury (12). Kistler *et al*. (15) reported that the release of pancreatic enzymes in septic shock caused tissue injury and generated cellular activators that were associated with organ dysfunction. A severe attack of panceatitis also induced splanchnic hypoperfusion leading to loss of intestinal mucosal integrity, intestinal barrier failure and subsequent bacterial translocation (22). Seerden *et al*. (25) and Kinnala *et al*. (14) also demonstrated that acute pancreatitis caused a loss of propulsive activity and contractility of intestine. Other studies provided evidence to suggest that pancreatitis-induced organ injury and dysfunction involved inflammatory mediators, activated leukocytes, cytokines storm (19), sepsis (5) and inducible nitric oxide synthase expression (4).

Nitric oxide (NO) represents a potential key mediator of the local and systemic manifestations of acute pancreatitis in different experimental models (17, 26). NO has been implicated to play a dual role (beneficial or detrimental effects) in organ injury due to different challenges (2, 3). NO is essential for the microvascular integrity and motor function of the gastrointestinal tract (20, 24, 36). Potential sources of NO in the gut include intrinsic intestinal tissue (mast cells, epithelium, smooth muscle and neural plexus) and resident and/or infiltrating leukocytes (neutrophils and monocytes) (10). Overproduction of NO could induce low reactivity of the intestines to vasoconstrictors, change intestinal motility and produce irritable bowel syndrome (10, 13). Decrease in vascular reactivity to vasoconstrictor after intestinal injury may contribute to the development of poor bowel function. Inducible nitric oxide synthase (NOS) is thought to play a role in gut motility disorders that occur under proinflammatory conditions (20, 24). Clinically, alterations in bowel motility may result from excessive production of NO in endotoxemia and inflammatory bowel disease (6, 24). These studies suggest that endogenous NO exerts a tonic relaxatory influence on the smooth muscle of the intestinal vessels and intestinal wall. The relationship among pancreatitis-induced low bowel motility, relaxative smooth muscle and NOS has not been yet clarified.

In this study, we aimed to examine the mesenteric vascular reactivity changes after intraperitoneal (i.p.) injection of pancreatic juice. We investigated possible alterations in mesenteric vascular reactivity to phenylephrine. In addition, the effects of NO were evaluated. The expression of NOS isoform was examined. The purpose was to elucidate the role of NO and NOS in the mesenteric vascular reactivity following i.p. injection of pancreatic juice.

Materials and Methods

Preparation of Animals

Male Sprague-Dawley rats (300 to 350 g, pathogen-free) were purchased from the National Animal Center. They were housed in a controlled environment at a temperature of 22 ± 1°C and under a 12 h/12 h light/dark cycle. Food and water were available *ad libitum*. Care and use of the animals were in accordance with the principles of the National Animal Center guidelines. The rats received an i.p. injection of pancreatic juice (2 ml of pancreatic juice per 100 g body weight). For the collection of pancreatic juice, one group of rats was anesthetized with pentobarbital (50 mg/kg i.p.). The abdomen was shaved. A midline vertical laparotomy was performed. Ligaments attaching the liver, diaphragm, abdominal wall and neighboring organs were divided. A catheter was inserted into the biliopancreatic duct to collect pancreatic juice. Fifteen hours after pancreatic injection, the rats were anesthetized with pentobarbital (50 mg/kg, i.p.). Femoral vein cannulation was used for blood sampling to determine changes in plasma levels of TNFα and NO. The superior mesenteric artery with its branches was isolated and perfused. Immunohistochemistry analysis was used to detect the protein expressions of endothelial NO synthase (eNOS) and inducible NO synthase (iNOS) in intestinal smooth muscle.

Measurement of NO by HPLC

We used a high-performance liquid chromatographic (HPLC) method for the determination of nitrite and nitrate anions as NO metabolites. After separation on a strong anion-exchange column (Spherisorb SAX, 250 × 4.6 mm I.D., 5 microns), two on-line post-column reactions occurred. The first involved nitrate reduction to nitrite on a copper-plated cadmium-filled column. In the second, diazotization-coupling reaction between nitrite and the Griess reagent (0.05% naphthylenediamine dihydrochloride plus 0.5% sulphanilamide in 5% phosphoric acid) took place, and the absorbance of the chromophore was read at 540 nm. Before injection into the chromatographic system (ENO-20, Eicom Nox Analyzer, Kyoto, Japan), the samples were diluted with methanol (30 µl plasma with 30 µl methanol). The precipitate was centrifuged at 15,000 × *g* for 10 min. The supernatant was injected into the HPLC and separated on a strong anion-exchange column (Spherisorb SAX, 250 × 4.6 mm I.D., 5 µm) and this separation was followed by two on-line post-column reactions. The first involved nitrate reduction to nitrite on a copper-plated cadmium-filled column. The second reaction

involved a diazotization-coupling reaction between nitrite and the Griess reagent (0.05% naphthylene-diamine dihydrochloride plus 0.5% sulphanilamide in 5% phosphoric acid). The absorbance of the chromophore was read at 540 nm. The method had a sensitivity of 30 pmol for both anions, as little as 0.05-0.1 ml sample volume was required and linearity was observed up to 60 nmol for each anion.

TNFα Measured by ELISA

TNFα concentration in blood samples was measured by an enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Woburn, MA, USA). Blood samples were collected in plasma separation tubes. All samples were stored at -70°C before testing. All reagents, samples and working standards were brought to room temperature and prepared for ELISA following the manufacturer's instructions. Each sample was performed in duplicate and the result was determined by an automated ELISA reader at 450 nm/540 nm wavelength.

Immunohistochemistry

Small intestinal tissues were dissected 15 h after pancreatic juice challenge for immunochemical analysis of the protein expression of eNOS and iNOS as follows: [1] Small intestinal tissues from pancreatic juice-treated and sham-operated rats were fixed in a tissue-fix buffer, embedded in Super-Tek OCT compound (catalog no: PS0001 and PS0002; Gene Research Laboratory, Taipei, Taiwan, ROC) and were frozen in liquid nitrogen. Sections (5-μm thickness) were cut on a cryostat (Leica CM1900), then thawed and mounted onto gelatin-coated slides. All 5-μm frozen intestinal sections from the juice and sham groups were used for immunohistochemical staining. [2] Small intestinal tissues were first incubated with blocking reagent, then with the appropriate dilution of primary antibody (mouse anti-rat eNOS or anti-rat iNOS monoclonal antibody at a titer of 1:50; Chemicon MAb, 13421, Temecular, CA, USA), and finally with an anti-mouse IgG-horseradish peroxidase (HRP) secondary antibody at a titer of 1:100. Sections were labeled and developed with HRP substrate solution and counterstained with a hematoxylin stain kit (PS003, Gene Research Laboratory, Taiwan, ROC). [3] To quantify immunohistochemical differences in rat small intestinal tissue sections without relying on subjective assessments, we used digital imaging and the Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA) microimaging package. Data were collected and analyzed by the method described in the user guide in the Counting, Measuring and Classifying sections. The overall fields from each section of each tissue were digitally captured with a high-resolution cooled CCD camera (ProgRes C14, Jenoptik Laser, Optik, System GmbH, Jena, Germany) and stored as 8-bit color images. The immunostained tissue cells were automatically highlighted by Image-Pro Plus, and the area covered by immunohistochemically positive cells (with brown color) was scored as positive and divided by the total area.

Reactivity Test of the Superior Mesenteric Artery (SMA)

Fifteen hours after receiving sham and pancreatic juice injection, the SMA was isolated and perfused with Tyrode's solution at a constant flow rate of 5 ml/min. The time interval (15 h) was taken in accordance to the previous study that acute lung injury occurred approximately 15 h after pancreatitis (33). The perfusion pressure was monitored from a side arm placed before the perfusion pump. The changes in perfusion pressure reflected the vascular response. Administration of phenylephrine (10^{-8} ~ 10^{-4} M) produced vasoconstriction in a dose-related manner in mesenteric vessels. The vasoconstrictor response to phenylephrine was compared between the mesenteric vascular beds from rats treated with pancreatic juice and the sham rats, and from pancreatic juice-treated rats with addition of L-NAME and aminoguanidine (AG).

Experimental Design

Animals were randomly divided into 3 groups. In the pancreatic juice group (n = 11), rats were given no treatment except i.p. injection of pancreatic juice. Rats in the sham group (n = 11) were prepared in the same manner as in the juice group without injection of juice. Pharmacologic intervention group (n = 11) were prepared by administration of L-NAME (20 mg/kg) one day before the i.p. injection of the pancreatic juice. The series of experiment included four groups of mesenteric perfusion preparation. There were sham (n = 10) and pancreatic juice (n = 30). The pancreatic juice group were then categorized into three subgroups with addition of saline, L-NAME and AG (n = 10 for each subgroup). Additional experiments were performed to test whether L-NAME, AG and L-ARG produced adverse effects. Ten rats were used for each agent.

Data Analysis

Data are expressed as means ± SEM. Comparisons among groups were made with one-way ANOVA and Scheffe's comparison. Comparisons within each group for a given parameter were made using paired Student's-*t* tests. Values of $P < 0.05$ were considered statistically significant.

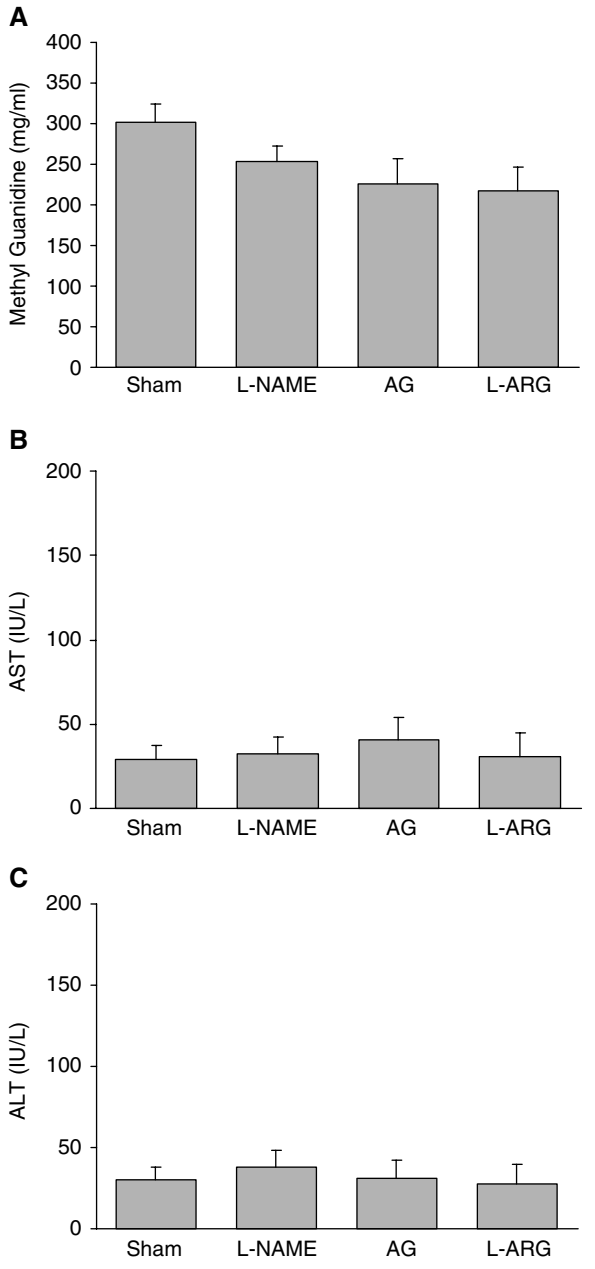

Fig. 1. Effects of L-NAME, AG and L-ARG on the plasma levels of methyl guanidine (A), AST (B) and ALT (C) in sham rats. There were no significant differences among the four groups (n = 10 for each group).

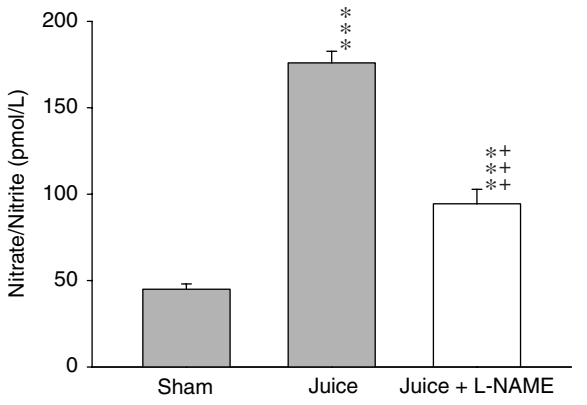

Fig. 2. Changes in plasma nitrite/nitrate, the metabolites of NO, in the sham (n = 6) and pancreatic juice-challenged groups (n = 7) (***$P < 0.001$, significantly different between the sham and juice-challenged groups). Non-specific NOS inhibitor, L-NAME, significantly attenuated the NO release ($^{+++}P < 0.001$) after i.p. injection of pancreatic juice.

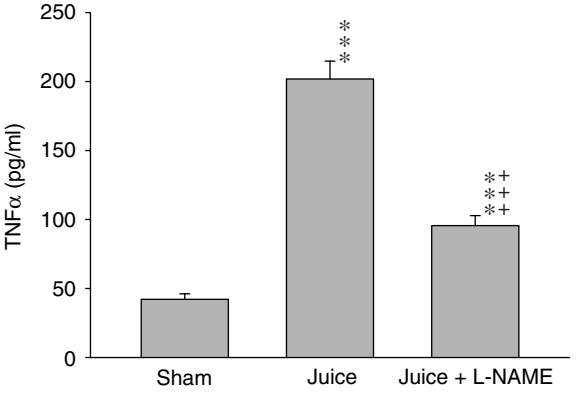

Fig. 3. Injection of pancreatic juice significantly increased the plasma TNFα level [***$P < 0.001$, significantly different between the sham (n = 6) and juice groups (n = 7)]. Non-specific NOS inhibitor, L-NAME, significantly attenuated the TNFα release after i.p. injection of pancreatic juice ($^{+++}P < 0.001$).

Results

The plasma levels of methyl guanidine, AST and ALT were not different among sham, L-NAME, AG and L-ARG groups. There were no significant adverse effects of NO inhibitors and precursor (Fig. 1). In a previous study, we also showed the NOS inhibitors or NO donors alone did not affect the lung weight changes and protein content in bronchoalveolar lavage in experiments using isolated rat's lungs (11).

Fig. 2 shows that nitrite/nitrate, the product of NO, increased significantly after pancreatic juice injection (***$P < 0.001$, significantly different between the juice and the sham groups). L-NAME significantly attenuated NO release ($^{+++}P < 0.001$) after the i.p. injection of pancreatic juice.

Pancreatic juice injection significantly increased the plasma level of tumor necrosis factor α (TNFα) (***$P < 0.001$, significantly different between juice and sham groups). L-NAME significantly attenuated the TNFα release ($^{+++}P < 0.001$) after the i.p. injection of pancreatic juice (Fig. 3).

The contractile responses to phenylephrine (PE) were attenuated in pancreatic juice-treated SMA. Addition of L-NAME (10^{-4} M) resulted in a full

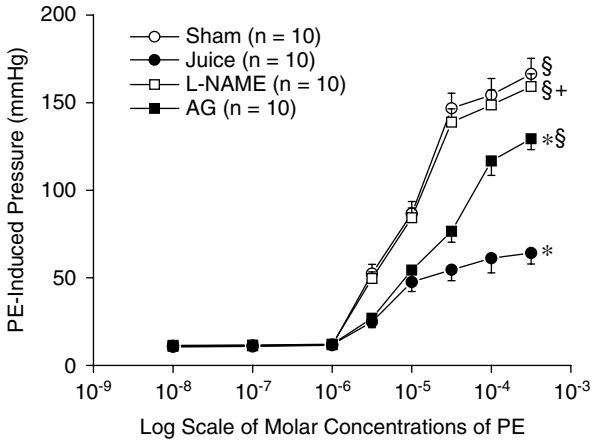

Fig. 4. Changes in perfusion pressure reflect the extent of vasoconstriction in the SMA bed perfused with constant flow. The contractile responses to PE were attenuated in pancreatic juice-treated SMA. Addition of L-NAME (10^{-4} M) resulted in full recovery of the responses to PE in SMA bed, while aminoguanidine (AG, 10^{-4} M) caused partial recovery (* *vs*. Sham; § *vs*. Juice; and + L-NAME *vs*. AG).

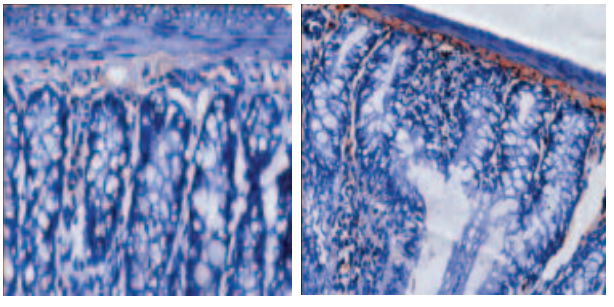

Fig. 5. Immunohistochemical staining of eNOS activity in the intestinal smooth muscle tissues of sham and juice-challenged groups. In the sham group, eNOS immunohistochemical staining was virtually absent. In contrast, the intestinal tissue in the juice-challenged group showed a marked increase in eNOS level (8.3 ± 1.2-fold increase, n = 3, $P < 0.001$) (×200).

recovery of the responses to PE in SMA bed, while AG (10^{-4} M) caused partial recovery (Fig. 4). The results suggest that eNOS and iNOS were involved in the low mesenteric reactivity by almost equal extent.

Fig. 5 shows the immunohistochemical expression of eNOS in intestinal tissues. The microphotographics revealed a marked increase in the expression of eNOS (8.3 ± 1.2-fold increase) in the juice group compared with the sham group. There was essentially no immunostaining for eNOS in the sham group.

Immunohistochemical expression of iNOS in intestinal tissues revealed a marked increase in the

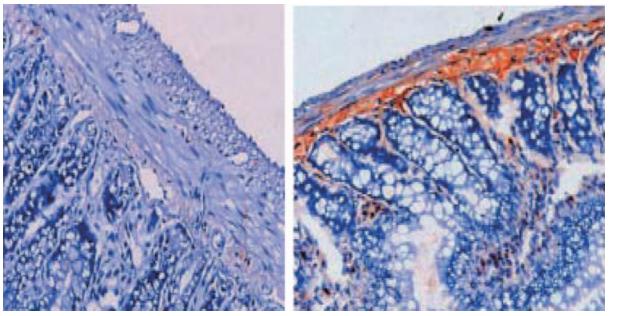

Fig. 6. Immunohistochemical staining of iNOS activity in the intestinal smooth muscle tissues of sham and pancreatic juice-challenged groups. In the sham group, iNOS immunohistochemical staining was virtually absent. In contrast, the intestinal tissue in the juice-challenged group showed a marked increase in the iNOS expression (11.4 ± 2.8-fold increase, n = 3, $P < 0.001$) (×200).

expression of iNOS (11.4 ± 2.8-fold increase) in the juice group compared with the sham group. There was essentially no immunostaining for iNOS in the sham group (Fig. 6). Administration of pancreatic juice, L-NAME, AG and L-ARG to the sham group did not significantly affect the eNOS and iNOS mRNA expression (Fig. 7).

Discussion

In this study, we demonstrated that i.p. injection of pancreatic enzymes induced low reactivity of the mesenteric vascular bed to the vasoconstrictor, PE. This low reactivity could be the result of increased protein expressions of eNOS and iNOS in the intestinal tissues. Thereby, a large increase in NO production reduced the reactiveness of intestinal vasculature to vasoconstrictor. It is possible that both eNOS and iNOS participated in the mesenteric low reactivity.

NO is a proposed mediator of nonadrenergic noncholinergic neural inhibition (23, 27). In addition to its role as a neuroeffector substance, studies have suggested that endogenous formation of NO maintains intestinal mucosal integrity, protecting the gut from blood-borne toxins and tissue-destructive mediators (18). Thus, NO plays a dual role in both gut smooth muscle relaxation and mucosal protection (7). NO is also involved in the function of endothelial cells, platelets, mast cells and macrophages within the digestive system (9). It is an endogenous neurotransmitter in human jejunal longitudinal smooth muscle, acting at least in part *via* a mechanism mediated by guanylyl cyclase (36). Overproduction of NO as a consequence of increased NOS expression could induce relaxation of gut smooth muscle (8), decrease in gut motility and occurrence of intestinal bloat or

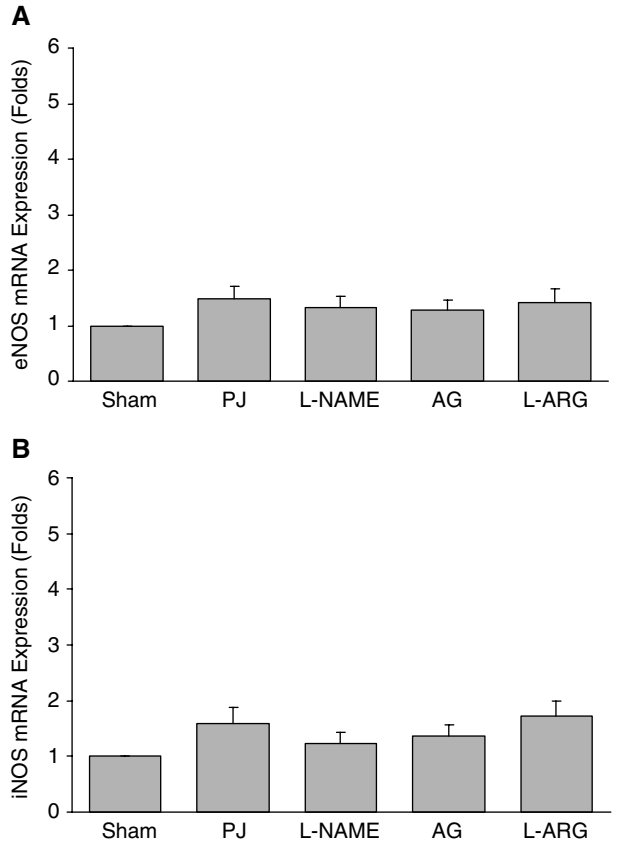

Fig. 7. mRNA expression of eNOS (A) and iNOS (B) was not significantly altered by the administration of pancreatic juice (PJ), L-NAME, AG and L-ARG to sham rats. The mRNA expression level in the sham group was taken as 1, and the changes in the other groups were calculated as folds increase (n = 10 for each group).

flatulence (1).

Inducible NOS is thought to play a role in gut motility disorders that occur under proinflammatory conditions. A great number of macrophages were found to be evenly distributed in the muscle layer of the gastrointestinal tract and produce effects on smooth muscle contraction and the initiation of inflammatory responses. After incubation with lipopolysaccharide (LPS), macrophages began to express iNOS and produced NO. Results indicated that resident macrophages in the muscle layer induced iNOS as an inflammatory reaction, affected smooth muscle contraction, and initiated immune response in the smooth muscle layer of the gastrointestinal tract, when activated by LPS (28). L-NAME could reduce basal intestinal blood flow and decrease intestinal motility in the dose-dependent manner. Overproduction of NO mediated by iNOS aggravates lung injury caused by acute necrotizing pancreatitis (16) and I/R-induced pancreatitis (29).

Clinically, ileus occurs after sepsis and shock-induced gut ischemia/reperfusion (I/R). Hassoun *et al*. demonstrated that iNOS is upregulated in the gut only after more severe ischemic insults, and ileus is mediated, at least in part, by iNOS under these conditions (10). It has been suggested that iNOS-induced NO production may promote inflammation and induce cell and tissue dysfunction and contributes to mucosal hyperemia. Inhibition of NO synthesis ameliorates barrier dysfunction during more advanced stages of inflammation (10). Less study addressed the involvement of eNOS on the bowel function and vascular reactiveness. However, we found that eNOS was also important in the mesenteric reactivity.

Pancreatitis induces leakage of pancreatic juice into peritoneal space, and activating factors could induce inflammation, cytokines release, NOS expression and hemodynamic changes. Intraperitoneal injection of pancreatic juice could induce increased inflammatory responses and changes in the reactivity of the mesenteric vascular bed. The possible reasons for these changes could be due to the release of mediators such as cytokines and NO, and inflammatory responses including inflammatory cells activation, oxidative stress and oxygen radicals release (35).

The possible mechanism of reduced intestinal vascular reactivity by NO, eNOS and iNOS may be operated by activation of NOS mRNA by pancreatic juice through an inhibition of vasoconstriction in the mesenteric bed and reduction of intestinal mobility in intestinal tissues (30, 36). We found that the mRNA expression of eNOS and iNOS was not significantly affected by the administration of pancreatic juice, L-NAME, AG and L-ARG to the sham rats. The results suggest that the mesenteric vascular reactivity may be mediated at the mRNA level. Further studies are required to clarify the ultimate mechanism of action.

In summary, pancreatic juice induced NOS expression in the mesenteric vascular bed and intestinal smooth muscle. Overproduction of NO reduced intestinal vascular smooth muscle contraction and reactivity to vasoconstrictors. Pancreatic juice-induced low reactivity of the superior mesenteric vascular bed of the rat involved protein expressions of eNOS and iNOS. Overproduction of NO resulted in low responsiveness of the SMA bed to vasoconstrictor. The juice-induced low reactivity of the superior mesenteric vascular bed was fully recovered by addition of L-NAME, whereas the reactivity was partially reversed by AG. The results suggest that both eNOS and iNOS are involved.

Acknowledgments

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