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INVOLVEMENT OF CYCLOOXYGENASE-DERIVED PROSTAGLANDIN E_2 AND NITRIC OXIDE IN THE PROTECTION OF RAT PANCREAS AFFORDED BY LOW DOSE OF LIPOPOLYSACCHARIDE

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Prostaglandins (PG), the products of arachidonate metabolism through cyclooxygenase (COX) pathway, protect the pancreas from the acute damage. The existence of two isoforms of COX was documented including: COX-1, present in normal tissues and COX-2, expressed at the site of inflammation, such as induced by bacterial lipopolysaccharide (LPS). Pretreatment with low dose of LPS and activation of nitric oxide (NO) synthase (NOS) has been shown to prevent the injury caused by caerulein-induced pancreatitis (CIP) in the rat. The aim of this study was to investigate the role of COX-1 and COX-2 in the LPS-induced protection of the pancreas against CIP and the involvement of NOS in the activation of COX-PG system in the rats with CIP. CIP was produced by subcutaneous (s.c.) infusion of caerulein (5 μg/kg-h for 5 h) to the conscious rats. Protective dose of LPS, from *Escherichia coli*, (1 mg/kg) was given intraperitoneally (i.p.) 15 min prior to the start of CIP. Nonselective inhibitor of COX; indomethacin (5 or 10 mg/kg), selective inhibitor of COX-1: resveratrol, or a highly selective inhibitors of COX-2: rofecoxib or NS-398 (2 or 10 mg/kg) were injected i.p. 15 min prior to the administration of LPS. COX-1 or COX-2 mRNA was determined by reverse transcription-polimerase chain reaction (RT-PCR) in the pancreatic tissue. Pancreatic blood flow (PBF) was measured by a laser Doppler flowmetry. PGE_2 content in the pancreas was measured by radioimmunoassay. CIP was manifested by an increase of pancreatic weight and plasma amylase activity (by 500% and 700%, respectively) and it was confirmed by histological examination. CIP slightly increased pancreatic PGE_2 generation (by 12%) and diminished PBF (by about 40%). LPS (1 mg/kg i.p.), given prior to the start of CIP, increased PGE_2 generation in the pancreas (by 45%), reversed the histological manifestations of pancreatitis, reduced the rise in amylase blood level and improved PBF. Administration of nonselective inhibitor of COX; indomethacin (5 or 10 mg/kg i.p.) prior to the injection of LPS abolished its protective effects on CIP and reduced pancreatic PGE_2 generation. Selective inhibitor of COX-1; resveratrol (10 mg/kg i.p.) given prior to the injection of LPS reversed its protective effects against CIP. Pretreatment with a selective inhibitors of COX-2: rofecoxib or NS-398 (10 mg/kg) attenuated LPS-induced pancreatic protection in the CIP rats. COX-1 expression was detected in the intact pancreas and was not significantly changed by CIP, LPS, indomethacin, NS-389 and their combination, while COX-2 mRNA expression appeared in the pancreas of rats

subjected to CIP and was significantly increased after LPS injection to these rats. Addition of selective COX-2 inhibitor; NS-389, or nonselective inhibitor of COX; indomethacin, enhanced COX-2 mRNA expression in the rats with CIP pretreated with LPS. Pretreatment of the rats with inhibitor of NOS; L-NNA (20 mg/kg i.p.), given together with LPS, 15 min prior to the start of caerulein overstimulation, resulted in complete reversion of LPS-induced pancreatic protection and decreased PGE_2 generation stimulated by LPS. Addition to L-NNA of the substrate for NOS; L-arginine (100 mg/kg i.p.), restored pancreatic protection afforded by low dose of LPS and increased pancreatic PGE_2 level in the rats with CIP. We conclude that: 1. increased pancreatic PGE_2 generation, induced by low dose LPS pretreatment, contributes to the pancreatic resistance to acute damage produced by caerulein overstimulation and 2. the NO-system is involved in above stimulation of PGE_2 generation and pancreatic protection against acute damage.

Key words: *caerulein-induced pancreatitis, lipopolysaccharide, prostaglandin, cyclooxygenase, indomethacin, resveratrol, rofexocib, NS-389; nitric oxide*

INTRODUCTION

Lipopolysaccharide (LPS, endotoxin) is the component of the cell wall of the gram-negative bacteria (1). Recently, bacterial endotoxin received attention as an activator of the tissue inflammatory response which might result in the septic shock and multiorgan dysfunction (2—4). Massive endotoxemia was observed in the severe pancreatitis with fatal outcome, and LPS, by itself at larger dose, has been shown to induce pancreatic damage (5—8).

In contrast to above harmful effects of large amounts of bacterial LPS, short-term application of low dose of this substance was reported to protect the pancreas from the injury produced by caerulein-overstimulation, as was demonstrated in our and other experimental studies (9, 10). Since LPS has been previously shown to stimulate the activity of nitric oxide synthase (NOS) in the numerous tissues, above beneficial effect of LPS on the pancreas has been attributed, at least in part, to the activation of nitric oxide synthase (NOS) and enhanced production of nitric oxide (NO) (10, 11).

Besides the activation of NO-system bacterial endotoxin is also able to induce the activity of cyclooxygenase (COX) and to enhanced biosynthesis of prostaglandins (PG) in isolated endothelial cells and in the stomach *in vivo* (12, 13). Two isoforms of COX were characterized one, constitutive enzyme (COX-1), present normally in various tissues and another inducible (COX-2), detected mainly in the tissues involved in the inflammatory process (12, 13). COX-1 may be selectively inhibited by resveratrol, derived from grapes and used in the cancer therapy (14), while, the novel anti-inflammatory drugs such as; rofecoxib or NS-389, have been shown to inhibit COX-2 (15). It is worthy to remember that both COX-1 and COX-2 enzymes could be activated by NO and LPS has been reported to activate both the COX-PG and NO-system in some cells (16—18).

PG of E and I series have been previously shown to diminish the severity of acute pancreatitis in experimental studies and the mechanism of their action on

the pancreas has been intensively studied during the last decade (19—22). This beneficial effect of PG on acute pancreatitis was attributed to such mechanisms as the improvement of pancreatic blood flow, the reduction in the vascular permeability and the limitation of digestive enzyme release from the pancreatic acinar cells (20—24). It is of interest that in acute pancreatitis the PG generation in platelets and in the endothelial cells probably involves the NO-dependent mechanism (25) and it is reasonably to assume that PG and NO interact on the modulation of the pancreatic inflammatory processes. Possible relationship between protective effects of LPS, activation of NO-system and PG biosynthesis in the pancreas remains an open question.

The aim of this study was: 1) to examine the involvement of endogenous PG in the rats subjected to caerulein-induced pancreatitis (CIP) without or with pretreatment with various doses of LPS and NO-donor, SNAP, 2) to assess the effects of nonselective inhibition of COX, (indomethacin), selective inhibition of COX-1 (resveratrol) and selective inhibition of COX-2 (NS-389 or rofecoxib) on the pancreatic protection afforded by low dose of LPS, 3) to examine mRNA expression for COX-1 and COX-2 in the inflammatory pancreatic tissue, and 4) to determine the involvement of endogenous NO in the activation of PG-system in LPS pretreated animals subjected to CIP.

MATERIAL AND METHODS

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

All animal use in this study was conducted in compliance with approved institutional animal care according to guidelines of Jagiellonian University Ethics Committee.

Following items were purchased: caerulein (Takus) from Pharmacia GmbH, Erlangen, Germany, LPS (serotype 0127;B8), N^G-nitro-L-arginine (L-NNA), a blocker of NO synthase, L-arginine, urecholine, trypsin inhibitor and nonselective inhibitor of COX; indomethacin from Sigma Chemical Co, (St Louis, MO, USA), selective inhibitor of COX-2; rofecoxib was from Merck, Sharp & Dohme, Warsaw, Poland, another inhibitor of COX-2; N[2-(cyclohexyloxy)--4-nitrophenyl]methane sulfonamide (NS-398), selective inhibitor of COX-2; resveratrol, NO-donor; S-nitroso-N-acetyl-penicillamine (SNAP) were from Cayman Chemical Co., (Ann Arbor, MI, USA), essential and nonessential amino acid mixture from SERVA Feinbiochemica (GmbH, Heidelberg, Germany), purified collagenase from Worthington Biochemical Co., (Freehold, NJ, USA), PGE_2 RIA kit was from NEN™ Life Science Products, (Boston, MA, USA).

During the experiment the rats were placed in individual Bollman cages. Acute caerulein-induced pancreatitis (CIP) was produced by s.c. infusion of caerulein to the conscious rats at a dose 5 µg/kg-h for 5 h. Caerulein was diluted in the saline and infused at a rate 1 ml/h. Experiments were carried out in four separate parts (I—IV).

Part I. To compare the effects of administration of LPS and of the donor of NO, S-nitroso-N-acetyl-penicillamine (SNAP), on the course of CIP and on PGE_2 generation in the

pancreas various doses of LPS (0.1, 1, 10 mg/kg) or SNAP (1.5 or 3, mg/kg) were dissolved in 0.5 ml of saline and administered i.p. to the animals as a bolus injection 15 min prior to the start of caerulein, or saline (control experiments) infusion.

Part II. The experiments were carried out to investigate the effect of suppression of endogenous PG generation on the pancreatic protection afforded by low doses of LPS in the rats subjected to CIP and to assess the possible role of COX-1 and COX-2 in this protection. The nonselective inhibitor of COX; indomethacin (5 or 10 mg/kg i.p.), the selective inhibitor of COX-1, resveratrol (2 or 10 mg/kg i.p.) and selective inhibitors of COX-2, NS-398 or rofecoxib (both at the doses 2 or 10 mg/kg i.p.) were applied to the rats 15 min prior to the administration of a standard dose of LPS (1 mg/kg i.p.). This LPS administration was followed 15 min later by the infusion of caerulein to induce CIP. The effects of indomethacin, resveratrol, or both inhibitors of COX-2, rofecoxib, or NS-398 (used at the doses mentioned above) on the course of CIP were also tested in the rats pretreated with saline (instead of LPS) 15 min prior to the induction of CIP.

Part III. For analysis of the mRNA for COX-1 and COX-2 in the pancreas rats were injected with LPS (1 mg/kg i.p.) with or without the addition of nonselective COX inhibitor; indomethacin (10 mg/kg i.p.) or selective COX-2 inhibitor, NS-389 (10 mg/kg i.p.). Indomethacin or NS-389 was administered to the rats 15 min prior to the injection of LPS followed 15 min later by the infusion of caerulein to induce CIP.

Part IV. To clarify whether endogenous NO is involved in the activation of COX-PG system in the pancreas of CIP-rats, the rats were injected first with a standard dose of LPS (1 mg/kg i.p.), and 15 min later N^G-nitro-L-arginine (L-NNA), a blocker of NO synthase (NOS) was applied (20 mg/kg i.p.) without or with a combination with L-arginine, a substrate for NOS (100 mg/kg). Caerulein or saline (control tests) infusion started 15 min after the injection of L-NNA or L-NNA combined with L-arginine to the animals.

The effects of various doses of LPS alone (0.1, 1, 10 mg/kg), SNAP alone (1.5, or 3 mg/kg) indomethacin, resveratrol, rofecoxib or NS-398 alone and L-NNA alone or combined with L-arginine were also tested in the rats receiving vehicle saline instead of caerulein infusion.

Determination of pancreatic blood flow (PBF) and pancreatic weight

Following 5 h injection of caerulein or vehicle saline (in control tests) animals were shortly anesthetized with Vetbutal, weighted and the abdominal cavity was opened. The pancreas was exposed for measurement of the pancreatic blood flow (PBF) by a laser Doppler flowmeter (LDF) using a Laserflo, model BPM Blood Perfusion Monitor (Vasamedics Inc., St Paul, MN, USA). PBF was measured in five different regions of the pancreas and was expressed as percent change from control value obtained from the rats injected with saline.

Determination of plasma amylase activity

Immediately after measurement of PBF, the inferior vena cava was exposed and blood was taken for plasma amylase determination. Plasma amylase was measured by an enzymatic method (Amylase reagent Dialab Diagnostic Ges. MBH, Wien, Austria) as described previously (26).

Determination of the PGE_2 generation in the pancreas

The samples of fresh pancreatic tissue (weighing about 50 mg) were taken for measurement of PGE_2 content in the pancreas by radioimmunoassay (RIA). Briefly, the samples were placed in pre-weighted Eppendorf vial and 1 ml of Tris buffer (50 mM/L, pH 9.3) was added to each vial. The sample was minced (about 15 s) with scissors, washed and centrifuged for 10 s, the pellet being

resuspended again in 1 ml of Tris. Then each sample was incubated on a Vortex mixer for 1 min and centrifuged for 15 s. The pellet was weighted and the supernatant was transferred to a second Eppendorf vial containing indomethacin (10 mM/L) to block any further PG formation and kept at −80°C until RIA. PGE_2 was measured in duplicate using RIA kit [E.I. Du Pont de Nemours and Co (Inc.), NEN™ Life Science Products, Boston, MA, USA] and the capacity of the pancreas to generate PGE_2 was expressed in ng/g of wet tissue weight.

RNA extraction and reverse-transcriptase polymerase chain reaction (RT-PCR) to detect mRNA for COX-1 and COX-2

Pancreatic tissue samples were taken from the control rats, from the rats with CIP, without or with pretreatment with LPS, from the rats injected with LPS alone, or LPS combined with selective COX-2 inhibitor; NS-389 and from the rats subjected to CIP and pretreated with combination of LPS+nonselective COX inhibitor; indomethacin or selective COX-2 inhibitor; NS-389. Samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Total RNA was isolated by a guanidinum isothiocyanate /phenol chlorophorm single-step extraction lit from Stratagene (Heidelberg, Germany). After precipitation the RNA was resuspended in RNAse-free TE buffer, and the concentration was estimated by measuring the absorbance at 260 nm. Single stranded cDNA was generated from total cellular RNA (5 µg) using 200 U Strata Script reverse transcriptase (Stratagene) and oligo-(dT)-primers (Stratagene). Briefly 5 µg of total RNA was uncoiled by heating and the cDNA was stored at −20°C until PCR.

Primers were synthetized by Biometra (Gottingen, Germany). The nucleotide sequences of the rat COX-1 and COX-2 were based on the published cDNA sequences encoding rat cycolooxygenases (27—30). The COX-1 sense primer was 5'-AGC CCC TCA TTC ACC CAT CAT TT, while the COX-1 antisense primer was 5' CAG GGA CGC CTG TTC TAC GG. The expected length of this PCR product was 561 bp. The oligonucleotide primer sequences for COX-2 were as follows: 5'ACA ACA TTC CTT CCT TC (sense) AND 5'CCT TAT TTC CTT TCA CAC C (antisense). The expected length of this PCR product was 201 bp. Concomitantly amplification of control rat β actin (ClonTech, Palo Alto, CA, USA) was performed on the same sample to assess the RNA integrity. Reaction mixtures for PCR contained cDNA templates, 50 pmol of each primer, and 2.5 U of Taq DNA polymerase (Serva, Heidelberg) in 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_{2.}$ 0.5 mM dNTPs in a volume of 50 µl. RT blanks (without RNA) and PCR blanks (without cDNA products) were included in each analysis. PCR products (8 µl) were detected by electophoresis on a 1.5% agarose gels containing ethidium bromide. Then visualization under UV light was performed. Location of predicted products was confirmed using GIBCO 100-bp ladder (GIBCO BRL/Life Technologies, Eggenstein, Germany) as a standard size marker. To compare the level of expression of COX-1 and COX-2 mRNA against the reference gene (β actin) mRNA data, the image analysis was employed. PCR products were analyzed using program Gel-Pro analyzer (Media Cybernetics, Silver Spring, Mass, 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 histological grading of edema, leukocyte infiltration or vacuolization was made using a scale ranging from 0 to 3, as described previously (10).

Statistical analysis

Comparison of the differences between the mean values of various groups of experiments were made by analysis of variance and the Student's t test for unpaired data. A difference with a p. value of < 0.05 was considered statistically significant. Results are expressed as means (±SEM).

RESULTS

PART I. COMPARISON OF THE EFFECTS OF LPS AND SNAP ON PGE_2 GENERATION IN THE PANCREAS AND ON THE COURSE OF CIP

Pancreatic edema, PBF and plasma amylase activity

Infusion of caerulein (5 μg/kg s.c. for 5 h) to the conscious fasted rats produced acute edematous pancreatitis (CIP) in all animals tested. The pancreatic edema was expressed as an increase of pancreatic weight that was almost doubled as compared to that of saline-treated control rats (*Table 1*). PBF, measured in the rats with CIP, was reduced to about 60% of the control value, whereas plasma amylase level dramatically increased, as compared to the level obtained from the control rats infused with saline instead of caerulein (*Table 1*). Administration of low doses of LPS (0.1, 1 or 10 mg/kg) to the rats prior to the start of caerulein infusion resulted in the significant reduction in both; pancreatic edema and plasma amylase activity and it was accompanied by a significant rise in the PBF comparing to the rats with acute pancreatitis without such LPS pretreatment (*Table 1*).

Table 1. The effects of pretreatment of the rats with various doses of LPS or SNAP on pancreatic weight, plasma lipase activity and pancreatic blood flow (PBF) in the rats with caerulein-induced pancreatitis (CIP). Mean ± SEM from 8—10 separate tests. Asterisk indicates significant difference as compared to the value obtained from the rats with CIP.

Test	Pancreatic weight mg/pancreas	Plasma lipase activity IU/l	PBF % of control
Control (vehicle saline)	790 ± 80	150 ± 8	100 ± 12
CIP	1558 ± 150	950 ± 170	51 ± 7
CIP + LPS 0.1 mg/kg	230 ± 60*	250 ± 30*	125 ± 15*
CIP + LPS 1 mg/kg	1080 ± 50*	305 ± 10*	119 ± 20*
CIP + LPS 10 mg/kg	1150 ± 100*	450 ± 50*	105 ± 18*
CIP + SNAP 1.5 mg/kg	1160 ± 120*	505 ± 90*	95 ± 10*
CIP + SNAP 3 mg/kg	980 ± 85*	450 ± 80*	87 ± 12*

Injection of the rats with low concentrations of NO donor, SNAP, that is known to release NO (1.5 or 3 mg/kg i.p.), diminished the pancreatic edema and the plasma amylase activity and increased PBF in the rats with CIP. This beneficial effect of SNAP on caerulein-pancreatitis was similar to that observed in the rats pretreated with low doses of LPS (*Table 1*). In control experiments, the administration of LPS at concentrations 0.1, 1, 10 mg/kg or SNAP (1.5 or

3 mg/kg) to the rats, receiving infusion of saline instead of caerulein, failed to affect significantly pancreatic weight, PBF or plasma amylase level and these results were omitted for the sake of clarity.

Histological examination

As expected, the infusion of caerulein (5 μg/kg × 5 h) produced pancreatic lesions in all tested rats (*Table 2*). The pancreas was grossly swollen and enlarged. Peritoneal fluid was present in all animals. Histologically the interlobular and intralobular edema was accompanied by perivascular infiltration of leukocytes and the presence of vacuolization in acinar cells. Morphological examination showed significant improvement in pancreatic histology in rats pretreated with low dose of LPS (1 mg/kg) prior to caerulein infusion; edema was markedly diminished (by 47%), infiltration was dramatically reduced (by 70%), and vacuolization was significantly decreased (by 63%) when compared to the changes observed in rats with CIP.

Table 2. Histological changes induced by caerulein-induced pancreatitis (CIP) alone, CIP combined with i.p. injection of SNAP (3 mg/kg), CIP combined with i.p. injection of LPS (1.0 mg/kg), CIP + i.p. injection of indomethacin (Indo) (10.0 mg/kg) combined with LPS (1.0 mg/kg), CIP + i.p. injection of Resverastrol (10 mg/kg) combined with LPS (1.0 mg/kg), CIP + i.p. injection of NS-398 (10 mg/kg) combined with LPS (1.0 mg/kg), CIP + i.p. injection of Rofecoxib (10 mg/kg) combined with LPS (1.0 mg/kg). Asterisk indicates significant change as compared to the value obtained with CIP alone.

	Edema 0—3	Leukocyte infiltration 0—3	Vacuolisation 0—3
Saline (control)	0	0	0
CIP	2.1 ± 0.3	1.17 ± 0.2	2.3 ± 0.3
SNAP 3 mg/kg + CIP	1.03 ± 0.2*	0.4 ± 0.1*	1.0 ± 0.2*
LPS 1 mg/kg + CIP	1.1 ± 0.2*	0.36 ± 0.1*	0.86 ± 0.15*
LPS 1 mg/kg + Indo 10 mg/kg + CIP	1.7 ± 0.3	1.3 ± 0.3	2.0 ± 0.5
LPS 1 mg/kg + Resveratrol 10 mg/kg + CIP	1.5 ± 0.3	1.0 ± 0.2	1.5 ± 0.2*
LPS 1 mg/kg + NS-398 10 mg/kg + CIP	1.2 ± 0.3*	0.8 ± 0.1	1.3 ± 0.5*
LPS 1 mg/kg + Rofecoxib 10 mg/kg + CIP	1.3 ± 0.3*	0.7 ± 0.1*	1.6 ± 0.3

Administration of low dose of SNAP (3 mg/kg) produced significant reduction in the pancreatic damage in rats with CIP and these protective effects were similar to those observed in rats pretreated with low dose (1 mg/kg) of LPS followed by caerulein infusion to produce CIP (*Table 2*).

Pancreatic PGE_2 generation

Fig. 1 shows the effects of low doses of LPS and SNAP on the pancreatic PGE_2 generation in rats subjected to acute CIP. In the pancreas of saline-treated control animals, the PGE_2 level was about 60.0 ± 10 ng/g of pancreatic tissue and this was taken as 100%. Following 5 h of caerulein infusion, to produce CIP, the ability of pancreatic tissue to generate PGE_2 was slightly but not significantly increased (*Fig. 1*). Increasing doses of LPS (0.1, 1, and 10 mg/kg) produced a dose-dependent rise in pancreatic PGE_2 content reaching, respectively, 125%, 160% and 175% of the control level. Protective dose of SNAP (3 mg/kg) followed by infusion of caerulein to produce CIP, resulted in an increase in pancreatic generation of PGE_2 to about 160% of control level that was similar to the value obtained with 1 mg/kg dose of LPS (*Fig. 1*).

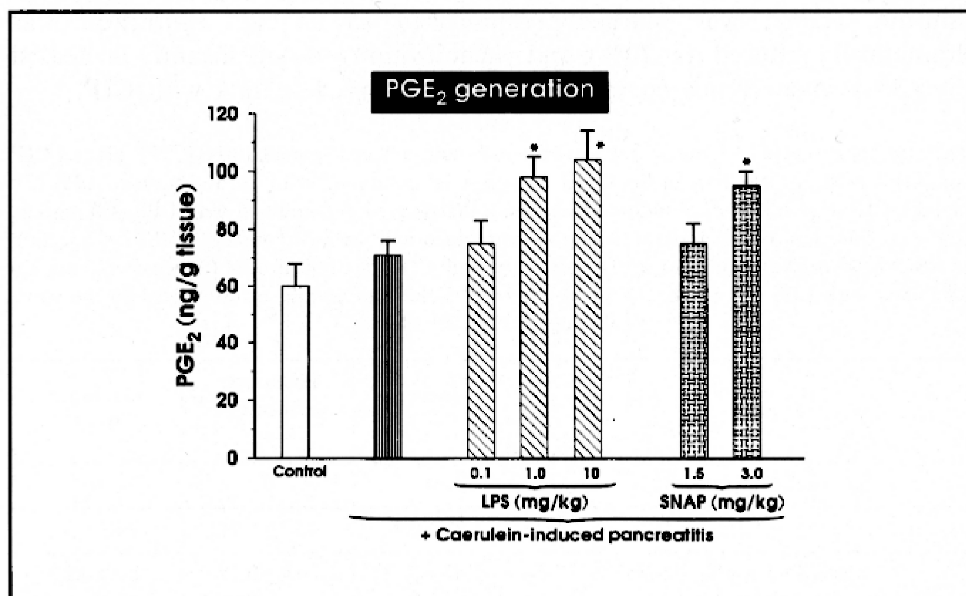

Fig. 1. The effects of increasing concentrations of LPS or SNAP on pancreatic PGE_2 generation in rats subjected to caerulein-induced pancreatitis. Asterisk indicates significant ($p < 0.05$) increase above the control value. Means$\pm$SEM of 6 separate tests.

PART II. EFFECTS OF THE SUPPRESSION OF COX ACTIVITY ON THE PANCREATIC PGE_2 GENERATION, AND THE PANCREATIC PROTECTION INDUCED BY LOW DOSE OF LPS IN THE COURSE OF CIP.

Pancreatic edema, PBF and plasma amylase activity

Pretreatment with lower dose of nonselective inhibitor of COX, indomethacin (5 mg/kg i.p.) or selective inhibitor of COX-1, resveratrol (2 mg/kg i.p.) together with standard dose of LPS (1 mg/kg i.p.) did not affect

significantly LPS-induced protection of CIP. When higher dose of indomethacin (10 mg/kg i.p.) was given prior to the protective dose of LPS (1 mg/kg i.p.) to the rats with CIP the beneficial effects of LPS on pancreatic edema, plasma amylase activity and PBF were completely reversed (*Figs 2, 3, 4*). Administration of high dose of selective inhibitor of COX-1, resveratrol (10 mg/kg i.p.) together with protective dose of LPS (1 mg/kg i.p.) also caused the attenuation of the beneficial effects of LPS on CIP; pancreatic weight and plasma amylase were higher, while PBF was reduced comparing to the values observed in the rats with CIP pretreated with LPS without addition of resveratrol. Higher doses of selective inhibitors of COX-2, NS-398 or rofecoxib (10 mg/kg) given together with low dose of LPS to the rats with CIP also resulted in the partial reversion of the LPS-induced protection against CIP (*Figs 2, 3, 4*) indicating that COX-2 could be also involved in this protection.

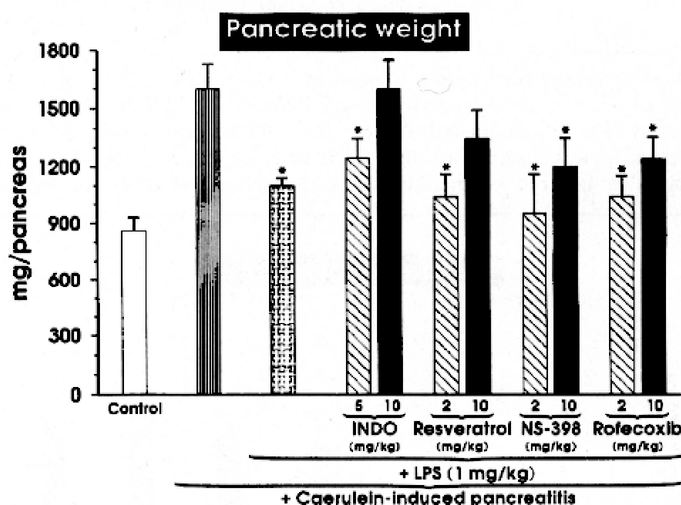

Fig. 2. The effects of various concentrations of nonselective inhibitor of COX; indomethacin (Indo), selective inhibitor of COX-1; resveratrol, selective inhibitors of COX-2; rofecoxib or NS-398 on pancreatic weight in rats pretreated with low dose of LPS prior to start of caerulein-induced pancreatitis. Asterisk indicates significant ($p < 0.05$) decrease below the value obtained form the rats with caerulein-induced pancreatitis alone. Means ± SEM of 8—10 separate tests.

Indomethacin, resveratrol, rofexoxib or NS-289 given to the control rats (injected with physiological saline) did not produce any significant alterations of the any parameters tested and these results were omitted for the sake of clarity.

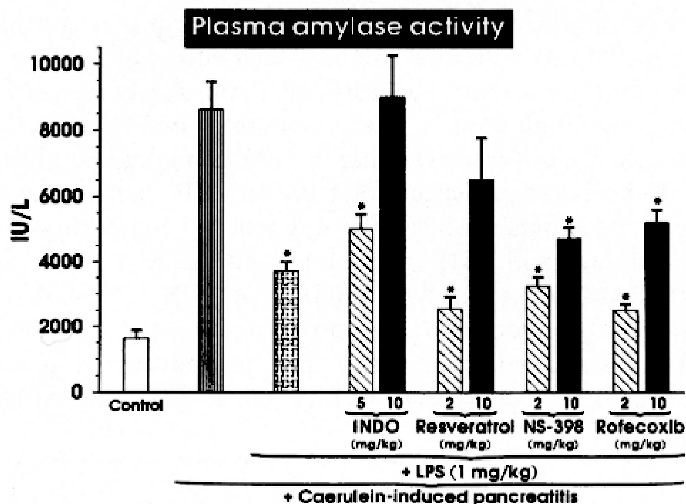

Fig. 3. The effects of various concentrations of nonselective inhibitor of COX; indomethacin (Indo), selective inhibitor of COX-1; resveratrol, selective inhibitors of COX-2; rofecoxib or NS-398 on plasma amylase activity in rats pretreated with low dose of LPS prior to start of caerulein-induced pancreatitis. Asterisk indicates significant ($p < 0.05$) decrease below the value obtained from the rats with caerulein-induced pancreatitis alone. Means ± SEM of 8—10 separate tests.

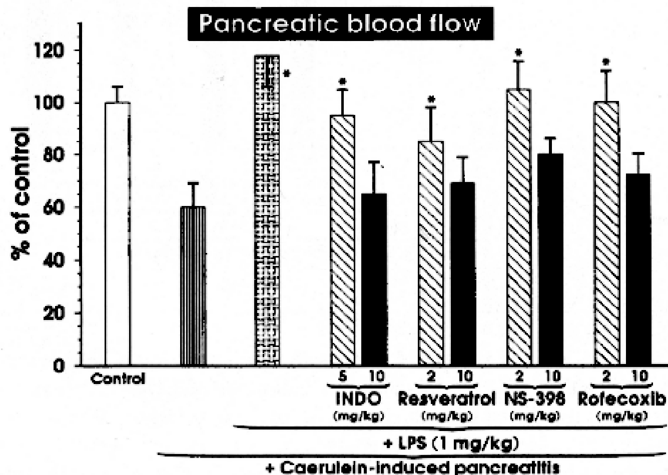

Fig. 4. The effects of various concentrations of nonselective inhibitor of COX; indomethacin (Indo) or selective inhibitor of COX-1; resveratrol, selective inhibitors of COX-2; rofecoxib or NS-398 on pancreatic blood flow in rats pretreated with low dose of LPS prior to start of caerulein-induced pancreatitis. Asterisk indicates significant ($p < 0.05$) increase above the value obtained from the rats with caerulein-induced pancreatitis alone. Means ± SEM of 8—10 separate tests.

Indomethacin, resveratrol, rofecoxib or NS-289 given to the rats with CIP also did not affect significantly pancreatic edema, increased plasma amylase activity and reduced PBF in the rats with CIP (*Table 3*).

Table 3. The effect of Indomethacin (Indo) (5 mg/kg or 10 mg/kg i.p.), Resveratrol (2 mg/kg or 10 mg/kg i.p.), NS-398 (2 mg/kg or 10 mg/kg i.p.) and Rofecoxib (2 mg/kg or 10 mg/kg i.p.) on pancreatic weight, plasma amylase activity, pancreatic blood flow (PBF) and pancreatic PGE_2 level in the rats without or with caerulein-induced pancreatitis (CIP). Mean ± SEM of 6 separate experiments.

	Pancreatic weight (mg/pancreas)	Plasma amylase activity (IU/1)	Pancreatic blood flow (% of control)
Control	820 ± 100	1640 ± 260	100 ± 10
CIP	1560 ± 150	8630 ± 850	60 ± 12
CIP + Indo 5 mg/kg	1700 ± 200	6940 ± 1280	80 ± 11
CIP + Indo 10 mg/kg	1620 ± 110	7360 ± 1400	70 ± 10
CIP + Resveratrol 2 mg/kg	1330 ± 240	6740 ± 370	70 ± 10
CIP + Resveratrol 10 mg/ kg	1540 ± 90	8530 ± 310	60 ± 10
CIP + NS-398 2 mg/kg	1430 ± 220	7100 ± 700	85 ± 20
CIP + NS-398 10 mg/kg	1500 ± 190	7600 ± 1100	75 ± 8
CIP + Rofecoxib 2 mg/kg	1220 ± 150	5480 ± 530	60 ± 19
CIP + Rofecoxib 10 mg/kg	1350 ± 140	7350 ± 1400	71 ± 4

Histological changes

Administration of nonselective inhibitor of COX, indomethacin or selective COX-1 inhibitor, resveratrol at a dose of 10 mg/kg completely abolished the protective effect of low dose LPS-pretreatment on CIP (*Table 2*). However when selective inhibitors of COX-2, NS-398 or rofecoxib (10 mg/kg i.p.) were given to the CIP rats pretreated with LPS only partial reversion of the protective effects of LPS on CIP was observed (*Table 2*).

Pretreatment with indomethacin (5 or 10 mg/kg i.p.), resveratrol (2 or 10 mg/kg i.p.), rofecoxib (2 or 10 mg/kg i.p.) or with NS-398 (2 or 10 mg/kg i.p.) did not produce any significant change of pancreatic tissue histology in control group of rats and did not affect significantly the histological picture of pancreatitis in the rats with CIP. Above results were omitted for the sake of clarity.

Pancreatic PGE_2 generation

Pretreatment with nonselective COX inhibitor, indomethacin (5 or 10 mg/kg i.p.) abolished the enhancement of pancreatic PGE_2 generation induced by LPS in the rats with CIP (*Fig. 5*). On the contrary the suppression of COX-2 by NS-398 (10 mg/kg i.p.), resulted only in the minor decrease of PGE_2 content in the pancreas, whereas lower dose of NS-398 (2 mg/kg i.p.) was completely ineffective (*Fig. 5*).

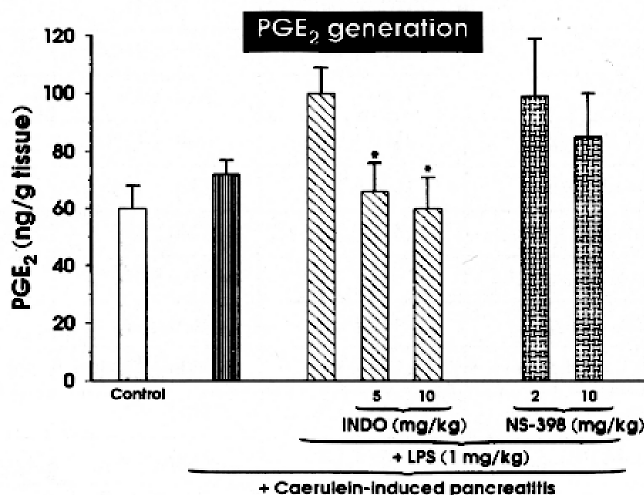

Fig. 5. The effects of various concentrations of nonselective inhibitor of COX; indomethacin (Indo) or selective inhibitor of COX-2; NS-398 on pancreatic PGE_2 generation in rats subjected to caerulein-induced pancreatitis. Asterisk indicates significant ($p < 0.05$) decrease below the value obtained with LPS-pretreatment in the rats with caerulein-induced pancreatitis. Means ± SEM of 6—8 separate tests.

Administration of indomethacin (5 or 10 mg/kg i.p.) or higher dose of NS-398 (10 mg/kg i.p.), followed by infusion of caerulein to produce CIP, resulted in the significant decrease of pancreatic PGE_2 generation much below the level observed after induction of CIP (*Table 4*).

Table 4. The effect of Indomethacin (Indo) (5 mg/kg or 10 mg/kg i.p.) and NS-398 (2 mg/kg or 10 mg/kg i.p.) on pancreatic PGE_2 level in the rats without or with caerulein-induced pancreatitis (CIP). Means ± SEM of 6 separate experiments. Asterisk (*) indicates significant decrease below the value obtained from the rats with CIP.

PGE₂ (ng/g of pancreatic tissue)					
		Indo 5 mg/kg	Indo 10 mg/kg	NS-398 2mg/kg	NS-398 10 mg/kg
Control	60±8	25±5	20±4	63±10	54±12
CIP	71±5	35±11*	21±5*	51±13	33±15*

Indomethacin given to the animals injected with physiological saline instead of caerulein produced significant decrease in the pancreatic PGE_2 content, whereas NS-398 failed to affect significantly pancreatic PGE_2 generation under normal conditions (*Table 4*).

PART III. THE EFFECT OF NONSELECTIVE INHIBITOR OF COX; INDOMETHACIN OR SELECTIVE INHIBITOR OF COX-2; NS-389 ON THE mRNA EXPRESSION FOR COX-1 AND COX-2 IN THE PANCREAS OF CIP RATS WITHOUT OR WITH PRETREATMENT WITH LPS

Fig. 6 shows the mRNA expression for β actin, COX-1 and COX-2 in the pancreas of control rats (infused with physiological saline instead of caerulein), in the rats infused with caerulein to induce CIP without or with pretreatment with standard dose of LPS (1 mg/kg i.p.), in the rats injected with LPS alone or LPS combined with selective inhibitor of COX-2; NS-389, or in the rats with CIP pretreated with nonselective inhibitor of COX; indomethacin or selective inhibitor of COX-2; NS-389 together with LPS.

The mRNA signal for β actin was well preserved in all samples and was similar in all types of experiments. The COX-1 mRNA was detected in all tested samples and was not significantly affected by caerulein infusion, LPS pretreatment, or addition of indomethacin or NS-389.

As shown on *Figs 6, 7* and *8* the COX-2 mRNA was not seen in the placebo (saline) treated control pancreas, in that pretreated with 1 mg/kg of LPS or LPS combined with NS-389. CIP resulted in the small increase of COX-2 mRNA. This mRNA expression for COX-2 in the pancreas of CIP rats was dramatically enhanced by pretreatment with LPS. In the rats with CIP pretreated with LPS combined with the nonselective inhibitor of COX; indomethacin or selective inhibitor of COX-2; NS-389 (10 mg/kg), resulted in the further increase in the COX-2 expression (*Figs 6* and *7*).

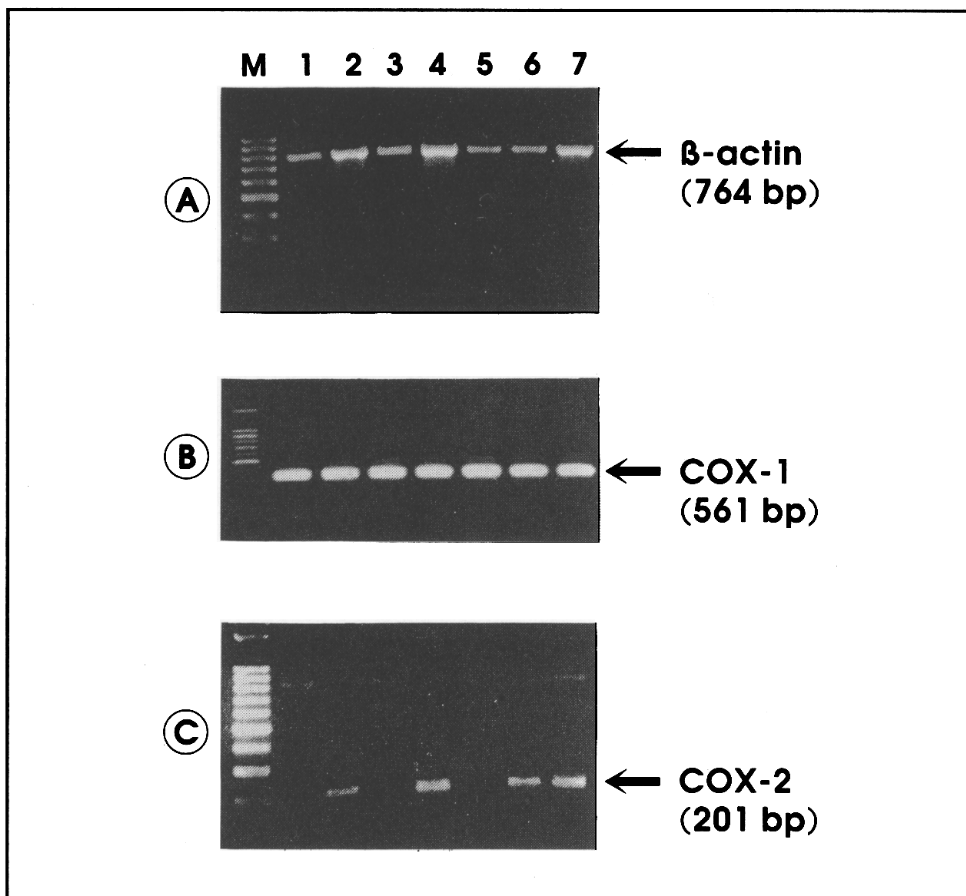

Fig. 6. Expression of mRNA for βactin (A), COX-1 (B) and COX-2 (C) in the pancreas of control rats (lane 1), rats subjected to caerulein-induced pancreatitis (lane 2), rats treated with; LPS (1 mg/kg i.p.), LPS + caerulein-induced pancreatitis, LPS + NS-398 (10 mg/kg i.p.), caerulein-induced pancreatitis + LPS + NS-398 (10 mg/kg i.p,) or caerulein-induced pancreatitis LPS + indomethacin (10 mg/kg i.p.) (lanes 3—7). M — size marker DNA, arrow — expected PCR product (bp)

PART IV. THE EFFECTS OF SUPPRESSION OF NOS BY L-NNA ON LPS-INDUCED GENERATION OF PGE_2 IN THE PANCREAS

Fig. 9. shows the effects of L-NNA without or with addition of L-arginine on pancreatic generation of PGE_2 stimulated by a standard (1 mg/kg) dose of LPS in the rats with CIP. In these animals pretreatment with L-NNA abolished the LPS-induced PGE_2 generation and this effect was restored when L-arginine, the substrate for NOS was added to L-NNA administration.

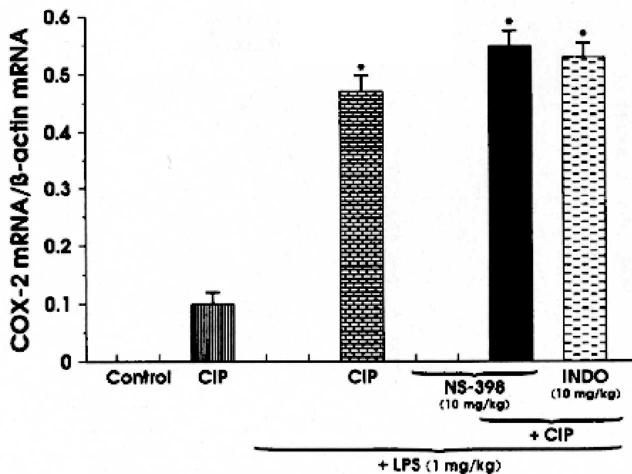

Fig. 7. The ratio of COX-2 over β actin mRNA in the pancreas of rats as in experiments on *Fig. 6.*

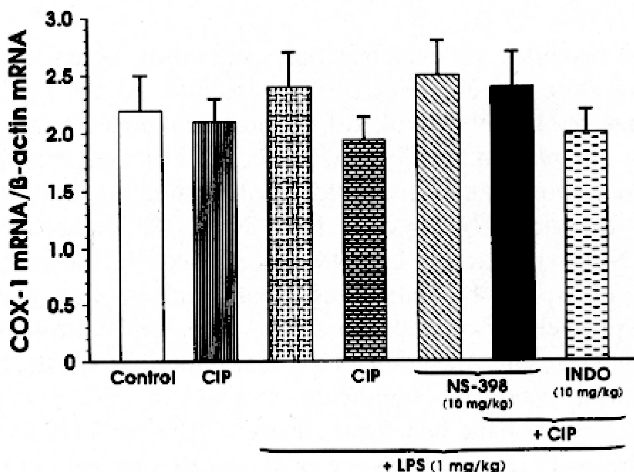

Fig. 8. The ratio of COX-1 over β actin mRNA in the pancreas of rats as in experiments on *Fig. 6.*

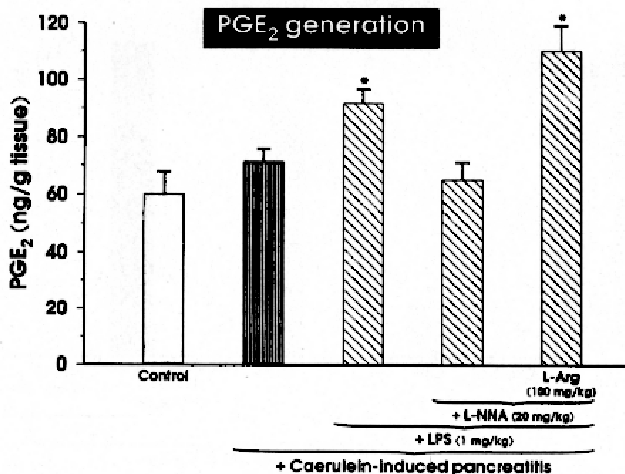

Fig. 9. Pancreatic PGE_2 generation in rats pretreated with LPS (1 mg/kg) combined with inhibitor of NO-synthase; L-NNA without or with the addition of L-arginine prior to the start of caerulein overstimulation. Asterisk indicates significant ($p < 0.05$) increase above the control value. Means ± SEM of 6 separate tests.

DISCUSSION

This study, provides an evidence that generation of endogenous PGE_2, induced by low doses of bacterial LPS, contributes to the protection of the pancreas against the injury provoked by caerulein-induced pancreatitis. As we have shown in our previous study, the protective effect of small doses of LPS against caerulein overstimulation could be attributed, at least in part, to the activation of L-arginine-NO-system (10). Now we demonstrate that the inhibition of NO synthase by L-NNA is followed by the reduction in the generation of PGE_2 in the pancreatic tissue and by the reversion of the LPS-induced protection of the pancreas. The implication of PGE_2 is supported by the fact, that the addition of L-arginine, the substrate for NOS to L-NNA injection, produced significant increase in the pancreatic PGE_2 generation that restored the beneficial effects of LPS on CIP. In addition, the marked increase of PGE_2 level in the pancreas by pretreatment with SNAP, an NO donor, given at low doses to the rats with CIP, produced the protective effects on the pancreas similar to those achieved with low doses of LPS. It seems very likely, therefore, that NO is able to activate the genes for COX resulting in increased PG generation in the pancreas and that both

arginine-NO and COX-PG systems interact on the LPS-induced protection of the pancreas against acute damage.

Recent reports demonstrated that in the stomach endotoxemia induced by the administration of LPS induces the activity of both; COX-1 and COX-2 and, thus, markedly increases the generation of PG in the gastric mucosa (13, 31). LPS has been found to induce COX-2 activity and to increase the formation of PGE_2 in the peritoneal macrophages (32). This may take a part in the modification of inflammatory process in the gastrointestinal tissue.

It is of interest that the induction of COX-2 expression in the pancreas of rats could be traced in rats subjected to simple CIP, using RT-PCR, but pretreatment with LPS alone failed to affect the COX-2 mRNA in the pancreas of control (saline-treated) rats. It could be explained that low, protective dose of LPS, that was used in our experiments is unable to affect COX-2 mRNA under normal conditions. On the contrary administration of low dose of LPS to the rats with CIP resulted in the marked increase of COX-2 signal comparing to that observed in the rats with CIP alone. It is likely that the stimulation of COX-2 expression by CIP is able to facilitate the effect of LPS on this expression producing the significant increase of COX-2 mRNA in the pancreas of CIP rats pretreated with LPS. Our observation in the pancreas resembles that in the stomach made by Davies and coworkers, who found the upregulation of COX-2 expression in the gastric mucosa of rats pretreated with COX inhibitors (indomethacin or aspirin) (33). It is likely that such upregulation of COX-2 expression in response to inhibition of COX-2 results from the local deficiency of PG due to the inhibition of prostaglandin synthesis because the addition of exogenous PG suppressed the COX-2 expression (33). In contrast to COX-2, COX-1 mRNA signal can be detected in the intact pancreas from the vehicle-treated control rats, (using RT-PCR method), and this signal for COX-1 remained unchanged in the rats with CIP without or with pretreatment with LPS, or combination of LPS with indomethacin or NS-389. This indicates that inhibition of prostaglandin synthesis does not affect the COX-1 gene expression in the pancreas of rats subjected to CIP.

It was previously reported that PG of E and I series are able to protect the pancreas and other tissues in the course of acute pancreatitis (17—21), however, extremely high doses of PGE_2 may aggravate the severity of inflammatory process in the pancreas (34). The mechanism of the protective effect of PG in acute pancreatitis is not clear and has been attributed to the stabilization of lysosomal membrane, decrease of the neutrophil sequestration and improvement of blood supply to the pancreas caused by PG (18, 21). In addition, PGE_2 and PGI were reported to inhibit secretion of pancreatic enzymes and thus could diminish tissue lesions produced by the limitation of digestive enzymes activated in the pancreatic tissue in the course of acute pancreatitis (22).

Recent reports shows that endotoxins interact with COX-PG-system in the release of sensory neuromediators from perivascular sensory nerves (35). On the other hand, exposition of macrophages and fibroblasts to LPS affects both NO and PG systems in these cells leading to NO-mediated activation of COX-1 as well as COX-2 and excessive PG biosynthesis (25).

In our present study the suppression of PG generation by nonselective COX inhibitor; indomethacin or selective inhibitor of COX-1 resveratrol, abolished the LPS-induced protection of the pancreas against CIP. Surprisingly, the highly specific inhibitors of COX-2; rofecoxib or NS-398 given to the rats with CIP pretreated with LPS also caused attenuation of the LPS-induced protection of the pancreas. This could be explained that LPS augments PG generation through the activation of both COX-1 and COX-2. Further studies are needed to clarify the implication of COX-1 and COX-2 in the LPS-induced pancreatic protection.

In the rats with caerulein-induced pancreatitis, pretreatment with LPS activated PG generation and significantly increased PGE_2 generation in the pancreatic tissue. This effect was reversed by pretreatment with L-NNA to block NOS activity indicating that in the pancreas of rats with CIP, LPS-stimulated PG generation could be mediated *via* an NO-dependent mechanism. This observation concerning the effect of NO on PG generation is in agreement with previous study showing that in acute pancreatitis the blockade of NOS produced the changes of PGF_1 and tromboxane B_2 levels (18).

In summary our report confirms the results of previous study that low dose of LPS from *Escherichia coli* is able to prevent the pancreatic damage induced by caerulein overstimulation (10). This protective effect is associated with the induction of PG biosynthesis, due to the activation of COX-1 and COX-2, both isoforms being involved in above pancreatic protection. The increase of PG generation caused by LPS in the pancreatic tissue invariably activates NO biosynthesis. Both NO-system and COX-PG systems take a part in the pancreatic prevention of acute damage afforded by low dose LPS.

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