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### Key Words

acute pancreatitis;  
 nitric oxide;  
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 rat

## Effects of Nitric Oxide Synthase Inhibitors on Retrograde Bile Salt-Induced Pancreatitis Rats

**Background.** The serum levels of proinflammatory cytokines have been reported to be significantly higher in severe acute pancreatitis compared with mild pancreatitis. Nitric oxide (NO) produced by cytokine-inducible NO synthase might be involved as the mechanisms for the progression of pancreatitis and the occurrence of systemic complications. The aim of the study was to evaluate the effects of a non-selective NO synthase inhibitor, nitro-L-arginine methyl ester (L-NAME), and an inducible NO synthase inhibitor, L-canavanine, on sodium taurodeoxycholate-induced acute necrotizing pancreatitis in rats.

**Methods.** Twenty-eight rats were randomized into 3 groups to receive L-NAME 5mg/kg/h, L-canavanine 20mg/kg/h, and equivalent volume of saline, respectively, i.v. infusion after the induction of pancreatitis for 5 hours. The serum levels of amylase and lipase and mean arterial pressure and heart rate at baseline and 5 hours, and cardiac output, systemic vascular resistance, the amount of ascites and pancreatic histopathology at 5 hours were examined.

**Results.** Five hours after induction of pancreatitis, all rats treated with L-canavanine and all but 1 treated with saline survived; however, all rats treated with L-NAME died. As compared with the control group, L-canavanine significantly reduced serum levels of amylase and lipase, the severity of pancreatic edema and necrosis, and the volume of ascites in 5 hours. In addition, L-canavanine significantly improved the reduction of mean arterial pressure and systemic vascular resistance at 5 hours.

**Conclusions.** L-NAME results in the mortality of acute necrotizing pancreatitis. L-canavanine reduces serum pancreatic enzymes and improves the changes of pancreatic histopathology and systemic hemodynamics at the early stage of acute pancreatitis. Inducible NO synthase inhibitor is beneficial for severe acute pancreatitis.

Acute necrotizing pancreatitis is a severe disease with high morbidity and mortality for which no specific therapy has proven effective. Systemic inflammatory response syndrome (SIRS) and progression to multiple organ failure syndrome (MOFS) are clinical entities associated with sepsis, trauma, burns, and other states of severe tissue inflammation, including pancreatitis.<sup>1</sup> It has been determined that the physiologic and cellular defects observed in SIRS and MOFS are mediated by cytokines produced by macrophages, leukocytes and other tissues in response to an initiating event.<sup>2</sup> The proinflammatory cytokines including tumor necrosis factor  $\alpha$ , interleukin-1 $\beta$ , interleukin-6 and interleukin-8

are involved.<sup>3-5</sup> A cascade of mediators is then produced, which leads to profound physiologic derangements. One of the final mediators is thought to be the diatomic gas nitric oxide (NO) that is produced dependent on the induction of NO synthase (NOS).<sup>6</sup> In physiological conditions, low concentrations of NO are produced by an endothelial constitutive NOS (cNOS), involved in the regulation of regional blood flows and arterial blood pressure. While stimulated by lipopolysaccharide and diverse cytokines, an inducible NOS (iNOS) is expressed by various cells including the vascular smooth muscle cells, which produces a lot of NO to make profound vasodilatation.<sup>6,7</sup>

Whether NOS inhibitor is protective or detrimental

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in acute pancreatitis is controversial.<sup>8-14</sup> NO produced by iNOS causes pancreatic tissue damage.<sup>15</sup> In such conditions, inhibition of iNOS might be useful as a therapeutic option. L-canavanine, a structural analogue of L-arginine, has shown to be a selective inhibitor of iNOS in vivo and in vitro.<sup>16,17</sup> There has been no report concerning about the effects of L-canavanine on experimental pancreatitis in the literature. The aim of the study was to evaluate the effects of nitro-L-arginine methyl ester (L-NAME, a non-selective NOS inhibitor) and L-canavanine on sodium taurodeoxycholate-induced acute necrotizing pancreatitis in rats. We compared the serum levels of amylase and lipase, pancreatic histopathology and systemic hemodynamics between the treated and control groups.

## METHODS

### Animal Preparation

Adult male Sprague-Dawley rats, weighing 250-300 g, were anesthetized with an intramuscular injection of ketamin-HCl 100 mg/Kg body weight. The left carotid artery and right jugular vein were cannulated with catheters (polyethylene tube, inner diameter 0.02 inches) for blood sampling, hemodynamic measurement and administration of various agents. One milliliter of blood was drawn for baseline and an equal volume of isotonic saline was replaced. The abdomen was opened and the common pancreaticobiliary duct was identified. The duodenal wall was punctured at its antimesenteric aspect with a 24-gauge i.v. catheter (Johnson-Johnson Medical K.K., Japan). Acute pancreatitis was induced by retrograde infusion of 0.2 mL of 2.5% sodium taurodeoxycholate (Sigma, St. Louis, MO, U.S.A.) over 3 minutes, using an infusion pump as previously described.<sup>18,19</sup> The pancreaticobiliary duct was clamped near the liver hilum throughout the intraductal infusion.

### Experimental Protocol

The rats were randomized into 3 groups to receive an i.v. infusion of L-NAME 5mg/kg/h (n = 8), L-canavanine 20 mg/kg/h (n = 10), or an equivalent volume of isotonic saline 0.5 mL/h as the controls (n = 10). The treatment started just after the induction of pancreatitis and continued for 5 hours. Serum amylase and lipase levels were

determined at baseline and 5 hours after the induction of pancreatitis. Mean arterial pressure and heart rate were measured at baseline and 5 hours. Cardiac output and systemic vascular resistance were assessed at 5 hours. All surviving rats were sacrificed by rapid bleeding through the indwelling arterial line at the end of experiments (5 hours). The pancreatic histopathology was evaluated at 5 hours or immediately after death between 3 and 5 hours after induction of pancreatitis. The abdomen was reopened. For light microscopy, the pancreas was removed, fixed in 10% formalin, embedded in paraffin and stained with hematoxylin-eosin.

### Biochemical Assay and Hemodynamic Measurement

Serum amylase levels were determined using the rate reaction method (Daiichi Pure Chemicals Co., Japan) and serum lipase activity using the colorimetric method (Olympus Optical Co., Japan). The measurement of systemic hemodynamic profiles including mean arterial pressure, cardiac index, systemic vascular resistance and heart rate was done as previously described.<sup>20</sup> The catheter in the carotid artery was connected to a pressure transducer (Spectramed Inc., Oxnard, CA), and the mean arterial pressure was recorded on a multi-channel recorder (Gould Electronics, CA) at baseline and 5 hours. The heart rate was determined from the recording. Cardiac output was measured using the thermodilution method. A thermistor was placed in the aortic arch just distal to the aortic valve, and the thermal indicator (0.1 mL of 5% dextrose in water) was injected into the right atrium through a polyethylene-50 catheter placed into the jugular vein. The aortic thermistor was connected to a cardiac output computer. Blood temperature was maintained at  $37.2 \pm 0.2$  °C. A typical thermodilution curve would have a peak temperature of approximately  $-0.3$  °C with a rapid upslope and a smooth decay. At least 3 thermodilution curves were obtained for each cardiac output measurement. The final cardiac output value was obtained from the arithmetic mean of the computer results of at least two thermodilution curves. The cardiac index ( $\text{mL} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$ ) was calculated as the cardiac output per 100 g body weight. The systemic vascular resistance ( $\text{mm Hg} \cdot \text{mL}^{-1} \cdot \text{min} \cdot 100 \text{ g}$ ) was calculated from the mean arterial pressure divided by the cardiac index.

### Histopathologic Analysis

Pancreatic histopathology was examined by a pathologist unaware of the type of treatment employed. The grading of histopathological changes was as the report of Van Ooijen *et al.*<sup>21</sup> The grading of necrosis referred to the approximate percentage of cell involved: 0 = absent, 1 = < 10%, 2 = 10-25%, 3 = 26-50%, 4 = > 50%. Interstitial edema was scored as follows: 0 = absent, 1 = mild, 2 = moderate, 3 = severe. Leukocyte infiltration was graded as 0 = scattered, 1 = mild, 2 = moderate, 3 = severe. Hemorrhage was scored as 0 = absent, 1 = small, 2 = large.

### Analysis of Data

Data were expressed as mean  $\pm$  SEM. Mann-Whitney test and Kruskal-Wallis test were used for statistical analysis. A  $p < 0.05$  was defined as statistically significant.

### RESULTS

All rats treated with L-NAME died from 3 to 5 hours after induction of pancreatitis. All rats treated with L-canavanine and all but 1 treated with normal saline survived 5 hours after induction of pancreatitis. L-canavanine significantly reduced serum levels of amylase ( $6261 \pm 576$  vs.  $11415 \pm 1211$  U/L,  $p < 0.005$ ) and lipase ( $512 \pm 74$  vs.  $1570 \pm 427$  U/L,  $p < 0.05$ ) as compared with the controls. The hemodynamic values of the L-canavanine and the control groups at baseline, and 5 hours after induction of pancreatitis are shown in Table 1. The reduction of mean arterial pressure and systemic vascular resistance was significantly improved by the administration of L-canavanine. Histopathological scores of the pancreas of L-NAME, L-canavanine and control groups

at 3-5 hours after induction of pancreatitis are shown in Table 2. The severity of pancreatic edema and necrosis was improved significantly by L-canavanine. The amount of ascites significantly decreased in the L-canavanine group, as compared with the controls ( $2.2 \pm 0.8$  vs.  $5.9 \pm 0.7$  mL,  $p < 0.005$ )

### DISCUSSION

NO has been implicated in the modulation of pancreatic enzyme secretion, the regulation of the pancreatic blood flow, the promotion of the capillary integrity, and the inhibition of leukocyte adhesion in experimental acute pancreatitis.<sup>10,11,22,23</sup> In contrast, NO seems to contribute multiorgan oxidative stress in severe acute pancreatitis.<sup>8</sup> The beneficial effect of NOS inhibitor on acute pancreatitis is still unclear. The present study investigated the effects of different NOS inhibitors on experimental acute necrotizing pancreatitis. We showed that L-canavanine, an iNOS inhibitor, was beneficial to acute necrotizing pancreatitis in rats; however, L-NAME, a non-selective NOS inhibitor, was detrimental to acute pancreatitis. The results support the theory that low level of NO, generated mostly by cNOS, exerts beneficial ef-

**Table 2. Histopathological scores of pancreas at 3-5 hours after induction of experimental pancreatitis**

	Leukocyte infiltration	Edema	Necrosis	Hemorrhage
L-NAME	$1.6 \pm 0.3$	$2.2 \pm 0.2$	$2.6 \pm 0.2$	$1.5 \pm 0.2$
L-canavanine	$1.3 \pm 0.2$	$1.5 \pm 0.2^*$	$2.0 \pm 0.2^*$	$1.1 \pm 0.2$
Control	$1.5 \pm 0.2$	$2.1 \pm 0.1$	$2.6 \pm 0.2$	$1.1 \pm 0.2$

\*  $p < 0.05$  vs. the control group. L-NAME = nitro-L-arginine methyl ester.

**Table 1. The hemodynamic values at baseline and 5 hours after induction of experimental pancreatitis**

	L-canavanine	Control	<i>p</i> value
Mean arterial pressure (mm Hg) at baseline	$133 \pm 3$	$133 \pm 3$	NS
Heart rate (beats/min) at baseline	$426 \pm 15$	$381 \pm 15$	NS
Mean arterial pressure (mm Hg) at 5 h	$117 \pm 3$	$102 \pm 5$	< 0.05
Heart rate (beats/min) at 5 h	$409 \pm 16$	$385 \pm 16$	NS
Cardiac index ( $\text{mL} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$ ) at 5 h	$23.8 \pm 1.1$	$25.5 \pm 1.1$	NS
Systemic vascular resistance at 5 hr ( $\text{mmHg} \cdot \text{mL}^{-1} \cdot \text{min} \cdot 100 \text{ g}$ )	$4.9 \pm 0.3$	$4.0 \pm 0.2$	< 0.05

fects in most of the tissues, including the pancreas, whereas iNOS is responsible for excessive production of NO from activated macrophages and lymphocytes resulting in tissue damage.<sup>7,24</sup>

We found that all rats treated with L-NAME died within 5 hours after induction of acute pancreatitis, however, all but one rats (90%) treated with normal saline survived 5 hours after induction of pancreatitis. We confirmed that non-selective inhibition of NOS had adverse effects on acute pancreatitis as reported in the literature.<sup>10-13</sup> In cerulein-induced acute pancreatitis, L-NAME showed to increase amylasemia and tissue myeloperoxidase activities, whereas NO donors reduced amylasemia and the histological damage of the pancreas.<sup>10</sup> Werner *et al.*<sup>11</sup> also demonstrated that L-NAME increased the severity of inflammation and decreased pancreatic tissue oxygenation in cerulein-induced acute pancreatitis in rats. L-NAME treatment aggravated hemorrhage, acinar cell necrosis, and microvascular thrombosis of the pancreas in closed duodenal loop-induced pancreatitis.<sup>12</sup> The administration of N<sup>G</sup>-nitro-L-arginine (L-NNA, a non-selective NOS inhibitor) also seemed to aggravate cellular injury of pancreatic tissue in cerulein-induced pancreatitis.<sup>13,25</sup> These results implicated that NO derived from cNOS may play a significant role in preventing the development and progression of acute pancreatitis.

In our study L-canavanine showed to significantly decrease serum levels of amylase and lipase, and the severity of pancreatic edema and necrosis at the early stage of bile salt-induced acute necrotizing pancreatitis. We also demonstrated that the reduction of mean arterial pressure and systemic vascular resistance 5 hours after induction of acute pancreatitis was significantly improved by the administration of L-canavanine. Selective iNOS inhibition may have beneficial effects on a variety of pathophysiological consequences in acute pancreatitis. Bacterial translocation is one of the most important factors in the development of septic complications and mortality in acute pancreatitis.<sup>26</sup> Specific iNOS inhibitor, S-methylisothiurea, seemed to inhibit bacterial translocation and ameliorate pancreatic damage in a rat model of acute pancreatitis.<sup>27</sup> Pretreatment with iNOS inhibitor, ONO-1714, attenuated diaphragmatic dysfunction, which may contribute to respiratory distress in cerulein-

induced pancreatitis in rats.<sup>28</sup> L-canavanine seemed to improve hemodynamic variables and survival in rodent endotoxic shock.<sup>17</sup> Lomis *et al.*<sup>9</sup> reported that NOS inhibitors, N-monomethylarginine and aminoguanidine, which are more powerful inhibitors of iNOS than of cNOS, reversed hypotensive insult in experimental pancreatitis.

In the normal rats iNOS was detected in vascular endothelial cells but not in acinar or ductal cells of pancreas. iNOS was positive in neutrophils, macrophages and injured acinar cells in experimental pancreatitis in rats.<sup>9,15</sup> Low doses of lipopolysaccharides were beneficial for cerulein-induced pancreatitis by enhancing the expression of cNOS and high doses of lipopolysaccharides failed to exhibit the protective effect due to iNOS over-expression.<sup>13</sup> The degree of pancreatic inflammation and tissue injury of cerulein-induced pancreatitis was markedly reduced in iNOS-deficient mice as compared with wild-type mice.<sup>29</sup> Therefore, it is conceivable that non-selective NOS inhibitor is harmful to acute pancreatitis and selective iNOS inhibitor is helpful to pancreatitis attack.

In conclusion, L-canavanine improves the changes of pancreatic histopathology and systemic hemodynamics at the early stage of acute necrotizing pancreatitis in rats. L-NAME increases the mortality of acute pancreatitis. Selective inhibition of iNOS may have clinical potential in the treatment of acute pancreatitis. iNOS may play an important role in the pathogenesis of severe acute pancreatitis.

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