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CENTRAL AND PERIPHERAL NEURAL ASPECTS OF GASTROPROTECTIVE AND ULCER HEALING EFFECTS OF LIPOPOLYSACCHARIDES

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Lipopolysaccharides (LPS) are major components of the outer membrane of Gram--negative bacteria playing a central role as potent endotoxins in the pathogenesis of endotoxic shock. Although large amounts of endotoxin may produce hemorrhagic lesions in the stomach, the possible gastroprotective effect of central or peripheral LPS against the acute gastric lesions has not been extensively studied. The aim of the present study was to compare the effect of intracerebroventricular (i.c.v.) and parenteral (i.p.) injection of LPS against gastric lesions induced by 100% ethanol. Male Wistar rats were treated either with a) vehicle (control); b) *E-coli*-LPS in various concentrations (1—10 μg/kg i.c.v or 0.1—40 mg/kg i.p.) followed 30 min later by 100% ethanol. The effects of pretreatment with nonselective inhibitor of nitric oxide synthase (L-NAME, 20 mg/kg i.g.) or selective inhibitor of inducible nitric oxide synthase, L-NIL (30 mg/kg i.g) on the gastroprotection induced by LPS was investigated. One hour after ethanol application, the gastric blood flow (GBF) and the area of gastric lesions were determined. In addition, the mucosal expression of iNOS, cNOS and leptin was assessed using RT-PCR. LPS applied i.c.v. or i.p. dose dependently reduced gastric lesions induced by ethanol and this effect was similar to that observed after the administration of NO donor (SNAP). LPS-induced protection was significantly abolished by L-NAME and significantly attenuated by the selective inhibitor of iNOS (L-NIL). The expression of cNOS was detected in vehicle treated gastric mucosa and did not change after LPS administration. iNOS was not detectable in intact mucosa but its expression dose-dependently increased after the LPS administration. The i.c.v. administration of LPS did not upregulate further the iNOS expression, and dose-dependently inhibited the leptin mRNA expression in gastric mucosa. We conclude that LPS applied centrally or peripherally protects gastric mucosa against ethanol-induced damage through an increase in gastric microcirculation mediated by NO due to overexpression of iNOS. Transcriptional downregulation of leptin in gastric mucosa is probably due to the increased leptin release induced by the intracerebroventricular application of lipopolysaccharide.

K e y w o r d s: *lipopolysaccharide, cNOS, iNOS, leptin, mucosal protection*

INTRODUCTION

LPS are major components of the outer membrane of Gram-negative bacteria playing a central role as potent endotoxins in the septic shock. LPS are

phosphorylated glycolipids consisting of 3 domains: outer polysaccharide moiety called O-specific chain, oligosaccharide core, and innermost component termed lipid A, which anchors the molecule in the bacterial outer membrane (1).

LPS as major mediators of endotoxemia cause massive disturbances in different organs, including gastrointestinal tract (2). It is well known that systemic administration of endotoxin provokes the disruption of mucosal architecture with a significant increase in mucosal permeability, which results in the development of hemorrhagic lesions in the stomach and intestine (3). On the other side, there is also an evidence that repeated exposures to LPS results in an increased resistance of gastric mucosa through the prostaglandin dependent mechanism (4).

More recently it has been shown that LPS acts through CD14 receptor which is expressed on macrophages, monocytes and neutrophils and also exists in soluble form in serum (5). Recent studies indicate that LPS acts also directly in the brain *via* upregulation of CD14 (6).

The present study was designed to compare the effect of intracerebroventricular (i.c.v.) and parenteral pretreatment with LPS on gastric lesions induced by 100% ethanol.

MATERIAL AND METHODS

Production of acute gastric lesions

Acute gastric lesions were induced in Wistar rats by an intragastric (i.g.) application of 100% ethanol (1.5 ml). 1h later, the animals were anesthesized, the stomach was exposed and the gastric blood flow (GBF) was measured by H_2-gas clearance method as described in our previous studies (7). Briefly, the double-needle electrodes connected to an electrolytic regional blood flow meter (Biomedical Science, Model RBF-2, Japan) were inserted through the serosa into the mucosa of the oxyntic gland area, one electrode being used for local generation of H_2 and the other for the measurement of tissue H_2 With this method the H_2 generated is carried away by the blood, and the polarographic current detector gives the decreasing tissue H_2 content as the clearance curve, which is then used to calculate absolute flow rate that was expressed as percent of control flow in the intact mucosa. Then the stomach was removed, and the area of necrotic lesions in the oxyntic mucosa was measured planimetrically (Morphomat, Carl Zeiss, Berlin, Germany). Studies were approved by the Ethic Committee for Animal Research of Jagiellonian University.

Effect of peripheral LPS and SNAP on gastric lesions induced by 100% ethanol

In the rats of this series intraperitoneal LPS (0.01—40 mg/kg) or SNAP (0.37—6 mg/kg) was administered in various doses 30 min before the

production of gastric lesions with 100% ethanol administered intragastrically in a volume of 1.5 ml. In control experiments vehicle (saline) was injected intraperitoneally in the same volume, and 30 min later 100% ethanol was administered. One hour after the ethanol administration the mean blood pressure (MBP) was determined, and then the rats were anesthetized with ether. The GBF was examined, and the area of the gastric lesions was measured as described above.

Effect of intracerebroventricular injection of LPS on gastric lesions induced by 100% ethanol

In the rats of this series, two different doses of LPS (1 and 10 μg) were administered centrally into the lateral cerebral ventricle. In the control group, vehicle (saline) injections were performed and 30 min later the rats received 100% ethanol in a volume 1.5 ml intragastrically. One hour after the ethanol, the rats were anesthetized with ether, the GBF was examined, and the area of the gastric lesions was measured as described above.

Determination of iNOS and cNOS mRNA expression using RT-PCR

The extraction of total RNA from gastric ulcerated tissues and control tissues was carried out as previously described (7). Briefly, total RNA was extracted from mucosal samples using a guanidium isothiocyanate/phenol chloroform single step extraction kit from Stratagene (Heidelberg, Germany) based on the method described by Chomczynski and Sacchi (8). Following precipitation, RNA was resuspended in RNAse-free TE buffer and its concentration was estimated by absorbance at 260 nm wavelength. Furthermore, the quality of each RNA sample was determined by running the agarose-formaldehyde electrophoresis. RNA samples were stored at -80°C until analysis.

Single stranded cDNA was generated from 5 μg of total cellular RNA using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Stratagene, Heidelberg, Germany) and oligo-(dT)-primers (Stratagene, Heidelberg, Germany). Briefly, 5 μg of total RNA was uncoiled by heating (65°C for 5 min) and then reversely transcribed into complementary DNA (cDNA) in a 50 μl reaction mixture that contained 50 U MMLV-RT, 0.3 μg oligo-(dT)-primer, 1 μl RNase Block Ribonuclease Inhibitor (40U/μl), 2 μl of a 100 mM/l mixture of deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP), 10 mM/l Tris-HCl (pH = 8.3), 50 mM KCl, 5 mM $MgCl_2$). The resultant cDNA (2 μl) was amplified in a 50 μl reaction volume containing 2 U Taq polymerase, dNTP (200 μM each) (Pharmacia, Germany), 1.5 mM $MgCl_2$, 5 μl

10 × polymerase chain reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH = 8.3) and specific primers used at final concentration of 1 mM (all reagents from Takara, Shiga, Japan). The mixture was overlaid with 25 μl of mineral oil to prevent evaporation. The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT) and the incubation and thermal cycling conditions were as followed: denaturation at 94°C for 1 min, annealing at 60°C for 45 sec and extension 72°C for 2 min. The number of cycles was 30 for β-actin, 35 for leptin and iNOS and 33 for cNOS. The nucleotide sequence of the primers were as follows: β-actin, sense 5‘-GAT CTT GAT CTT CAT GGT GCT AGG-3‘; antisense 5‘-TTG TAA CCA ACT GGG ACG ATA TGG-3‘, leptin (*ob* gene), sense 5‘-CGA GAA GAT GAC CCA GAT CAT G-3‘; antisense 5‘-AGT GAT CTC CTT CTG CAT CCT G-3‘; cNOS sense 5‘-TAC TTG AGG ATG TGG CTG-3‘ ; antisense 5‘-GTC TTC TTC CTG GTG ATG-3‘ and iNOS sense 5’-CAG TGG CAA CAT CAG GTC-3’; iNOS antisense 5’-GGT CTC GGA CTC CAA TCT-3’. The primer sequences for β-actin, leptin, cNOS and iNOS were based on the sequences of the published cDNAs and were synthesized by GIBCO BRL/Life Technologies (Eggenstein, Germany) (7).

Polymerase chain reaction products were detected by electrophoresis on a 1,5% agarose gel containing ethidium bromide. Location of predicted products was confirmed by using 100-bp ladder (Takara, Shiga, Japan) as a standard size marker. The gel was then photographed under UV transillumination. The intensity of PCR products was measured using video image analysis system (Kodak Digital Science). The signal for leptin, cNOS and iNOS mRNA was standardized against that of the β-actin mRNA from each sample and the results were expressed as leptin or cNOS or iNOS/β-actin mRNA ratio.

Studies on the role of endogenous NO in LPS induced protection

To test possible involvement of endogenous NO in gastroprotective action of LPS, a non selective inhibitor of NO-synthase (NOS), L-N^G-nitro--L-arginine-methyl ester (L-NAME)(20 mg/kg intravenously), or a preferential inhibitor of inducible NOS (iNOS), L-N^G-(1-iminoethyl)-lysine (L-NIL) (30 mg/kg i.v.) was used. L-NAME was administered intravenously either alone or in combination with L-arginine (300 mg/kg i.v.), which is known to serve as a substrate for NOS, or D-arginine, which is not a substrate for this enzyme. Injection of L-NAME or L-arginine or D-arginine was performed 15 min before LPS administration, which was followed 30 min later by 100% ethanol.

In tests with L-NIL with or without LPS, the mucosal NO production was quantified indirectly as nitrate (NO_3^-) or nitrite (NO_2^-) levels in the gastric contents. This method is based on the Griess reaction and generation of

chromophore absorbing at 595 nm, in accordance with the original procedure described previously (9). Since NO released by gastric mucosa into the gastric lumen is quickly transformed into NO_3^- and NO_2^-, 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 after the intragastric 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, USA).

Statistical analysis

Results are expressed as means ± standard error of the mean. The statistical significance of differences between groups of parametric data was determined by one-way analysis of variance followed by the Bonferronit test. Comparisons between groups of nonparametric data were made with the Mann-Whitney U test. A p value of less than 0.05 was taken to indicate statistical significance.

RESULTS

Effect of peripheral LPS and SNAP on gastric lesions induced by 100% ethanol and gastric blood flow

Fig. 1 shows the effect of LPS given intraperitoneally 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 MBP and GBF. With increasing doses of LPS from 0.1 to 1 mg/kg, a dose dependent decrease in the area of ethanol--induced lesions was observed, reaching maximum at a dose of 1 mg/kg. This effect was accompanied by a significant increase in the GBF but without significant alterations in MBP.

Pretreatment with LPS at higher dose (10 mg/kg), which produced a small but significant decrease in MBP, failed to decrease the area of ethanol lesions below the value observed at a dose 1 mg/kg i.p. With further increase in the dosage of LPS (20 and 40 mg/kg i.p.), the protective effect of endotoxin against ethanol damage disappeared progressively. This was accompanied by decrease of the GBF and the MBP.

For comparison with LPS, the effect of NO donor (SNAP) in various doses on gastric lesions, GBF and MBP were determined. SNAP injected intraperitoneally caused a dose-dependent decrease in the mean area of gastric lesions inducing the minimal lesion area at a dose 3 mg/kg. This reduction in

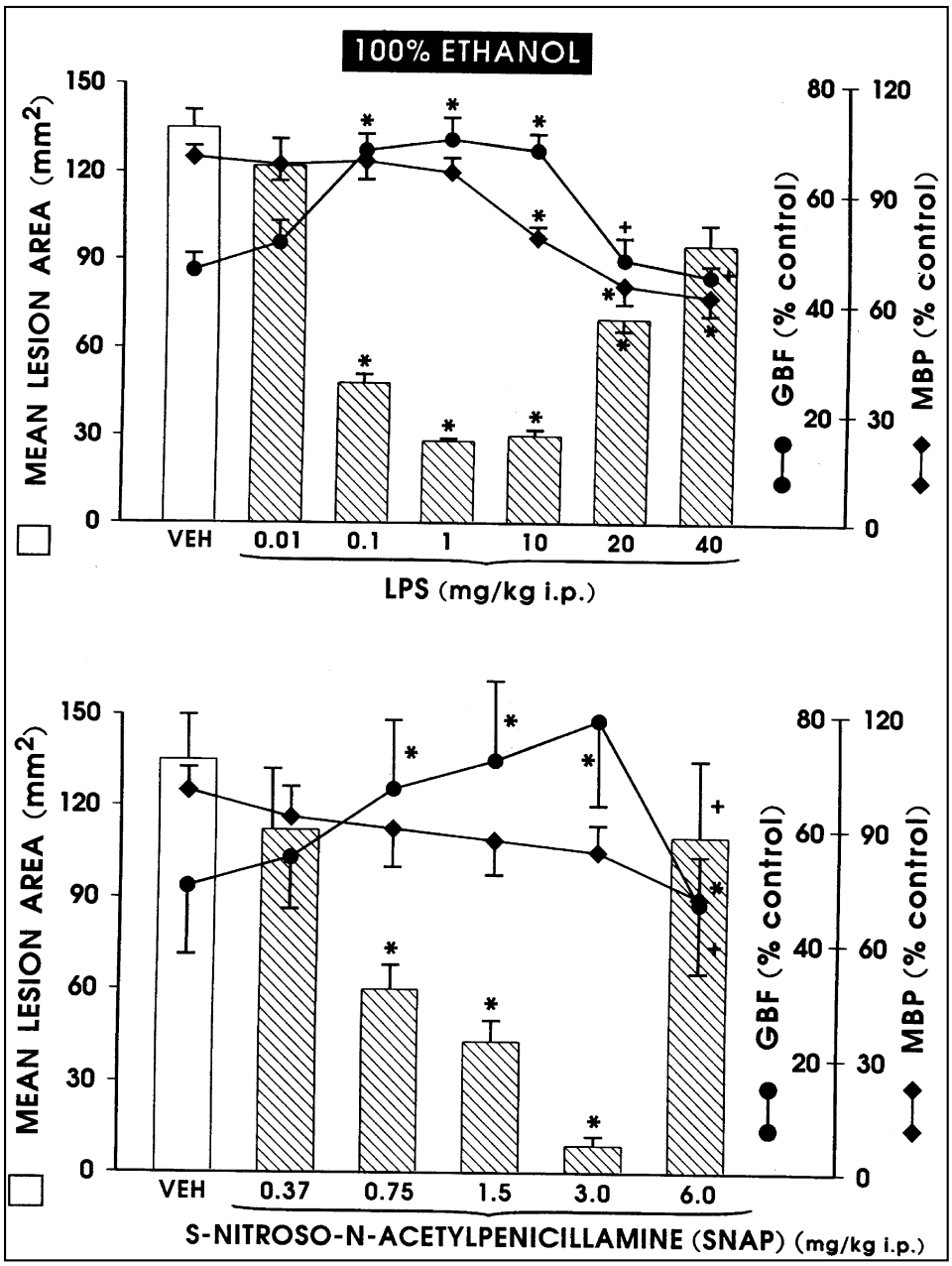

Fig. 1. Effect of lipopolysaccharide (LPS) applied i.p. in various doses ranging from 0.01 to 40 mg/kg and SNAP applied i.p. in various doses from 0.037 to 6 mg/kg on the area of gastric lesions induced by 100% ethanol, the gastric blood flow and mean blood pressure (MBP). Mean ± standard error of the mean of eight rats. Asterisk indicates significant change as compared with the values recorded in control animals. The cross indicates a significant change as compared with the value recorded in rats pretreated with LPS at doses 1 mg/kg.

mucosal damage was accompanied by a gradual increase in GBF in the ethanol treated gastric mucosa. After the administration of SNAP at a dose of 6 mg/kg, there was no significant change in area of ethanol-induced gastric lesions as compared with that in vehicle-treated animals. This was accompanied by a significant reduction in GBF and MBP.

Effect of intracerebroventricular injection of LPS on gastric lesions induced by 100% ethanol, gastric blood flow and gene expression of cNOS, iNOS and leptin

Centrally injected LPS at dose 1 and 10 μg i.c.v. dose-dependently reduced the mean area of ethanol-induced gastric lesions and this effect was accompanied by increase in GBF. However, these last changes were not statistically significant. The maximal protective effect of centrally injected LPS was observed at a dose 1 μg i.c.v. LPS injected at a higher dose of 10 μg/kg i.c.v. failed to cause further reduction in the mean lesion area induced by ethanol (*Fig. 2*).

Fig. 3 shows the expression of cNOS and iNOS mRNA assessed by RT-PCR technique in intact gastric mucosa and in the mucosa of rats injected centrally with vehicle or (LPS 1 or 10 μg) followed 30 min later by the administration of 100% ethanol. The expression of cNOS was detected in all

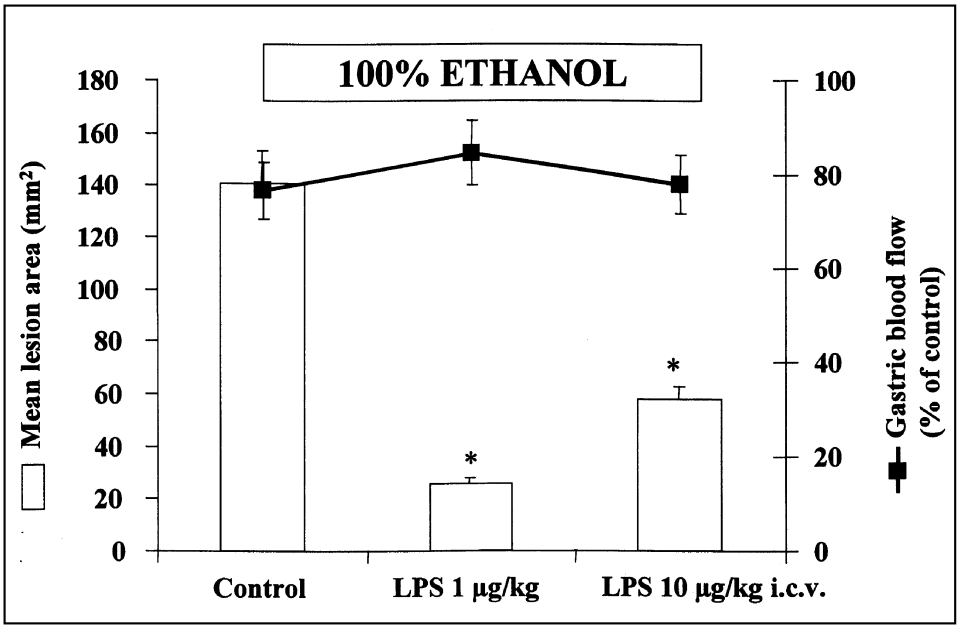

Fig. 2. Effect of lipopolysaccharide (LPS) applied i.c.v. in various doses ranging from 1 to 10 μg on the area of gastric lesions induced by 100% ethanol and the gastric blood flow. Mean ± standard error of the mean of eight rats. Asterisk indicates significant change as compared with the values recorded in control animals.

tested groups and the pretreatment with centrally injected LPS did not significantly affect the expression of cNOS. In contrast, a very weak signal for iNOS was detected in the intact gastric mucosa. In vehicle-pretreated rats, the administration of 100% ethanol was accompanied with a significant increase in iNOS mRNA expression. The pretreatment with LPS at a dose 1 μg i.c.v. induced a further increase in mRNA expression for iNOS mRNA, however, this change was not statistical significant. The pretreatment with a higher dose of LPS (10 μg) was not accompanied by a further increase in iNOS mRNA expression as compared to the vehicle-pretreated group.

As shown on *Fig. 4*, the expression for leptin was detected in intact gastric mucosa. In vehicle-pretreated rats, the administration of 100% ethanol was accompanied with a significant two-fold increase in leptin mRNA expression. The pretreatment with LPS (10 μg i.c.v.) was accompanied by a dose-dependent decrease in leptin mRNA expression.

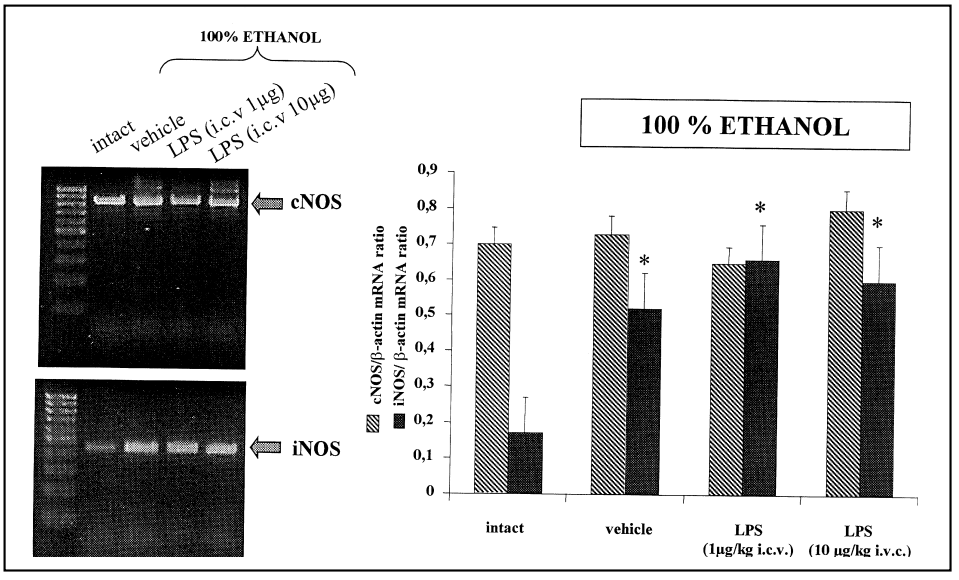

Fig. 3. Expression of iNOS and cNOS mRNA in gastric mucosa of rats injected centrally with vehicle or LPS (1 or10 μg) and 30 min later administered with 100% ethanol (1.5 ml i.g.). Asterisk indicates a significant increase above the value recorded in the contact gastric mucosa.

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

In the intact gastric mucosa the iNOS mRNA expression was not detected. Following the exposure to LPS we observed a dose dependent increase in mRNA expression of iNOS. The mRNA expression for cNOS was detected in intact gastric mucosa and did not significantly change after exposure to LPS (*Fig. 5*).

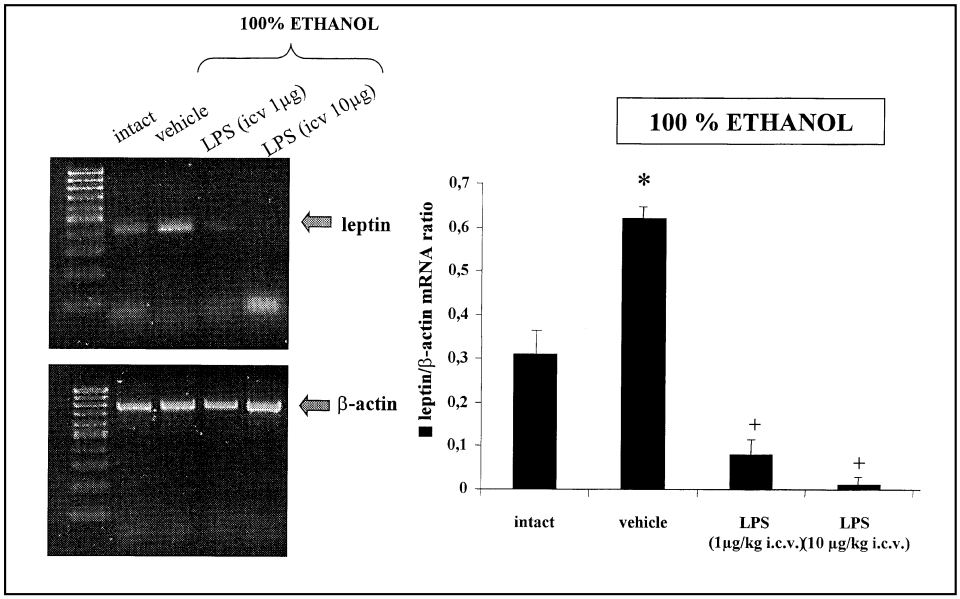

Fig. 4. Expression of leptin mRNA in gastric mucosa of rats injected centrally with vehicle or LPS (1 or10 μg) and 30 min later administered with 100% ethanol (1.5 ml i.g.). Asterisk indicates a significant change as compared with the value obtained in intact gastric mucosa. Cross indicates a significant change as compared with the value obtained in gastric mucosa treated with vehicle plus ethanol.

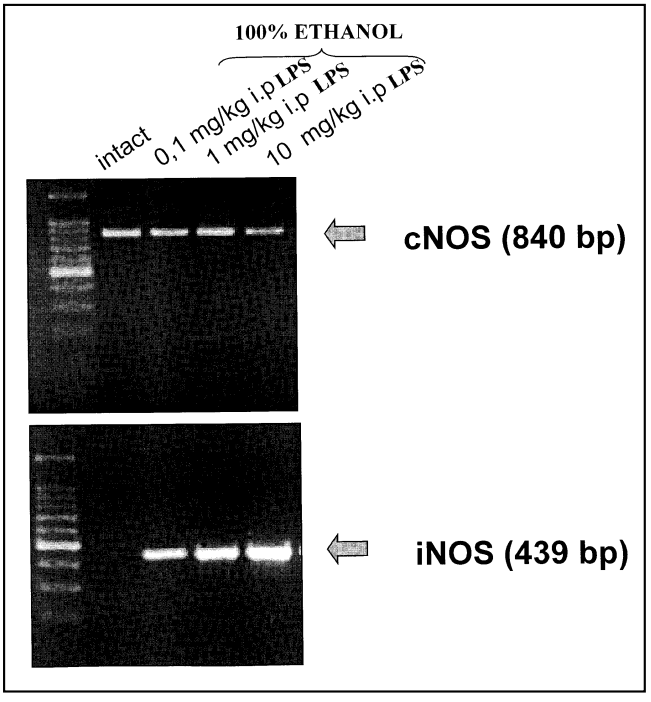

Fig. 5. Expression of iNOS and cNOS mRNA in intact gastric mucosa and in gastric mucosa of rats injected peripherally with various doses of LPS (0.1; 1 or 10 mg/kg i.p.) and then exposed to 100% ethanol.

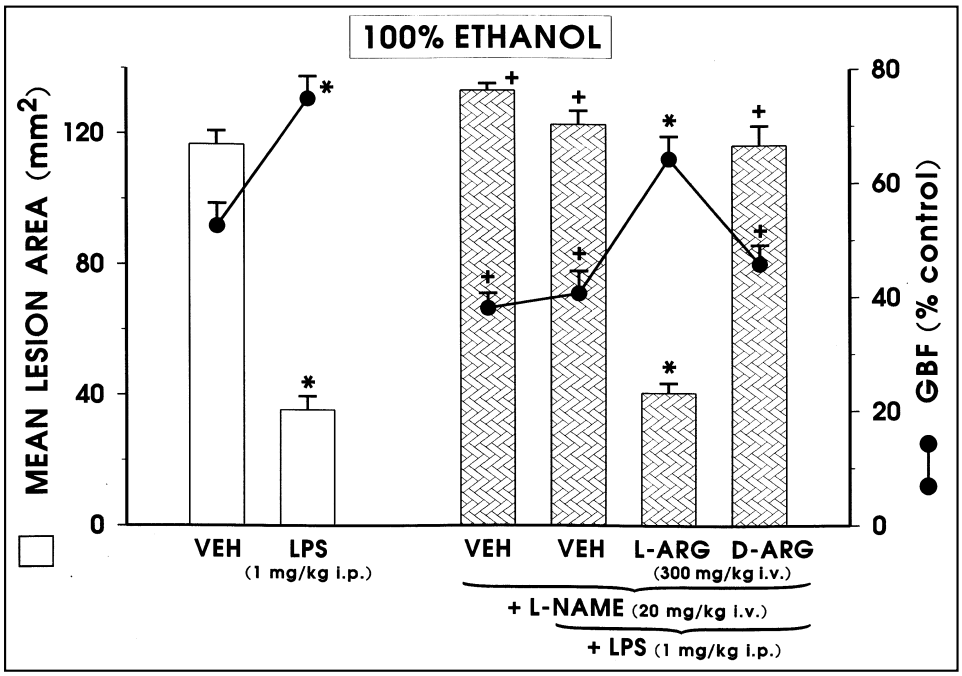

Fig. 6. The area of ethanol-induced gastric lesions and gastric blood flow in rats treated with LPS (1 mg/kg i.p.) without or with pretreatment with L-NAME with or without addition of L-arginine or D-arginine. Mean ± standard error of the mean of eight rats. Asterisk indicates significant change as compared with the values recorded in control animals. Crosses indicate significant changes as compared with the value recorded in similar tests without pretreatment with L-NAME.

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

The effects of L-NAME (20 mg/kg), a nonspecific inhibitor of NOS activity, applied intravenously without or with L-arginine or D-arginine, on the LPS-induced protection and the accompanying changes in the GBF are shown on *Fig. 6*. In this series of experiments, LPS administered at a dose 1 mg/kg i.p. caused a reduction in the area of ethanol-induce gastric lesions and this effect were accompanied by increase in GBF. The pretreatment with L-NAME, which by itself significantly aggravated ethanol lesions, completely reversed the LPS-induced protection and accompanying increase in GBF. Addition of L-arginine, but not D-arginine, to L-NAME restored the protection and the accompanying increase in GBF induced by LPS in gastric mucosa treated with 100% ethanol.

The pretreatment with L-NIL (30 mg/kg i.g.) did not cause any significant changes in ethanol-induced gastric lesions or GBF or NO production in the

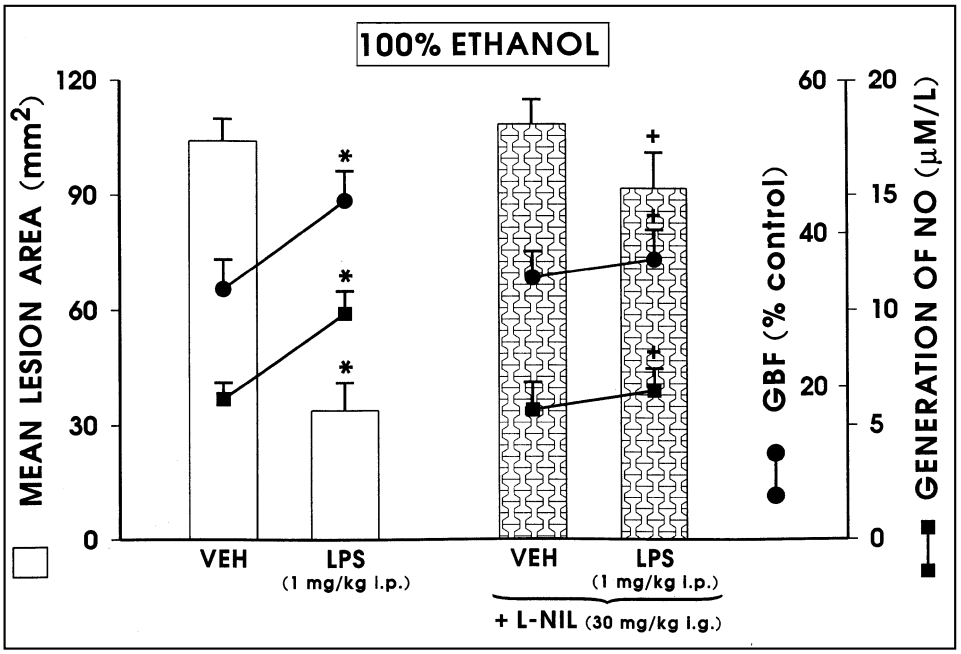

Fig. 7. The area of ethanol-induced gastric lesions, gastric blood flow and generation of NO in luminal content in rats treated with LPS (1 mg/kg i.p.) without or with pretreatment with L-NIL. Mean ± standard error of the mean of eight rats. Asterisk indicates significant change as compared with the values recorded in control animals. Crosses indicate significant changes as compared with the value recorded in similar tests without pretreatment with L-NIL.

vehicle-treated rats. In the LPS treated stomach, L-NIL strongly reversed the mucosal protection induced by LPS and significantly reduced the increase in GBF and NO production by gastric mucosa (*Fig. 7*).

DISCUSSION

The present study indicates that LPS applied centrally and peripherally protects gastric mucosa against the damage from 100% ethanol. The results of our study suggest that the gastroprotective actions of LPS are mediated, at least in part, through the NO-arginine system. This is supported by the fact that the suppression of endogenous cNOS and iNOS activity with non-specific blocker of NO-synthase, L-NAME, almost completely abolished the LPS-induced protection. Further support for this hypothesis comes from the results of the selective blockade of iNOS, which effectively attenuated the gastroprotective effects of LPS. In addition, the pretreatment with peripherally applied LPS was associated with a dose-dependent increase in iNOS mRNA expression in gastric mucosa. Taken together, these observations indicate that LPS protects gastric mucosa via stimulation of NO production, mainly by iNOS. These

observations are in agreement with previous findings suggesting an importance of NO in the gastric protection (10).

In the present study we showed for the first time that LPS applied centrally also shows gastroprotective action on gastric mucosa. However, the exact mechanisms by which centrally injected LPS is gastroprotective remains unclear. The increased expression of iNOS mRNA in gastric mucosa and increased gastric blood flow in rats injected centrally with LPS indicate the involvement of L-arginine-NO systems.

The key question remains whether LPS-induced gene expression of iNOS and excessive production of NO are the sole factors involved in gastroprotection afforded by endotoxin. In the present study we attempted to mimic the action of endogenous NO induced by LPS by using SNAP, a chemical donor of NO, which release NO spontaneously (11). We confirmed that the pretreatment with SNAP protected the gastric mucosa against mucosal damage. This is a further support for the importance of L-arginine-NO system in the gastroprotection afforded by LPS. However, other factors may also play some role in this phenomenon. The studies performed by Ferraz *et al.* and our group demonstrated an importance of COX-2 derived prostaglandins in the protection induced by LPS (4, 12).

Recently, the possible role of leptin in the gastroprotection has been considered (13, 14). Our own data have shown that exogenous leptin protects gastric mucosa against injury induced by ethanol or aspirin (15). In addition, our previous studies provided an evidence that leptin is produced in gastric mucosa and that the damage of the gastric mucosa is associated with the local upregulation of gastric leptin synthesis. Furthermore, leptin accelerates the healing of gastric ulcers (7). In agreement with our previous data, we demonstrated an upregulation of leptin mRNA in gastric mucosa of rats after the intragastric administration of ethanol. Interestingly, in rats injected centrally with LPS and then exposed to 100% ethanol, the leptin mRNA in gastric mucosa decreased dose-dependently. One possible explanation could be the increase release of leptin observed during endotoxemia (16, 17), could lead to the transcriptional downregulation of this factor in gastric mucosa. In line with this finding is our own data, which showed that the application of exogenous LPS to rats with chronic ulcers leads to the decreased expression of this cytokine in the gastric mucosa (7).

In conclusion, LPS applied centrally and peripherally protects gastric mucosa against ethanol-induced damage through an increase in gastric microcirculation mediated by NO due to overexpression of iNOS. The centrally applied LPS leads to the dose-dependent downregulation of leptin mRNA expression, possibly due to the increased release and elevated levels of this cytokine in plasma.

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