Peptidergic and cholinergic neurons and mediators in peptone-induced gastroprotection: role of cyclooxygenase-2

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Ehrlich, Karlheinz, Susanne Plate, Thomas Stroff, Britta Gretzer, Michael Respondek, and Brigitta M. Peskar. Peptidergic and cholinergic neurons and mediators in peptone-induced gastroprotection: role of cyclooxygenase-2. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G955–G964, 1998.—This study investigates the neural pathways, mediators, and cyclooxygenase isoenzymes involved in the gastroprotection conferred by peptone in rats. Intragastric perfusion with 8% peptone protected against gross and histological damage induced by subsequent perfusion with 50% ethanol. The gastroprotective effect of peptone was near maximally inhibited by gastrin immunoneutralization, inactivation of capsaicin-sensitive afferent neurons, calcitonin gene-related peptide (CGRP), or somatostatin receptors, and by the nitric oxide (NO) synthase inhibitor L-nitro-arginine methyl ester and was partially (46%) counteracted by atropine. Indomethacin and the selective cyclooxygenase-2 inhibitors NS-398 and L-745,337 dose dependently (50% inhibitory dose, 4.2, 0.8, and 1.5 mg/kg, respectively) attenuated the peptone-induced protection. Dexamethasone was ineffective. These results indicate that protective effects of peptone involve endogenous gastrin and possibly somatostatin and are mediated by capsaicin-sensitive afferent, cholinergic, and bombesin/GRP neurons. CGRP, NO, and prostaglandins participate as essential mediators. The study provides evidence that prostaglandins derived from a constitutive cyclooxygenase-2 contribute to mucosal defense in the presence of ulcerogens and thus participate in homeostatic functions of the stomach.

The present study was designed to examine the hormonal and neural pathways through which peptone in the stomach increases the resistance of the mucosa to injury and to characterize the mediators involved. The results provide further evidence that the protective response to peptone involves endogenous gastrin and possibly somatostatin and is mediated by capsaicin-sensitive afferent neurons, cholinergic neurons, and bombesin/GRP neurons. CGRP, NO, and prostaglandins are involved as essential mediators of the protective effect. The selective COX-2 inhibitors NS-398 [N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (6) and L-745,337 [5-methanesulfonamido-2-(2,4-difluorothio-phenyl)-1-indanone] (2) counteract the protection conferred by peptone, suggesting that COX-2-derived prostaglandins contribute to protective effects and thus have a function in gastrointestinal homeostasis.
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MATERIALS AND METHODS

Experimental Protocol

Male Wistar rats (weighing 180–220 g) were deprived of food for 24 h with free access to tap water. Rats were kept on a 12:12-h light-dark cycle and under conditions of controlled temperature (22 ± 1°C). All experimental protocols were approved by the Animal Care Committee of Ruhr-University of Bochum. For gastric perfusion experiments anesthesia was induced by injection of pentobarbital (50 mg/kg ip).

Protective effect of peptone. Rats were anesthetized and tracheotomized, and the abdomen was opened by a midline incision. The gastric lumen was perfused at a rate of 0.9 ml/min via a needle injected into the stomach lumen through the forestomach. The perfusate was collected through a cannula (3 mm diameter) inserted from the duodenum through the pylorus. Perfusions were performed using isotonic peptone solution (8% peptone dissolved in H2O, 300 mosmol/l) for 20 min followed by perfusion with 8% peptone dissolved in 50% ethanol for 15 min. Controls were perfused with saline for 20 min followed by 50% ethanol in saline for 15 min. After the ethanol-perfusion period, rats were killed by cervical dislocation. The stomach was removed, and gross mucosal damage was assessed in a blinded manner by calculation of a lesion index using a 0–3 scoring system based on the number and length of lesions, as described previously (31).

For histological study, a strip of the stomach wall parallel to the limiting ridge was processed using routine methods, stained with hematoxylin and eosin, and examined under a light microscope. Six rats per group were studied. One full-length portion of tissue across the entire corpus was cut from each stomach (section length, 3.2 ± 0.07 cm, n = 48). The sections were evaluated in a randomized blinded fashion by a histologist who was unaware of the experimental protocol. The following two grades of histological injury were assessed. Grade 1 was classified as superficial damage confined to the surface epithelium, and grade 2 was deep damage extending beyond the surface epithelium into the region of the pits and glands. For each stomach strip the length of mucosal areas showing superficial and deep damage was determined and expressed as a percentage of the total section length studied.

Functional ablation of afferent neurons. Rats were treated with a subcutaneous injection of capsaicin (25 and 50 mg/kg on day 1, 50 mg/kg twice on day 2). Capsaicin was dissolved in 10% ethanol, 10% Tween 80, and 80% saline (vol/vol). Control animals received equal volumes (2 ml/kg) of vehicle. All injections were performed under ether anesthesia, and to counteract the respiratory impairment associated with the administration of capsaicin, the rats were pretreated with terbutaline (0.2 mg/kg), atropine (0.2 mg/kg), and theophylline (20 mg/kg) before capsaicin injection. Rats were used for experiments 14 days after this treatment. On the day before the experiments, inactivation of afferent neurons was ascertained by evaluating the reduction of the protective wiping movements in response to intracural instillation of a 0.1 mg/ml solution of capsaicin.

Effect of atropine and methyl atropine. Rats were treated with atropine (1 mg/kg iv) 10 min before the intragastric perfusion with 8% peptone. After a 20-min perfusion period, the lumen was perfused with 8% peptone in 50% ethanol for a further 15 min. Controls received the vehicle (0.5 ml/kg of saline). To differentiate between a central and peripheral mechanism of the atropine effect, experiments were also performed with methyl atropine (1 mg/kg iv), which does not cross the blood–brain barrier.

Effect of anti-CGRP antibodies. Globulin aliquots were prepared by ammonium sulfate precipitation from 2 ml of a polyclonal anti-rat CGRP antiserum raised in a rabbit or from 2 ml of serum obtained from nonimmunized rabbits. Details of the immunization procedure, preparation of globulin fractions, and the characterization of the antiserum have been described previously (16). Globulin aliquots were injected intravenously 10 min before starting the intragastric peptone perfusion.

Effect of human CGRP-(8–37). Groups of rats received the CGRP1 receptor antagonist human (h) CGRP-(8–37) (500 pmol · kg−1 · min−1 for 40 min) or vehicle (0.1% BSA in saline) by intravenous infusion at a rate of 20 µl/min. Infusions of hCGRP-(8–37) or vehicle were started 5 min before the intragastric peptone perfusion and were continued until the end of the experiment.

Effect of inhibition of NO biosynthesis. Groups of rats were pretreated 10 min before intragastric peptone perfusion with intravenous injection of the NO biosynthesis inhibitor N5-nitro-arginine methyl ester (L-NAME); 10 mg/kg) or its inactive enantiomer D-NAME (10 mg/kg). Additional groups of rats received L-arginine or D-arginine (each 400 mg/kg iv) immediately before L-NAME.

Effect of antigastrin antibodies. Globulin aliquots were prepared by ammonium sulfate precipitation from 2 ml of a polyclonal anti-rat gastrin-17 antiserum raised in a rabbit. The immunization procedure and preparation of globulin fractions were identical to those described previously for the anti-CGRP antibodies (16). Globulin aliquots were injected intravenously 10 min before starting the intragastric peptone perfusion. Previous experiments have shown that injection of the antigastrin antibodies abolished the protective effect of intravenously infused gastrin-17 (data not shown).

Effect of blockade of gastrin receptors. Groups of rats were treated with intravenous injections of the selective gastrin (CCK-B) receptor antagonist CAM-1028 [4-[(2-[3H-indol-3-yl]-2-methyl-1-oxo-2-((1,7,7-trimethyl-bicyclo(2.2.1)hept-2-yl)-oxy(aryl)amino)propyl]amino]1-phenylethyl] amino-4-oxo-15-oxa-5(15)6-butoanotate N-methyl-D-glucamine (0.3–3 mg/kg) or of the CCK-A receptor antagonist CAM-1481 [N-(α-methyl-N-[tricyclo(3.3.1.1^3,7)dec-2-yl-oxoxy(aryl)carbonyl]-L-tryptophyl)-D-3-(phenylmethyl)-β-alanine] (3 mg/kg) 10 min before starting the intragastric peptone perfusion. Previous studies have shown that CAM-1028, but not CAM-1481, abolished the protective effect of intravenous infusions of gastrin-17 (data not shown).

Effect of blockade of bombesin/GRP receptors. Groups of rats were treated with intravenous injections of the selective bombesin/GRP receptor antagonist D-22213 [RC-3095, d-Tp4,Leu14-bombesin-(6–14)] and b (25). Injections (180–720 nmol/kg) were administered 10 min before injection of bombesin (6 nmol/kg iv) or 1 min before and 15 min after starting the intragastric peptone perfusion. These studies showed that a single injection of D-22213 at 540 nmol/kg abolished the protective effect of bombesin. The effect was, however, extremely short-lived, and suppression of bombesin-induced protection lasting for a period of 40 min (as necessary to counteract the effect of a 35-min intragastric peptone perfusion) was only achieved when D-22213 was injected twice during this period. Additional groups of rats were treated with intravenous injection of D-22213 (360 and 720 nmol/kg) 5 min before and 10 min after starting an intravenous infusion of gastrin-17 (50 pmol · kg−1 · min−1 for 20 min). Fifteen minutes after starting the gastrin perfusion, 1 ml of 70% ethanol was instilled orally, and mucosal damage was assessed 5 min later.
Effect of blockade of somatostatin receptors. Groups of rats were treated with the somatostatin receptor antagonist cycl(7-aminooctanoyl-Phe-o-Trp-Lys-Thr[BZL]) (5) (0.1–3 nmol/kg iv) 10 min before starting the intragastric perfusion with 8% peptone. Additional groups of rats received the somatostatin receptor antagonist (3 and 10 nmol/kg iv) 10 min before an intravenous infusion of gastrin-17 (50 pmol·kg⁻¹·min⁻¹ for 20 min). Ethanol (1 ml, 70%) was instilled orally 15 min after starting the gastric infusion, and mucosal damage was assessed 5 min later.

Effect of inhibition of COX-1 and COX-2. To assess the role of prostaglandins generated via the COX-1 and COX-2 pathways, groups of rats were treated orally with the dual COX-1 and COX-2 inhibitor indomethacin (0.4–20 mg/kg), the selective COX-2 inhibitors NS-398 (0.04–3 mg/kg) and L-745,337 (0.7–5 mg/kg), or dexamethasone (3 mg/kg). COX inhibitors were administered 60 min before the peptone perfusion started and dexamethasone was administered 24 and 2 h before the peptic perfusion. Controls received the vehicle (0.25% methylcellullose, 2.5 ml/kg).

To exclude unspecific damage-modifying effects, all pretreatments used in the study were examined in rats treated with intragastric ethanol perfusion in the absence of peptone.

Measurement of Gastric Mucosal Blood Flow

After induction of anesthesia, rats were tracheotomized and the stomach was exposed by a midline incision. A plastic cannula (3.5 mm diameter) was inserted in the forestomach and tied in place to allow free access to the gastric lumen for the measurement of blood flow by laser-Doppler flowmetry. A Teflon-coated laser optic probe (standard probe, 0.25 mm fiber separation; PeriFlux PF 409, Perimed) was inserted into the gastric lumen via the forestomach cannula and allowed to rest on the corpus mucosa. After a resting period of 60 min, gastric mucosal blood flow was recorded continuously with a laser-Doppler flow sensor (diode laser of wavelength 780 nm; PeriFlux 4001 Master, Perimed). The jugular vein was cannulated for continuous infusion of saline (1.5 ml/h) to avoid dehydration. Then the gastric lumen was perfused with 8% peptone in H₂O for 20 min followed by perfusion with 8% peptone in 50% ethanol for 15 min, and gastric mucosal blood flow was monitored until the end of the experiment. Controls were perfused with saline instead of peptone. To exclude an interference of the intragastric peptone solution with the measurement of gastric mucosal blood flow, the hyperemic response to intragastric perfusion with capsaicin (320 µM, 80 µl/min for 20 min) was compared in the absence and presence of luminal 8% peptone.

Measurement of Systemic Arterial Blood Pressure

In anesthetized rats, the carotid artery was cannulated for measurement of mean systemic arterial blood pressure (Isotec pressure transducer; Hugo Sachs, Hugstetten, Germany). After a resting period of 60 min, the stomachs were perfused with 8% peptone in H₂O for 20 min followed by perfusion with 8% peptone in 50% ethanol for 15 min, and mean arterial blood pressure was monitored until the end of the experiment. Controls received intragastric perfusions with saline instead of peptone.

Assessment of Eicosanoid Formation

Groups of rats were perfused with saline or 8% peptone for 20 min. Then the stomach was removed and opened, and mucosal fragments from the corpus region were excised, blotted, and weighed. Two aliquots of 40 mg of tissue were incubated in oxygenated Tyrode solution for 10 min at 37°C.

After incubation, the medium was removed and analyzed for the content of prostaglandin E₂ and 6-ketoprostaglandin F₁α using RIA. Additional groups of rats were treated intravenously with atropine or orally with indomethacin (20 mg/kg), NS-398 (3 mg/kg), or L-745,337 (5 mg/kg). Controls received the corresponding vehicles. Sixty minutes later, the stomach was removed and mucosal fragments were incubated as described above.

Statistical Analysis

All data are expressed as means ± SE of n values. Comparisons between groups were made using Student’s t-test for paired or unpaired data as appropriate or the Wilcoxon rank test for nonparametric data. P < 0.05 was considered significant.

Drugs

D-22213 (RC-3095) was donated by Dr. J. Engel (Asta Medica, Dresden, Germany). L-745,337 was a generous gift from Dr. A. W. Ford-Hutchinson (Merck-Frosst, Montreal, Canada). CAM-1481 and CAM-1028 were donated by Dr. D. Horwell (Neuroscience Research Center, Parke-Davis, Cambridge, UK). NS-398 was from Cayman Chemical (Ann Arbor, MI). Atropine sulfate, atropine methyl bromide, and all other chemicals were purchased from Sigma Chemical (St. Louis, MO). Gastrin-17 and hCGRP-(8—7) were dissolved in 0.1% BSA in saline and kept frozen until use. All dilutions were freshly prepared before each experiment.

RESULTS

Effect of Functional Ablation of Afferent Neurons

Perfusion of the gastric lumen with 50% ethanol produced extensive mucosal damage in rats with intact afferent neurons (lesion index 34 ± 2, n = 7) or with capsaicin-induced functional ablation of afferent neurons (lesion index 36 ± 6, n = 6). In rats with intact afferent neurons, perfusion with peptone (8%) prevented mucosal injury caused by a subsequent ethanol perfusion (lesion index 9 ± 1, n = 7; P < 0.001). In rats pretreated with a neurotoxic dose of capsaicin to induce functional ablation of afferent neurons, the protective effect of intraluminal peptone was lost (lesion index 33 ± 2, n = 8) (Fig. 1).

Effect of Atropine and Methyl Atropine

Atropine had no effect on gastric mucosal damage induced by perfusion with 50% ethanol in the absence of peptone but significantly (P < 0.001) attenuated the protective effect of intragastric peptone by 46% against a subsequent ethanol perfusion (lesion index 20 ± 3 vs. 9 ± 1, with (n = 10) or without atropine (n = 7), respectively). Identical inhibition of peptone-induced protection was observed with methyl atropine, which has no central activity (lesion index 25 ± 4, n = 6; P < 0.001) (Fig. 1).

Effect of Peptone on Gastric Mucosal Blood Flow

Basal values for gastric mucosal blood flow did not significantly change from the initial control value throughout the observation period. Perfusion of the gastric lumen with peptone for 20 min had only minor effects on gastric mucosal blood flow (13% increase over
Effect of Peptone on Gastric Mucosal Blood Flow

Table 1. Effect of peptone on gastric mucosal blood flow

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>% Increment of Gastric Mucosal Blood Flow</th>
</tr>
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<tbody>
<tr>
<td>Saline</td>
<td>5</td>
<td>5 ± 6</td>
</tr>
<tr>
<td>Peptone</td>
<td>9</td>
<td>13 ± 6*</td>
</tr>
<tr>
<td>Saline + ethanol</td>
<td>5</td>
<td>36 ± 9</td>
</tr>
<tr>
<td>Peptone + ethanol</td>
<td>6</td>
<td>28 ± 8</td>
</tr>
</tbody>
</table>

Values are means ± SE of n rats/group. Rats were perfused with peptone (8%) in H2O for 20 min followed by perfusion with 8% peptone in 50% ethanol for 15 min. Controls were perfused with saline for 20 min followed by perfusion with 50% ethanol in saline for 15 min. Gastric mucosal blood flow was monitored from 10 min before starting gastric perfusion until end of experiment. Values represent maximum % increment of gastric mucosal blood flow observed during the corresponding perfusion period compared with pretreatment values. *P < 0.01 vs. pretreatment values.

Effect of Anti-CGRP Antibodies

Serum obtained from the immunized rabbit recognized rat α-CGRP and rat β-CGRP (89% cross reaction), whereas serum obtained from nonimmunized rabbits did not recognize the two forms of CGRP. Intravenous injection of globulin aliquots prepared from serum of the immunized rabbit abolished the protective effect of peptone (lesion index 31 ± 2, n = 8). Globulin aliquots prepared from nonimmunized rabbits had no effect (lesion index 8 ± 1, n = 4) (Fig. 2).

Effect of Anti-Gastrin Antibodies

As shown in Fig. 4, intravenous injection of globulin aliquots prepared from serum of the rabbit immunized against rat gastrin neutralized and CGRP1 receptor blockade on protective effect of peptone. Peptone was perfused through the gastric lumen for 20 min followed by perfusion with 50% ethanol in peptone for 15 min. Groups of rats were pretreated (10 min) with intravenous injections of globulin fractions prepared from serum of a rabbit immunized against rat α-CGRP or from normal rabbit serum (NRS) obtained from nonimmunized rabbits. Other rats were treated with intravenous infusion of the CGRP1 receptor antagonist hCGRP-(8–37) (500 pmol·kg−1·min−1) iv for 40 min without pretreatment. Controls were perfused with saline instead of peptone. Bars are means ± SE of 6–9 rats. *P < 0.001 vs. saline-perfused controls; *P < 0.001 vs. rats perfused with peptone without pretreatments.

Effect of Inhibition of NO Biosynthesis

Treatment with L-NAME (10 mg/kg iv) augmented gastric mucosal damage caused by ethanol perfusion of the gastric lumen from 30 ± 1 in vehicle-treated rats to 36 ± 2 (n = 6/group; P < 0.05) and antagonized the protective effect of peptone (lesion index 29 ± 2, n = 5; P < 0.001 vs. control). d-NAME (10 mg/kg) was inactive (lesion index 9 ± 2, n = 5; P < 0.001 vs. L-NAME), indicating stereospecificity. Concurrent treatment with L-arginine (400 mg/kg) reversed the effect of L-NAME (lesion index 15 ± 2, n = 6), whereas d-arginine had no effect (lesion index 40 ± 3) (Fig. 3).

Effect of Anti-Gastrin Antibodies

As shown in Fig. 4, intravenous injection of globulin aliquots prepared from serum of the rabbit immunized against rat gastrin neutralized and CGRP1 receptor blockade on protective effect of peptone. Peptone was perfused through the gastric lumen for 20 min followed by perfusion with 50% ethanol in peptone for 15 min. Groups of rats were pretreated (10 min) with intravenous injections of globulin fractions prepared from serum of a rabbit immunized against rat α-CGRP or from normal rabbit serum (NRS) obtained from nonimmunized rabbits. Other rats were treated with intravenous infusion of the CGRP1 receptor antagonist hCGRP-(8–37) (500 pmol·kg−1·min−1) iv for 40 min without pretreatment. Controls were perfused with saline instead of peptone. Bars are means ± SE of 6–9 rats. *P < 0.001 vs. saline-perfused controls; *P < 0.001 vs. rats perfused with peptone without pretreatments.
against rat gastrin-17 significantly ($P < 0.001$) attenuated the protective effect of peptone ($\text{lesion index } 25 \pm 2.5, n = 8$). Globulin aliquots prepared from nonimmunized rabbits had no effect (see Fig. 2).

**Effect of Blockade of Gastrin Receptors**

Pretreatment with the selective gastrin (CCK-B) receptor antagonist CAM-1028 dose dependently counteracted the protective effect of peptone ($50\%$ inhibitory dose ($\text{ID}_{50}$), $0.5 \text{ mg/kg}$). CAM-1028 at a dose of $1 \text{ mg/kg}$ abolished the protection. In contrast, the selective CCK-A receptor antagonist CAM-1481 at a dose of $3 \text{ mg/kg}$ was ineffective ($\text{lesion index } 10 \pm 1$ vs. $9 \pm 1$, rats treated with CAM-1481 vs. treated with peptone only, respectively; $n = 6$ for each group). Results are shown in Fig. 4.

**Effect of Blockade of Bombesin/GRP Receptors**

Pretreatment with the selective bombesin/GRP receptor antagonist D-22213 administered as a single intravenous injection dose dependently ($\text{ID}_{50}$, $320 \text{ nmol/kg}$) inhibited the protective effect of bombesin ($6 \text{ nmol/kg iv}$) and at a dose of $540 \text{ nmol/kg}$ abolished the protection. Similarly, the protective effect of intragastric perfusion with peptone was dose dependently inhibited by D-22213 ($\text{ID}_{50}$, $438 \text{ nmol/kg}$), although due to the extremely short half-life of biological activity, D-22213 had to be injected twice to antagonize the protective effect of peptone. At the dose of $720 \text{ nmol/kg}$, however, D-22213 completely blocked the protective activity of intragastric peptone. D-22213 (720 nmol/kg, injected twice) partially attenuated the protective activity of gastrin-17 ($50 \text{ pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) (Fig. 5).

**Effect of Blockade of Somatostatin Receptors**

Intravenous injection of the somatostatin receptor antagonist cyclo(7-aminoheptanoyl-Phe-D-Trp-Lys-Thr-[BZL]) ($0.1–3 \text{ nmol/kg iv}$) counteracted the protective effect of intragastric perfusion with $8\%$ peptone in a dose-dependent manner ($\text{ID}_{50}$, $0.7 \text{ nmol/kg}$). The highest dose studied abolished the peptone-evoked protection. In contrast, the same somatostatin receptor antagonist up to a dose of $10 \text{ nmol/kg}$ had no significant effect on the protective activity of gastrin-17 ($50 \text{ pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) (Fig. 6).

**Effect of Inhibition of COX-1 and COX-2**

Indomethacin ($0.4–20 \text{ mg/kg}$), which inhibits both COX-1 and COX-2 dose dependently, antagonized the protective effect of intragastric peptone perfusion ($\text{ID}_{50}$, $4.2 \text{ mg/kg}$). Indomethacin at $10 \text{ mg/kg}$ reduced the peptone-induced protection by $66\%$ ($\text{lesion index } 22 \pm 2$ vs. $10 \pm 1$, with ($n = 7$) vs. without indomethacin ($n = 6$), respectively; $P < 0.001$). Likewise, the selective COX-2 inhibitors NS-398 ($0.04–3 \text{ mg/kg}$) and L-745,337 ($0.7–5 \text{ mg/kg}$) dose dependently inhibited the protective effect of intraluminal peptone ($\text{ID}_{50}$, $0.8$ and $1.5$...
mg/kg, respectively). The maximum inhibition of peptone-evoked protection was 55% with NS-398, reached at a dose of 1 mg/kg, and 73% with L-745,337, reached at a dose of 2 mg/kg. Increasing the doses of indomethacin or of the selective COX-2 inhibitors did not augment the inhibitory effect. Dexamethasone did not affect peptone protection (Fig. 7). None of the drugs significantly affected ethanol-induced gastric mucosal damage in the absence of peptone.

**Histological Injury**

Treatment with peptone prevented the development of grade 2 deep histological mucosal injury extending beyond the surface epithelium into the region of the pits and glands in rats with intact afferent neurons but not in rats with functional ablation of these nerves. In rats with intact afferent neurons, histological damage of the mucosa was significantly higher in rats treated before peptone with anti-CGRP antibodies, L-NAME (10 mg/kg iv), D-22213 (720 nmol/kg iv), NS-398 (1 mg/kg po), or L-745,337 (2 mg/kg po) before intragastric peptone perfusion were compared with rats perfused with peptone alone. Controls were perfused with saline. Afferent denervation was induced by pretreatment with capsaicin 2 wk before experiments were performed. Values are means ± SE of 6 experiments per group. *P < 0.001 vs. control; •P < 0.02, •P < 0.01, •P < 0.002 vs. rats perfused with peptone alone; •P < 0.001 vs. peptone in rats with intact afferent neurons.

No significant effects on the extent of grade 1 superficial damage (data not shown).

**Eicosanoid Formation and Effect of COX Inhibitors and Atropine**

Perfusion of the gastric lumen with 8% peptone for 20 min did not increase mucosal synthesizing capacity for prostaglandin E2 and 6-ketoprostaglandin F1α. Pretreatment with indomethacin (20 mg/kg) suppressed ex vivo release of prostaglandin E2 and 6-ketoprostaglandin F1α, from gastric mucosal tissues by 93% and 96%, respectively. In contrast, pretreatment with NS-398 (3 mg/kg) and L-745,337 (5 mg/kg) did not inhibit gastric mucosal prostaglandin synthesizing capacity (Table 2). Likewise, pretreatment with atropine (1 mg/kg) did not reduce mucosal formation of prostaglandin E2 (1,485 ± 320 vs. 1,195 ± 230 pg·mg⁻¹·10 min⁻¹ in controls) and 6-ketoprostaglandin F1α (730 ± 90 vs. 690 ± 95 pg·mg⁻¹·10 min⁻¹ in controls, n = 6/group). Qualitatively identical results were obtained when the effects of COX inhibitors and atropine were assessed using methanol extraction of gastric mucosal fragments to determine prostaglandin tissue levels instead of synthesizing capacity (data not shown).

**DISCUSSION**

Perfusion of the gastric lumen with peptone markedly reduced damage caused by subsequent exposure of the mucosa to ethanol, confirming previous observations (15). Because the 8% solution of peptone used in the study is isotonic, the protective effect cannot be attributed to hyperosmolality. The protection conferred by peptone depended on intact afferent neurons and was not observed in rats pretreated with a high neurotoxic dose of capsaicin to induce inactivation of afferent neurons.
Table 2. Gastric mucosal formation of prostaglandin E₂ and 6-ketoprostaglandin F₁₂ and effect of COX inhibitors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Prostaglandin E₂, pg/mg</th>
<th>6-Ketoprostaglandin F₁₂, pg/mg</th>
</tr>
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<tbody>
<tr>
<td>Saline</td>
<td>601 ± 191</td>
<td>943 ± 57</td>
</tr>
<tr>
<td>Peptone</td>
<td>588 ± 212</td>
<td>1,281 ± 613</td>
</tr>
<tr>
<td>Oral bolus administration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>705 ± 103</td>
<td>1,084 ± 134</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>49 ± 7*</td>
<td>39 ± 6*</td>
</tr>
<tr>
<td>NS-398</td>
<td>682 ± 121</td>
<td>1,002 ± 122</td>
</tr>
<tr>
<td>L-745,337</td>
<td>669 ± 98</td>
<td>907 ± 98</td>
</tr>
</tbody>
</table>

Values are means ± SE of 6 experiments/group. For gastric perfusion experiments rats were perfused intragastrically with saline or peptone (8%) for 20 min. Then the stomachs were removed, and mucosal fragments from the corpus region were excised and incubated in Tyrodesolution for 10 min at 37°C. Release of prostaglandins into incubation medium was determined using RIA. For oral bolus administration experiments rats were treated orally with indomethacin (20 mg/kg), NS-398 (3 mg/kg), or L-745,337 (5 mg/kg). Controls received vehicle (0.25% methylcellulose, 2.5 ml/kg). Sixty minutes later, the stomachs were removed and mucosal fragments were incubated for 10 min at 37°C to determine release of prostaglandins into the medium. COX, cyclooxygenase. *P < 0.001 vs. vehicle-treated control rats.

neurons. Capsaicin-sensitive afferent neurons represent an important line of mucosal defense. The gastric mucosa is densely innervated with such neurons, particularly in the vicinity of mucosal and submucosal arterial vessels that control blood flow to the mucosa (9). Activation of afferent neurons by intragastric administration of low concentrated capsaicin or agents that weaken the gastric mucosal barrier, thus leading to acid back-diffusion, was found to confer protection against various noxious agents (9). Stimulation of afferent nerves results in release of CGRP, the most abundant neuropeptide contained in these neurons, which then promotes NO generation. NO seems to be the exclusive mediator of CGRP-induced protection because inhibition of NO biosynthesis completely blocks the effect (16, 23). In contrast, CGRP and NO do not participate in afferent nerve-independent gastroprotection elicited by sulfhydryl-donating or -blocking agents, metals, or sodium salicylate (28). In vitro incubation experiments of rat antrum mucosa have shown that intraluminal peptone stimulates release of CGRP, indicating activation of afferent neurons (19). In our study, CGRP immunoneutralization or CGRP receptor blockade suppressed the protective effect of intraluminal peptone. Furthermore, pretreatment with the NO synthase inhibitor L-NAME abolished the protective activity of peptone, confirming previous observations (15). Simultaneous administration of L-arginine, which acts as a substrate for NO synthase, reversed the effect of L-NAME. This indicates that the attenuation of peptone-induced protection by L-NAME is due to inhibition of NO biosynthesis and not properties of L-NAME to act as a muscarinic antagonist in the cholinergic transmission (1). Taken together, these findings provide evidence for a crucial role of primary afferent neurons in mediating the protective activity of peptone. This proposal is in keeping with a study demonstrating that gastric acid secretory responses induced by peptone involve capsaicin-sensitive afferent neurons (26).

Activation of afferent neurons by intragastric capsaicin or acid back-diffusion elicits an increase in gastric mucosal blood flow (9). This hyperemic reaction has been suggested to underlie afferent nerve-mediated protection (9). Both CGRP and NO are potent vasodilating mediators that contribute to the gastric mucosal hyperemia evoked by afferent nerve stimulation (10, 34). In our study, peptone perfusion of the gastric lumen under experimental conditions that near-maximally reduced ethanol-induced mucosal damage did not influence mean arterial blood pressure and had only minor effects on gastric mucosal blood flow. Thus gastric mucosal blood flow was increased by 13% during peptone perfusion in the absence of ethanol. Perfusion of the gastric lumen with ethanol elevated gastric mucosal blood flow by 28% in peptone-treated rats and 36% in saline-treated rats. In both groups, the effect did not reach the level of statistical significance. From these findings it seems unlikely that the protection evoked by intragastric peptone is the result of increased gastric mucosal blood flow. Afferent nerve-mediated protective effects are not necessarily associated with an increase in gastric mucosal blood flow. Thus we have recently shown that analogs of the tachykinin neurokinin A protect the rat gastric mucosa against ethanol-induced damage by a mechanism involving activation of afferent neurons, release of CGRP, and biosynthesis of NO (29). Tachykinin-evoked protective effects occurred despite a marked reduction in gastric mucosal blood flow resulting from a potent local vasoconstrictor action of neurokinin A derivatives in the gastric microcirculation (29). Similarly, in the rat stomach, protective actions of CGRP against endothelin-induced mucosal damage were associated with a decrease in mucosal blood flow (18). These findings suggest that under certain experimental conditions effects not related to vasodilation underlie the protective activity of CGRP and NO.

In vivo and in vitro studies in various species have shown that peptone and other protein instillates stimulate gastrin secretion (4). In rats, intravenous injection of exogenous gastrin-17 caused near-maximal protection against ethanol-induced mucosal damage (31). As demonstrated for peptone-evoked protection, the protective activity of gastrin-17 involved stimulation of afferent neurons, release of CGRP, and activation of NO biosynthesis (31). Circulating gastrin levels reached after injection of a protective dose of exogenous gastrin-17 were in the same order of magnitude as those measured during perfusion of the gastric lumen with 8% peptone (31). This could imply that endogenously released gastrin mediates the protective effect of peptone. This proposal is substantiated by the finding that gastrin immunoneutralization or blockade of gastrin (CCK-B), but not CCK-A, receptors inhibited the protective effect of intraluminal peptone. Reversal of peptone-
induced protection by a CCK-B receptor antagonist was also reported by Konturek et al. (15).

In previous studies (27) using the vascularly perfused rat stomach, it was shown that peptone stimulates gastrin secretion by activating intramural cholinergic and noncholinergic bombesin/GRP neurons. This was concluded from the fact that the peptone-evoked gastrin release was fully blocked by TTX and partially inhibited by atropine or a selective bombesin/GRP receptor antagonist (27). The gastrin response to peptone was abolished by a combination of the bombesin/GRP receptor antagonist with atropine. This observation was taken as evidence that activation of cholinergic and bombesin/GRP neurons accounts for the gastrin response to peptone (27). The present study shows that both atropine and the selective bombesin/GRP receptor antagonist D-22213 antagonize the protective effect of peptone. The effect of atropine was mimicked by methyl atropine, which does not cross the blood-brain barrier, indicating that it is mediated by a peripheral mechanism. Whereas atropine partially inhibited the peptone-induced protection, antagonism of bombesin/GRP receptors completely counteracted the effect. Endogenous bombesin/GRP may have a more important function in peptone-evoked gastrin release in the intact rat compared with the isolated in vitro perfused stomach. In addition, activation of bombesin/GRP receptors could contribute to the protective effect of endogenous gastrin. This contention is in keeping with previous findings that bombesin exerts potent gastroprotective effects (30) and with results of the present study that blockade of bombesin/GRP receptors partially attenuates the protection induced by gastrin-17.

Pretreatment with the somatostatin receptor antagonist cyclo(7-aminohexanoyl-Phe-d-Trp-Lys-Thr[BZL]) counteracted the protective effect of peptone in a dose-dependent manner. In contrast, the somatostatin receptor antagonist did not affect the protection induced by gastrin-17, indicating that gastrin does not exert its protective effect through release of endogenous somatostatin. Exogenously administered CGRP and endogenous CGRP released on stimulation of primary afferent neurons were found to promote secretion of somatostatin from the isolated perfused rat stomach (11). Whereas numerous studies have shown that in the rat stomach bombesin/GRP is a potent stimulator for the release of somatostatin (see Ref. 8), both stimulation (17) and inhibition (27) of somatostatin release have been described for peptone. Furthermore, in our study we failed to demonstrate that exogenous somatostatin exerts protection (data not shown), although Karmeli et al. (14) have described such an effect.

It is well established that the vagus nerve plays an important role in gastric mucosal defense. Thus central vagal stimulation induced by intracerebral injection of RX-77368, the stable analog of thyrotropin-releasing hormone, prevented ethanol-induced gastric lesions in rats by a muscarinic-mediated mechanism (35). The protective effect of vagal stimulation was associated with increased formation of prostaglandin E2 in the stomach and abolished by pretreatment with indomethacin and atropine, suggesting that vagal activation may play a role in the control of gastric mucosal integrity against injury through cholinergic prostaglandin release (35).

Biosynthesis of prostaglandins is rate limited by the initial conversion of arachidonic acid to prostaglandin G and prostaglandin H by the COX enzyme. Two major isoforms of COX have been described, the constitutive COX-1 and the inducible COX-2 (3, 21). High levels of COX-1 protein and mRNA are detected in most normal tissues (13, 22). Whereas COX-2 protein is lower or even undetectable in most normal tissues, the COX-2 isoform can be induced rapidly in vitro by cytokines, growth factors, mitogens, and endotoxin. It can also be induced in vivo by proinflammatory stimuli in a variety of animal models of inflammation, in synovial tissues from patients with rheumatoid arthritis, and in human and animal tumors (for review, see Ref. 21). From these findings it has been proposed that prostaglandins generated via the COX-1 pathway are essential for physiological functions such as platelet homeostasis and maintenance of renal hemodynamics and gastrointestinal mucosal integrity, whereas prostaglandins generated by the COX-2 isoform have a role in pathophysiological events, in particular inflammation, pain, pyrexia, and tumor growth (21, 33). In contrast to nonselective nonsteroidal anti-inflammatory drugs that block both COX-1 and COX-2, selective COX-2 inhibitors do not produce gastric lesions in rats (2, 6, 20); it has thus been suggested that COX-1-derived, but not COX-2-derived, prostaglandins are involved in gastric mucosal defense reactions against injury (33).

NS-398 (6, 20) and L-745,337 (2) are selective inhibitors of the COX-2 isoform. Studies performed in our laboratory have revealed that, whereas indomethacin produced severe gastric mucosal lesions, neither NS-398 (unpublished results) nor L-745,337 (26a) caused damage to the gastric mucosa, confirming previous reports (2, 6, 20). However, NS-398 and L-745,337 dose dependently antagonized the protective effect of peptone. The ID50 was 0.8 and 1.5 mg/kg for NS-398 and L-745,337, respectively. The maximal effective doses reduced the protective activity of peptone by 55% and 73%, respectively. The effects of the selective COX-2 inhibitors were comparable to those of the nonselective COX inhibitor indomethacin (ID50, 4.2 mg/kg; maximal inhibition of protection, 66%). Prostaglandins are not involved in the increase in mucosal resistance after afferent nerve stimulation (16). Furthermore, atropine inhibited the protective effect of peptone to an extent comparable to that of nonselective and selective inhibitors of prostaglandin formation. From these findings it is tempting to speculate that prostaglandins contribute to the cholinergic part of peptone-evoked protection. However, the attenuating effects of atropine and inhibition of prostaglandin formation were additive (data not shown), suggesting that peptone-induced protective effects in addition to stimulation of cholinergic neurons are prostaglandin dependent.

Dexamethasone did not affect the protection elicited by peptone. This finding is consistent with the concept
that a constitutive, not an inducible, COX-2 enzyme generates the prostaglandins essential for peptone-evoked protection. This proposal is further supported by the finding that COX-2-sensitive protective effects of intragastric peptone occur within 20 min, a time period that seems rather short for effective enzyme induction.

Gastric mucosal formation of prostaglandin E₂ or 6-ketoprostaglandin F₁α was not increased to a measurable extent by perfusion of the gastric lumen with peptone and was not reduced by pretreatment with atropine. Whereas treatment with indomethacin substantially inhibited gastric mucosal formation of both prostaglandins, NS-398 and L-745,337 at doses that maximally blocked peptone-induced protection did not reduce prostaglandin formation in the gastric mucosa. The lack of measurable inhibitory activity on gastric prostaglandin generation indicates that NS-398 and L-745,337 do not counteract the peptone-induced protection as a result of inhibition of COX-1-derived prostaglandins. We have recently shown (7) that in the rat stomach pretreatment with NS-398 or L-745,337 abolishes the protective effect of 20% ethanol. Similar to the findings described in this study, we could demonstrate neither measurable increases in mucosal production of prostaglandin E₂ and 6-ketoprostaglandin F₁α after instillation of the mild irritant nor reduction of prostaglandin generation after treatment with selective COX-2 inhibitors (7). The failure to demonstrate inhibition of gastric prostaglandin formation by selective COX-2 inhibitors may be due to the fact that COX-2-derived prostaglandins represent only a minority of the gastric prostaglandin pool. With the background of large amounts of prostaglandins generated via the COX-1 isoform it may be difficult to demonstrate changes in the small COX-2-catalyzed prostaglandin compartment. The finding that NS-398 and L-745,337 did not affect dimercaprol-evoked protection (data not shown), which is not prostaglandin mediated and is insensitive to indomethacin, supports the hypothesis that the inhibitory effect of these agents on peptone-induced protection is indeed due to suppression of COX-2-derived prostaglandins. However, at present, we cannot completely rule out the possibility that effects of NS-398 and L-745,337 not related to inhibition of COX-2 interfere with the protective properties of peptone.

Constitutively expressed levels of COX-2 in addition to COX-1 are present in normal unstimulated tissues of various species. Thus the occurrence of COX-2 mRNA and COX-2 protein in small but possibly strategic areas in normal rat gastric mucosa with particularly strong expression of COX-2 in the endothelia of gastric mucosal microvessels has recently been described (32). Immunoreactivity for COX-2 has been found in the surface mucous cells in both the fundic and pyloric regions of the rat stomach (12). Using immunoblot analysis, expression of low but detectable levels of COX-2 protein could be demonstrated in microsomes prepared from gastrointestinal tissues of normal rats (13). Finally, in the human stomach approximately equivalent levels of COX-1 and COX-2 mRNA were present when RT-PCR was applied to quantitate the COX isoforms (22).

In conclusion, the results show that a complex system including several neural pathways and chemical mediators underlies the gastroprotective effect of peptone. The findings of this and previous studies (4, 11, 16, 27, 31, 35) suggest the following interactions of the systems involved. Peptone in the gastric lumen promotes the release of gastrin by stimulating intramural cholinergic and noncholinergic, bombesin/GRP-containing neurons. Gastrin then activates primary afferent neurons, thus inducing release of CGRP. CGRP in turn stimulates biosynthesis of NO, which acts as the final mediator of the afferent nerve-induced gastromotion. In addition, activation of bombesin/GRP and afferent neurons releases somatostatin and/or by an unknown mechanism contributes to or facilitates the protective effect of gastrin. Activation of cholinergic neural pathways contributes to peptone-evoked protection indirectly by promotion of gastrin release as well as possibly by a direct mechanism resulting from stimulation of prostaglandin generation in the stomach. The precise mechanisms by which NO and prostaglandins mediate protective effects have not been elucidated so far but obviously do not rely on elevated gastric mucosal blood flow. Our findings also show that, although selective inhibitors of COX-2 do not produce gastric mucosal lesions, they significantly attenuate the protective activity of intragastric peptone, suggesting that prostaglandins mediating the protection are generated via the COX-2 isoform. These findings provide evidence that prostaglandins biosynthesized by a constitutive COX-2 enzyme are crucially involved in physiological functions that are essential for homeostasis reactions in the gastrointestinal tract.

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REFERENCES


