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PROTECTIVE ROLE OF ENDOGENOUS NITRIC OXIDE (NO) IN LIPOPOLYSACCHARIDE — INDUCED PANCREATIC DAMAGE. (A NEW EXPERIMENTAL MODEL OF ACUTE PANCREATITIS)

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Lipopolysaccharide (LPS) derived from the bacterial cell wall activates the inflammatory response in the tissue but the role of LPS in the pathogenesis of pancreatic damage and in the activation of NO system in the pancreas has not been fully explained. The aim of this study was to investigate the effect of repeated administration of LPS to the rats on the integrity of the pancreas, on the ability of isolated pancreatic acini to secrete the amylase and on the plasma level of tumor necrosis factor alpha (TNFα). The role of NO in the pancreatic resistance to the damage was assessed in animals subjected to repeated administration of LPS. To induce pancreatic damage one group of rats received intraperitoneal (i.p.) injection of LPS (from E. coli) every day during 5 consecutive days (10 mg/kg — day). Another groups of animals were given N^G-nitro-L-arginine (L-NNA), an inhibitor of NO synthase (NOS) (20 mg/kg i.p.) alone or in combination with L-arginine (100 mg/kg i.p.), 30 min prior to each LPS injection. Plasma level of TNFα was determined by ELISA kit. Repeated administration of LPS produced mild pancreatic inflammation that was most pronounced at day 5 of LPS treatment and manifested as edema, neutrophil infiltration and hemorrhage of the pancreas. The survival rate after 5 days treatment with LPS was 87.5%. Pancreatic weight, plasma levels of TNFα and amylase, pancreatic blood flow (PBF) and NO generation by pancreatic acini were markedly increased in rats subjected to repeated administration of LPS whereas the amylase response of isolated pancreatic acini to pancreatic secretagogues was significantly attenuated. Suppression of NOS by L-NNA resulted in a dramatic increase in the mortality of the animals reaching 50% and significantly increased inflammatory changes in the pancreatic tissue, decreased PBF, abolished the ability of pancreatic acini to release NO and to secrete amylase. Pancreatic weight and plasma levels of amylase and TNFα significantly increased in the group of rats treated with combination of LPS + L-NNA as compared to the animals received LPS alone. Addition of L-arginine to L-NNA + LPS administration reversed all harmful effects produced by L-NNA in the pancreas. We conclude that repeated administration of high doses of bacterial LPS to the rats could induce pancreatic tissue damage by itself, however, it is not able to produce severe pancreatitis. Suppression of NO generation significantly aggravates the pancreatic lesion produced by LPS leading to the dramatic mortality in treated rats. The rise of plasma level of TNFα corresponds to the severity of pancreatic inflammation.

K e y w o r d s: Lipopolysaccharides, nitric oxide, tumor necrosis factor alpha, isolated pancreatic acini, pancreatic inflammation

INTRODUCTION

Lipopolysaccharide (LPS, endotoxin) released from the bacterial outer membrane is a major factor that contributes to the pathophysiology of bacterial infection (1, 2). Enteric bacteria were found in pancreatic necrosis which is often complicated by septic shock and multiorgan dysfunction and failure (3, 4). Endotoxemia was proposed as a one of the prognostic factors of the severity of acute pancreatitis in clinical studies (5). Administration of endotoxin to the animals aggravated pancreatic tissue damage caused by acute inflammation (6). Injection of LPS by itself could produce hyperamylasemia and ultrastructural changes of the acinar cells leading to the development of acute pancreatitis (7, 8). In contrast to above harmful effects produced by endotoxemia, short-term application of LPS to the rats failed to affect significantly pancreatic cell morphology and function (9, 10).

The mechanism of the noxious effects of LPS on the pancreas is not fully explored. It is well known that endotoxin activates macrophages to release the inflammatory mediators such as; reactive oxygen species (ROS) and various cytokines; including tumor necrosis factor alpha (TNFα), interleukin-1β (IL-1β, interleukin-6 (Il-6), interleukin-8 (Il-8) and platelet activating factor (PAF) (7, 11—13). Increased production of TNFα in acute pancreatitis was demonstrated in clinical as well as in experimental studies (14, 15). According to recent findings TNFα produced by pancreatic acinar cells could contribute to the regulation of pancreatic apoptosis in acute inflammation (16). However, to our knowledge, there are no reports concerning the correlation between pancreatic lesion caused by chronic administration of endotoxin and the production of TNFα.

Nitric oxide (NO) derived from L-arginine by inducible from of nitric oxide synthase (iNOS) is mostly involved in cellular processes of inflammation as an effective molecule of macrophase activity (17). LPS stimulates iNOS activity and leads to the increased generation and release of NO (17). In the pancreas NO generation has been demonstrated in various cells such as; endothelial cells, intrinsic neurons and also in pancreatic acinar cells (18—20). Endogenous NO generated in the pancreas has a beneficial influence on the gland mostly by enhancing capillary perfusion and promoting tissue integrity. The blockade of NOS potentiated the inflammatory changes caused by acute pancreatitis (20—22).

The aim of this study was to investigate the effect of repeated administration of LPS on the pancreatic integrity, pancreatic blood flow and the ability of pancreatic acinar cells to secrete the enzyme and to generate NO. To asses the role of endogenous NO in the pancreatic resistance to damage an inhibitor of NOS; N^G-nitro-L-arginine (L-NNA) was used in the rats subjected to repeated endotoxin administration. The changes of TNFα in plasma were compared

with the severity of pancreatic lesions of LPS-treated rats without or with administration of L-NNA.

MATERIAL AND METHOD

Studies were performed on male Wistar rats weighing 150—200 g. Animals were housed in the cages under standard conditions, at room temperature with a 12-h light and dark cycle. Food (commercial pelleted chow) and drinking water were available *ad libitum*.

Following items were purchased: LPS (serotype 0127; B8), N^G-nitro-L-arginine (L-NNA), N^G-nitro-L-arginine (L-NNA), a blocker of NO synthase, L-arginine, urecholine, trypsin inhibitor from Sigma Chemical Co, (St Louis, MO, USA), caerulein (Takus) from Pharmacia GmbH, Erlangen, Germany, NO assay commercial kit was from Cayman Chemical Co., (Ann Arbor, MI, USA), essential and nonessential amino acid mixture from SERVA Feinbiochemia (GmbH, Heidelberg, Germany), purified collagenase from Worthington Biochemical Co., (Freehold, NJ, USA, and TNFα solid phase enzyme linked immunosorbent assay kit were from BioSource International, Inc. (Camarillo, CA, USA).

LPS (10 mg/kg-day) was dissolved in 0.5 ml of saline and administered intraperitoneally (i.p.) as a bolus injection during 5 consecutive days. In some experiments the rats were injected first with LPS, and 15 min later N^G-nitro-L-arginine (L-NNA), a blocker of NO synthase (NOS) (20 mg/kg i.p.), L-arginine, a substrate for NOS (100 mg/kg) or combination of above was applied. In control experiments vehicle saline was administered into the rats instead of tested substances.

Experimental groups:

Rats were randomly divided into five main groups:

A. Control (0.5 ml of vehicle saline i.p.),
B. LPS (10 mg/kg i.p.),
C. LPS (10 mg/kg i.p.)+L-NNA (20 mg/kg i.p.),
D. LPS (10 mg/kg i.p.)+L-NNA (20 mg/kg i.p.)+L-arginine (100 mg/kg i.p.),
E. LPS (10 mg/kg i.p.)+L-arginine (100 mg/kg i.p.).

The animal groups were injected respectively, with saline (group A), with LPS (group B), with the combination of LPS+L-NNA (group C), with the combination of LPS+L-NNA+L-arginine (group D) and LPS+L-arginine (group E) once daily during 5 consecutive days. The rats from each group were sacrificed at 1, 3, or 5 day of the experiment. Eight to twelve animals from each treatment group were used for each day test. Data of control rats (group A) were expressed as the value at time 0.

The effects of L-NNA alone or combined with L-arginine were also tested in the rats receiving vehicle saline instead of LPS injection.

Examination of the pancreatic blood flow (PBF)

4 hours after i.p. injection of LPS, LPS+L-NNA, LPS+L-NNA+L-arginine, LPS+L-arginine or vehicle saline (in control tests) animals were shortly anesthetized with ether, weighted and the abdominal cavity was opened. The pancreas was exposed for measurement of the blood flow by a laser Doppler flowmeter (LDF) using a Laserflo, model BPM Blood Perfusion Monitor

(Vasamedics Inc., St. Paul, MN, USA). Blood flow was measured in five different regions of the pancreas. PBF was presented as percent change from control value obtained from the rats injected with saline.

Determination of plasma amylase concentration

Immediately after measurement of PBF, the venous blood was taken from inferior vena cava for plasma amylase determination by an enzymatic method (Amylase reagent, Dialab Diagnostic Ges. MBH, Wien, Austria) as described previously (21). The values were expressed as percent change from control value obtained in rats infused with saline.

Testing of pancreatic secretion and NO release in vitro:

The pancreas was carefully dissected of from its attachment to the stomach, the duodenum and the spleen. Fat and excess tissue were trimmed away. The pancreas was rinsed with saline, blotted on paper and weighted. Pancreatic secretion of amylase and generation of NO was measured using dispersed pancreatic acini prepared as described in previous paper (23). Acinar suspensions were incubated for 30 min in 37°C in shaking bath with or without addition of caerulein (10^{-10} M) or urecholine (10^{-4} M) at the concentrations, which were shown in our previous study to produce maximal amylase secretion (21). After incubation the amylase content was determined using the method of Bernfeld (23) and expressed as percent of total amylase release per 30 min.

Spontaneous NO release by the pancreatic acini was measured in the supernatant after incubation of the acini for 30 min in 37°C without addition of pancreatic secretagogues. The supernatant was taken separated from the pellet by centrifugation at 1000 rpm for 5 min. NO release by pancreatic acini was quantified as nitrite (NO_2^-) and nitrate (NO_3^-) levels in the supernatant and was determined according to the method that is based on the Griess reaction (24) using the commercially available kit (Cayman Chemicals Assay Kit, Cayman Chemical Co., Ann Arbor, MI, USA).

Histological examination

Pieces of the pancreas were excised from the body portion, fixed in 10% formalin and stained with haematoxylin and eosin (H&E). The slides were examined histologically by technician without the knowledge of the treatment given. The histological grading of edema was made using a scale ranging from 0 to 3:0 = no edema, 1 = interlobular edema, 2 = interlobular edema and moderate intralobular edema, 3 = interlobular edema and severe intralobular edema. Neutrophil infiltration was also graded from 0 (absent) to 3 = maximal alterations (diffuse infiltration on the entire pancreatic gland). Grading of hemorrhage was based on the perceived involvement of the examined area: 0 = absent, 1 = less than 25%, 2 = 25—30%, and 3 = more than 50% of area involved.

TNFα measurement:

Serum TNFα was determined using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol. In brief, a 48-well microtiter plate with wells precoated with anti-rat TNFα antibody were used to capture rat TNFα from standards and test samples. After washing the plates to remove unbound material, a peroxidase-conjugated polyclonal TNFα antibody which bind to captured rat TNFα was added. To remove unbound material, each well was washed again and a substrate solutions were added to initiate a peroxidase catalyzed color

reaction which was stopped by acidification. Absorbance was measured at 450 nm for both standards and experimental samples. Concentrations of rat TNFα was determined using the standard curve and expressed as pg/ml.

Statistical analysis

Comparison of the difference between the mean values of various groups of experiments was made by analysis of variance or the Wilcoxon's rank-sum test. A difference with a p value of < 0.05 was considered statistically significant. Results are expressed as means ($\pm$SEM).

RESULTS

Survival rate

Survival of the animals in each tested group at day 5 of experiments is present on *Fig. 1*. In the group of rats injected with LPS alone at a dose of 10 mg/kg i.p. (group B) the survival at day 5 was 87.5%. In the group treated with
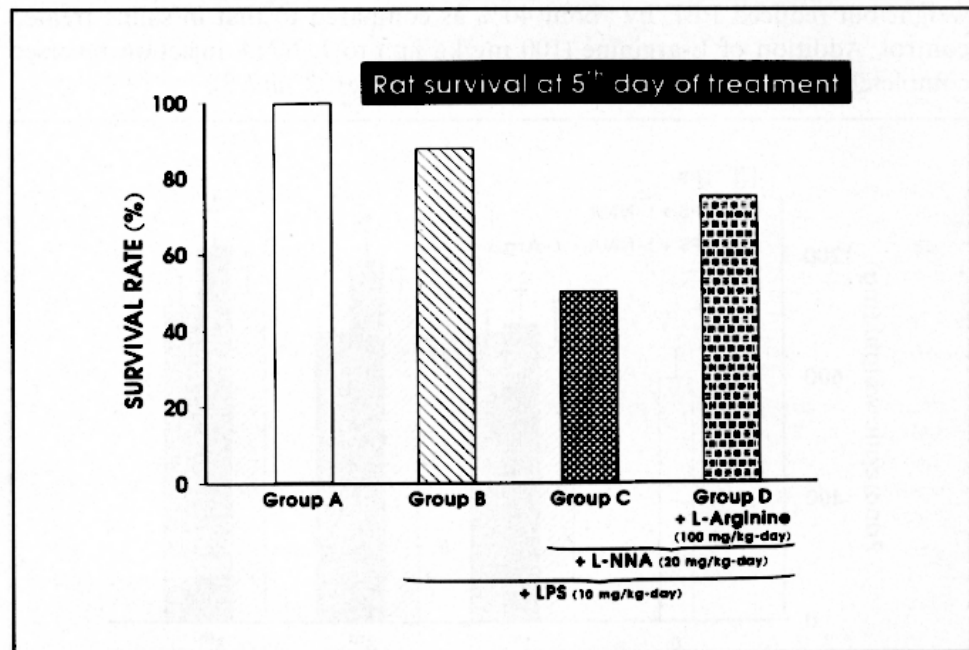

Fig. 1. Effect of repeated i.p. administration of LPS alone (10 mg/kg-day), LPS combined with i.p. injection of L-NNA (20 mg/kg-day i.p.), or LPS given with L-NNA plus L-arginine (100 mg/kg — day i.p.) on rat survival at day 5 of treatment.
Group A = untreated control; n = 6, survived = 6.
Group B = LPS treatment; n = 8, survived = 7.
Group C = LPS + L-NNA treatment; n = 12, survived = 6.
Group D = LPS + L-NNA + L-arginine treatment; n = 12, survived = 9.
(n = number of rats at the beginning of each test)

the combination of LPS plus L-NNA (group C) a dramatic increase in the mortality was observed, reaching 50% of animals at day 5 of the study. Addition of L-arginine to the LPS + L-NNA injection (group D) significantly increased the survival rate to 75% at day 5 of the experiment. In control group injected with saline (group A) all animals survived (100%) and similar survival was observed in the group of rats treated with L-NNA alone, LPS + L-arginine or with combination of L-NNA + L-arginine but these results were not included for the sake of clarity.

Pancreatic blood flow (PBF) and pancreatic weight

Single administration of LPS (10 mg/kg i.p.) did not affect significantly pancreatic weight, and PBF (*Figs 2 and 3*). L-NNA (20 mg/kg i.p.) combined with single administration of LPS also did not affect significantly pancreatic weight but reduced PBF by about 40% as compared to that in saline-treated control. Addition of L-arginine (100 mg/kg i.p.) to L-NNA injection reversed completely above effect of L-NNA on PBF (*Figs 2 and 3*).
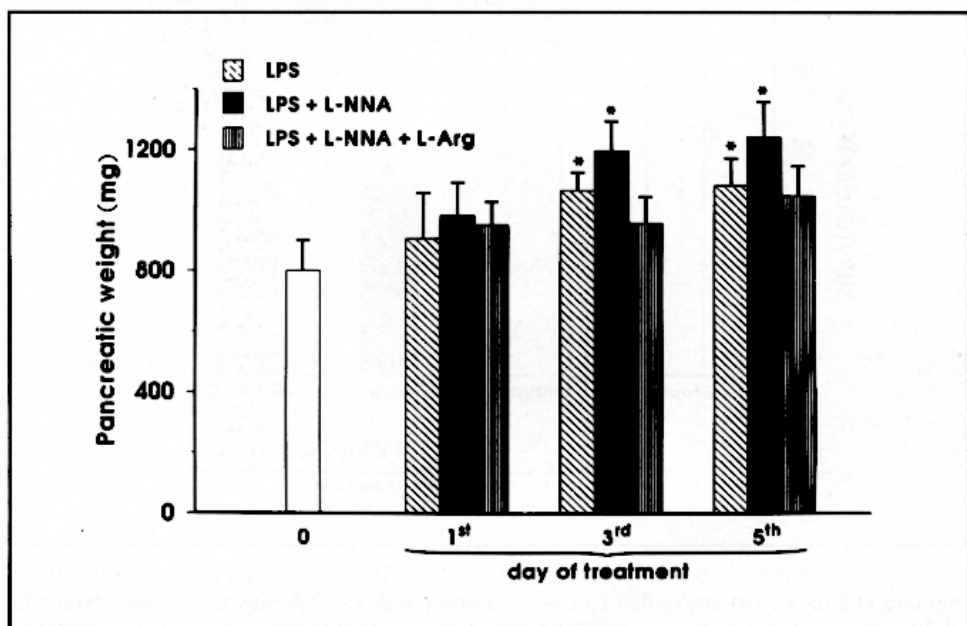

Fig. 2. Effect of repeated administration of LPS alone (10 mg/kg — day i.p.), LPS combined with injection of L-NNA (20 mg/kg — day i.p.), or LPS given with L-NNA plus L-arginine (100 mg/kg — day i.p.) on pancreatic weight at day 1, 3, and 5 of treatment. Means ± SEM of 8—12 rats in each group. Asterisk (*) indicates significant increase above the control value obtained from untreated rats at time 0.

With repeated administration of LPS significant rise of pancreatic weight (by about 35%) at day 3 and 5 of the test was observed (*Fig. 2*). This was accompanied by a significant increase of PBF (by 40%) (*Fig. 3*). Combination of L-NNA with LPS tended to cause further increase in the pancreatic weight, and resulted in a significant fall in PBF at day 3 and 5 of experiment. Addition of L-arginine of L-NNA injection completely reversed all changes produced by L-NNA in LPS treated rats (*Figs. 2 and 3*).

Addition of L-arginine to LPS injection did not affect significantly the changes of pancreatic weight and PBF produced by administration of LPS to the rats at days 1—5 of the test and these results were omitted for the sake of clarity.
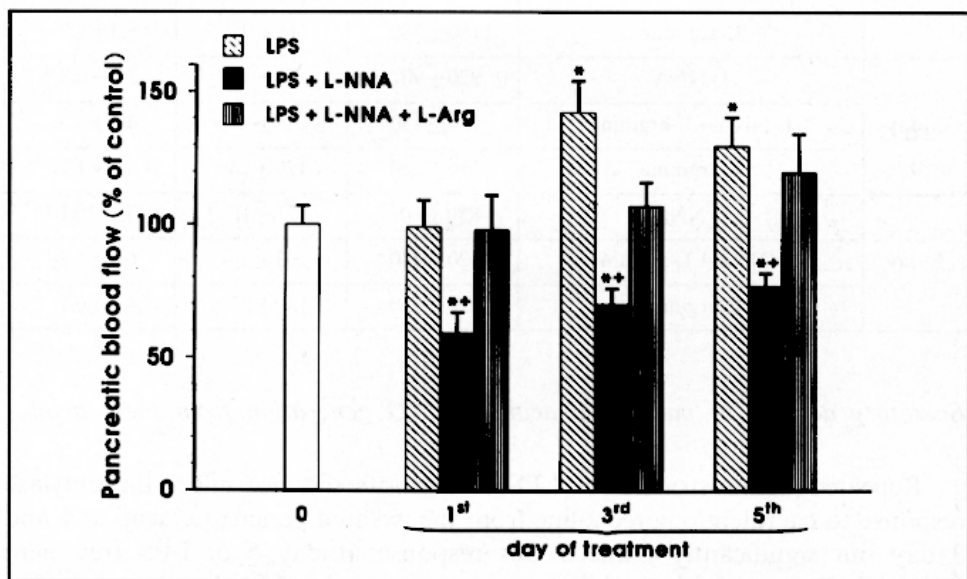

Fig. 3. Effect of repeated administration of LPS alone (10 mg/kg — day i.p.), LPS combined with injection of L-NNA (20 mg/kg — day i.p.), or LPS given with L-NNA plus L-arginine (100 mg/kg — day i.p.) on pancreatic blood flow at day 1, 3, and 5 of treatment. Means $\pm$ SEM of 8—12 rats in each group. Asterisk (*) indicates significant change as compared to the untreated control at time 0. Cross indicates significant decrease below the value obtained from the rats treated with LPS alone.

Administration of L-NNA alone (20 mg/kg — day for 5 days) diminished PBF by about 30% at days 1—5 of treatment, whereas pancreatic weight tended to increase but this was not significant (*Table 1*). Addition of L-arginine to L-NNA reversed these changes of PBF produced by L-NNA. L-arginine alone did not affect significantly pancreatic weight or PBF at any day (*Table 1*).

Table 1. The effects of repeated administration of L-NNA alone (20 mg/kg — day i.p.), L-arginine alone (100 mg/kg — day i.p.) or combination of L-NNA + L-arginine at day 1, 3 and 5 of experiment on pancreatic weight, pancreatic blood flow (PBF) and generation of NO from isolated pancreatic acini. Results are means ± SEM from 3—6 separate experiments. Asterisk (*) indicates significant ($p < 0.05$) change as compared to the control value.

		Pancreatic weight mg/pancreas	PBF (% of control)	NO_2^-/NO_3^- (μM/30 min)
	Control	800 ± 100	100 ± 8	4.5 ± 0.5
1 day	L-NNA	1050 ± 170	68 ± 11 *	3.2 ± 0.4 *
	L-NNA + L-arginine	730 ± 150	106 ± 10	5.2 ± 1.0
	L-arginine	1180 ± 280	115 ± 9	6.3 ± 1.2
3 day	L-NNA	920 ± 90	72 ± 12 *	2.5 ± 0.8 *
	L-NNA + L-arginine	750 ± 150	98 ± 9	4.1 ± 0.3
	L-arginine	110 ± 220	120 ± 15	5.7 ± 1.1
5 day	L-NNA	880 ± 60	77 ± 16	3.5 ± 0.1 *
	L-NNA + L-arginine	1120 ± 280	103 ± 6	6.0 ± 1.2
	L-arginine	1020 ± 130	130 ± 25	4.0 ± 0.7

Secretory activity of pancreatic acini and NO generation from these acini

Repeated administration of LPS to the rats did not affect the amylase response to caerulein or urecholine from the isolated pancreatic acini at 1 and 3 day but significantly reduced this response at day 5 of LPS treatment (*Table 2*). The generation of NO by these acini significantly increased after single injection of LPS at day 1 and showed further increase at day 3 and 5 of LPS administration (*Fig. 4*). In the group of rats injected with LPS + L-arginine the generation of NO and secretion of amylase were similar to those observed in the group treated with LPS alone and these results were omitted for the sake of clarity. In the rats injected with the combination of LPS plus L-NNA the secretion of amylase from pancreatic acini in response to caerulein or urecholine was significantly diminished at day 3 and 5 of treatment as compared to that obtained with LPS-alone at these days (*Table 2*). NO generation by isolated pancreatic acini obtained from the rats treated with LPS plus L-NNA was reduced to the level similar to that observed in the control vehicle-treated animals (*Fig. 4*). L-arginine added to L-NNA plus LPS treated rats reversed all changes in amylase secretion and NO generation produced by L-NNA (*Table 2* and *Fig. 4*).
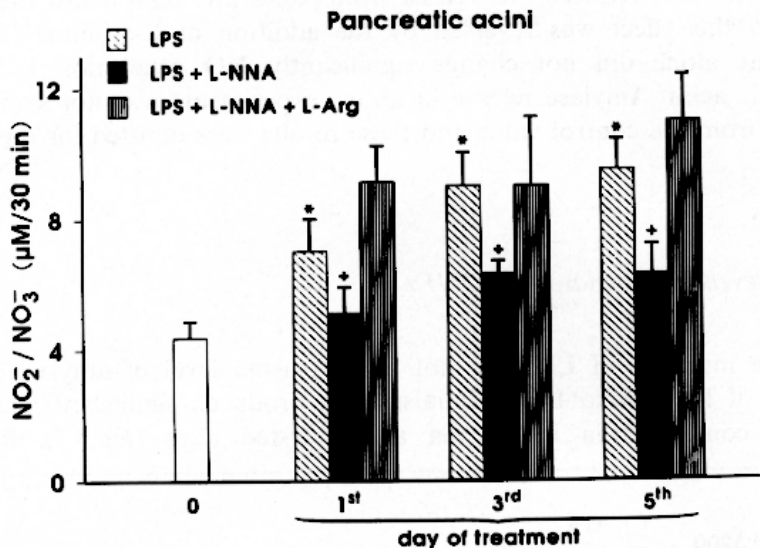
Fig. 4. Generation of NO in isolated pancreatic acini obtained from the rats treated with LPS alone (10 mg/kg — day i.p.), LPS combined with i.p. injection of L-NNA (20 mg/kg — day i.p.), or LPS given with L-NNA plus L-arginine (100 mg/kg — day i.p.), at day 1, 3, and 5 day of treatment. Means $\pm$ SEM of 8—12 rats in each group. Asterisk (*) indicates significant change as compared to the untreated control at time 0. Cross($^+$) indicates significant decrease below the value obtained from the rats treated with LPS alone.

Table 2. The release of amylase from isolated pancreatic acini (% of total) stimulated by caerulein (10^{-10} M) or urecholine (10^{-4} M) added to the incubation medium of isolated acini obtained from the pancreas of rats treated with LPS (10 mg/kg — day i.p.), LPS + L-NNA (20 mg/kg — day i.p.) or LPS + L-NNA + L-arginine (100 mg/kg — day i.p.) at 1, 3 or 5 day of treatment. Results are means $\pm$ SEM from 3 separate experiments. Asterisk (*) indicates significant decrease below the control value. Cross ($^+$) indicates significant decrease below the value obtained with LPS alone.

		Caerulein 10^{-10} M	Urecholine 10^{-4} M
	Control	14.0 ± 1.5	15.5 ± 2.0
1 day	LPS alone	15.3 ± 1.8	14.3 ± 1.5
	LPS + L-NNA	12.2 ± 1.7	11.6 ± 1.6
	LPS + L-NNA + L-arginine	14.7 ± 2.0	16.0 ± 2.1
3 day	LPS alone	12.4 ± 1.6	11.8 ± 1.6
	LPS + L-NNA	6.0 ± 1.0 *$^+$	7.6 ± 0.8 *$^+$
	LPS + L-NNA + L-arginine	13.4 ± 0.5	15.1 ± 1.5
5 day	LPS alone	10.4 ± 1.1 *	9.1 ± 0.8 *
	LPS + L-NNA	6.3 ± 0.4 *$^+$	6.5 ± 0.9 *$^+$
	LPS + L-NNA + L-arginine	10.9 ± 0.6 *	9.6 ± 1.5 *

L-NNA alone reduced NO release from pancreatic acini below the control level and this effect was reversed by the addition of L-arginine (*Table 1*). L-arginine alone did not change significantly NO generation in isolated pancreatic acini. Amylase release in above experiments was not significantly different from the control value and these results were omitted for the sake of clarity.

Plasma levels of amylase and TNFα

Single injection of LPS did not affect plasma level of amylase but the addition of L-NNA to LPS administration produced significant increase of amylase concentration in plasma at all tested days (*Fig. 5*). Repeated
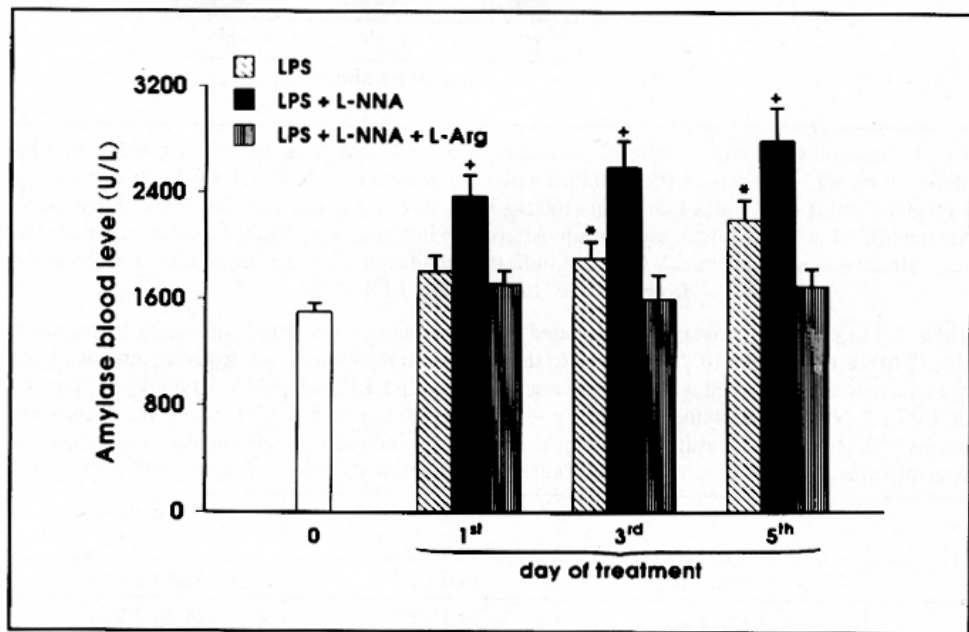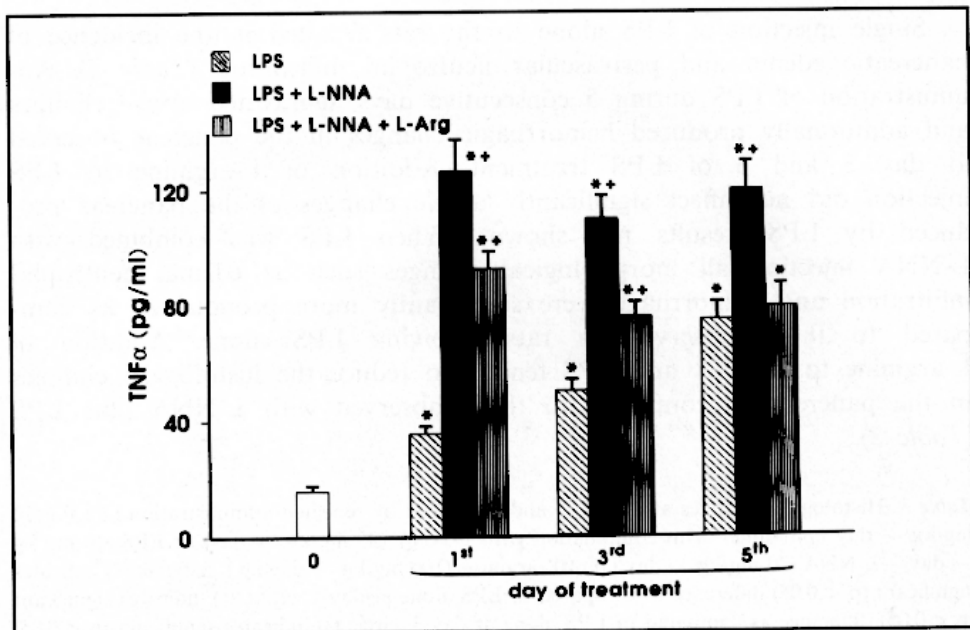

Fig. 5. Effect of repeated administration of LPS alone (10 mg/kg — day i.p.), LPS combined with injection of L-NNA (20 mg/kg — day i.p.), or LPS given with L-NNA plus L-arginine (100 mg/kg — day i.p.) on amylase blood level at day 1, 3, and 5 of treatment. Means±SEM of 8—12 rats in each group. Asterisk (*) indicates significant change as compared to the untreated control at time 0. Cross (+) indicates significant increase above the value obtained from the rats treated with LPS alone.

administration of LPS resulted in significant elevation of plasma amylase at day 3 and 5 achieving, respectively 120% and 145% of the control level. Combination of LPS plus L-NNA significantly augmented the level of amylase

in plasma above that observed in LPS-treated rats at all days of experiment. These effects were reversed by the addition of L-arginine to the L-NNA injection (*Fig. 5*). Addition of L-arginine to LPS administration did not affect significantly the changes of amylase observed after injection of LPS alone (results not shown).

Fig. 6. Changes of TNF α level in plasma in rats subjected to repeated administration of LPS alone (10 mg/kg-day i.p.), LPS combined with injection of L-NNA (20 mg/kg-day i.p.), or LPS given with L-NNA plus L-arginine (100 mg/kg-day i.p.) at day 1, 3, and 5 of treatment. Means $\pm$ SEM of 8—12 rats in each group. Asterisk (*) indicates significant change as compared to the untreated control at time 0. Cross (+) indicates significant change as compared to the value obtained from the rats treated with LPS alone

TNFα level in the plasma gradually increased with repeated administration of LPS and achieved the highest level (about four fold increase comparing to the control level) at day 5 of LPS treatment (*Fig. 6*). L-arginine added to LPS did not affect significantly above changes of plasma level of TNFα produced by LPS (results not shown). L-NNA added to LPS administration produced dramatic rise of plasma TNFα level above that observed after treatment with LPS alone. Addition of L-arginine (100 mg/kg i.p.) to L-NNA plus LPS injection partially reversed the rise in TNFα level produced by the combination of LPS plus L-NNA (*Fig. 6*). L-NNA alone, L-arginine alone or their combination given without LPS did not affect significantly

any of above parameters and these results were omitted for the sake of clarity.

Morphological changes

Single injection of LPS alone to the rats resulted in the incidence of pancreatic edema and perivascular neutrophil infiltration (*Table 3*). Administration of LPS during 5 consecutive days aggravated above changes and additionally produced hemorrhagic changes in the pancreas observed at day 3 and 5 of LPS treatment. Addition of L-arginine to LPS injection did not affect significantly above changes of the pancreas produced by LPS (results not shown). When LPS was combined with L-NNA injection all morphological changes such as edema, neutrophil infiltration and hemorrhage were significantly more pronounced as compared to those observed in rats receiving LPS alone. Addition of L-arginine to L-NNA and LPS tended to reduce the histological changes in the pancreas as compared to those observed with L-NNA plus LPS (*Table 3*).

Table 3. Histological changes at days 1, 3 and 5 induced by repeated administration of LPS (10 mg/kg — day i.p.) alone, or in combination with L-NNA (20 mg/kg — day) or LPS (10 mg/kg — day)+L-NNA (20 mg/kg — day i.p.)+L-arginine (100 mg/kg — day i.p.). Asterisk (*) indicates significant ($p < 0.05$) difference as compared to LPS alone at day 1, cross (+) indicates significant ($p < 0.05$) difference as compared to LPS alone at day 3, circle (°) indicates significant ($p < 0.05$) difference as compared to LPS alone at day 5.

		Edema 0—3	Neutrophil infiltration 0—3	Hemorrhage 0—3
	Control	0	0	0
1 day	LPS alone	1.0 ± 0.1	1.0 ± 0.0	0
	LPS+L-NNA	1.9 ± 0.1 *	1.7 ± 0.1 *	0.8 ± 0.1 *
	LPS+L-NNA+L-arginine	1.3 ± 0.2	1.0 ± 0.1	0.2 ± 0.0
3 day	LPS alone	1.8 ± 0.1	2.0 ± 0.2	0.3 ± 0.1
	LPS+L-NNA	2.0 ± 0.2	2.7 ± 0.2 +	1.0 ± 0.1 +
	LPS+L-NNA+L-arginine	1.3 ± 0.1	2.0 ± 0.13	0.2 ± 0.1
5 day	LPS alone	1.9 ± 0.1	2.3 ± 0.1	0.6 ± 0.1
	LPS+L-NNA	2.3 ± 0.1 °	2.8 ± 0.1 °	0.9 ± 0.1 °
	LPS+L-NNA+L-arginine	1.6 ± 0.1	2.0 ± 0.0	0.3 ± 0.0

In rats given L-NNA alone without LPS only mild edema and neutrofil infiltration was observed without hemorrhage (*Table 4*). Addition of L-arginine reduced significantly the histological changes induced by L-NNA. L-arginine alone did not affect significantly the morphology of the pancreatic tissue (*Table 4*).

Table 4. The effects of repeated administration of L-NNA alone (20 mg/kg — day i.p.), L-arginine alone (100 mg/kg — day i.p.) or their combination on the morphology of pancreatic tissue at day 1, 3 and 5 of treatment. Asterisk (*) indicates significant (p < 0.05) change as compared to the control value.

		Edema 0—3	Neutrophil infiltration 0—3	Hemorrhage 0—3
	Control	0	0	0
1 day	L-NNA	0.5±0.2	0.1±0.05	0
	L-NNA+L-arginine	0	0	0
	L-arginine	0.3±0.2	0	0
3 day	L-NNA	1.2±0.3 *	1.0±0.2 *	0.2±0.1
	L-NNA+L-arginine	0.4±0.2	0.5±0.2	0
	L-arginine	0.6±0.2	0	0
5 day	L-NNA	1.6±0.4 *	1.33±0.2 *	0
	L-NNA+L-arginine	0.5±0.2	0.5±0.2	0.1±0.05
	L-arginine	0.2±0.1	0.33±0.1 *	0

DISCUSSION

The results of the present study demonstrate that repeated administration of bacterial LPS to the rats produces the mild inflammatory changes in the pancreatic tissue, increases generation of NO in isolated pancreatic acini, leads to the hyperamylasemia and decreases the secretory response of these acini to pancreatic secretagogues. In rats treated with LPS, the addition of L-NNA suppresed NO generation, resulted in the dramatic increase in the mortality of animals, and aggravated pancreatic secretory and morphological changes produced by LPS in these animals. The plasma TNFα correlates with the severity of pancreatic inflammation produced by LPS and greatly increased following the addition of L-NNA to LPS.

Clinical studies demonstrated that infection with enteric bacteria is one of the major causes of mortality in acute pancreatitis (25, 26). Pancreatic inflammation is associated with an increase of the intestinal permeability

and under these conditions enteric bacteria could be translocated into the circulation leading to the endotoxemia and pancreatic infection (4, 27). In spite of the observations showing that endotoxemia frequently occurs in the patients with severe pancreatitis (5, 25, 26) the reports concerning the effects of endotoxins on the pancreas are limited and controversial. Previous study demonstrated that chronic infusion of bacterial LPS to the rats produced pancreatic tissue lesion and modulated pancreatic enzyme secretion (7). Single injection of LPS was reported to produce acute pancreatitis in rabbits (8), whereas LPS in rats did not affect pancreatic morphology and function (9, 10).

The results of our present study confirms previous observation that repeated administration of LPS to the rats results in an impairment of pancreatic amylase secretion, produces an increase in plasma amylase activity and causes morphological changes of pancreatic tissue such as edema, neutrophil infiltration and hemorrhage (7). These changes induced by LPS were markedly aggravated by suppression of NO generation by L-NNA.

It is well documented that endogenous NO exerts a beneficial effect against acute pancreatitis, and that the inhibition of NOS augments the inflammatory changes of the gland (6, 20—22, 29). The mechanism of this protective action of NO on pancreatitis could be related to the improvement of pancreatic microcirculation and decreased accumulation of the neutrophils in the pancreatic tissue as well as to the reduction of the vascular permeability caused by NO in the pancreas (17, 21, 22, 30, 31).

Administration of LPS to the rat enhanced NO production in several tissues including pancreas (32). It was previously shown that pancreatic acini are able to release NO and that this NO could contribute to the protection of the pancreas against acute damage (20).

An important finding of the present paper is the observation that LPS increases NO generation in the pancreatic acini, whereas the blockade of NOS by L-NNA completly abolishes this LPS-stimulated NO release from the pancreatic acinar cells. As we demonstrated in this study LPS markedly increases pancreatic blood flow *in vivo* and this effect was prevented by the suppression of NO by L-NNA. It is likely that NO generated by pancreatic acini could diffuse to the blood vessels, cause vasodilatation and this could improve pancreatic microcirculation leading to the amelioration of the inflammatory cells from the pancreatic tissue (31). The vascular mechanism plays a crucial role in the pathogenesis of acute pancreatitis and impairment of pancreatic circulation could by itself produce pancreatic damage (33, 34). The suppression of the NO production in LPS-treated rats enhanced leukocyte adhesion to the endothelium leading to the infiltration of the pancreatic tissue with neutrophils.

The results of our study support previous observation that long-term administration of LPS to the animals produces inflammatory changes in the

pancreas (17). In our rats with endotoxemia, the suppression of NO by L-NNA produced a significant aggravation of pancreatic damage and resulted in an increased mortality of animals treated with L-NNA together with LPS. It is of interest that administration of endotoxin without the addition of L-NNA produced only mild inflammatory changes of the pancreas. The treatment of the rats with L-NNA alone reduced only the pancreatic blood flow but also failed to produce significant pancreatic lesion. This paper demonstrated that the combination of above noxious agents such as suppression of NO generation by L-NNA and the repeated administration of endotoxin is needed to produce pancreatic inflammation and to result in animal death.

In the pancreas LPS has been reported to activate the neutrophils, to release the number of inflammatory cytokines including TNFα and to induce the apoptosis of the pancreatic acini (35—37). Recent study demonstrated that TNFα has been produced and released by pancreatic acinar cells, and that this TNFα might contribute to the development of pancreatitis and cell apoptosis (16). However, in contrast to the observation showing that LPS increased TNFα secretion in the macrophages (38), the release of TNFα from the pancreatic acini was not stimulated by LPS (16). It is likely that the increase of TNFα level in the plasma in response to LPS that was observed in our study and reported in the previous papers (5, 26), originates from the neutrophils which infiltrated the pancreas and not from the acinar cells. As we observed in our study, repeated administration of LPS produces dose-dependent increase of TNFα level in the plasma, whereas suppression of NO by L-NNA caused dramatic rise of the plasma level of this cytokine. The suppression of NO generation aggravated pancreatic tissue lesion, and potentiated the infiltration of the pancreas with inflammatory cells. Our study clearly shows that in rats subjected to repeated LPS administration plasma level of TNFα correlated with the severity of pancreatic lesion.

Hyperamylasemia is a commonly used indicator of acute pancreatitis (6, 10, 14, 20, 21, 29, 30), though the increase in amylase level is not in parallel to the severity of pancreatic damage (39). Previous studies showed that endotoxemia produced the impairment of pancreatic enzyme secretion leading to an increase of the plasma amylase level (7, 28). The mechanism of this effect of LPS on the pancreatic enzyme secretion remained an unanswered question. Our present study clearly demonstrates that repeated administration of LPS decreases the secretory responsiveness of isolated acini to pancreatic secretagogues and the pretreatment with L-NNA together with LPS abolished the ability of these acini to secrete the amylase. Repeated administration of LPS to the rats resulted in the moderate changes of plasma amylase. It is likely that this hyperamylasemia could be due to the pancreatic cell damage caused by LPS. The direct effect of LPS on pancreatic acini could not be excluded, however, this possibility requires further study.

We can conclude from the present report that repeated administration of LPS to the rats could induce by itself mild pancreatic damage, however, it is not able to produce severe pancreatitis. Suppression of NO generation in the LPS-treated rats aggravated pancreatic inflammation leading to the dramatic increase in the mortality of the treated animals. This detrimental effect of the suppresion of NO synthesis was probably related to the impairment of the pancreatic blood flow caused by L-NNA in the rats with endotoxemia. Our study presents a new model of the pancreatic inflammation which could be a used as an experimental pancreatitis.

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