Involvement of Capsaicin-Sensitive Afferent Nerves and Cholecystokinin 2/Gastrin Receptors in Gastroprotection and Adaptation of Gastric Mucosa to Helicobacter pylori-Lipopolysaccharide

Tomasz Brzozowski, Peter C. Konturek, Anthony P. Moran, Robert Pajdo, Slawomir Kwiecien, Stanislaw J. Konturek, Zbigniew Sliwowski, Danuta Drozdowicz, Wieslaw W. Pawlik, and Eckhart G. Hahn

Department of Physiology, Jagiellonian University School of Medicine, Cracow, Poland (T.B., R.P., S.K., S.J.K., Z.S., D.D., W.W.P.); Department of Medicine I, University of Erlangen-Nuremberg, Erlangen, Germany (P.C.K., E.G.H.); and Department of Microbiology, National University of Ireland, Galway, Ireland (A.P.M.)

Received January 8, 2004; accepted March 15, 2004

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, gastric blood flow (GBF), expression of mRNA and protein of leptin and plasma leptin, gastrin, interleukin-1β, and tumor necrosis factor-α levels were examined. Single (once) or repeated (five times) 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, up-regulated leptin mRNA and protein, and increased plasma leptin and gastrin levels. Hp-LPS significantly reduced the ASA-induced gastric damage and the accompanying decline in the GBF, and these effects were significantly attenuated by capsaicin denervation and selective antagonism of cholecystokinin-B (CCK2) receptors by RPR-102681 [N-(metoxy-3 phenyl) N-(N-methyl N-phenyl-carbamylmethyl) carbamoylmethyl][3 ureido]-3 phenyl]-2 proproniique 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 and the expression and release of leptin and gastrin exerting 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.

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 Marshall, 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 in addition to ammonia being cytotoxins released by Hp strains expressing the vacuolating cytotoxin A and cytotoxin-associated gene A proteins, Hp-derived lipopolysaccharides (Hp-LPSs), and the enhanced generation of reactive oxygen species (Megraud et al., 1992; Crabtree, 1996; Figura and Tabaqchali, 1996; Moran, 2001a,b). 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,b). 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 (Slo-
C. jejuni, and enterocolitica enter LPSs isolated from enteric bacteria such as ethanol (Pepperman and Soper, 1994; Konturek et al., 1998a,b; 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 controls food intake and energy expenditure (Friedman and Halaas, 1998). Recent studies documented the presence of leptin in the plasma of experimental animals, such as mice and rats, as well as in humans (Shalev et al., 1997; Barbier et al., 1998). 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 (Pepperman 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 (ASA). Finally, the physiological significance of gut hormones such as leptin and gastrin in the adaptation of gastric mucosa, developed by daily injections of endotoxins such as Hp-LPS, requires 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 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 B. fragilis, Y. enterocolitica, and C. 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)-1β and tumor necrosis factor (TNF)α in gastric mucosal integrity, and possible gastric mucosal adaptation afforded by Hp-LPS.

Materials and Methods

Three major series (A, B, and C) consisting of 200 male Wistar rats weighing 180 to 220 g were used. All procedures have been carried out in accordance with the Declaration of Helsinki and were accepted by the 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 orogastic tube (series A). In series B, gastric mucosa was subjected to single or repeated exposures to vehicle (saline) or LPS isolated 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), 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 that 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 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 that were not available to us; therefore, only parenteral administration of this LPS was employed in the present study. The animals received Hp-LPS once and LPS isolated from intestinal bacteria (1 mg/kg) by i.p. route for comparison, or they were treated repeatedly by the same route with Hp-LPS and that of other intestinal bacteria for 4 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 (five 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.

Three hours after 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 a percentage of 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 (five times) with Hp-LPS or LPS isolated from intestinal bacteria were placed in individual Bollman cages (to which the animals were well conditioned) at day 4 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 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 was rinsed gently with 5 to 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.) was given once or administered at the same dose for 4 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 administered 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.p.); 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.g.) and CCK-8 (10 µg/kg) i.p. followed 120 min later by ASA; 5) 8% peptone meal (1 ml per 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 CCK, and CCK 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 CCK, and CCK receptors with RPR-102681 was studied in rats with or without Hp-LPS applied i.p. 2 h prior to ASA was studied in rats with or without Hp-LPS applied i.p. 2 h prior to ASA. 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). Aliquots of RNA samples were stored at ~80°C until analysis.

**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.p. 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). Aliquots of RNA samples were stored at ~80°C until analysis.

**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% SDS, 100 mmol dithiothreitol) by the addition of 1:20 dilution of aprotinin and 1:50 dilution of 100 mmol phenylmethylsulfonyl fluoride. 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 (Bio-Rad,
Hercules, CA) by electroblotting. Skim fast milk powder (5% w/v) in Tris-buffered saline/Tween 20 buffer (137 mmol NaCl, 20 mmol Tris-HCl, pH 7.4, 0.1% Tween 20) was used to block filters for at least 1 h at room temperature. As a primary antibody, 1:500 dilution of specific goat polyclonal antiserum against leptin (Santa Cruz Biochemicals, Santa Cruz, CA) or 1:1000 dilution of rabbit polyclonal anti-β-actin (Sigma Aldrich) antiserum was added to the membrane, followed by an anti-goat or anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:2000; Santa Cruz). Incubation of the primary antibody was followed by three washes with Tris-buffered saline/Tween 20 buffer for 10 min. Incubation of the secondary antibody was followed by four washes for 10 min. Nonisotopic visualization of immunocomplexes was achieved by chemiluminescence using BM chemiluminescence blotting substrate (Boehringer Ingelheim Gmbh, Ingelheim, Germany). Thereafter, the developed membrane was exposed to an X-ray film (Kodak, Wiesbaden, Germany).

Statistical Analysis. Results are expressed as means ± S.E.M. Statistical analysis was done using analysis of variance and a two way analysis of variance test with post hoc Tukey’s honestly significant difference test. Differences of p < 0.05 were considered 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 five daily injections on gastric acid secretion in conscious rats with chronic gastric fistula. The basal gastric acid output in rats treated with vehicle (saline) reached the value of 158 ± 14 μmol/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 with 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 μmol/ml) compared with those in vehicle-control animals (volume of gastric juice, 2.8 ± 0.3 ml/30 min; gastric H⁺ concentration, 56.4 ± 8 μmol/ml). In rats injected daily (five 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 μmol/ml) were significantly lower compared with those in animals injected once with Hp-LPS (volume of gastric juice, 2.0 ± 0.3 ml/30 min; gastric H⁺ concentration, 32.5 ± 4 μmol/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 with that recorded in Hp-LPS-treated animals (Table 1). Five-time daily injections of LPSs derived from B. fragilis, Y. enterocolitica, and C. jejuni caused a 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 CCK₁ receptor antagonist, or RPR-102681, a CCK₂ receptor antagonist, failed to significantly influence basal gastric acid output compared with 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 μmol/30 min in vehicle-control versus 32 ± 4 μmol/30 min in Hp-LPS-treated). The reduction in the acid output induced by Hp-LPS applied five times was not influenced significantly by loxiglumide (gastric acid output, 32 ± 4 μmol/30 min in Hp-LPS-treated versus 38 ± 5 μmol/30 min in loxiglumide plus Hp-LPS applied five times). Administration of RPR-102681 reversed the attenuation of the gastric acid output induced by five daily injections of Hp-LPS (32 ± 4 μmol/30 min in Hp-LPS versus 114 ± 8 μmol/30 min in RPR-102681 plus Hp-LPS applied five 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 five times and failed to significantly alter the GBF when compared with 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 with vehicle treatment. In vehicle-pretreated rats, acidified ASA resulted in multiple gastric lesions and a significant decline in the GBF by about 30% compared with 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 the accompanying decline in the GBF 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 with and without 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 five times with vehicle resulted in 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 five times with Hp-LPS and then subsequently exposed to ASA, there was a significant attenuation of the gastric mucosal injury compared with those treated five times with vehicle and then exposed to ASA (Fig. 2D).

Hp-LPS applied in a single dose of 1 mg/kg i.p. significantly reduced 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 gas-

<table>
<thead>
<tr>
<th>Test</th>
<th>Acid Output μmol per 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>One administration Vehicle</td>
<td>158 ± 14</td>
</tr>
<tr>
<td>Hp-LPS</td>
<td>65 ± 7*</td>
</tr>
<tr>
<td>B. fragilis-LPS</td>
<td>74 ± 9*</td>
</tr>
<tr>
<td>Y. enterocolitica-LPS</td>
<td>69 ± 6*</td>
</tr>
<tr>
<td>C. jejuni-LPS</td>
<td>78 ± 7*</td>
</tr>
<tr>
<td>Five daily injections Vehicle</td>
<td>149 ± 12</td>
</tr>
<tr>
<td>Hp-LPS</td>
<td>32 ± 4**</td>
</tr>
<tr>
<td>B. fragilis-LPS</td>
<td>41 ± 6**</td>
</tr>
<tr>
<td>Y. enterocolitica-LPS</td>
<td>39 ± 3**</td>
</tr>
<tr>
<td>C. jejuni-LPS</td>
<td>48 ± 5**</td>
</tr>
</tbody>
</table>
trin 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 (five 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 significantly raised the GBF (Table 2). As shown in Fig. 1, the repeated parenteral application of these LPSs also significantly reduced the lesions induced by acidified ASA. The protective effects against ASA-induced gastric lesions of these endotoxins injected repeatedly were

---

**Fig. 1.** Effect of single (once) and repeated (five times) administrations of Hp-LPS and LPS of intestinal bacteria such as C. jejuni, Y. enterocolitica, and B. fragilis given i.p. at a dose of 1 mg/kg on the area of ASA-induced gastric lesions and the alterations in GBF. Data represent the mean ± S.E.M. of 8 to 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. 2.** A–D, representative photomicrographs showing the gross appearance of intact rat gastric mucosa (A), the gastric mucosa exposed to acidified ASA (150 mg/kg in 0.2 N HCl i.g.) (B) or treated five times with Hp-LPS (1 mg/kg i.p.) (C), or that treated repeatedly (five 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) compared with intact stomach (B versus A). Repeated treatment with Hp-LPS, which by itself induced only a few mucosal lesions (C), produced a marked attenuation of the ASA-induced gastric injury (D versus B).
accompanied by a significant rise in GBF compared with the respective values obtained in gastric mucosa exposed to ASA alone (Fig. 1).

Figure 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 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, which increased the plasma leptin levels by 2- to 3-fold and significantly raised 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 decline in GBF as those presented in Figs. 1 and 2. The area of these lesions and the accompanying decline in GBF were significantly reduced in rats injected once with Hp-LPS or daily (five times) with this endotoxin. Suppression of CCK_1 receptors with loxiglumide by itself failed to significantly influence the ASA-induced gastric damage and the accompanying decline 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 CCK_2 receptors, which by itself also failed to influence the ASA-

<table>
<thead>
<tr>
<th>Type of Test</th>
<th>Mean Lesion Area</th>
<th>GBF % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (saline)</td>
<td>74 ± 11</td>
<td>58 ± 6</td>
</tr>
<tr>
<td>B. fragilis-LPS</td>
<td>54 ± 8*</td>
<td>69 ± 4*</td>
</tr>
<tr>
<td>Y. enterocolitica-LPS</td>
<td>48 ± 5*</td>
<td>73 ± 5*</td>
</tr>
<tr>
<td>C. jejuni-LPS</td>
<td>52 ± 7*</td>
<td>70 ± 3*</td>
</tr>
</tbody>
</table>

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 ± S.E.M. of six examinations on six rats. The asterisk indicates a significant change in value compared with that obtained with vehicle or LPS alone.

Fig. 3. Effect of single (once) and repeated (five times) administrations of Hp-LPS given i.p. on the area of gastric lesions induced by ASA (150 mg/kg i.g.) and alterations in plasma gastrin and leptin levels. Data represent the mean ± S.E.M. of 8 to 10 rats. The asterisk indicates a significant change compared with the value obtained with vehicle (saline) control. The asterisk and cross indicate a significant change compared with the respective values in animals treated once with Hp-LPS.

Fig. 4. Mean area of ASA-induced gastric lesions and accompanying changes in 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, with 8% peptone meal, or with Hp-LPS (1 mg/kg i.p.). Data represent the mean ± S.E.M. of 8 to 10 rats. The asterisk indicates a significant change compared with the value obtained with vehicle (control).
induced gastric damage, almost completely abolished 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 (five times) significantly reduced the area of ASA-induced gastric lesions and significantly raised the GBF compared with 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 significantly influence GBF but resulted in almost complete elimination of the protective and hyperemic effects induced by Hp-LPS injected once or daily (five times) (Fig. 6).

Effect of Single and Repetitive Treatment with Hp-LPS on Plasma IL-1β and TNF-α Levels. As shown in Table 3, the plasma levels of both proinflammatory cytokines (IL-1β 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-1β 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 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 the 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

TABLE 3

<table>
<thead>
<tr>
<th>Type of Treatment</th>
<th>IL-1β (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>8 ± 0.5</td>
<td>0.6 ± 0.03</td>
</tr>
<tr>
<td>One administration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hp-LPS</td>
<td>43 ± 3.2*</td>
<td>3.8 ± 0.5*</td>
</tr>
<tr>
<td>ASA (150 mg/kg i.g.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Vehicle</td>
<td>116 ± 9.4*</td>
<td>18.3 ± 2.6*</td>
</tr>
<tr>
<td>+ Hp-LPS</td>
<td>68 ± 6.6*</td>
<td>9.4 ± 1.3*</td>
</tr>
<tr>
<td>Five daily injections</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hp-LPS</td>
<td>45 ± 3.4*</td>
<td>3.9 ± 0.8*</td>
</tr>
<tr>
<td>ASA (150 mg/kg i.g.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Vehicle</td>
<td>109 ± 6.2*</td>
<td>16.8 ± 2.1*</td>
</tr>
<tr>
<td>+Hp-LPS</td>
<td>32 ± 2.9*</td>
<td>4.2 ± 0.8*</td>
</tr>
</tbody>
</table>

Fig. 5. Mean area of ASA-induced gastric lesions and GBF in rats treated with vehicle or Hp-LPS (1 mg/kg i.p.) applied once or administered five times with or without pretreatment with loxiglumide (30 mg/kg i.p.) or RPR-102681 (10 mg/kg i.p.). Data represent the mean ± S.E.M. of 8 to 10 rats. The asterisk indicates a significant change compared with the value obtained in control (vehicle) rats. The single cross indicates a significant change compared with 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 five times.
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, which have been proven 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 up-regulation 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 that an increase in the 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 and thereby emphasizes 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 agrees with the original finding of Tepperman and Soper (1994), who showed protection of the rat gastric mucosa against ethanol induced damage by 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 the 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 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. Some of these effects are believed to be mediated through cytokines and hormones (for instance, leptin and prolactin), and this process involves the activation of peripheral autonomic nerves such as effenter 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-1β because a soluble IL-1β 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 the 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 the release of the vasoactive calcitonin gene-related peptide from the primary sensory afferents due to sensitizing their terminals. The mechanism by which enhancement in the plasma IL-1β 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, and heat shock proteins released via overexpression of cyclooxygenase-2, inducible nitric-oxide synthase, and heat shock protein 70 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 gastric level. The importance of gastrin in the observed protection and hyperemia seems to be particularly significant because the antagonism of receptors for gastrin (CCK2) with RPR-102681 completely blocked the LPS-induced increase in the levotolin levels (Francis et al., 1999). Thus, we can conclude that Hp-LPS-induced protection and adaptation resulting in the 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 the release of the vasoactive calcitonin gene-related peptide from the primary sensory afferents due to sensitizing their terminals. The mechanism by which enhancement in the plasma IL-1β 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, and heat shock proteins released via overexpression of cyclooxygenase-2, inducible nitric-oxide synthase, and heat shock protein 70 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 gastric level. The importance of gastrin in the observed protection and hyperemia seems to be particularly significant because the antagonism of receptors for gastrin (CCK2) with RPR-102681 completely blocked the LPS-induced increase in the levotolin levels (Francis et al., 1999). Thus, we can conclude that Hp-LPS-induced protection and adaptation resulting in the 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 the release of the vasoactive calcitonin gene-related peptide from the primary sensory afferents due to sensitizing their terminals. The mechanism by which enhancement in the plasma IL-1β 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, and heat shock proteins released via overexpression of cyclooxygenase-2, inducible nitric-oxide synthase, and heat shock protein 70 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 gastric level. The importance of gastrin in the observed protection and hyperemia seems to be particularly significant because the antagonism of receptors for gastrin (CCK2) with RPR-102681 completely blocked the LPS-induced increase in the levotolin levels (Francis et al., 1999). Thus, we can conclude that Hp-LPS-induced protection and adaptation resulting in the 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 the release of the vasoactive calcitonin gene-related peptide from the primary sensory afferents due to sensitizing their terminals. The mechanism by which enhancement in the plasma IL-1β 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, and heat shock proteins released via overexpression of cyclooxygenase-2, inducible nitric-oxide synthase, and heat shock protein 70 mRNA, as reported recently (Brzozowski et al., 2003).

Address correspondence to: Dr. T. Brzozowski, Department of Physiology, Jagiellonian University School of Medicine, 16 Grzegorzecka Str., 31-531 Cracow, Poland. E-mail: mbrzozo@cyf-kr.edu.pl