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T. BRZOZOWSKI, P.CH. KONTUREK, Z. ŚLIWOWSKI, D. DROZDOWICZ, R. PAJDO, J. STACHURA, E.G. HAHN, S.J. KONTUREK

LIPOPOLYSACCHARIDE OF *HELICOBACTER PYLORI* PROTECTS GASTRIC MUCOSA *VIA* GENERATION OF NITRIC OXIDE

Institute of Physiology, Jagiellonian University Medical School, Cracow, Poland
Department of Medicine I, University of Medicine, Erlangen-Nuremberg, Germany

Lipopolysaccharide (LPS) has been proposed to act as the major virulence factor in *Helicobacter pylori* (Hp)-infected stomach but its action on mucosal integrity has been little studied. We determined the effects of LPS of Hp, expressing cytotoxic antigens *CagA* and *VacA* on acute gastric lesions induced by 100% ethanol, mucosal blood flow (GBF) and expression of constitutive nitric oxide (NO) synthase (cNOS) mRNA and inducible NO synthase (iNOS) mRNA in gastric mucosa using RT-PCR. Two major series (A and B) of rats were employed; A — with suppressed NOS activity by nonspecific NOS inhibitor, such as N^G-nitro-L-arginine methyl ester, (L-NAME) (5 mg/kg i.v.), or by specific iNOS inhibitor, N^G-(1-Immunoethyl) lysine (L-NIL) (30 mg/kg i.g.), or with inhibited induction of NOS activity by dexamethasone (2 mg/kg i.p.) and series B — vehicle (saline)-treated controls. LPS (0.01-1.0 mg/kg) given i.p. attenuated dose-dependently ethanol-induced mucosal lesions and this protective effect was accompanied by a rise in the GBF and excessive mucosal production and luminal release of NO. LPS (1 mg/kg i.p.) administered at lower dose (1 mg/kg i.p.) to rats without ethanol instillation significantly elevated GBF and luminal release of NO, while higher doses of LPS (20 and 40 mg/kg i.p.) or SNAP (6 mg/kg), which produced systemic hypotension, were not protective. Suppression of NOS activity by pretreatment with standard dose of L-NAME or L-NIL and inhibition of NOS induction by treatment with dexamethasone reversed the protective and hyperemic effects of LPS and this reversal was significantly antagonized by the addition of the substrate for cNOS, L-arginine, but not D-arginine. Administration of L-NAME, L-NIL or dexamethasone, completely abolished the enhanced mucosal NO production and the hyperemia induced by LPS in rats without or with topical application of ethanol. Expression of cNOS was detected by RT-PCR in the intact mucosa but intense signals for expression of both cNOS and iNOS were detected by RT-PCR in the gastric mucosa of LPS-treated rats. We conclude that parenteral LPS protects gastric mucosa from acute ethanol-induced damage *via* an increase in mucosal microcirculation mediated by NO due to the overexpression of iNOS and activation of arginine-NO-system.

Key words: *Helicobacter pylori, lipopolysaccharide, gastroprotection, nitric oxide, nitric oxide synthase, iNOS-mRNA, gastric blood flow*

INTRODUCTION

Helicobacter pylori (Hp) is now considered as the primary etiologic factor in the development of gastritis and gastroduodenal ulcers (1, 2). The mechanism of the damaging action of Hp has not been fully explained but it has been attributed to the production of large amounts of ammonia (3) and cytotoxins such as cytotoxin associated gene A (Cag A) (4), the stimulation of cytokines, including tumor necrosis factor (TNF_α) and various interleukins (IL) (5), and the excessive release of endotoxin such as lipopolysaccharide (LPS) from the outer cell membrane of this bacteria (6). LPS originating from Hp was shown to impair the mucosal integrity due to binding to laminin, one of the important component of extracellular matrix (7, 8). Moreover, endotoxemia associated with the administration of bacterial LPS is known to induce the septic shock and the multiorgan dysfunctions due to the activation of mediators of tissue injury such as macrophage-derived cytokines and free oxidant species (9, 10). LPS of Hp was shown to inhibit gastric mucus secretion (11), to interfere with somatostatin receptor (12) and to enhance pepsinogen secretion (13) suggesting that it might deteriorate gastric mucosal integrity during the Hp infection of the human stomach.

Nitric oxide (NO) released due to the induction of the Ca^{2+}-independent isoform of NO synthase (iNOS) by bacterial endotoxin is considered to be deleterious to gastrointestinal mucosa by inducing capillary leakage and direct damage to gastrointestinal cells (14—16). Recent studies revealed, however, that LPS deriving from *Escherichia coli* stimulated iNOS activity leading to excessive production of NO but resulting in the protection of the gastric mucosa against lesions induced by ethanol (17). In another report, small doses of bacterial endotoxin injected to rats exhibited significant protection against damage induced by various ulcerogens and this effect was attributed to the activation of certain protective cytokines such as interleukin-1 (18) or augumentation of inducible isoform of nitric oxide (NO) synthase (iNOS) (17). These results are in contrary to the previous reports indicating that iNOS, whose induction by LPS has been identified in gastric epithelial cells (15), exhibited cytotoxic action on the gastrointestinal mucosa (14—16), possibly due to the formation of excessive amounts of NO. Thus, it remains unclear whether an augmentation of NOS activity and resulting excessive NO production contribute to the protective or damaging effect of LPS on gastric mucosa.

If LPS acts on the gastric mucosa predominantly *via* induction of iNOS activity and upregulation of iNOS transcription, it is expected that the effect of this endotoxin on the gastric mucosa should be mimicked by the administration of potent chemical NO releaser such as S-nitroso-N-acetyl-penicillamine (SNAP). Indeed, it has been shown that SNAP exerts protective

influence on the gastric mucosa mainly due to release of an excess of NO (19). The role of LPS originating from Hp in the mucosal integrity and gastric microcirculation has not been studied extensively and it remains unknown whether LPS obtained from fresh clinical isolates or culture of Hp could protect the gastric mucosa against the damage induced by strong irritants. This study was designed 1) to compare the effect of topical and systemic pretreatment with LPS originating from Hp with that exhibited by nitrosothiol NO donor, SNAP on the formation of acute gastric lesions induced by 100% ethanol and accompanying changes in the gastric blood flow; 2) to determine the involvement of endogenous NO in the action of LPS on gastric mucosa and 3) to assess the role of gene expression of cNOS- and iNOS-mRNA in the gastric mucosa of rats treated with LPS of Hp.

MATERIAL AND METHODS

Preparation of LPS from H. pylori

LPS was isolated from antral mucosal biopsy specimens of patients with duodenal ulcer confirmed by gastroduodenoscopy and with upper abdominal complaints according to the procedure described previously (20). The Hp isolates were cultured on Skirrow's medium in a microaerophilic atmosphere (5% O_2, 10% CO_2 and 85% N_2) at 37°C for 5 days. The harvested bacteria were washed with distilled water, treated with ethanol and acetone, dried and subjected to homogenization with liquid phenol-chlorophorm-petroleum ether (2:5:8, by vol) as originally described (20). The suspension was centrifuged at 50 000 rpm for 15 min and the resulting supernatant containing LPS was filtered through the filter paper while bacteria residue was re-extracted again. Petroleum ether and chlorophorm were removed from the pooled supernatant solution on a rotary evaporator and the LPS precipitated by addition of water. The precipitate was centrifuged, washed with 80% phenol solution followed by ether, and finally dried. The dry residue was dissolved oin a small volume of water at 45°C and centrifuged at 100 000 g for 4 hr. The resulting LPS sediment was then redissolved in water and freeze-dried (12, 20). Analyses indicated that LPS preparation was essentially free of nucleic acids as determined by absorption at 260 nm, and its protein content, measured by BCA protein assay kit, was less than 0.15%.

Production of gastric lesions

Acute gastric lesions were induced in Wistar male rats weighing 180-220 g, by an intragastric (i.g.) application of 100% ethanol in a volume of 1.5 ml using an orogastric tube. One hour afterwards, the mean arterial blood pressure (MBP) was recorded in conscious rats without or with Hp LPS or SNAP pretreatment using the Rat Tail Blood Flow Pressure System (RTBP 1001, Kent Scientific Corporation, Litchfield, CT, USA). This system offers suitable method for such pressure measurement in conscious rats and is based on the principle that distension caused by arterial blood pulses can be detected by a highly sensitive piezoelectric sensor placed on animal tail. The signals are recorded on a storage oscilloscope or chart recorder and analyzed by computer program. The MBP was recorded and the values were calculated and expressed as a percentage of the value recorded in vehicle (saline) pretreated control rats.

Following measurement of MBP, the animals were anesthetized with ether, the stomach was exposed and the gastric blood flow (GBF) was measured by H_2-gas clearance technique as described in our previous studies (22, 23). The area of necrotic lesions in the oxyntic mucosa was measured planimetrically (Morphomat, Carl Zeiss, Berlin, Germany). All macroscopic examination were performed by the person unaware of the treatment given. For histological assessment two longitudinal sections of gastric mucosa were fixed in 10% buffered formalin and embedded in paraffin. The paraffin sections were stained with routine hematoxillin and eosin method and used for quantitative histological evaluation (morphometry) using Nikon microscope equipped with Microplan II. The disruption of surface of the mucosal strips, the denudation of epithelium and the deep necrotic lesions penetrating the mucosa were measured and expressed as a percent of total.

Effect of Hp LPS or SNAP on gastric lesions induced by 100% ethanol

In rats of this series, intraperitoneal (i.p.) or intragastric (i.g.) LPS (0.01 up — 40 mg/kg) or SNAP (0.32 — 6 mg/kg) was administered in various doses about 30 min before the production of gastric lesions with 100% ethanol applied i.g. a volume of 1.5 ml. In control experiments, vehicle (saline) was injected i.p. in the same volume and 30 min later 100% ethanol was administered. In addition, the effect of LPS alone, administered i.p. in a dose of 1 mg/kg on the GBF and NO production was tested in intact animals not exposed to ethanol administration.

Studies on the role of endogenous NO in LPS-induced protection

To test the possible involvement of endogenous NO in gastroprotective action of LPS, a nonselective inhibitor of NO-synthase (NOS), L-N^G-nitro-L- arginine-methyl ester (L-NAME) (20 mg/kg i.v.) (24,25) or more selective inhibitor of iNOS L-N^G-(1-Iminoethyl)-lysine (L-NIL) (26) was employed. L-NAME was administered i.v. before LPS either alone or in the combination with L-arginine (300 mg/kg i.v.), which is known to serve as a substrate for cNOS or D-arginine, which is not a substrate for this enzyme (25). Injection of L-NAME without or with L-arginine or D-arginine was performed 15 min before administration of LPS (1 mg/kg i.p.) and this was followed 60 min later by 100% ethanol. The following groups of rats were included in these series: 1) vehicle (1 ml of saline) or LPS (1 mg/kg i.p.) alone; 2) vehicle followed 60 min later by 100% ethanol; 3) LPS (1 mg/kg i.p.) followed 60 min later by 100% ethanol; 4) L-NAME (20 mg/kg i.v.) followed 15 min later by LPS (1 mg/kg i.p.); 5) L-NAME (20 mg/kg i.v.) followed 15 min later by LPS (1 mg/kg i.p.) and then 60 min later by 100% ethanol; 6) L-arginine or D-arginine (300 mg/kg i.v.) followed 15 min later by L-NAME (20 mg/kg i.v.) and then followed 15 min later by LPS (1 mg/kg i.p.) and 7) L-arginine or D-arginine (300 mg/kg i.v.) followed 15 min later by L-NAME (20 mg/kg i.v.) and then followed 15 min later by LPS (1 mg/kg i.p.) and finally, by 100% ethanol.

In another group of rats, the pretreatment with N^G-(1-Iminoethyl) lysine (L-NIL) was employed 60 min before the i.p. administration of LPS in tests without or with i.g. application of 100% ethanol. L-NIL is a recently described selective iNOS inhibitor (26), which is approximately 30-folds more selective for the inducible than for the constitutive form of NOS (27).

In separate group of rats the effects of pretreatment with dexamethasone (1 mg/kg i.p.) (to suppress the iNOS induction) (14) on LPS-treated gastric mucosa in rats without ethanol administration and on the protection afforded by LPS against ethanol-lesions were studied. The following experimental groups of animals were included; 1) vehicle (saline) followed 60 min later by 100% ethanol; 2) LPS (1 mg/kg i.p.) followed 60 min later by 100% ethanol; 3) dexamethasone (2 mg/kg i.p.) followed 30 min later by LPS (1 mg/kg i.p.); 4) dexamethasone (2 mg/kg i.p.) followed 30 min later by vehicle and then 60 min later by 100% ethanol and; 5) dexamethasone (2 mg/kg i.p.) followed 30 min later by LPS (1 mg/kg i.p.) and finally, 60 min later, by 100% ethanol. In addition,

the effect of LPS alone applied in a standard dose of 1 mg/kg i.p. on the GBF and NO production was studied in rats not exposed to ethanol but pretreated with L-NAME or dexamethasone.

In tests with dexamethasone or L-NIL without or with LPS, the mucosal NO—production was quantified indirectly as nitrate (NO_3^-) and nitrite (NO_2^-) levels in the gastric contents (28, 29). This method is based on the Griess reaction and generation of chromophore absorbing at 595 nm, according to original procedure described previously (29). Since NO released by epithelial cells into the gastric lumen is quickly transformed into NO_3^-- and NO_2^- (28), we measured photometrically the sum of both these substances as an index of production of NO by the gastric mucosa. For this purpose, the gastric content was aspirated just before the removal of the stomach following the i.g. injection of 1 ml of saline to wash out the luminal content. After centrifugation for 10 min at 3000 rpm, the samples were mixed with Griess reagent from the commercially available kit (Cayman Chemical assay kit, Cayman Chemical Company, Ann Arbor, MI, USA). In all tests including those with LPS, the GBF was measured in the oxyntic mucosa in each group of animals in similar manner as mentioned before and expressed as percent change from the control value recorded in intact-vehicle treated animals.

Studies on gene expression for cNOS and iNOS, in the gastric mucosa by reverse-transcriptase polymerase chain reaction (RT-PCR)

After the rats have been killed, the stomachs were removed, and the gastric oxyntic mucosa (about 500 mg) was scraped off on ice using glass slide and then immediately snap frozen on dry ice and stored at −70°C. Total RNA was isolated from the gastric oxyntic mucosa using a rapid guanidinum isothiocyanate/phenol chloroform single step extraction kit from Stratagene. Following precipitation, the RNA was resuspended in RNase-free TE buffer and the concentration was estimated by absorbance at 260 nm wavelength. Samples were frozen at −80°C until analysis. First-strand cDNA was synthesized from total cellular RNA (5 %g) using 200 U StrataScript TM reverse transcriptase (Stratagene, La Jolla USA) and oligo(dt)primers (Stratagene, La Jolla, USA). After the reaction, the transcriptase activity was destroyed by heating and the products were then stored at −20°C until PCR.

Primers were synthesized by Biometra (Gottingen, Germany). The nucleotide sequences of the rat cNOS and iNOS were based on the published cDNA sequences encoding rat cNOS and iNOS (30, 31). The cNOS sense primer was

5'TAC GGA GCA GCA AAT CCA C3'

while the cNOS antisense primer was

5'CAG GTC GCA GTC CTT TGA TC3'.

The oligonucleotide primer sequences for iNOS were

5'CAC AAG GCC ACA TCG GAT TTC 3' (sense)

and

5'TGC ATA CCA CTT CAA CCC GAG 3' (antisense).

Concomitantly, amplification of control rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ClonTech, Palo Alto, CA) was performed on the same samples to assess RNA integrity. The oligonucleotide primers sequences for GAPDH were

5'TGA AGG TCG GTG TCA ACG GAT TTG GC3' (sense)

and

'CAT GTA GGC CAT GAG GTC CAC CAC3' (antisense).

Reaction mixtures for PCR contained cDNA templates, 50 pmol of each primer, and 2.5 U of Taq DNA polymerase (Promega) 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 (no RNA included) and PCR blanks (no cDNA products included) were included in each analysis. To maximize the amplification specificity, hot start PCR was performed in a Perkin Elmer Cetus DNA thermal Cycler for 33 cycles (94°C for 1 min, 60°C for 45 sec, 72°C for 2 min) using Ampli Wax PCR Gem 50 beads.

Briefly, after adding primers, buffer and dNTPs, a Ampli Wax PCR Gem was added and heated to 80°C for 10 min. Then, the DNA Taq polymerase, cDNA sample and buffer was pipetted into the mixture. Evaluation of amplified PCR products on 1.5% agarose gels, staining with ethidium bromide and visualization under UV light was performed. Location of predicted PCR-product was confirmed by using DNA digest PhiX 174/Hae III as a standard size marker. The gel was photographed under UV transilumination. Oligonucleotides primer sequences are specific as ascertained by computer assisted search of updated versions of GeneBank. In addition to size analysis by agarose gel electrophoresis, the specificity of primer pair for cNOS and iNOS was assessed by sequencing of PCR products. For quantification, we determined the intensity of polymerase chain reaction products on the negative film of the gel photographs according to Griffiths *et al* (30). Expression of the products was quantified using video image analysis system (LKB Ultrascan, Pharmacia, Sweden). An index of messenger RNA expression was determined in each sample using the following equotation according to Tanoue *et al* (31): Index = Area (Unit) x Intensity (Unit). The index of cNOSmRNA or iNOSmRNA signal was standardized against that of the GAPDH signal from the same RNA and expressed as cNOS or iNOS over GAPDH ratio.

Statistical analysis

Results are expressed as means ± S.E.M. Statistical comparisons were made by analysis of variance and, where appropriate, by unpaired Student t-test with p value > 0.05 considered significant.

RESULTS

Effect of LPS on gastric lesions induced by 100% ethanol and changes in mean arterial blood pressure (MAB) and gastric blood flow (GBF)

Table 1 shows the effect of LPS given i.p. or i.g. in graded doses ranging from 0.01 up to 40 mg/kg, on the area of gastric lesions provoked by 100% ethanol and related changes in the GBF. LPS given i.p. at a dose of 0.01 mg/kg failed to alter gastric lesions induced by 100% ethanol and also did not affect the fall in GBF caused by ethanol. With increasing doses of LPS from 0.1 to 10 mg/kg, a dose-dependent decrease in the area of ethanol induced lesions was observed, reaching maximum at a dose of 1 mg/kg. LPS applied i.p. in doses ranging from 0.01 up to 1 mg/kg did not affect the MBP but following larger doses such as 10, 20 and 40 mg/kg, there was a significant reduction in MBP respectively, by about 15, 33 and 45% as compared to vehicle-treated controls.

Table 1. Effect of vehicle (saline) or Hp LPS injected i.p. in various doses (0.01—40 mg/kg) on the area of ethanol lesions, gastric blood flow (GBF) and mean arterial blood pressure (MBP). Results are means ± S.E.M. of 6—8 rats. Asterisk indicates significant change as compared to the value recorded in vehicle-control animals. Cross indicates significant change as compared with the value obtained with LPS given i.p. in the doses 0.1—10 mg/kg.

Type of test	Mean lesion area (mm^2)	GBF (% Control)	MBP (% Control)
Vehicle	137 ± 12	46 ± 4	98 ± 2
LPS (mg/kg i.p.)			
0.01	129 ± 9	49 ± 5	98 ± 2
0.1	55 ± 8	58 ± 6	96 ± 3
1	23 ± 4 *	65 ± 8 *	95 ± 4
10	26 ± 3 *	72 ± 6 *	84 ± 6 *
20	59 ± 5 +	56 ± 5 +	66 ± 4 +
40	99 ± 8 +	48 ± 3 +	54 ± 3 +

Pretreatment with LPS at a higher dose (10 mg/kg i.p.), which produced a small but significant decrease in MBP in rats subsequently exposed to ethanol (*Table 1*), failed to decrease the area of ethanol lesions below the value attained with 1 mg/kg of this endotoxin. With further increase in the dosage of LPS (20 and 40 mg/kg i.p.), the progressive disappearance of protective effect of LPS against ethanol damage was observed. The GBF was also decreased following injection of larger doses of LPS, so the GBF in these animals reached similar values to those recorded in vehicle-pretreated animals exposed to 100% ethanol. In contrast, topical i.g. application of LPS in gradually increasing doses ranging from 0.01 up to 40 mg/kg failed to affect the ethanol-induced gastric lesions or to alter the fall in the GBF produced by 100% ethanol and these results have not been included for the sake of clarity.

Table 2. Quantitative histology of gastric mucosa in rats treated with LPS (1 mg/kg i.p.) or vehicle (saline) without or with i.g. application of 100% ethanol. Results are means ± S.E.M. of 6 examinations on six rats. Asterisk indicates a significant change as compared to the value obtained with vehicle or LPS alone. Cross indicates a significant change as compared with the value obtained in rats treated with vehicle plus 100% ethanol.

Type of test	Denuded surface (%)	Deep necrosis (%)
Vehicle (saline)	2 ± 0.3	1.2 ± 0.02
LPS (mg/kg i.p.)	3 ± 0.5	1.8 ± 0.04
Vehicle + 100% ethanol	84 ± 7 *	32 ± 4 *
LPS (1 mg/kg i.p.) + 100% ethanol	41 ± 3 *	8 ± 2 +

Histologically, the application of 100% ethanol caused massive desquamation of surface epithelium and deep necrosis of about 32% of samples (*Table 2*). Pretreatment with a standard dose of LPS reduced significantly the extent of superficial and almost completely prevented deep mucosal damage.

Table. 3. Mean area of gastric lesions induced by 100% ethanol and accompanying changes in GBF and MBF in rats pretreated with S-nitroso-N-acetylpenicillamine (SNAP) administered i.p. in graded doses ranging from 0.37—6.0 mg/kg. Mean ± SEM of 6—8 rats. Asterisk indicates the significant change as compared to the value obtained in rats treated with vehicle. Cross indicates significant change as compared to the value recorded with lower doses of SNAP (0.37—3 mg/kg i.p.).

Type of test	Mean lesion area (mm^2)	GBF (% Control)	MBP (% Control)
Vehicle	129 ± 12	49 ± 5	98 ± 2
SNAP (mg/kg i.p.)			
0.37	101 ± 9	54 ± 6	96 ± 3
0.75	58 ± 6 *	68 ± 4 *	95 ± 4
1.5	32 ± 5	78 ± 6 *	92 ± 7
3.0	15 ± 2	81 ± 8	90 ± 6
6.0	92 ± 8 +	47 ± 6 +	67 ± 4 +

Table 3 shows the effect of SNAP applied i.p. in various doses on gastric lesions, GBF and MBP. SNAP injected i.p. produced a dose-dependent decrease in the mean area of gastric lesions reaching the maximum at a dose of 3.0 mg/kg. The attenuation of damage induced by SNAP was accompanied by a gradual decrease in GBF in the ethanol-treated gastric mucosa, reaching peak at a dose of 3.0 mg/kg. When SNAP was applied i.g. in higher dose of 6 mg/kg i.g. the lack of protective effect against ethanol lesions was observed and this was accompanied by a significant fall in the GBF and MAB as compared to those obtained in rats pretreated with lower doses of this NO-donor.

Effect of the inhibition of cNOS and iNOS activity on LPS induced gastroprotection against ethanol lesions

The effects of LPS alone applied (without ethanol) in a standard dose of 1 mg/kg i.p. without or with the combination with L-NAME, an inhibitor of NOS activity, with L-NIL, a selective inhibitor of iNOS and with dexamethasone, an inhibitor of iNOS transcription, on GBF and NO production are summarized in *Table 4*. LPS applied i.p. in a standard dose (1 mg/kg) by itself failed to induce gastric lesions but produced a significant rise in the GBF and enhanced significantly mucosal production of NO as determined by the measurement of the final products of NO degradation *in vivo* (NO_3^- and NO_2^-) using Griess reagents (*Table 4*). Pretreatment with

L-NAME or dexamethasone significantly attenuated the increase in GBF and NO production induced by LPS. The hyperemic activity and the rise in luminal NO production provoked by LPS were completely abolished by pretreatment with L-NIL.

Table 4. Effect of vehicle of LPS (1 mg/kg) applied i.p. without or with the pretreatment with L-NAME 15 mg/kg i.v.), L-NIL (30 mg/kg i.g.) or dexamethasone (2 mg/kg i.p.) on the GBF and luminal release of NO. Mean ± SEM of 6—8 rats. Asterisk indicates the significant change as compared to the value recorded in vehicle-control rats. Cross indicates significant decrease as compared to the value obtained in rats treated with LPS.

Type of test	GBF (%Control)	Production of NO μM/L
Vehicle (saline)	100	4.5 ± 0.1
LPS	119 ± 4 *	56.7 ± 7.4 *
LPS + L-NAME	94 ± 4 +	18.5 ± 3.6 +
LPS + L-NIL	98 ± 5 +	19.2 ± 2.8 +
LPS + Dexamethasone	98 ± 6	27.6 ± 4.9 +

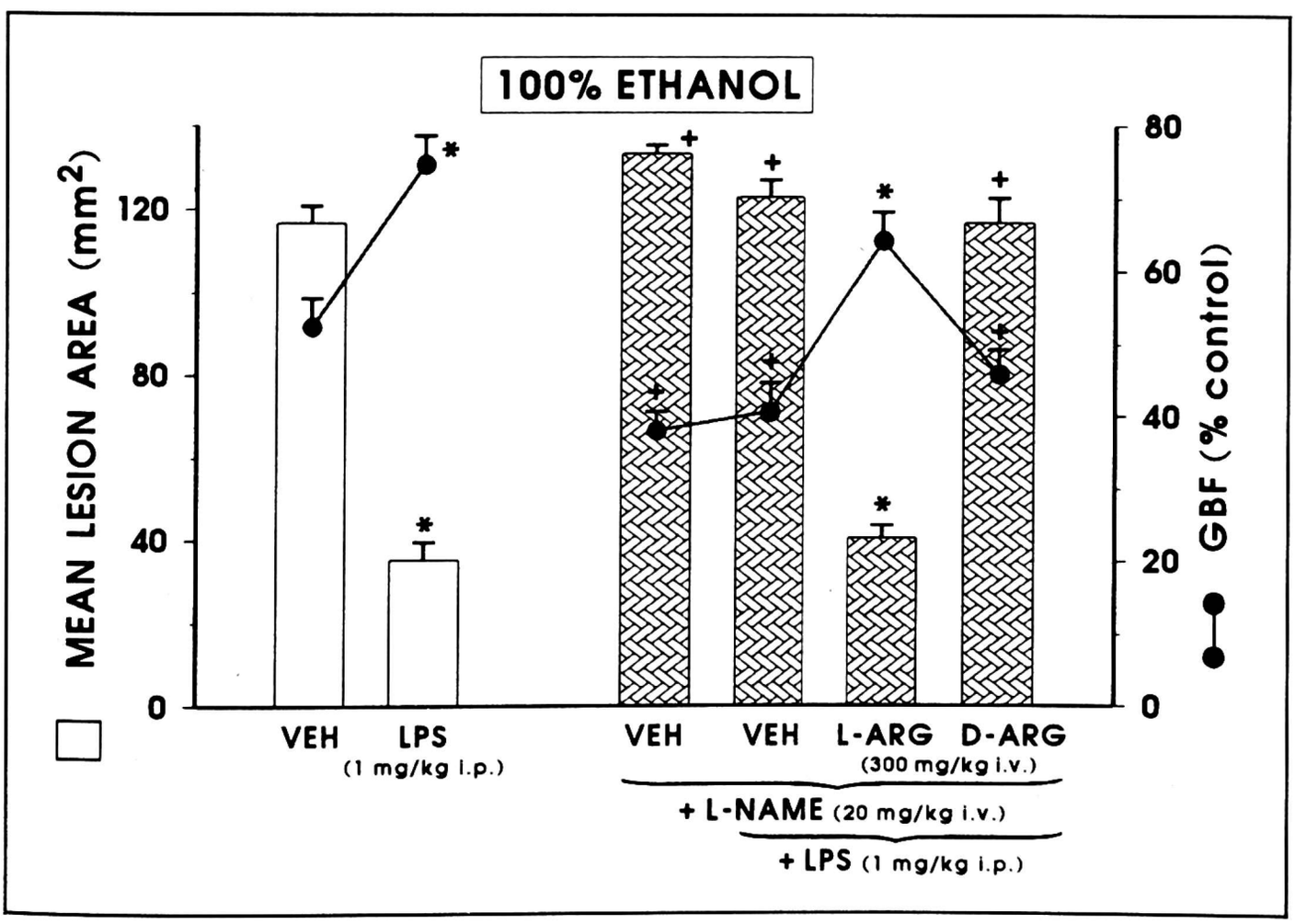

Fig. 1. The area of ethanol-induced gastric lesions and the gastric blood flow (GBF) in rats treated with LPS (1 mg/kg i.p.) without or with pretreatment with L-NAME combined with or without of L-arginine or D-arginine. Means ± S.E.M. of 8 — 10 rats. Asterisk indicates significant change as compared to the values obtained in vehicle-control. Cross indicates significant change as compared to the values obtained in similar tests without pretreatment with L-NAME.

Fig. 1 shows the effect of NOS activity inhibitor, L-NAME (20 mg/kg), applied i.v. without or with L-arginine or D-arginine, on the LPS-induced protection against ethanol injury and accompanying changes in the GBF. In this series of experiments LPS administered i.p. in a standard dose of 1 mg/kg caused similar reduction in the area of gastric lesions and similar changes in the GBF to those shown in *Table 1*. The pretreatment with L-NAME which by itself failed to influence significantly ethanol lesions, almost completely reversed the LPS-induced protection and the accompanying rise in GBF. Addition of L-arginine to L-NAME restored the protective and hyperemic effects of LPS on gastric mucosa treated with ethanol, whereas D-arginine under the same experimental conditions remained without any influence.

The effects of pretreatment with L-NIL (30 mg/kg i.g.), a selective inhibitor of iNOS, on LPS-induced protection and rise in the GBF are presented in *Fig. 2*. LPS attenuated ethanol damage and the protective and hyperemic effects of LPS were accompanied by the significant increase in luminal release of NO and the rise in GBF. In LPS-treated stomach, L-NIL almost completely reversed the mucosal protection induced by LPS and significantly reduced the rise in GBF and NO production by the gastric mucosa (*Fig 2*).

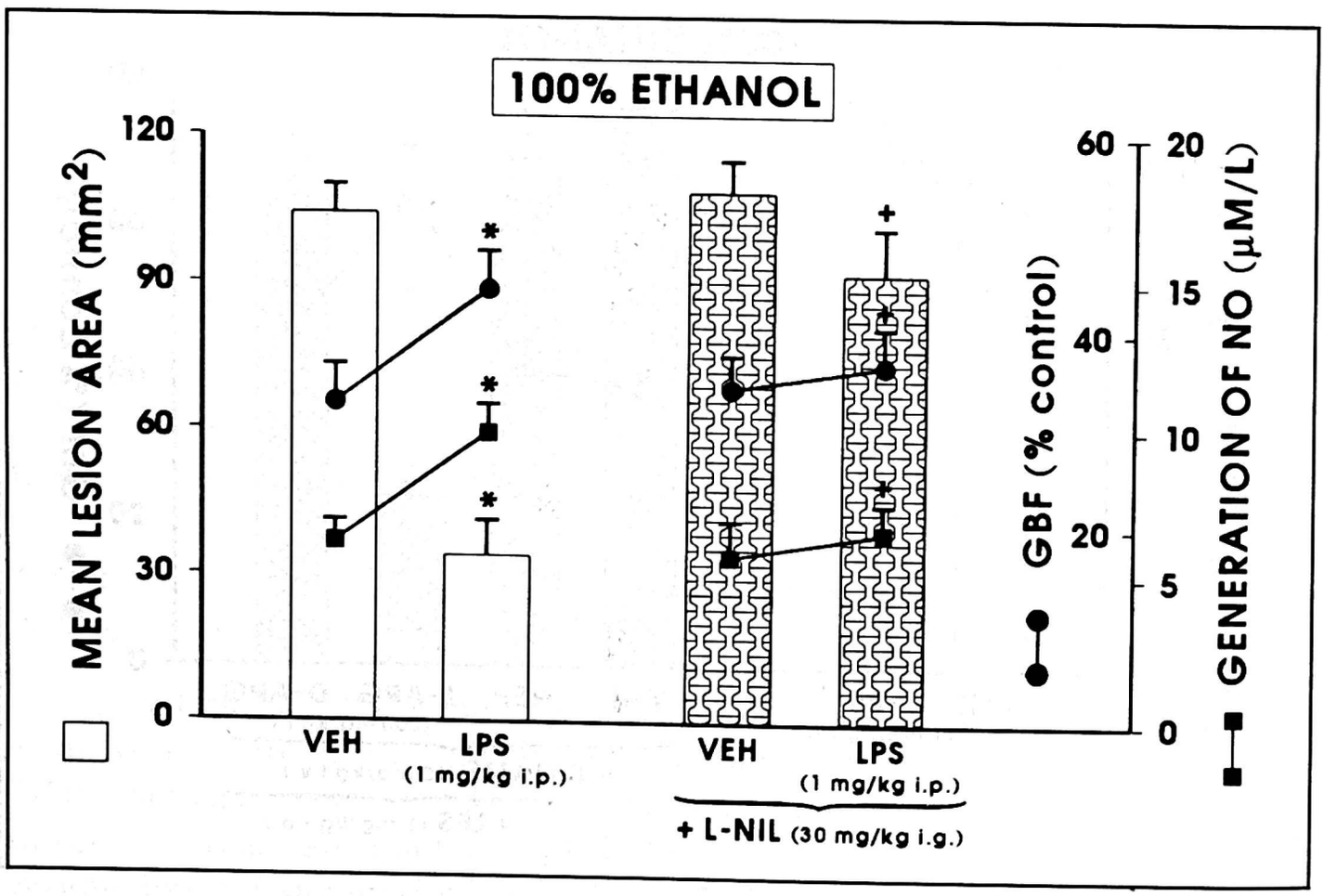

Fig 2. The area of gastric lesions, gastric blood flow and generation of NO in luminal content in rats without or with pretreatment with L-NIL (30 mg/kg i.g.). Means ± S.E.M. of 8—10 rats. Asterisk indicates significant change as compared to the vehicle-control. Cross indicates a significant change as compared to the value obtained in similar tests without the pretreatment with L-NIL.

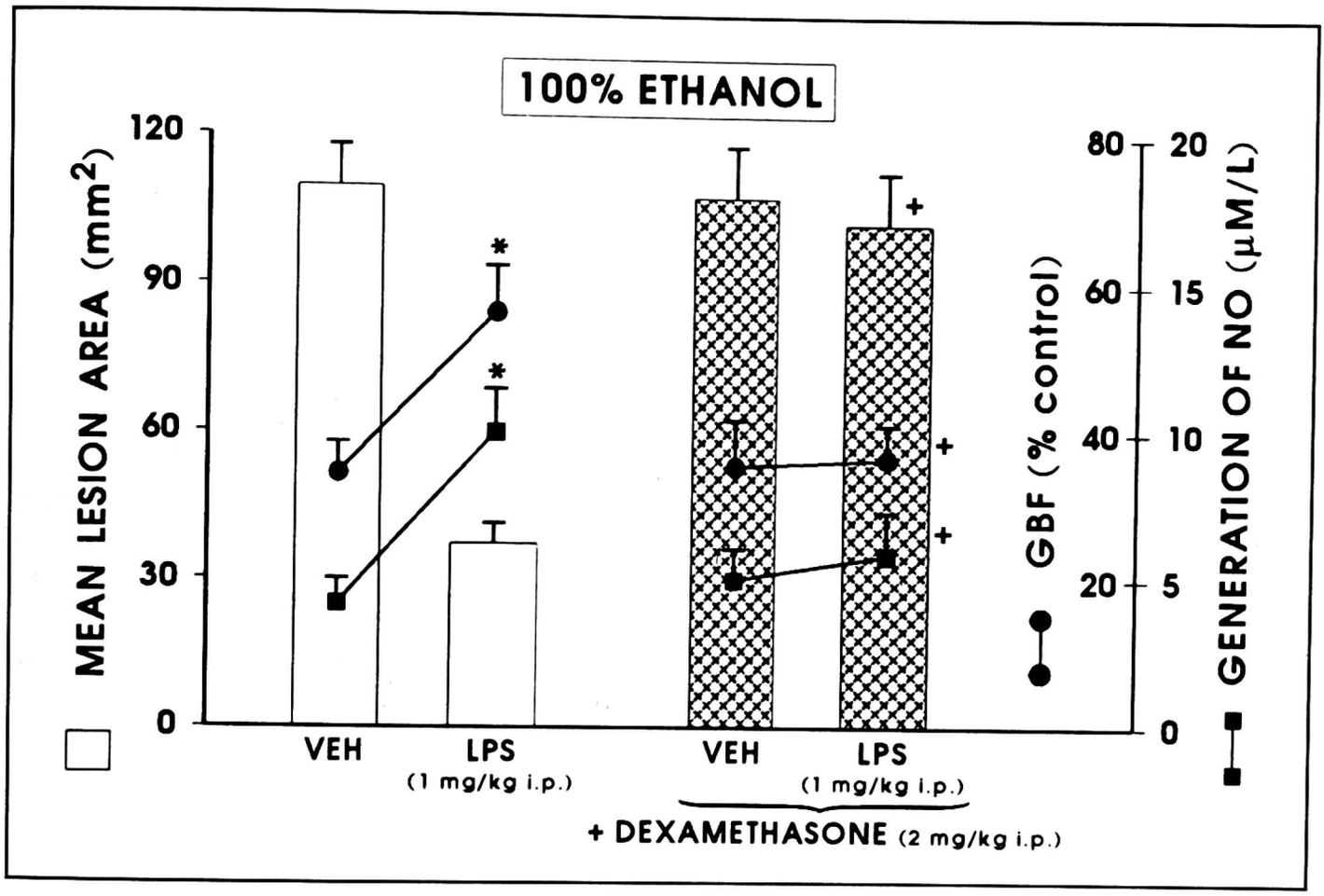

Fig 3. The area of gastric lesions, gastric blood flow (GBF) and generation of NO in luminal content in rats without or with pretreatment with dexamethasone. Means ± S.E.M. of 8—10 rats. Asterisk indicates significant change as compared to the vehicle-control. Cross indicates a significant change as compared to the value obtained in similar tests without the pretreatment with dexamethasone.

Fig. 3 summarizes the results of LPS without or with pretreatment with dexamethasone, the potent inhibitor of iNOS transcription (14) on ethanol induced gastric lesions, GBF and and production of NO in the stomach. The protective and circulatory effects of LPS (1 mg/kg i.p.) were accompanied by about 40% increase in GBF and about 35% rise in mucosal production of NO. Pretreatment with dexamethasone (2 mg/kg i.p.), which in vehicle-treated rats failed to influence ethanol lesions and production of NO in gastric lumen, completely reversed the LPS-induced protection and gastric hyperemia and accompanying rise in NO production.

Effect of LPS on the expression of cNOS- and iNOS-transcripts in the gastric mucosa

Fig 4 shows the expression of cNOS using RT-PCR technique presented as the ratio of cNOS mRNA over GAPDH mRNA after administration of vehicle (saline) or LPS applied i.p. in doses of 0.1, 1 or 10 mg/kg. The expression of cNOS mRNA was detected in vehicle-treated gastric mucosa and at each dose of LPS tested. The ratio of cNOS mRNA over GAPDH was significantly

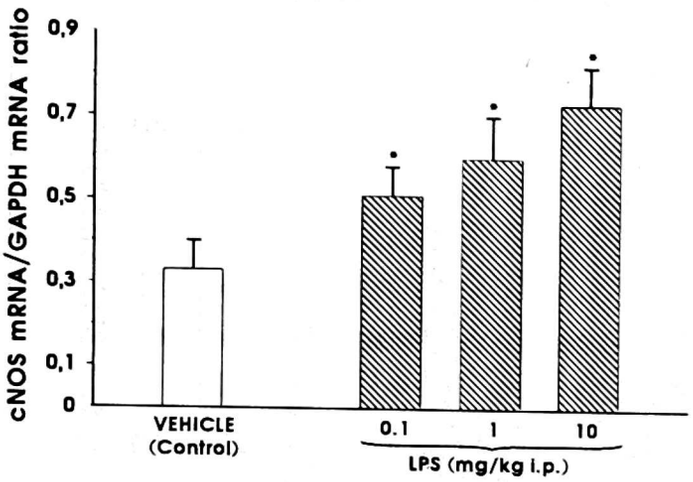

Fig 4. Expression of cNOS mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis in the gastric mucosa of intact-vehicle control rat and rat treated i.p. with LPS at the dose of 0.1 mg/kg, 1 mg/kg and 10 mg/kg. Asterisk indicates significant change as compared to value obtained with vehicle.

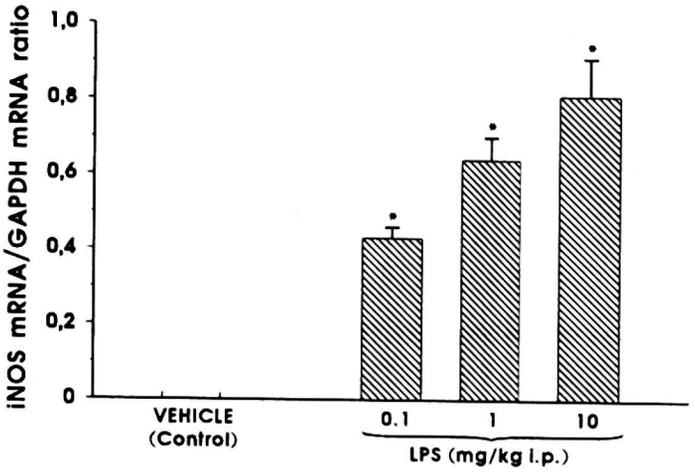

Fig 5. Expression of iNOS mRNA by RT-PCR analysis in the gastric mucosa of rat treated with vehicle and rat treated i.p. with LPS 0.1 mg/kg, 1 mg/kg and 10 mg/kg. Asterisk indicates significant change as compared to the value obtained in gastric mucosa treated with vehicle.

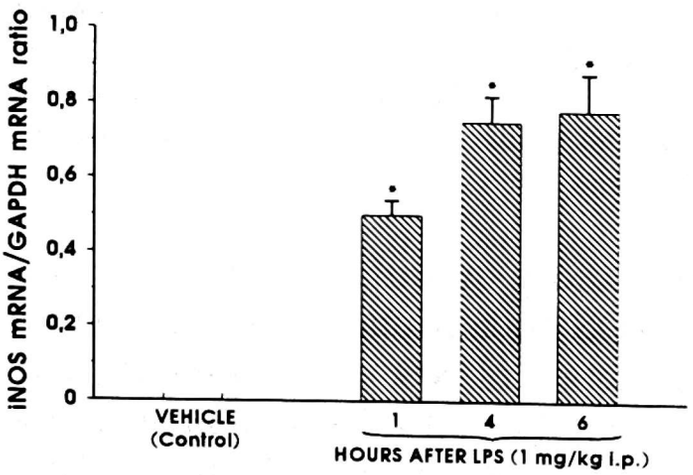

Fig. 6. Expression of iNOSmRNA by RT-PCR analysis in gastric mucosa of vehicle control rat and in gastric mucosa of LPS-treated animals at 1, 4 and 6 h after the administration of LPS (1 mg/kg i.p.). Asterisk indicates significant change as compared to the value obtained with vehicle (control).

increased at each dose of LPS tested reaching the peak at the dose of 10 mg/kg. In contrast, the iNOS mRNA (*Fig. 5*) was undetectable by RT-PCR in the intact gastric mucosa of vehicle-treated animals but was detected after administration LPS at each dose tested, with the most intense signal observed at the dose of 10 mg/kg. As shown in *Fig. 6* the expression of iNOSmRNA using RT-PCR technique was undetectable in the intact gastric mucosa of vehicle-treated rats but was detected at 1, 4 and 6 h following the administration of LPS given i.p. at the dose of 1 mg/kg. As a positive control, the rat GAPDH was assayed in all mucosal specimens to verify efficiency of cDNA synthesis from extracted RNA (*Fig 7*). The ratio of iNOS mRNA over GAPDH showed a significant rise at 1, 4 and 6 h after LPS administration (*Fig. 6*).

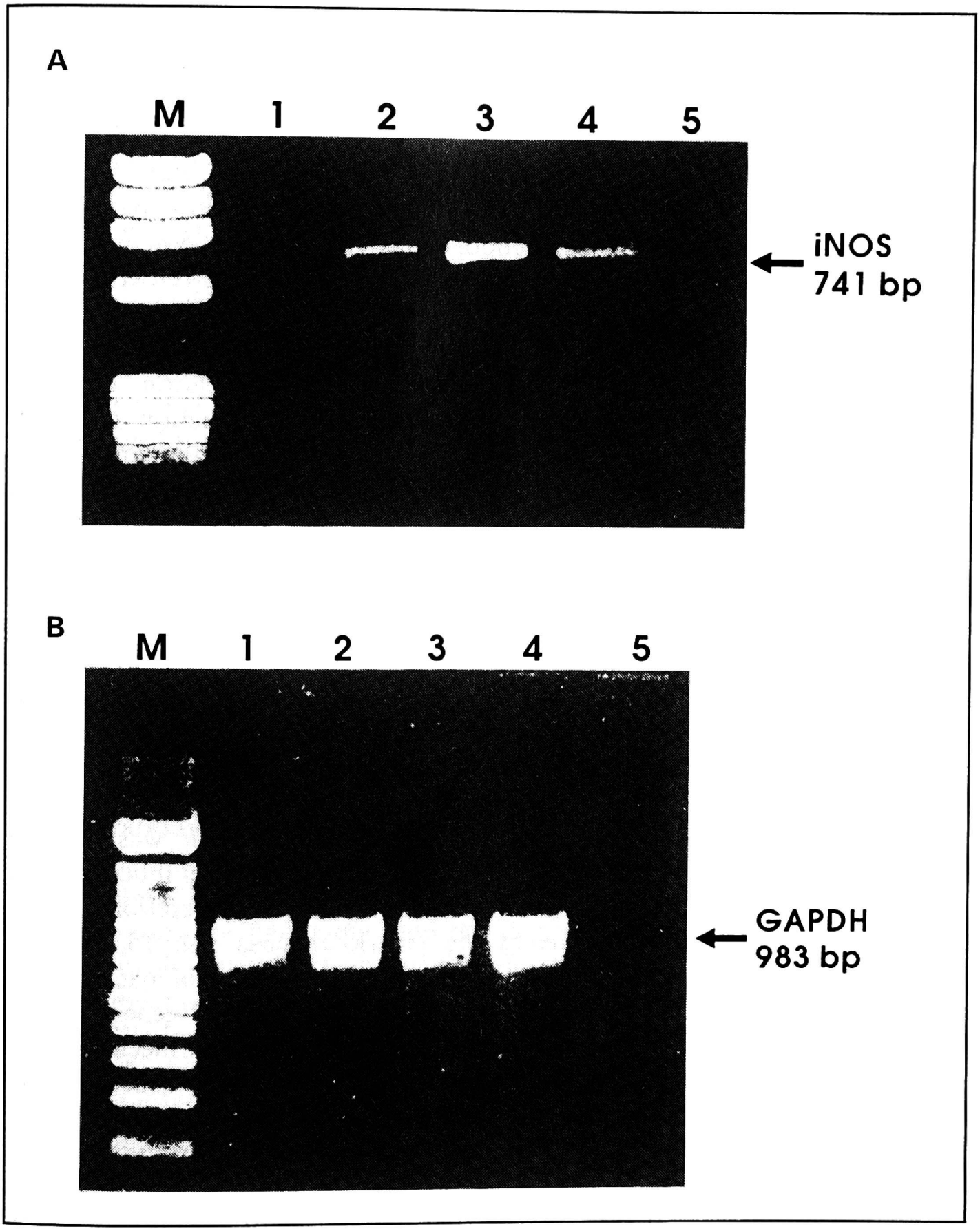

Fig. 7A. RT-PCR expression of iNOSmRNA in intact (vehicle-control) gastric mucosa (lane 1) and in gastric mucosa at 1, 4 and 6 h after the administration of LPS (1 mg/kg i.p.) (lanes 2—4). Lane 5 represents negative control. M is Lambda/Hind II phi 174/Hae III molecular weight marker. The size of the predicted amplified product iNOS (741 bp) is indicated on the right.

Fig. 7B. RT-PCR expression of GAPDHmRNA in experiments with time sequence after LPS administration as shown in *Fig 6.* M is Lambda/Hind II phi 174/Hae III molecular weight marker. The size of the predicted amplified product GAPDH (983 bp) is indicated on the right.

DISCUSSION

This study provides an evidence that LPS, originating from Hp and administered parenterally at a dose that does not affect systemic circulation, protects rather than damages the gastric mucosa exposed to 100% ethanol. This protection is accompanied by a significant rise in gastric blood flow probably involving, at least in part, endogenous NO-arginine system because the suppression of NOS activity by non specific inhibitor, L-NAME, or by selective iNOS inhibitor L-NIL, significantly attenuated the LPS-induced protection and the rise in the GBF. Both L-NAME and L-NIL reduced also the mucosal production of NO induced by the application of LPS. Furthermore, the inhibition by dexamethasone of the induction of iNOS completely abolished the prevention of ethanol-lesions and accompanying rise in gastric blood flow afforded by LPS. SNAP, at potent NO donor, resembled protective and hyperemic action of LPS. At the higher doses both, LPS and SNAP were less effective in the protection against ethanol damage possibly due to their systemic hypotensive effect and the fall in gastric microcirculation. It is of interest that intense signals for the expression of cNOS-mRNA and iNOS-mRNA were detected by RT-PCR in the gastric mucosa following the application of gastroprotective doses of LPS indicating that an overexpression of NO transcription and excessive production and release of NO in gastric mucosa contributed significantly to the mucosal protection and hyperemia afforded by LPS.

Bacterial endotoxin, LPS, originating from *Escherichia coli*, was recently shown to induce Ca^{2+}-independent iNOS activity in rat gastric mucosal cells and this was also associated with the inhibition of gastric acid secretion and the formation of hemorrhagic lesions in the gastric mucosa (14, 15, 32, 33). Molecules such as LPS, mainly associated with the surface of bacteria are known to induce cytokine production (34). Stimulation of NO generation by the bacteria cytokine products has been considered as the major mechanism of the deleterious action of bacteria on the gastrointestinal mucosa (35). Pretreatment with LPS stimulated NO production *via* the enhancement of the activity of iNOS, leading to hepatic injury and this was attributed to the formation of highly toxic species such as peroxynitrate generated during hepatic reperfusion (36). It is of interest that these deleterious effects of iNOS induction by LPS were partially antagonized by L-NMMA (36).

Recent studies revealed, however, that LPS in the rat stomach actually triggers the protective mechanisms against deleterious agents but the nature of these mechanisms has not been clarified (17). In the present study, we found that LPS of Hp applied i.p. in low doses, induced typical cytoprotection against gross and microscopic necrotic mucosal damage of gastric mucosa induced by ethanol. This protection disappeared when LPS was applied in higher doses

possibly due to its well-known endotoxic activity and significant reduction in the systemic blood pressure with subsequent fall in gastric microcirculation. The protection and accompanying hyperemia induced by LPS observed at lower doses were reversed by the blockade of NOS activity by L-NAME or by L-NIL, a specific inhibitor of iNOS which is about 30 times more selective for the iNOS than cNOS (27), indicating that endogenous NO is involved in these gastroprotective and mucosal circulatory effects of LPS. The involvement of NO-arginine system in LPS induced protection is supported by our finding of an increased expression of genes encoding cNOS-mRNA and iNOS-mRNA in the LPS-treated gastric mucosa using the technique of RT-PCR. It was shown that this expression of iNOS-mRNA in gastric mucosa occurs already after 60 min after the administration of LPS. This early expression of iNOS mRNA was followed by a significant rise in NO production and gastric luminal release as measured by nitrate and nitrite concentration in the gastric contents, confirming that the excessive production of NO by gastric mucosa contributes to the protective effect of LPS. Most studies to date described longer time (e.g. 2—4 h) required for the induction of iNOS but the early phase of the induction of iNOS has also been reported. Bateson *et al.* (37) demonstrated that endotoxemia by LPS is preceded by an extremely rapid increase in iNOS-mRNA in myocardium as detected by RT-PCR in their study already within 30 min after the administration of LPS. They concluded that rapid induction of iNOS may contribute to the protection of the heart muscle against microbial infection (37). Our observation is in keeping with this finding by the demonstration that iNOS mRNA was detected after 60 min following the LPS injection and at the same time the protection of gastric mucosa against ethanol was revealed.

This raises the major question whether LPS induced gene expression of iNOS is really deleterious or beneficial for the gastric mucosa and indirectly whether the mucosal infection with Hp has only abnoxious influence on gastric mucosa. We found that such LPS-stimulated expression of iNOS transcription was rapidly upregulated resulting in a profound gastroprotection against ethanol injury. We attempted in this study to mimic the action of endogenous NO produced by LPS using SNAP, that is known to release spontaneously NO and to exert biphasic influence on gastric mucosa, a cytoprotective effect at lower doses and damaging action at the higher doses (19). It was confirmed that such pretreatment with SNAP prevented the gastric mucosa against ethanol damage but similarly as in experiments with LPS, larger dose of SNAP that produced hypotension was not protective. This strongly supports our notion that NO released in moderate amounts due to induction of iNOS is essential for the gastroprotective and microcirculatory effects of LPS against ethanol damage but excessive induction of this enzyme may result in opposite effects of this endotoxin.

It should be emphasized that LPS applied topically to the gastric mucosa was completely deprived of any influence on the mucosa suggesting that Hp in the mucus covering the mucosa may have limited action on this mucosa from the luminal side unless its products are absorbed and act inside the mucosa.

Our finding is in keeping with recent observations (17) that activation of Ca^{2+}-independent NO-synthase activity by LPS derived from *Escherichia coli* reduced the gastric mucosal damage in response to intragastric instillation of ethanol. The results of this study are consistent with the notion that NO produced by the iNOS has protective actions on the gastric mucosa. Our present study employing LPS originating from Hp also supports other observations that pretreatment with LPS may be highly effective in the protection of the gastric mucosa against the lesions produced by various ulcerogens including ethanol, nonsteroidal antiinflammatory agents (NSAIDs) and stress (18). The protection against acid-dependent lesions such as those induced by NSAIDs or stress could be attributed, at least in part, to the potent antisecretory activity of LPS as suggested previously (18). Endogenous PG were not tested, however, in the present study, but they could contribute to both the antisecretory and protective effects of LPS because they were eliminated by indomethacin suggesting the involvement of the products of arachidonate-metabolism in the action of LPS on the stomach (18). Furthermore, recent studies revealed that LPS-induced inhibition of gastric secretion results not from its toxic activity but direct inhibitory action on parietal cells in the stomach (38). The role of the suppression of gastric secretion by LPS in gastroprotection should be further tested but it appears unlikely that antisecretory effect of LPS plays a major role in protection against acid-independent lesions produced by strong irritants such as 100% ethanol employed in our study.

Our present results indicate that in contrast to the toxic effect of some endotoxins reported previously (35, 36, 39), LPS of Hp may have also beneficial effect on the host stomach. This could explain the fact that despite widespread prevalence of Hp infection, reaching 70—90% in developing countries, only small percentage of infected subjects develop gastroduodenal ulcerations or gastric cancer (1, 2). Furthermore, there is also a paradoxical increase in the mucosal tolerance to NSAID in patients infected by Hp as compared to those without such an infection (40).

Dexamethasone was first pharmacologic agent that was found *in vitro* to selectively block iNOS activity, whereas COX-1 expression was still unaffected (15, 41). In our present study, dexamethasone abolished almost completely the protective activity of LPS suggesting that the blockade of iNOS results in the depletion of NO (and PG) generated by LPS in the mucosa leading to the removal of the gastroprotection by this endotoxin. This is in agreement with the previous study that dexamethasone reduced both *in vivo* and *in vitro* iNOS

activity (15, 28, 31) and attenuated the gastroprotective action of LPS against ethanol injury (17), again, reinforcing the notion that endogenous NO plays a crucial role in the LPS-induced gastroprotection. Since cNOS activity by itself is inhibited by strong irritants such as ethanol (17), the induction of iNOS with subsequent production of excessive amounts of NO could be a reasonable explanation for the protective effect of LPS against ethanol injury observed in our present study.

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Author's address: S.J. Konturek, Institute of Physiology University School of Medicine, Grzegórzecka 16, 31-531 Kraków, Poland.