

**Involvement of capsaicin sensitive afferent nerves
and CCK₂/gastrin receptors in gastroprotection and adaptation
of gastric mucosa to *Helicobacter pylori*-lipopolysaccharide (*Hp*-LPS)**

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Abbreviations:

Helicobacter pylori -----*Hp*

Lipopolysaccharide -----LPS

Gastric blood flow -----GBF

Tumor necrosis factor alpha-----TNF- α

Interleukin-1 beta -----IL-1 β

Aspirin-----ASA

ABSTRACT

Lipopolysaccharide (LPS) is one of the virulence factors in the *Helicobacter pylori* (*Hp*)-infected stomach, but it remains unknown whether single and prolonged pretreatment with *Hp*-LPS can affect the course of gastric damage induced by aspirin (ASA). We compared the effects of *Hp*-LPS with those induced by LPSs isolated from intestinal *Bacteroides fragilis*, *Yersinia enterocolitica* and *Campylobacter jejuni* applied for 4 days on acute ASA-induced gastric lesions in rats. The area of ASA-induced gastric lesions, the gastric blood flow (GBF), the expression of mRNA and protein of leptin and plasma leptin, gastrin, interleukin-(IL)-1 β and tumor necrosis factor- (TNF-) α levels were examined. Single (once) or repeated (5 times) intraperitoneal (i.p.) injections of *Hp*-LPS (1 mg/kg) or intestinal LPSs failed to produce macroscopic gastric damage and did not affect the GBF when compared with vehicle. *Hp*-LPS injected repeatedly suppressed the gastric acid secretion, upregulated leptin mRNA, and protein and increased plasma leptin and gastrin levels. *Hp*-LPS reduced significantly the ASA-induced gastric damage and the accompanying fall in the GBF, and these effects were significantly attenuated by capsaicin-denervation and selective antagonism of cholecystokinin-B (CCK₂) receptors by RPR-102681, but not by loxiglumide, an antagonist of CCK₁ receptors. We conclude that 1) daily application of *Hp*-LPS enhances gastric mucosal resistance against ASA damage due to the increase of GBF, expression and release of leptin and gastrin, exerting the trophic and gastroprotective effects, and 2) this enhanced resistance to ASA damage in *Hp*-LPS adapted stomach is mediated by the sensory afferents and specific CCK₂/gastrin receptors.

Introduction

Helicobacter pylori (*Hp*) is now generally accepted as a major cause of chronic gastritis and an important risk factor for peptic ulcer disease and gastric cancer (Warren and Marshal, 1983; Konturek et al., 1999), but it remains unknown whether the gastric mucosa is capable of adapting to repeated *Hp* insults and whether such *Hp*-adaptation might alter the mucosal resistance to the injurious action of strong irritants.

Various pathogenic factors originating from *Hp* have been implicated in the damaging effect of this bacterium on the gastric mucosa, the most important besides ammonia, being cytotoxins released by *Hp*-strains expressing the vacuolating cytotoxin A (VacA) and cytotoxin-associated gene A (CagA) proteins, *Hp*-derived lipopolysaccharides (*Hp*-LPS) and the enhanced generation of reactive oxygen species (Megraud et al., 1992; Figura and Tabaqchali, 1996; Crabtree, 1996; Moran, 2001a; Moran, 2001b). *Hp*-LPS exhibits a low immunological activity and this property has been assumed to play an important role in the persistency of *Hp* infection in the human stomach (Moran, 2001a; Moran, 2001b). Nevertheless, the deleterious action of LPS derived from *Hp* in the stomach includes an interaction of this endotoxin with laminin (Valkonen et al., 1994), its influence on the gastric mucus formation and composition (Slomiany et al., 1992) and expression of proinflammatory cytokines (Crabtree et al., 1994; Moran, 2001a). Recent evidence suggests, however, that LPS derived from *Escherichia coli* applied parenterally, also induces gastroprotective activity against lesions induced by strong topical irritants such as ethanol (Tepperman and Soper, 1994; Konturek et al., 1998a; Konturek et al., 1998b; Ng et al., 2002) and results in mucosal adaptation to topical irritants after prolonged administration (Ferraz et al., 1997).

Leptin is accepted as a protein product of the *ob* gene acting directly and through the sensory afferent on central leptin receptors (*Ob-R*) in the hypothalamus that control food intake and energy expenditure (Friedman and Halaas, 1998). Recent studies documented that leptin is present in the plasma of experimental animals, such as mice and rats, as well as in humans (Barbier et al., 1998; Shalev et al., 1997). Leptin is believed to be secreted mainly by adipocytes and the placenta, but recent studies have revealed that leptin messenger RNA (mRNA) and leptin protein can also be detected in the rat gastric oxyntic mucosa, suggesting that the gastric corpus may be another important source of leptin (Bado et al., 1998; Brzozowski et al., 1999).

The importance of leptin in the action of bacterial LPS has been supported by evidence that reduced levels of leptin during starvation increased animal susceptibility to endotoxic shock (Faggioni et al., 2000). Since parenteral LPS was shown to attenuate ethanol-induced gastric damage (Tepperman and Soper, 1994; Konturek et al., 1998a; Brzozowski et al., 2003) the question remains whether leptin, which exhibits gastroprotective activity in the stomach (Brzozowski et al., 1999), can also contribute to the LPS-induced protection against mucosal damage induced by aspirin. Finally, the physiological significance of gut hormones such as leptin and gastrin in adaptation of gastric mucosa, developed by daily injections of endotoxins such as *Hp*-LPS, requires an elucidation.

This study was designed to determine the effect of single or repeated parenteral applications of *Hp*-LPS on acute gastric lesions induced by intragastric (i.g.) administration of acidified aspirin (ASA) and accompanying changes in the gastric blood flow (GBF), gastric secretion, and the gene expression and release of leptin. An attempt was made to compare the effects of five daily injections with *Hp*-LPS with those exhibited by different LPSs isolated from enteric bacteria such as *Bacteroides fragilis*, *Yersinia enterocolitica* and

Campylobacter jejuni on gastric acid secretion and ASA-induced gastric damage.

Furthermore, we attempted to compare the effects of *Hp*-LPS with those of exogenous leptin, CCK and peptone meal, a potent releaser of both CCK and leptin, and to examine the involvement of specific CCK₁ (for CCK) and CCK₂ (for gastrin) receptors, sensory nerve activity, proinflammatory cytokines such as interleukin- (IL-) 1 β and tumor necrosis factor- (TNF-) α in gastric mucosal integrity and possible gastric mucosal adaptation afforded by *Hp*-LPS.

Methods

Three major series (A, B & C) consisting of 200 male Wistar rats weighing 180-220 g were used. All procedures have been carried out in accordance with the Declaration of Helsinki and were accepted by Local Ethical Committee at the Jagiellonian University. Acute gastric lesions were induced by an i.g. application of acidified ASA (150 mg/kg in 0.15 N HCl) in a volume of 1.5 ml by means of a metal orogastric tube (series A). In series B, gastric mucosa was subjected to single or repeated exposures to vehicle (saline) or LPS isolated from *Hp* (Moran, 1992). Series C was designed to determine the effect of single and repeated parenteral injections of LPS derived from the intestinal bacteria *B. fragilis*, *Y. enterocolitica* and *C. jejuni*. *B. fragilis* NCTC 9343 was obtained from the National Collection of Type Cultures (London, UK), *C. jejuni* ATCC 43431 was purchased from the American Type Culture Collection (Manassas, Virginia, USA) and *Y. enterocolitica* IY-9 was a clinical isolate originating from a human with diarrheal disease.

Induction of gastric adaptation to *Hp*-LPS. Gastric adaptation was achieved by daily parenteral administration of *Hp*-LPS or vehicle (saline) to normally fed rats for the

entire period of the study. The parenteral route of LPS administration was chosen based on our previous observations (Konturek et al., 1998a, Brzozowski et al., 2003) that gastric mucosa directly exposed to LPS applied in a dose of 1 mg/kg (i.g.), failed to adapt to this endotoxin and such LPS applied i.g. also failed to influence the mucosal lesions induced by strong irritants (*e.g.*, ethanol, ASA and stress). Since LPS produced by the bacteria contaminating the gastrointestinal lumen and adherent to mucosal cells under the mucus layer covering the surface epithelium, may penetrate the mucosa and reach the general circulation, we decided to employ parenteral injection rather than the intragastric route as the route of bacterial LPS administration in order to mimic the fate of this systemic LPS. Our preliminary observation with intragastric daily application of *Hp*-LPS at a dose of 10 mg/kg also exerted gastroprotection against ASA-induced gastric damage, but such an investigation required large doses of *Hp*-LPS which were not available to us, and therefore, only parenteral administration of this LPS was employed in the present study. The animals received *Hp*-LPS once and, for comparison, LPS isolated from intestinal bacteria (1 mg/kg) by intraperitoneal route (i.p.), or were treated repeatedly by the same route with *Hp*-LPS and that of other intestinal bacteria for four consecutive days as described in detail in our previous studies with ASA-induced gastric adaptation (Konturek et al., 1994; Brzozowski et al., 1995). Rats with single or repeated daily (5 times) injections of *Hp*-LPS were sacrificed, the stomach was quickly removed, opened along the greater curvature, and the gastric mucosa was photographed to subsequently measure the area of gastric lesions by two observers using planimetry (Morphomat, Carl Zeiss, Berlin, Germany). The gastric mucosa of separate overnight-fasted rats treated repeatedly with vehicle, *Hp*-LPS, or intestinal bacterial LPS was then challenged 120 min after the last dose of vehicle, *Hp*-LPS or intestinal bacterial LPS with acidified ASA applied i.g. in a volume of 1.5 ml.

At 3 h upon the ASA application, the animals were lightly anesthetized with ether, their abdomen was opened by the midline incision and the stomach was exposed for the measurement of GBF by means of the H₂-gas clearance technique (Konturek et al., 1994; Konturek et al., 2001a). The measurements were made in three areas of the oxyntic mucosa and the mean values of the measurements were calculated and expressed as percent changes of those recorded in the vehicle (saline)-treated animals.

The alterations of gastric secretions in rats treated with the vehicle (saline), *Hp*-LPS or LPS derived from intestinal bacteria applied once or given repeatedly, were tested in a separate group of 60 fasted rats, surgically equipped with chronic gastric fistulas as described in our earlier studies (Brzozowski et al., 2000a). The rats that have been treated once with *Hp*-LPS or injected repeatedly (5 times) with *Hp*-LPS or LPS isolated from intestinal bacteria, were placed at day 4 in individual Bollman cages (to which the animals were well-conditioned) to prevent coprophagy and to maintain the necessary restraint. In addition, the effect of five daily injections with *Hp*-LPS with or without loxiglumide, an inhibitor of CCK₁ receptors, and N-(methoxy-3 phenyl) N-(N-methyl N-phenyl-carbamylmethyl) carbamoylmethyl]-3 ureido}-3 phenyl}-2 propronique (RPR-102681), the selective CCK₂ receptor antagonist, was determined (Konturek et al., 1995; Brzozowski et al., 2000b). Each fistula was then opened, and the stomach rinsed gently with 5-8 ml of tap water at 37°C. Basal gastric secretion was collected for 120 min, during which time all animals received saline at a rate of 4 ml/h subcutaneously. The gastric juice was collected every 30 min, the volume was measured, and then the acid concentration and output were determined and expressed as the output per 30 min as described previously (Brzozowski et al., 2000a).

Experimental groups and treatments. Vehicle or *Hp*-LPS (dose of 1 mg/kg i.p.) were given once or were administered at the same dose for four consecutive days. After five daily injections with *Hp*-LPS or vehicle, the gastric mucosa was challenged with acidified ASA. The protective effect of *Hp*-LPS applied i.p. 2 h prior to ASA was compared with known gastroprotective agents (Brzozowski et al., 1999) such as those of leptin and CCK administered i.p. (dose of 10 µg/kg) or 8% peptone meal applied i.g. in a volume of 1 ml per rat.

The following groups of rats were used: 1) vehicle (1 ml of saline i.p.) followed 120 min later by acidified ASA (150 mg/kg i.g.); 2) *Hp*-LPS (1 mg/kg i.p.) followed 120 min later by ASA; 3) *B. fragilis*-LPS, *Y. enterocolitica*-LPS and *C. jejuni*-LPS (1 mg/kg i.p.) followed 120 min later by ASA; 4) leptin (10 µg/kg i.p.) and CCK-8 (10 µg/kg i.p.) followed 120 min later by ASA; 5) 8% peptone meal (1 ml/rat i.g.) followed 120 min later by ASA; 6) vehicle (saline) or *Hp*-LPS (1 mg/kg i.p.) administered daily for 4 days with or without the challenge with ASA applied at day 4; and 7) *B. fragilis*-LPS, *Y. enterocolitica*-LPS and *C. jejuni*-LPS (1 mg/kg i.p.) administered daily for 4 days with or without the challenge with ASA applied at day 4.

Effect of suppression of CCK₁ and CCK₂ receptors and sensory nerves on gastroprotection and adaptation induced by *Hp*-LPS. To check whether gastrin/CCK is involved in the action of bacterial LPS on mucosal integrity, separate subgroups of rats were used and the effects of inhibition of CCK₁ and CCK₂ receptors with loxiglumide and RPR-102681, respectively, on the protection and adaptation induced by LPS were examined. RPR-102681 is a novel, non-peptide selective antagonist of the CCK₂/gastrin receptor, which has been shown to display nanomolar affinity of about 2000-fold greater selectivity for CCK₂ than

CCK₁ receptors (Bohme et al., 1997). [Loxiglumide was a generous gift of Dr. Rovati (Milan, Italy), and RPR-102681 was purchased from Rhone-Poulenc, Rorer S.A., (Vitry-sur-Seine, France).] In subsequent studies, two series of experiments were carried out.

Series (i) was used to examine the effect of *Hp*-LPS applied (i.p.) against the mucosal lesions induced by ASA in rats without or with the blockade of CCK₁ receptors with loxiglumide (30 mg/kg i.p.) or CCK₂ receptors with RPR-102681 (30 mg/kg i.p.) (Brzozowski et al., 2000b). The dose of loxiglumide and RPR-102681 was selected on the basis of our previous studies in rats which showed that loxiglumide attenuated the gastroprotective and secretory effects of CCK by having no influence on gastroprotection and secretory activity of gastrin and leptin, whereas RPR-102681 reversed the leptin-induced gastroprotection without significant effect on that induced by CCK (Konturek et al. 1995, Brzozowski et al., 2000b).

The involvement of sensory nerves (series ii) in gastroprotection and adaptation by *Hp*-LPS, was studied in rats with or without deactivation of afferent nerves with a neurotoxic dose of capsaicin as described previously (Brzozowski et al., 1996). For this purpose the animals were pretreated with capsaicin (Sigma Co., St. Louis, MO) injected subcutaneously (s.c.) for three consecutive days at respective doses of 25, 50 and 50 mg/kg (total of 125 mg/kg) about 2 weeks before the experiment. All injections of capsaicin were performed under ether anesthesia to counteract the respiratory impairment associated with injection of this agent. Control rats received vehicle injections. All animals pretreated with capsaicin showed a negative wiping movement test, thereby confirming the functional denervation of the capsaicin-sensitive afferent fibers and the loss of corneal reflex.

The following groups of rats, each consisting of 6 to 8 animals, that had been exposed to *Hp*-LPS applied once or administered 5 times were used: 1) vehicle (saline) or *Hp*-LPS (1

mg/kg i.p.) applied once or given 5 times and followed 2 h later by acidified ASA without or with capsaicin-denervation; 2) loxiglumide or RPR-102681 (30 mg/kg i.p.) followed 60 min later by vehicle applied once or given 5 times, and then followed 2 h later by acidified ASA; 3) loxiglumide and RPR-102681 (30 mg/kg i.p.) followed 60 min later by *Hp*-LPS (1 mg/kg i.p.) applied once or given 5 times and then finally followed 2 h later by acidified ASA. Subsequently, rats were anesthetized, the GBF was measured and the area of gastric lesions was determined by planimetry in a similar manner to that mentioned above.

Determination of plasma leptin levels by radioimmunoassay (RIA) and plasma IL-1 β and TNF- α by ELISA. At the termination of some experiments after treatment with vehicle or *Hp*-LPS, leptin, CCK-8 and 8% peptone meal administration followed 2 h later by acidified ASA, the rats were anesthetized with ether and the blood samples (about 3 ml) were taken from the *vena cava* for the measurement of plasma leptin by RIA as described previously (Brzozowski et al., 1999) and determination of plasma IL-1 β and TNF- α levels by ELISA (Endogen Inc., Cambridge, MA, USA) as described earlier (Brzozowski et al., 2000a). For comparison, intact rats that had been fasted overnight and only given i.p. vehicle saline were also anaesthetized with ether, and blood samples were collected for the determination of control values of leptin, IL-1 β and TNF- α in plasma. For determination of plasma leptin, blood samples collected in heparin-coated polypropylene tubes were centrifuged at 3000 g for 20 min at 4°C, and the clear supernatant plasma was then stored at -80°C until analysis. Measurement of plasma leptin was by using a RIA-kit for rat leptin from Linco Research Inc. (St. Charles, Missouri, USA). Briefly, this RIA involved the competition of a rat leptin sample with ¹²⁵I-rat leptin tracer for binding to a specific rabbit anti-leptin polyclonal

antibody. The limit of assay sensitivity was 0.5 ng/ml; the intra-assay variation was less than 7% and the interassay variation less than 9%.

Reverse-transcriptase-polymerase chain reaction (RT-PCR) for detection of mRNA of leptin. Stomachs were removed from rats treated with vehicle (control) and those treated with *Hp*-LPS, with or without i.g. application of ASA for the determination of leptin mRNA expression by RT-PCR with specific primers (Brzozowski et al., 2000b). Gastric mucosa was scraped off from the oxyntic gland area using a slide glass and immediately snap frozen in liquid nitrogen and stored at -80°C until analysis. Total RNA was extracted from mucosal samples by a guanidinium isothiocyanate/phenol chloroform method using a kit from Stratagene® (Heidelberg, Germany). Aliquoted RNA samples were stored at -80°C until analysis.

Single-stranded cDNA was generated from 5 µg of total cellular RNA using StrataScript™ reverse transcriptase and oligo-(dT)-primers (Stratagene, Heidelberg, Germany). The nucleotide sequences of the primers for leptin and β -actin were based on published cDNA-encoding leptin and β -actin, respectively (Bado et al., 1998; Brzozowski et al., 1999). The sense primer for leptin was CTG CTC AAA GCC ACC ACC TCT G, and the antisense primer was CCT GTG GCT TTG GTC CTA TCT G. The sense primer for β -actin was TTG TAA CCA ACT GGG ACG ATA TGG, and for antisense was GAT CTT GAT CTT CAT GGT GCT AGG. The primers were synthesized by GIBCO BRL/Life Technologies (Eggenstein, Germany).

Polymerase chain reaction (PCR) products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Location of predicted products was confirmed by using a DNA 100-bp ladder (GIBCO, Eggenstein, Germany) as a standard size marker.

Protein extraction and analysis of leptin expression in the gastric mucosa by Western blotting

Shock frozen tissue from rat stomach was homogenized in lysis buffer (100 mmol Tris-HCl, pH 7.4, 15% glycerol, 2mmol EDTA, 2% sodium dodecyl sulfate (SDS), 100 mmol D,L-dithiothreitol (DDT) by the addition of 1:20 dilution of aprotinin and 1:50 dilution of 100 mmol phenylmethylsulfonyl fluoride (PMSF). Insoluble material was removed by centrifugation at 12000g for 15 min. Approximately 100 µg of cellular protein extract were loaded into a well, separated electrophoretically through a 13.5% SDS-polyacrylamide gel and transferred onto Sequi-Blot™ PVDF membrane (BioRad, USA) by electroblotting. Skim fast milk powder (5% w/v) in TBS-Tween-20 buffer (137 mmol NaCl, 20 mmol Tris-HCl, pH7.4, 0.1% Tween-20) was used to block filters for at least 1 h at room temperature. As primary antibody 1:500 dilution of specific goat polyclonal antiserum against leptin (Santa Cruz, USA) or 1:1000 dilution of rabbit polyclonal anti-β-actin (Sigma Aldrich, Germany) antiserum was added to the membrane, followed by an anti-goat or anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:2000, Santa Cruz, USA). Incubation of primary antibody was followed by 3 washes with TBS-Tween-20 buffer for 10 min. Incubation of the secondary antibody was followed by 4 washes for 10 min. Non-isotopic visualization of immunocomplexes was achieved by chemiluminescence using BM Chemiluminescence Blotting Substrate (Boehringer, Mannheim, Germany). Thereafter, the developed membrane was exposed to an X-ray film (Kodak, Wiesbaden, Germany).

Statistical analysis. Results are expressed as means \pm SEM. Statistical analysis was done using analysis of variance and a two way ANOVA test with post hoc Tukey HSD test. Differences of $p < 0.05$ were considered as significant.

Results

Effect of single and five daily injections of *Hp*-LPS and bacterial LPSs on gastric acid secretion. Table 1 shows the effects of vehicle, *Hp*-LPS and LPSs derived from intestinal bacteria applied once or as 5 daily injections on gastric acid secretion in conscious rats with chronic GF. The basal gastric acid output in rats treated with vehicle (saline) reached the value of 158 ± 14 $\mu\text{mol}/30$ min. When *Hp*-LPS was applied once at a dose of 1 mg/kg i.p., the gastric acid output was significantly reduced compared to that obtained in vehicle control animals (Table 1). At such a dose, *Hp*-LPS significantly reduced the volume of gastric juice (2.0 ± 0.1 ml/30 min) and the gastric H^+ concentration (32.5 ± 4 $\mu\text{mol}/\text{ml}$) as compared with those in vehicle-control animals (volume of gastric juice, 2.8 ± 0.3 ml/30 min; gastric H^+ concentration, 56.4 ± 8 $\mu\text{mol}/\text{ml}$). In rats injected daily (5 times) with *Hp*-LPS (1 mg/kg i.p.), the gastric acid secretion was significantly more suppressed than after a single application of LPS. In *Hp*-LPS injected five times, the volume of gastric juice (1.5 ± 0.4 ml/30 min) and the gastric H^+ concentration (21.4 ± 2 $\mu\text{mol}/\text{ml}$) were significantly lower as compared with those in animals once injected with *Hp*-LPS (volume of gastric juice, 2.0 ± 0.3 ml/30 min and the gastric H^+ concentration, 32.5 ± 4 $\mu\text{mol}/\text{ml}$). For comparison, the single parenteral application of LPSs derived from *B. fragilis*, *Y. enterocolitica* and *C. jejuni* resulted in a similar decrease in the gastric acid outputs compared to that recorded in *Hp*-LPS treated animals (Table 1). Five times daily injections of LPSs derived from *B. fragilis*, *Y. enterocolitica* and *C. jejuni* caused significantly stronger reduction in the gastric acid outputs than that observed with a single dose and with an extent similar to that obtained in rats treated repeatedly with *Hp*-LPS. Administration of loxiglumide, a CCK_1 receptor antagonist or RPR-102681, a CCK_2 receptor antagonist, failed to significantly influence basal gastric

acid output as compared to that in vehicle-treated animals. Following the five daily injections with *Hp*-LPS, a significant decrease in the gastric acid output was observed (158 ± 14 $\mu\text{mol}/30$ min in vehicle-control vs 32 ± 4 $\mu\text{mol}/30$ min in *Hp*-LPS treated). The reduction in the acid output induced by *Hp*-LPS applied 5 times was not influenced significantly by loxiglumide (gastric acid output, 32 ± 4 $\mu\text{mol}/30$ min in *Hp*-LPS treated vs 38 ± 5 $\mu\text{mol}/30$ min in loxiglumide plus *Hp*-LPS applied 5 times). Administration of RPR-102681 reversed the attenuation of the gastric acid output induced by five daily injections of *Hp*-LPS (32 ± 4 $\mu\text{mol}/30$ min in *Hp*-LPS vs 114 ± 8 $\mu\text{mol}/30$ min in RPR-102681 plus *Hp*-LPS applied 5 times).

Effect of *Hp*-LPS and bacterial LPSs applied once or five times daily on ASA-induced gastric lesions with accompanying alterations in GBF, plasma leptin and gastrin levels. As shown in Fig. 1, the parenteral application of *Hp*-LPS (1 mg/kg i.p.) produced negligible macroscopic injury in the stomach when applied once or injected daily 5 times, and failed to alter significantly the GBF when compared to that recorded in vehicle-control rats. Similarly, single or repeated injections of LPSs from *B. fragilis*, *Y. enterocolitica* and *C. jejuni* produced only small gastric mucosal lesions and failed to influence GBF compared to vehicle treatment. In vehicle-pretreated rats, acidified ASA resulted in typical multiple gastric lesions and a significant fall in the GBF by about 30%, compared to the respective value recorded in animals pretreated with vehicle alone without ASA (Fig. 1). Five daily injections with each endotoxin significantly reduced ASA-induced gastric damage and accompanying fall in the GBF as compared with those in vehicle-pretreated rats exposed to ASA. Representative gross macroscopic evidence of the ASA-induced gastric damage and the reduction in these lesions in the animal stomach without and

with the daily injections of *Hp*-LPS is presented in Fig. 2A-D. Compared with the intact gastric mucosa, the exposure of gastric mucosa to ASA in the rat treated 5 times with vehicle, resulted in a multiple gastric lesions localized mainly in the oxyntic mucosa (Fig. 2A and B). In contrast, the repeated treatment with *Hp*-LPS alone produced only a few gross gastric mucosal lesions (Fig. 2C). In rats injected daily 5 times with *Hp*-LPS, and then subsequently exposed to ASA, there was a significant attenuation of the gastric mucosal injury as compared to those treated 5 times with vehicle and then exposed to ASA (Fig. 2D).

Hp-LPS applied in a single dose of 1 mg/kg i.p. reduced significantly the ASA-induced gastric lesions, and these protective effects were accompanied by a significant rise in GBF and an elevation of plasma immunoreactive leptin and gastrin levels (Fig. 3). In intact animals without ASA, the plasma gastrin concentration averaged 52 ± 5 pmol/l and plasma leptin reached a value of 0.65 ± 0.04 ng/ml; these values remained relatively unchanged in animals treated with vehicle (Fig. 3). In rats injected once with *Hp*-LPS, both plasma leptin and gastrin concentrations showed a several fold increase, being significantly higher than those in vehicle-treated animals. In rats injected daily with *Hp*-LPS, the plasma leptin and gastrin concentrations gave a further significant rise compared with that recorded in vehicle-treated animals or those exposed to single treatment with this endotoxin (Fig. 3). The ASA-damage was significantly attenuated in the gastric mucosa of rats exposed to single or repeated (5 times) injections of *Hp*-LPS, and these effects were accompanied by a significant elevation of plasma gastrin and leptin increments (Fig. 3).

The single parenteral application of *B. fragilis*-LPS, *Y. enterocolitica*-LPS and *C. jejuni*-LPS applied i.p. at a dose of 1 mg/kg, also resulted in the attenuation of ASA-induced gastric damage and raised significantly the GBF (Table 2). As shown in Fig. 1, the repeated parenteral application of these LPSs also reduced significantly the lesions induced by

acidified ASA. The protective effects against ASA-induced gastric lesions of these endotoxins injected repeatedly were accompanied by a significant rise in GBF, compared with the respective values obtained in gastric mucosa exposed to ASA alone (Fig. 1).

Fig. 4 shows the results of parenteral administration of leptin, CCK-8, *Hp*-LPS and 8% peptone meal on the mean area of ASA-induced gastric lesions and the accompanying changes in the GBF and plasma leptin levels. Exogenous leptin and CCK-8, both given in a single dose of 10 µg/kg i.p., or i.g. application of 8% peptone meal, that increased the plasma leptin levels by 2-3 folds and raised significantly GBF, resulted in a significant attenuation of gastric lesions induced by ASA, with an extent similar to those achieved with single parenteral injection of *Hp*-LPS.

Effect of pretreatment with loxiglumide and RPR-102681 and deactivation of sensory nerves on the ASA-induced gastric lesions in rats. As shown in Fig. 5, the i.g. application of acidified ASA produced similar gastric lesions and a similar fall in GBF to those presented in Figs. 1 and 2. The area of these lesions and the accompanying fall in GBF were significantly reduced in rats injected once with *Hp*-LPS or daily (5 times) with this endotoxin. Suppression of CCK₁ receptors with loxiglumide by itself failed to influence significantly the ASA-induced gastric damage, and the accompanying fall in GBF. Loxiglumide also failed to affect the reduction in the area of ASA-induced gastric damage and the accompanying increase in GBF attained with *Hp*-LPS applied once or administered repeatedly. Pretreatment with RPR-102681, to suppress specific CCK₂ receptors, which by itself also failed to influence the ASA-induced gastric damage, abolished almost completely the decrease in the area of these lesions and the accompanying rise in the GBF evoked by single or repeated treatment with this endotoxin.

Hp-LPS applied once or injected daily (5 times) reduced significantly the area of ASA-induced gastric lesions and raised significantly the GBF compared to those recorded in rats treated with ASA without endotoxin administration (Fig. 6). Capsaicin-denervation failed to enhance the area of ASA-induced gastric damage and failed to influence significantly GBF, but resulted in almost complete elimination of the protective and hyperemic effects induced by *Hp*-LPS injected once or daily (5 times) (Fig. 6).

Effect of single and repetitive treatment with *Hp*-LPS on plasma IL-1 β and TNF- α levels. As shown in Table 3, the plasma levels of both proinflammatory cytokines (IL-1 β and TNF- α) in the intact animals were negligible, but they were significantly increased in *Hp*-LPS treated animals, and further dramatically raised in rats exposed to acidified ASA that caused widespread acute gastric mucosal lesions. In rats injected once or daily with *Hp*-LPS and later exposed to ASA, a significant decrease in plasma IL-1 β and TNF- α levels was recorded, although the levels in plasma of these cytokines reached significantly higher values than those obtained in intact gastric mucosa.

Determination of leptin mRNA and protein by RT-PCR and Western Blotting in the gastric mucosa of rats treated once or repeatedly with *Hp*-LPS. The internal control with the β -actin mRNA and protein showed intense signals in all the samples tested, indicating a high integrity of RNA that was isolated from the gastric mucosa of vehicle-control rats, as well as from those injected once or daily with *Hp*-LPS (Fig. 7, left and right panels). Expression of leptin mRNA was detectable in intact mucosa not exposed to *Hp*-LPS and in that treated with vehicle or *Hp*-LPS injected once or given daily (Fig. 7, left panel).

No trace of leptin was recorded in rats serving as the negative control (saline) and this result has been omitted for the sake of clarity. In rats injected daily with *Hp*-LPS, the strong signal for leptin mRNA was greater than that in the vehicle-treated gastric mucosa. A weak signal for leptin protein was detected in the vehicle-treated gastric mucosa (Fig. 7, right panel). In contrast, an increased expression of leptin protein occurred in gastric mucosa of rats treated with *Hp*-LPS injected once or daily five times.

Discussion

The present study demonstrates that gastric mucosa can adapt in a relatively short period to multiple parenteral administration of *Hp*-LPS and shows for the first time that this adaptation enhances the mucosal resistance to subsequent acid-dependent gastric mucosal lesions, induced by acidified ASA. It is noteworthy that repeated treatment with LPS derived from other intestinal bacteria mimicked the protective action of *Hp*-LPS against ASA-induced gastric damage, suggesting that adaptive efficacy of gastric mucosa to endotoxins of different bacterial origin is not specifically related to *Hp*, and that it could contribute to strengthening the mucosal integrity resulting in attenuation of the damage produced by acid-dependent ulcerogens such as ASA. Furthermore, we found that both single and repeated injections of *Hp*-LPS produced a marked rise in plasma hormones such as gastrin and leptin, that have been proved to exert the gastroprotective and ulcer healing activity (Konturek et al., 1995; Bado et al., 1998; Brzozowski et al., 2000b; Konturek et al., 2001a). The protective effects of *Hp*-LPS were accompanied by a rise in plasma gastrin and were significantly attenuated by pretreatment with RPR-102681, a highly specific inhibitor of mucosal gastrin receptors (CCK₂), but not influenced by loxiglumide, an antagonist of receptors for CCK (CCK₁), whose plasma level was not affected by *Hp*-LPS (unpublished observation). These

results emphasize the importance of gastrin and CCK₂, rather than CCK and CCK₁, receptors in the protective and adaptive response of *Hp*-LPS. Both the protection and adaptation to *Hp*-LPS in ASA-injured mucosa were accompanied by upregulation of leptin at the level of both mRNA and protein and subsequent release of leptin, indicating that this hormone, indeed, contributes to *Hp*-LPS-induced attenuation of ASA-induced gastric damage. Also, in rats with intact gastric mucosa injected with *Hp*-LPS, a marked increase in plasma leptin was observed. Thus, this study shows for the first time, an increase in expression of leptin at the levels of mRNA and protein with subsequent plasma release of this peptide occurs in rats injected once or daily with *Hp*-LPS thereby emphasizing that leptin, which has been shown previously to exert a protective effect against gastric mucosal injury by strong irritants (Bado et al., 1998; Brzozowski et al., 1999), might contribute to the enhanced resistance of gastric mucosa of rats treated with *Hp*-LPS against damage induced by acidified ASA. This notion is in agreement with the original finding of Tepperman et al. (1994) who showed protection of the rat gastric mucosa against ethanol lesions after parenteral administration of *E.coli*-LPS. The present study is also consistent with the evidence of Ferraz et al. (1997), and our own recent observation (Brzozowski et al., 2003), that animals treated repeatedly with *E.coli*- or *Hp*-derived LPS developed mucosal tolerance to these endotoxins, and that this adaptive response enhanced gastric mucosal resistance to ethanol-induced gastric damage. Our data also agrees with the observations by Sugiyama et al. (2001) who demonstrated that the extent of ethanol-induced damage of gastric mucosa was greatly limited in an experimental model of *Hp*-infection in the stomach of Mongolian gerbils. These authors concluded that *Hp*-infection, possibly due to release of endotoxin and the mild irritant effects of these cytotoxins, exhibits an apparent paradoxical (protective) effect on gastric mucosal integrity by enhancing the resistance of this mucosa to damage induced by necrotizing irritants (Sugiyama et al.,

2001). This “protective” action of *Hp* infection against ethanol lesions has been attributed to the increased generation of prostaglandin E₂ derived from cyclooxygenase-2 (COX-2) overexpression in *Hp*-infected stomach and has been confirmed recently in a model using daily treatment with LPS (Konturek et al., 2001b).

It is known that LPS produces several neuroendocrine effects, and some of these effects are believed to be mediated through cytokines and hormones, for instance leptin and prolactin, and that this process involves the activation of peripheral autonomic nerves such as efferent and afferent vagal nerves (Mastronardi et al., 2001). This prompted our study to determine the role of leptin, neuropeptides released from sensory afferents, and gastric hormones such as gastrin in the mechanism of enhancing the resistance of gastric mucosa to ASA damage as induced by repetitive treatment with *Hp*-LPS. In a hamster model of Gram-negative bacterial infection, systemic leptin was increased after prolonged administration of LPS, and this was considered to enhance host response to endotoxemia (Grunfeld et al., 1996). Turrin et al. (2001) have shown that LPS applied i.p. activated cytokine production in the brain and at the periphery including in adipose tissue, liver and spleen. In another study, LPS-induced leptin release was mediated through IL-1 β because a soluble IL-1 β receptor antagonist completely blocked the LPS-induced increase in the leptin levels (Francis et al., 1999). Thus, we can conclude that *Hp*-LPS induced protection and adaptation resulting in limitation of ASA damage may depend upon leptin expression and release, and, as shown in this study, could also be mediated by neuropeptides released from sensory afferent nerves. The latter is supported by our present observation that the capsaicin-induced functional ablation of sensory nerves abolished the protective and hyperemic effects of single and repeated administration of *Hp*-LPS. It is suggested that endotoxins such as *Hp*-LPS can affect sensory afferent nerves that in turn may activate the brain-gut axis resulting in

limitation of ASA-induced gastric damage. This conclusion agrees with the observation by Hua et al. (1996) that endotoxin treatment enhanced release of vasoactive CGRP from the primary sensory afferents due to sensitizing their terminals. The mechanism by which enhancement in the plasma IL-1 β and TNF- α induced by ASA was reduced in rats treated repeatedly with *Hp*-derived endotoxin remains to be elucidated, but it could be due to the suppressive action on these cytokines of prostaglandins, nitric oxide (NO) and heat shock proteins (HSP) released *via* overexpression of COX-2, inducible NO synthase (iNOS) and HSP70 mRNA as reported recently (Brzozowski et al., 2003).

The major finding of the present study is that *Hp*-LPS is capable of inhibiting gastric acid secretion, while showing a significant rise in plasma gastrin level. The importance of gastrin in the observed protection and hyperemia appears to be of particular significance as the antagonism of receptors for gastrin (CCK₂) with RPR-102681 completely reversed the inhibition of gastric secretion, gastroprotection and adaptation of gastric mucosa, and the rise in GBF afforded by *Hp*-LPS, whereas the blockade of CCK₁ receptors by loxiglumide eliminated the action of CCK, was ineffective.

It is now becoming evident that non-steroidal anti-inflammatory drugs (NSAIDs) such as ASA may influence the pathogenic effects of *Hp* as a result of possible direct interaction with this microorganism (Wang et al., 2003). However, this was not the case in our study, because the single or repeated parenteral injections with *Hp*-derived endotoxin actually increased mucosal resistance to the damaging effect of ASA in animals treated repeatedly with this endotoxin. The major drawback of this study is that only parenteral administration of LPS but not its intragastric administration was employed. Although our previous studies documented that LPS administered intragastrically in a relatively small dose (1 mg/kg) failed to influence the lesions provoked by acidified ASA, our preliminary observations with a large

intragastric dose of LPS (10 mg/kg i.g.) was found effective. This difference required further explanation but it could be that small *Hp*-LPS doses given into the stomach lumen may not be able to penetrate the thick mucus covering of the surface epithelium to activate the mucosa protective mechanism. However, when *Hp* remains under the mucus layer and is in direct contact with the epithelial cells, it could activate the protective mechanism by local release of its endotoxins such as LPS. Further studies are needed to determine whether *Hp*-LPS present in the gastric lumen can reach the gastric circulation sufficiently to mimic the changes observed after repeated parenteral *Hp*-LPS injections.

Our results with antisecretory effects of *Hp*-LPS and other non-*Hp* related LPSs agree with those of Uehara et al. (1990) and Konturek et al. (2001) that peripheral and central applications of LPS derived from *E.coli* to rats produces profound dose-dependent inhibition of gastric acid output. Since ASA damage depends upon gastric acidity it is reasonable to assume that suppression of gastric acid secretion by this endotoxin could contribute to the limitation of this damage. As shown in the present study, a marked increase in plasma gastrin in rats treated repeatedly with *Hp*-LPS, which is known to exert gastroprotective influence on the gastric mucosa, could result from hypochlorhydria and could contribute to the greater tolerance of this mucosa to ASA-induced gastric damage. This was confirmed in the present study by blocking the receptors for gastrin (CCK₂) which resulted in attenuation of the protective effects of *Hp*-LPS and the related rise in the plasma gastrin levels.

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Legends for Figures

Fig. 1. Effect of single (once) and repeated (5 times) administrations of *Hp*-LPS and LPS of intestinal bacteria such as *C. jejuni*, *Y. enterocolitica* and *B. fragilis* given intraperitoneally (i.p.) at a dose of 1 mg/kg on the area of ASA-induced gastric lesions and the alterations in gastric blood flow (GBF). Data represents the mean \pm SEM of 8-10 rats. The asterisk indicates a significant change compared with the value obtained with rats treated with vehicle and various LPSs. The cross indicates a significant change compared with the value obtained in ASA-treated rats.

Fig. 2A,B,C,D. Representative photomicrographs showing the gross appearance of intact rat gastric mucosa (A), the gastric mucosa exposed to acidified aspirin (ASA; 150 mg/kg in 0.2 N HCl i.g.) (B) or treated 5 times with *Hp*-LPS (1 mg/kg i.p.) (C) or that treated repeatedly (5 times) with *Hp*-LPS and then exposed to ASA (D). Please note: ASA produced gross gastric mucosal lesions localized predominantly in the oxyntic mucosa (arrows) as compared with intact stomach (B vs. A). Repeated treatment with *Hp*-LPS, which by itself induced only few mucosal lesions (C), produced a marked attenuation of the ASA-induced gastric injury (D vs. B).

Fig. 3. Effect of single (once) and repeated (5 times) administrations of *Hp*-LPS given intraperitoneally (i.p.) on the area of gastric lesions induced by acidified aspirin (ASA; 150 mg/kg i.g.) and alterations in plasma gastrin and leptin levels. Data represents the mean \pm SEM of 8-10 rats. The asterisk indicates significant change compared with the value obtained with vehicle (saline) control. The asterisk and cross indicate a significant change compared to the respective values in animals treated with *Hp*-LPS once.

Fig. 4. Mean area of ASA-induced gastric lesions and accompanying changes in gastric blood flow (GBF) and leptin levels in plasma of rats treated with vehicle (saline), exogenous

leptin and CCK-8 applied i.p. at a dose of 10 µg/kg, 8% peptone meal or with *Hp*-LPS (1 mg/kg i.p.) Data represents the mean ± SEM of 8-10 rats. The asterisk indicates a significant change compared with the value obtained with vehicle (control).

Fig. 5. Mean area of ASA-induced gastric lesions and gastric blood flow (GBF) in rats treated with vehicle or *Hp*-LPS (1 mg/kg i.p.) applied once or administered 5 times without or with pretreatment with loxiglumide (30 mg/kg i.p.) or RPR 102681 (10 mg/kg i.p.). Data represents the mean ± SEM of 8-10 rats. The asterisk indicates a significant change compared with the value obtained in control (vehicle) rats. The asterisk and cross indicates a significant change compared to the values obtained with *Hp*-LPS applied once. A single cross indicates a significant change compared with the values obtained with *Hp*-LPS applied once or given 5 times.

Fig. 6. Mean area of ASA-induced gastric lesions and gastric blood flow (GBF) in rats treated with vehicle or *Hp*-LPS (1 mg/kg i.p.) applied once or administered 5 times without or with the pretreatment with capsaicin to induce functional ablation of sensory nerves. Data represents the mean ± SEM of 8-10 rats. The asterisk indicates a significant change compared to the value obtained with vehicle (control). The asterisk and cross indicates a significant change compared to the value obtained with *Hp*-LPS applied once. A single cross indicates a significant change compared to the values obtained with *Hp*-LPS applied once or given 5 times.

Fig. 7. Expression of leptin and β-actin mRNA and protein determined by RT-PCR (left panel) and Western Blotting (right panel) in the gastric mucosa of rats injected with vehicle (line 1), *Hp*-LPS (1 mg/kg i.p.) injected once (line 2) and *Hp*-LPS injected 5 times (line 3). M= DNA marker (Gibco 100 bp ladder).

Table 1.

Effect of single (once) i.p. injection and daily treatments (5 times) with vehicle (saline), *Hp*-LPS and LPSs derived from *B. fragilis*, *Y. enterocolitica* and *C. jejuni* (1 mg/kg) on basal gastric acid output in rats equipped with a chronic gastric fistula (GF). Results are means \pm S.E.M. of 8-10 rats. The asterisk indicates a significant change compared to the value recorded in vehicle-control animals. A cross indicates a significant change compared with the value obtained in rats treated once with endotoxins.

Test	Acid output (μ mol/30 min)
One administration	
<i>Vehicle</i>	158 \pm 14
<i>Hp</i> -LPS	65 \pm 7*
<i>B. fragilis</i> -LPS	74 \pm 9*
<i>Y. enterocolitica</i> -LPS	69 \pm 6*
<i>C. jejuni</i> - LPS	78 \pm 7*
Five daily injections	
<i>Vehicle</i>	149 \pm 12
<i>Hp</i> -LPS	32 \pm 4* ⁺
<i>B. fragilis</i> -LPS	41 \pm 6* ⁺
<i>Y. enterocolitica</i> -LPS	39 \pm 3* ⁺
<i>C. jejuni</i> - LPS	48 \pm 5* ⁺

Table 2.

The effect of a single administration of LPSs from *B. fragilis*, *Y. enterocolitica* and *C. jejuni* (1 mg/kg i.p.) on acidified ASA (150 mg/kg i.g.)-induced gastric lesions and the accompanying changes in GBF. Results are means \pm S.E.M. of six examinations on six rats. The asterisk indicates a significant change in value compared to that obtained with vehicle or LPS alone.

Type of test	Mean lesion area (mm ²)	GBF (% control)
Vehicle (saline)	74 \pm 11	58 \pm 6
<i>B. fragilis</i> -LPS	54 \pm 8*	69 \pm 4*
<i>Y. enterocolitica</i> -LPS	48 \pm 5*	73 \pm 5*
<i>C. jejuni</i> -LPS	52 \pm 7*	70 \pm 3*

Table 3.

Effect of single or daily (5 times) administrations of vehicle and *Hp*-LPS (1 mg/kg i.p.) on plasma IL-1 β and TNF- α levels in rats exposed to acidified aspirin (ASA; 150 mg/kg i.g.).

Data represents the mean \pm SEM of 8-10 rats. The asterisk indicates a significant change compared to the values obtained in intact rats. The single cross indicates a significant change compared with the value obtained in rats pretreated once with vehicle. The double cross indicates a significant change compared with the value obtained in rats with single administration of *Hp*-LPS.

Type of treatment	IL-1 β (pg/ml)	TNF α (pg/ml)
Intact	8 \pm 0.5	0.6 \pm 0.03
<i>One administration</i>		
<i>Hp</i> -LPS	43 \pm 3.2*	3.8 \pm 0.5*
ASA (150 mg/kg i.g.)		
+ Vehicle	116 \pm 9.4*	18.3 \pm 2.6*
+ <i>Hp</i> -LPS	68 \pm 5.6 ⁺	9.4 \pm 1.3 ⁺
<i>Five daily injections</i>		
<i>Hp</i> -LPS	45 \pm 3.4*	3.9 \pm 0.8*
ASA (150 mg/kg i.g.)		
+ Vehicle	109 \pm 6.2*	16.8 \pm 2.1*
+ <i>Hp</i> -LPS	32 \pm 2.9 ⁺⁺	4.2 \pm 0.8 ⁺⁺

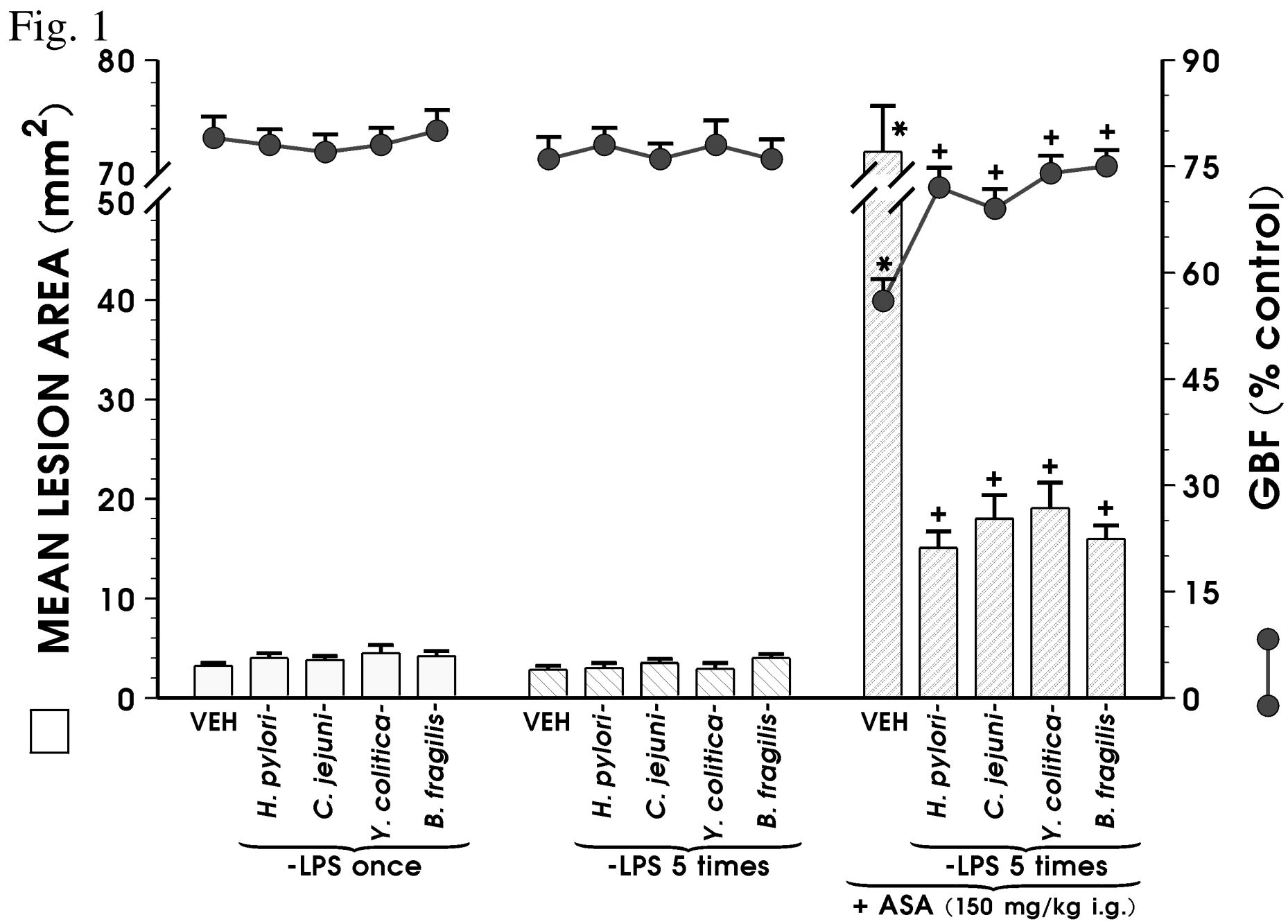


Fig. 2
A, B,
C, D

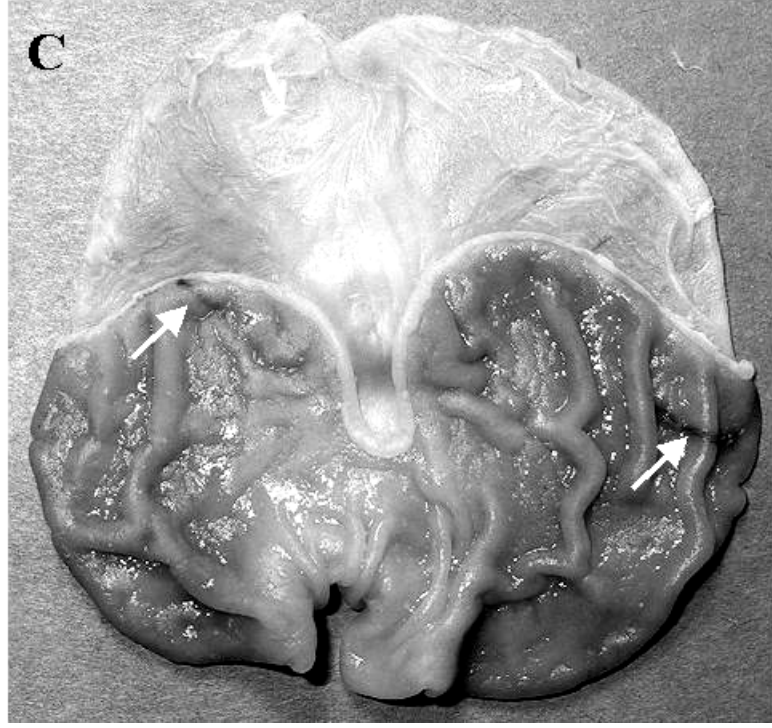
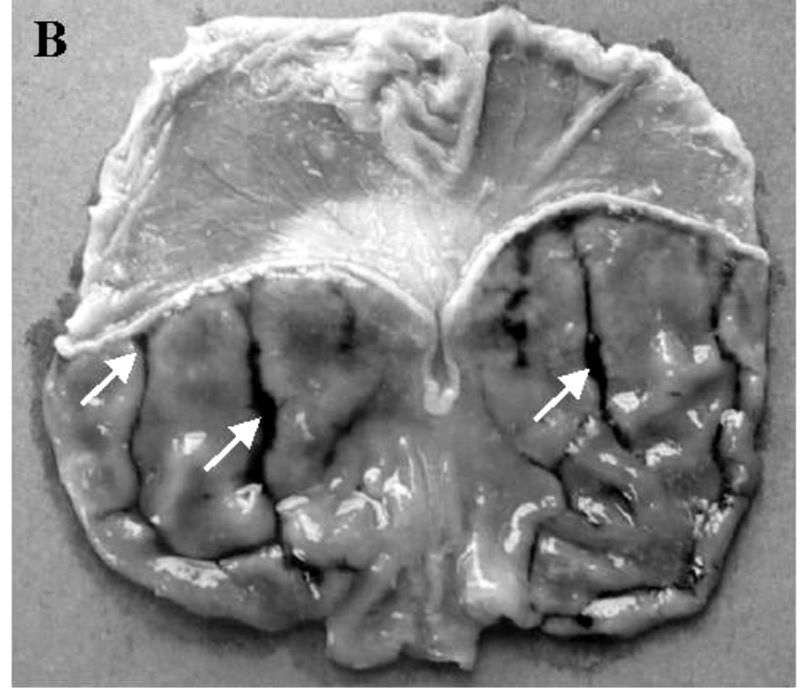
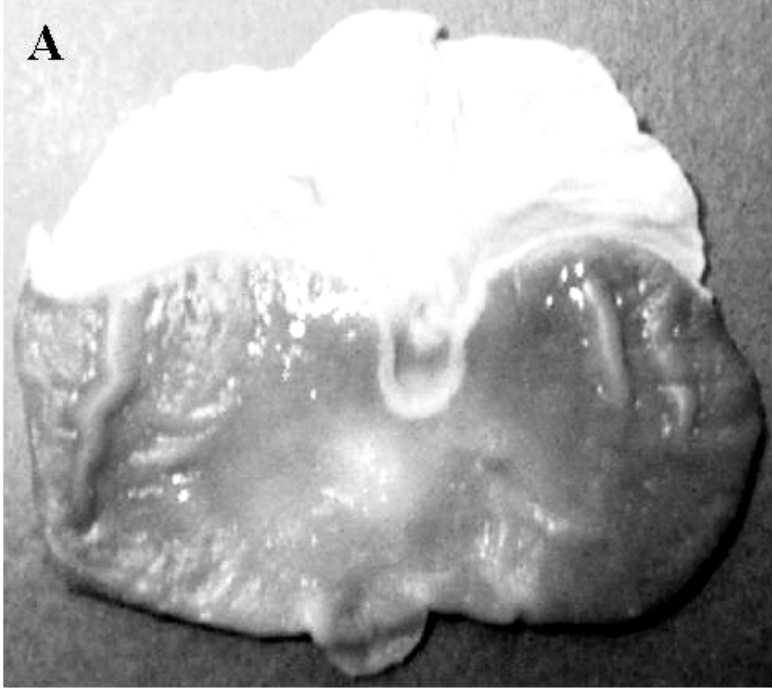


Fig. 3

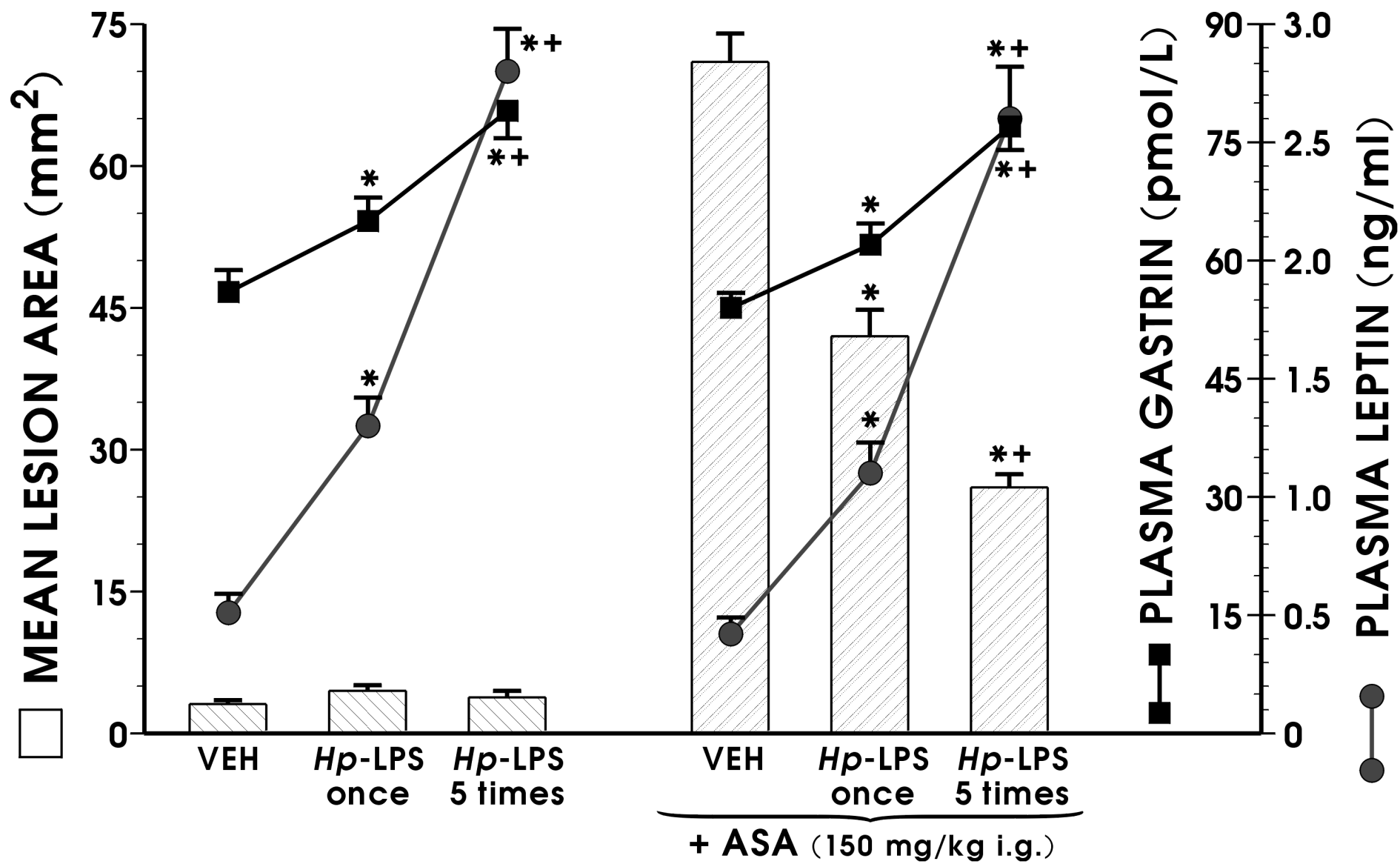


Fig. 4

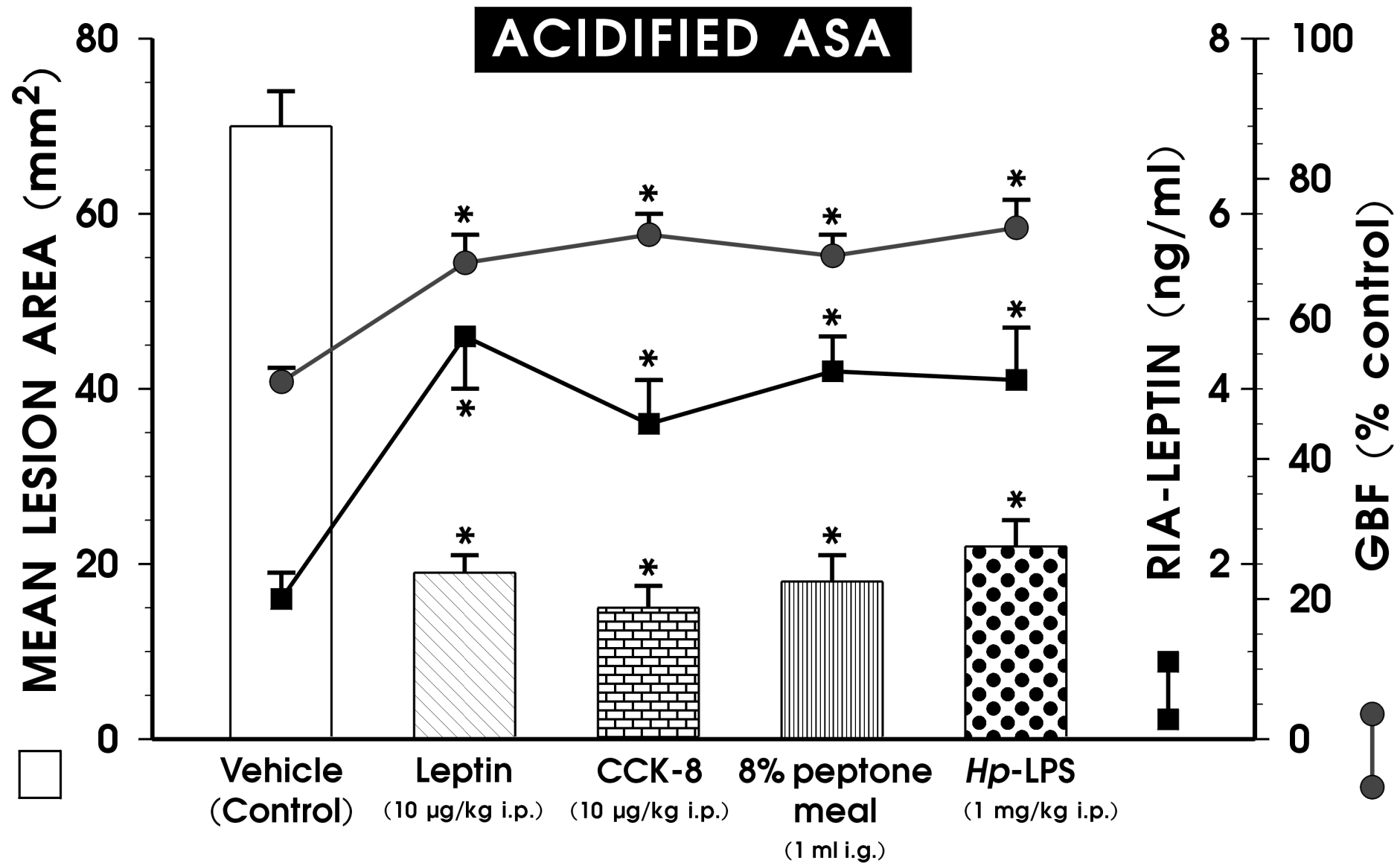


Fig. 5

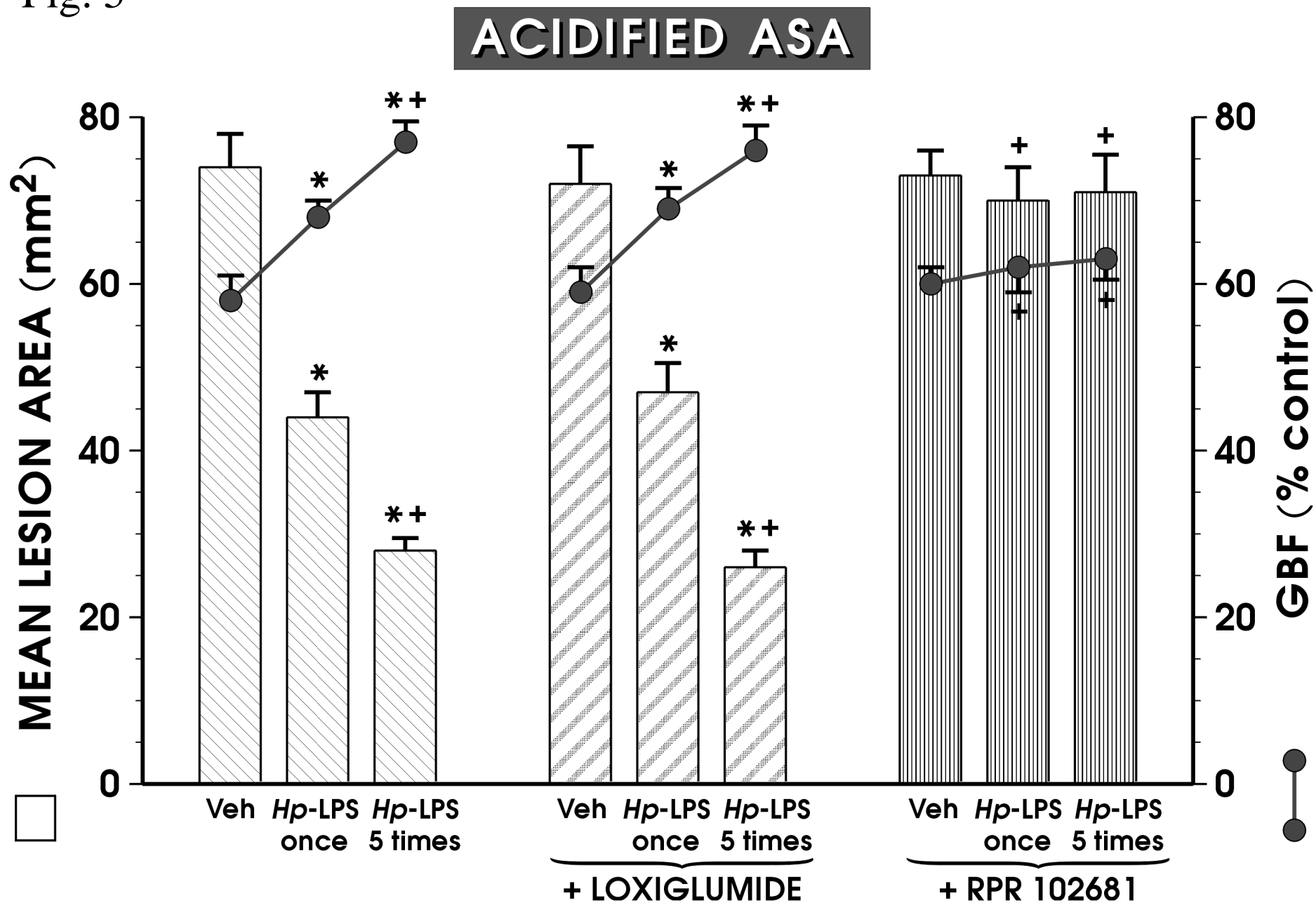


Fig. 6

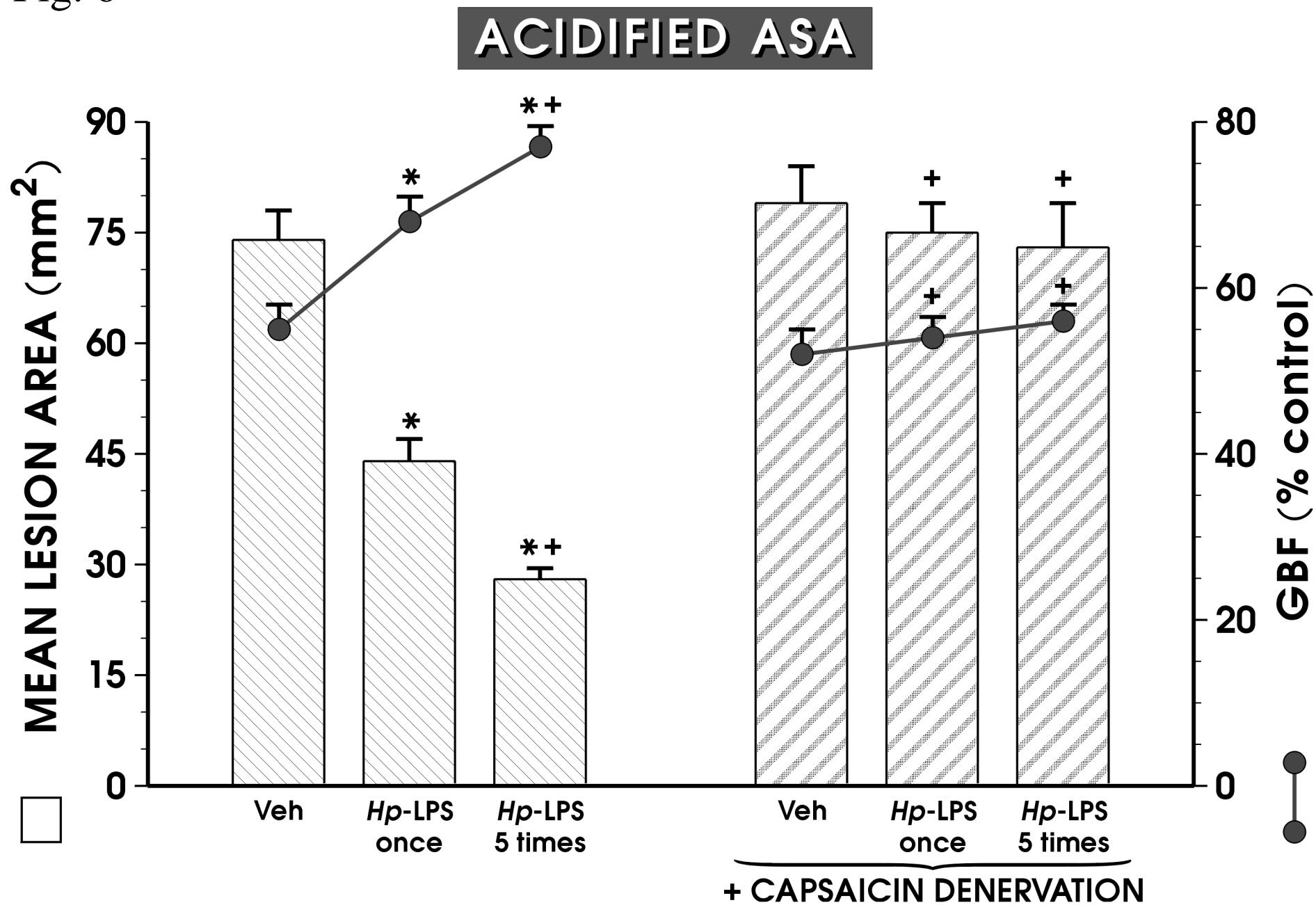


Fig. 7

