Prostaglandin/Cyclooxygenase Pathway in Ghrelin-Induced Gastroprotection against Ischemia-Reperfusion Injury

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ABSTRACT

Ghrelin is involved in the control of food intake, but its role in gastroprotection against the formation of gastric mucosal injury has been little elucidated. We studied the effects of peripheral (i.p.) and central (i.c.v.) administration of ghrelin on gastric secretion and gastric mucosal lesions induced by 3 h of ischemia/reperfusion (I/R) with or without inhibition of ghrelin growth hormone secretagogue type 1a receptor (GHS-R1a) by using ghrelin antagonist, d-Lys3-GHRP-6; blockade of cyclooxygenase (COX)-1 (indomethacin, SC560 [5-(4-chloro-phenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole]) and COX-2 (rofecoxib); and bilateral vagotomy or capsaicin denervation. I/R produced typical gastric erosions, a significant fall in the gastric blood flow (GBF), an increase in gastric myeloperoxidase (MPO) activity and malonyldialdehyde (MDA) content, and the up-regulation of mucosal ghrelin mRNA. Ghrelin dose-dependently increased gastric secretion and significantly reduced I/R-induced gastric erosions, while producing a significant rise in the GBF and mucosal PGE2 generation and a significant fall in MPO activity and MDA content. The protective and hyperemic activities of ghrelin were significantly attenuated in rats pretreated with d-Lys3-GHRP-6 and capsaicin denervation and completely abolished by vagotomy. Indomethacin, SC560, and rofecoxib, selective COX-1 and COX-2 inhibitors, attenuated ghrelin-induced protection that was restored by supplying the methyl analog of prostaglandin (PG) E2. The expression of mRNA for COX-1 was unaffected by ghrelin, but COX-2 mRNA and COX-2 protein were detectable in I/R injured mucosa and further up-regulated by exogenous ghrelin. We conclude that ghrelin exhibits gastroprotective and hyperemic activities against I/R-induced erosions, the effects that are mediated by hormone activation of GHS-R1a receptors, COX-PG system, and vagal-sensory nerves.

Ghrelin is a recently described 28-amino acid peptide that has been discovered in rat and human gastrointestinal tract, particularly in gastric mucosa, as an endogenous ligand for growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999). Ghrelin stimulates food intake and body weight gain exerting a modulating effect on energy expenditure acting throughafferent nerves and directly on hypothalamic feeding centers (Tschoop et al., 2000). This peptide was also shown to enhance the gastric motility and gastric secretion (Masuda et al., 2000; Date et al., 2001).

Little is known about the factors that might affect ghrelin release in the stomach. A recent study revealed that endocrine Gr cells of the stomach are a major source of circulating ghrelin. Gastrectomy produces a dramatic fall in plasma ghrelin levels, whereas fasting and anorexia nervosa are accompanied by elevated plasma ghrelin levels (Ariyasu et al., 2001; Kojima and Kangawa, 2002). On the other hand, infection with Helicobacter pylori, which is now considered the causal factor in the pathogenesis of gastritis and peptic ulcer, was found to attenuate the expression of ghrelin and to reduce appetite (Tatsuguchi et al., 2004).

Previous studies revealed that two GHS-R subtypes are generated by alternative splicing of a single gene: the full-length type 1a receptor (GHS-R1a) and a carboxyl-terminally truncated GHS-R1b (McKee et al., 1997). The GHS-R1a is the functionally active, signal transducing form of the growth hormone secretagogue receptor; NO, nitric oxide; PG, prostaglandin; COX, cyclooxygenase; I/R, ischemia/reperfusion; GF, gastric fistula; GBF, gastric blood flow; MPO, myeloperoxidase; MDA, malonyldialdehyde; SC560, 5-(4-chloro-phenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole; RIA, radioimmunoassay; RT, reverse transcriptase; PCR, polymerase chain reaction; CGRP, calcitonin gene-related peptide; i.e., intragastrically; 4HNE, 4-hydroxynonenal; i.c.v., intracerebroventricular; i.p., intraperitoneal.

ABBREVIATIONS: GHS-R, growth hormone secretagogue receptor; NO, nitric oxide; PG, prostaglandin; COX, cyclooxygenase; I/R, ischemia/reperfusion; GF, gastric fistula; GBF, gastric blood flow; MPO, myeloperoxidase; MDA, malonyldialdehyde; SC560, 5-(4-chloro-phenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole; RIA, radioimmunoassay; RT, reverse transcriptase; PCR, polymerase chain reaction; CGRP, calcitonin gene-related peptide; i.e., intragastrically; 4HNE, 4-hydroxynonenal; i.c.v., intracerebroventricular; i.p., intraperitoneal.
GHS-R, whereas the GHS-R1b is devoid of high-affinity ligand binding and signal transduction activity. Ghrelin molecules, produced by endocrine cells of gastric glands, exist in two major molecular forms, ghrelin and des-\(\text{n}-\text{octanoyl ghrelin}\) (des-acyl ghrelin) (Hosoda et al., 2000; Date et al., 2001).

The role of ghrelin in the mechanism of gastric mucosal defense and gastroprotection has been little investigated except for the reports of Sibilia et al. (2003) and our group (Brzozowski et al., 2004a; Konturek et al., 2004), revealing that central and peripheral administration of ghrelin reduces the formation of lesions induced by ethanol and cold stress.

It was proposed that NO and sensory neuropeptides may mediate these gastroprotective effects because the blockade of both NO synthase activity with \(\text{N}^{\text{G}}\text{-nitro-L-arginine methyl ester}\) and the functional ablation of sensory afferent nerves with capsaicin were both found to attenuate them (Brzozowski et al., 2004a). It remains unknown whether the observed gastroprotective activity of ghrelin is due to a direct activation of GHS-1a receptors or involves other mediators or receptors, as yet uncharacterized and distinct from the GHS-R.

Recently, endogenous prostaglandins (PGs) have been implicated in the control of food intake and appetite (Luquet et al., 2000), revealing their role as well as the importance of expression of cyclooxygenase (COX)-1 and COX-2 in the possible gastroprotective activity of ghrelin. This prompted our interest in endogenous PGs because the formation of lesions induced by ethanol and cold stress. It was documented that 30 min of ischemia (I) by clamping of the celiac artery followed by 3 h of reperfusion (R) (as originally described by Wada et al. (1996) and our group (Brzozowski et al., 2000a)) in the stomach was followed by the development of lesions.

Gastric Secretory Studies. The effects of acylated ghrelin (Bachem AG, Bubendorf, Switzerland), on gastric acid secretion were examined in 50 conscious rats weighing 200 to 230 g and equipped with a gastric probe in various doses i.c.v. in a volume of 5 \(\mu\text{l}\) using a 10-\(\mu\text{l}\) Hamilton microsyringe. Vehicle (saline applied in a volume of 1 ml i.g. or 5 \(\mu\text{l}\) i.c.v.) or ghrelin was injected i.p. or i.c.v. in gradually increasing doses ranging from 2.5 to 40 \(\mu\text{g/kg}\) and 150 to 2400 \(\mu\text{g/rat}\), respectively, each dose being administered on a separate test day. The effectiveness of i.c.v. administration was verified by injecting 10 \(\mu\text{g}\) of dye (0.1% toluidine blue). The visualization of dye on the walls of the stomach was detected using a camera system. The rats were fasted overnight before the experiment, and the stomach was infused with a solution of 0.9% saline in a volume of 1 ml i.g. or 5 \(\mu\text{l}\) i.c.v. The volume and acid concentrations of each collected sample of gastric juice were measured, and acid outputs (expressed in terms of micromoles of acid per 30 min) were determined as described previously (Brzozowski et al., 1999).

Gastroprotective Studies and Measurement of GBF. Acute gastric lesions were induced in 120 rats by exposing of their stomach for 30 min of ischemia (I) by clamping of the celiac artery followed by 3 h of reperfusion (R). Several groups of rats, each consisting of six to eight animals, were pretreated 30 min before the I/R with vehicle (saline); ghrelin (standard dose, 20 \(\mu\text{g/kg}\) i.p.); \(\text{D-Lys}^{3}\text{-GHRP-6}\) (100 \(\mu\text{g/kg}\) i.p.), the ghrelin receptor antagonist (Peeters, 2005); \(\text{SC560}\) (5 \(\mu\text{g/kg}\) i.g.), a selective COX-1 inhibitor (Brzozowski et al., 2004a); \(\text{Takeuchi et al., 2004}\); \(\text{rofexoib} (10 \mu\text{g/kg i.g.)}, the highly selective COX-2 inhibitor (Ehrich et al., 2000); hydroxybenzoic acid (1 \(\mu\text{g/kg i.g.)}, 5 \(\mu\text{g/kg i.g.)}, a nonselective COX-1 inhibitor (Chen et al., 1995). The dose of ghrelin receptor antagonist, \(\text{D-Lys}^{3}\text{-GHRP-6}\), was selected on the basis of maximum inhibition of gastric acid secretion. The effects of treatment with nonselective (indomethacin and selective COX-1 (SC560) and COX-2 (rofexoib) inhibitors on I/R-induced gastric lesions and accompanying changes in the GBF and gastric mucosal generation of PGs were studied. The GBF was measured using a 10-\(\mu\text{l}\) Hamilton microsyringe and the head of the stomach was clamped by a clip. The GF was opened, and the stomach was rinsed gently with a small amount of saline. The washings were collected and the volume and PGs measured. The visualization of dye on the walls of the stomach was detected using a camera system. The rats were fasted overnight before the experiment, and the stomach was infused with a solution of 0.9% saline in a volume of 1 ml i.g. or 5 \(\mu\text{l}\) i.c.v. The volume and acid concentrations of each collected sample of gastric juice were measured, and acid outputs (expressed in terms of micromoles of acid per 30 min) were determined as described previously (Brzozowski et al., 1999).
basis of our preliminary studies in rats pretreated with this antigen
with or without the combination with ghrelin against formation of
gastric damage induced by ethanol. In addition, this GHS-R1a
antagonist applied i.p. in graded doses starting from 10 up to 200
µg/kg i.p. dose-dependently inhibited food intake stimulated by gh-
relin in rats with GF. At the dose used in the present study, indo-
methacin has been shown previously to inhibit gastric PGE2 gener-
ation by ~90% without itself causing any mucosal damage (Konturek et al., 1987). The doses of SC560 and rofecoxib were
selected on the basis of previous studies showing that these agents
almost completely suppress PGE2 generation in exudate of air pouch
inflammation and inhibit gastric PGE2 production in mucosa with
preexisting gastric ulcer (Lesch et al., 1998; Brzozowski et al., 2001).
SC560 (Cayman Chemical Co., Ann Arbor, MI) was first dissolved in
absolute ethanol to obtain a stock solution of 50 mg/ml and then
diluted to the desired concentration with isotonic saline. Rofecoxib
(Sharp and Dhome, Warsaw, Poland) was first dissolved in methanol
and allowed to dissolve in the saline to obtain stock solution of 75
mg/ml and then diluted to the desired concentration with isotonic saline. Control rats received the corre-
sponding vehicle. Our preliminary studies (data not shown) showed
that none of the COX inhibitors used in this study produced by itself
any gastric lesions at the doses tested.

In another group of animals subjected to I/R and treated with
COX-1 and COX-2 inhibitors with or without ghrelin administration,
the effect of prostaglandin replacement therapy using 16,16-di-
methyl PGE2 (Upjohn, Kalamazoo, MI) applied i.g. in a dose of 1
µg/kg was examined. This dose of dimethyl PGE2 was found in our
preliminary study to be without any influence on gastric lesions
caused by I/R and accompanying fall in GBF. For this reason, syn-
thetic PGE2 analogs were administered together with each COX-1 or
COX-2 inhibitor with or without ghrelin administration starting 30
min before I/R.

Upon the termination of reperfusion after 3 h, the animals were
anesthetized with pentobarbital, their abdomen was opened by mid-
line incision, and the stomach was exposed for measurement of GBF
by means of the H2 gas clearance technique as described previously
(Konturek et al., 1995; Brzozowski et al., 2000a). For this purpose,
two electrodes of an electrolytic regional blood flowmeter (model
RBF-2; Biotechnical Science, Osaka, Japan) were inserted into the
gastric mucosa. The measurements were made in three areas of the
oxyntic mucosa, and the mean values were calculated and expressed
as percent changes of those recorded in the control animals. After
GBF measurement, the stomach was removed, rinsed with saline,
and pinned open for macroscopic examination. The area of gastric
lesions was determined by computerized planimetry (Morphomat;
Carl Zeiss GmbH, Jena, Germany) (Brzozowski et al., 2004b) by a
researcher blind to the experimental grouping.

Effect of Ghrelin on the I/R Lesions and Accompanying
Changes in the GBF: Involvement of the COX-PG System in
Ghrelin-Induced Gastroprotection. The involvement of the
COX-PG system in the protective effects of ghrelin was studied in
rats pretreated 60 min before the ghrelin injections with (or without)
indomethacin, a nonselective COX-1 and COX-2 inhibitor, and with
SC560, a selective COX-1 inhibitor or rofecoxib, a specific COX-2
inhibitor. In the gastroprotection study, the following groups of rats,
each consisting of six to eight animals, were used: vehicle (saline 1 ml
i.p.), followed 30 min later by I/R; ghrelin (2.5–40 µg/kg i.p.), fol-
lowed 30 min later by I/R; ghrelin (150–2400 ng/rat i.v.), followed 30
min later by I/R; ghrelin (standard dose; 20 µg/kg i.p. or 1200
ng/rat), followed 30 min later by I/R; indomethacin (5 mg/kg i.p.),
followed 60 min later by ghrelin (20 µg/kg i.p.) and then followed
30 min later by I/R; and rofecoxib (10 mg/kg i.g.), followed 60 min later
by ghrelin (20-µg/kg i.p.) and then 30 min later by I/R.

Samples of the oxyntic gland area were taken by biopsy (approx-
imately 100 mg) immediately after the animals were sacrificed to
determine the mucosal generation of PGE2 by specific radioimmu-
noassay (RIA) as described previously (Konturek et al., 1995). PGE2
was measured in duplicate using RIA kits (New England Nuclear,
Munich, Germany). The mucosal generation of PGE2 was expressed
in nanograms per gram of wet tissue weight.

Effect of Ghrelin on Gastric Mucosal MPO Activity and
MDA Concentration in Rats Exposed to I/R. Because lipid per-
oxidation is a well established mechanism of cellular injury induced
by reactive oxygen metabolites generated by I/R, we measured the
changes in MPO activity, as an index of neutrophil activation and
superoxide radical accumulation and the MDA as an indicator of the
lipid peroxidation in gastric mucosa exposed to I/R without and with
the treatment with ghrelin. The MPO concentration was determined
using enzyme immunoassay kit (Oxis International, Inc., Portland,
OR). For the measurement of lipid peroxidation, the tissue was
weighed, transferred to the ice-cooled test tube, and homogenized
in 400 µl of 20 mM Tris buffer, pH 7.4, containing 5 mM butylated
hydroxytoluene to prevent new lipid peroxidation that can occur
during the homogenization. The homogenate was then centrifuged
at 4°C for 10 min, and resulting supernatant (200 µl) was stored in
−80°C until an assay of lipid peroxidation. The content of lipid
peroxidation was measured at 37°C by spectrophotometer at wave-
length of 586 nm and compared with the absorbance of purified MDA
as the standard as described in details previously (Pohle et al., 2001).

Determination of Plasma Ghrelin Levels by Radioimmuno-
assay. At the termination of some experiments with ghrelin alone
applied i.p. and ghrelin i.p. injection followed 30 min later by I/R, the
rats were anesthetized with pentobarbital and blood samples (ap-
proximately 3 ml) taken from the vena cava for the measurement of
plasma ghrelin levels by RIA as described previously (Date et al.,
2001; Brzozowski et al., 2004a). Intact rats fasted overnight and
given only vehicle (saline) i.p. were measured similarly to determine
control values for plasma ghrelin concentration. Blood samples were
collected in heparin-coated polypropylene tubes and centrifuged at
3000 rpm for 20 min at 4°C. The supernatant was then stored at
−80°C until measurement of plasma ghrelin levels using an RIA kit
for rat ghrelin (Bachem AG). In brief, the ghrelin RIA involved the
competition of a rat ghrelin sample with 125I-rat ghrelin tracer for
binding to a specific rabbit anti-ghrelin polyclonal antibody. The
limit of assay sensitivity was 4 pg/tube, the intra-assay variation was
less than 9%, and the interassay variation was less than 6%.

Effect of Vagotomy and Sensory Denervation on Gastro-
protection by Ghrelin against I/R-Induced Gastric Erosions.
The involvement of vagal nerves in gastroprotection by central and
peripheral ghrelin was studied in rats with or without vagotomy,
performed as described previously (Brzozowski et al., 2000b). Ap-
proximately 30 min before the experiment, rats were anesthetized
with ether, and the abdomen was opened by a small incision. Both
branches of vagal nerves were identified and transected. The control
rats were treated similarly except that the vagi were uncovered and
left intact. Vagotomized and sham-operated rats received ghrelin (20
µg/kg i.p. or 1200 ng/rat i.c.v.), and 30 min later, the I/R was per-
formed as described above.

The role of sensory afferent nerves in gastroprotection by ghrelin
was tested in rats with capsaicin-induced deactivation of these
nerves. For this purpose, the animals were pretreated with capsaicin
(Sigma Co., St. Louis, MO) injected subcutaneously for 3 consecutive
days at a dose of 25, 50, and 50 mg/kg about 2 weeks before the
experiment (Holzer et al., 1991; Brzozowski et al., 1996). All injec-
tions of capsaicin were performed under ether anesthesia to coun-
teract the respiratory impairment associated with injection of this
agent. To check the effectiveness of the capsaicin denervation, a drop
of 0.1 mg/ml solution of capsaicin was placed in the eye of each rat,
and the protective wiping movements were counted as described
previously (Holzer et al., 1991). Control rats received vehicle injec-
tions. All animals pretreated with capsaicin showed negative wiping
movement tests, confirming functional denervation of the capsaicin-
sensitive nerves. At 3 h after the termination of standard I/R with
or without ghrelin administration, vagotomized and sensory dener-
vated rats were sacrificed, and areas of gastric lesions and GBF were
measured as above.
Reverse Transcriptase-Polymerase Chain Reaction for Detection of mRNA for Ghrelin and COX Enzymes in Rats Exposed to I/R. The stomachs were removed from intact rats and from those treated with vehicle (control) or ghrelin with or without exposure to I/R for the determination of ghrelin, COX-1, and COX-2 mRNA expression using specific primers by reverse transcriptase-polymerase chain reaction (RT-PCR). Gastric mucosal specimens were scraped off from oxyntic mucosa 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 guanidium isothiocyanate/phenol chloroform method using a kit from Stratagene (Heidelberg, Germany). Single-stranded cDNA was generated from 5 μg of total cellular RNA using Stratascript reverse transcriptase and oligo(dT) primers (Stratagene). The polymerase encoding ghrelin and the β-actin (sense) was selected on the basis of the published cDNA sequences of the primers for ghrelin and β-actin were selected on the basis of the published cDNA sequences of ghrelin and β-actin, respectively (Konturek et al., 2004). The sense primer for ghrelin was 5′-TTGAGCCAGAGGCACAGGAAA-3′, and the antisense primer was 5′-AGTTGGAGGAGGGCCAGAAGCT-3′. The oligonucleotide sequences for β-actin were TTG TAA CCA ACT GGG ACG ATA TGG (sense) and GAT CTT GAT CTT CAT GGT GCT AGG (antisense). The nucleotide sequences of the primers for COX-1 and COX-2 were identical to those published by our group previously (Brzozowski et al., 2001, 2004b). The primers were synthesized by GIBCO BRL/Life Technologies (Eggenstein, Germany). The signals for ghrelin mRNA were standardized against the β-actin signal for each sample, and results were expressed as the ratio of ghrelin, COX-1, or COX-2 mRNA to β-actin mRNA.

Protein Extraction and Analysis of COX-1 and COX-2 Expression in the Gastric Mucosa by Western Blot. Flash-frozen gastric tissue was homogenized in lysis buffer (100 mmol Tris-HCl, pH 7.4, 15% glycerol, 2 mmol EDTA, 2% SDS, and 100 mmol of DDT) by the addition of 1:20 dilution of aprotinin and 1:50 dilution of 100 mmol of phenylmethylsulfonyl fluoride as described in detail previously (Konturek et al., 2004). Insoluble material was removed by centrifugation at 12,000g for 15 min. Approximately 100 μg of cellular protein extract was loaded into a well, separated electrophoretically through a 13.5% SDS-polyacrylamide gel, and transferred onto a Sequi-Blot TMPVDF membrane (Bio-Rad, Hercules, CA) by electroblotting. The specific primary rabbit polyclonal antibody against COX-1 and COX-2 (dilution, 1:500; Santa Cruz Biochemicals, Santa Cruz, CA) or against β-actin was added to the membrane, followed by an anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (dilution, 1:2000; Santa Cruz Biochemicals). Nonisotopic visualization of immunocomplexes was achieved by chemiluminescence using BM Chemiluminescence Blotting Substrate (Boehringer, Mannheim, Germany), and the developed membrane was exposed to an X-ray film (Kodak, Wiesbaden, Germany). Comparisons between different treatment groups including rats with intact sensory nerves and those with capsaicin denervation of sensory nerves with or without ghrelin administration were made by determining the COX-1 and COX-2/β-actin ratio of the immunoreactive area by densitometry.

Statistical Analysis. Results are expressed as means ± S.E.M. Statistical analysis was done using analysis of variance and two-way analysis of variance test with Tukey post hoc test where appropriate. Differences of p < 0.05 were considered significant.

Results

Effects of Exogenous Ghrelin on Gastric Acid Secretion, I/R-Induced Gastric Lesions, and the Alterations in the GBF and Plasma Ghrelin. Ghrelin applied i.p. or i.c.v. in graded doses ranging from 2.5 up to 40 μg/kg i.p. or from 150 ng/rat up to 2400 ng/rat i.c.v. resulted in dose-dependent increase in gastric acid secretion from the GF in conscious rats from basal vehicle value of 112 ± 6 t o 162 ± 6 μmol/30 min at the highest dose of i.c.v. ghrelin and to 154 ± 5 μmol/30 min at the highest dose of i.c.v. ghrelin. Results with smaller doses of ghrelin on gastric acid secretion were omitted for the sake of clarity.

The results of administration of ghrelin on the area of gastric lesions induced by I/R, and accompanying changes in the GBF and plasma ghrelin levels are presented in Fig. 1. Exposure of the gastric mucosa to I/R, although producing gastric lesions, caused a significant increase in the plasma ghrelin levels (121 ± 12 pM in sham control animals without I/R versus 246 ± 18 pM in vehicle control rats exposed to I/R).

Fig. 1. The mean area of gastric erosions induced by I/R, GF, and plasma immunoreactivity of ghrelin in rats treated with vehicle (saline) or with various doses of ghrelin (5–40 μg/kg i.p.). Means ± S.E.M. of six to eight rats. * indicates change compared with the vehicle control values.
TABLE 1

The effect of vehicle (saline) and ghrelin applied i.c.v. in graded doses ranging from 150 up to 2400 ng/rat on the mean area of I/R-induced gastric erosions and accompanying alterations in GBF and plasma ghrelin levels

<table>
<thead>
<tr>
<th>Type of Test</th>
<th>Mean Lesion Area (mm²)</th>
<th>% Control</th>
<th>Plasma Ghrelin (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (Control)</td>
<td>37 ± 5.3</td>
<td>68 ± 3</td>
<td>12 ± 6</td>
</tr>
<tr>
<td>Ghrelin (ng/rat i.c.v.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>34 ± 4.2</td>
<td>72 ± 5</td>
<td>116 ± 7</td>
</tr>
<tr>
<td>300</td>
<td>28 ± 3.4*</td>
<td>76 ± 4*</td>
<td>120 ± 6*</td>
</tr>
<tr>
<td>600</td>
<td>21 ± 2.3*</td>
<td>79 ± 5*</td>
<td>128 ± 9*</td>
</tr>
<tr>
<td>1200</td>
<td>18 ± 1.5*</td>
<td>83 ± 4*</td>
<td>135 ± 8*</td>
</tr>
<tr>
<td>2400</td>
<td>10 ± 1.1*</td>
<td>85 ± 5*</td>
<td>138 ± 11*</td>
</tr>
</tbody>
</table>

* Significant change as compared with the value recorded in the vehicle-treated control rats.

Such pretreatment with i.p. ghrelin reduced dose-dependently the area of gastric lesions evoked by I/R with the threshold reduction occurring at a dose of 5 μg/kg and with the ID₅₀ averaging approximately 8 μg/kg ghrelin. The protective effects of ghrelin were accompanied by a significant and dose-dependent rise in the GBF and plasma ghrelin levels (Fig. 1). In rats pretreated i.p. with vehicle (saline) and exposed to I/R, a significant reduction in GBF of approximately 35% was recorded compared with that recorded in sham control animals without I/R. With gradually increasing doses of i.p. ghrelin before I/R, when the area of I/R-induced gastric lesions was significantly attenuated, a concomitant and significant increase in GBF was observed starting with a dose of 5 μg/kg ghrelin (Fig. 1). The maximal increase in the plasma ghrelin levels was achieved at a dose of 40 μg/kg i.p. Further application of higher doses of ghrelin (80 μg/kg i.p.) failed to alter the area of I/R lesions significantly; for clarity, these results have been omitted. When ghrelin was injected i.c.v. in graded doses ranging from 150 up to 2400 ng/rat, it also dose-dependently attenuated gastric lesions induced by I/R with ID₅₀, being approximately 1200 ng/rat (Table 1). The decrease in the area of I/R lesions induced by i.c.v. ghrelin was accompanied by a dose-dependent increase in GBF and plasma ghrelin levels (Table 1).

Effect of Pretreatment with Ghrelin Receptor Antagonist on Ghrelin-Induced Protection against I/R-Induced Gastric Lesions. As shown in Fig. 2, treatment with ghrelin significantly decreased the area of I/R-induced gastric erosions and significantly raised the GBF compared with those obtained in vehicle-pretreated rats. Treatment with D-Lys³-GHRS-6 (100 μg/kg i.p.), a ghrelin receptor antagonist, by itself failed to affect the area of I/R-induced gastric erosions and accompanying fall in GBF. The decrease in the area of these erosions and accompanying rise in the GBF induced by ghrelin was significantly attenuated by concurrent treatment with the GHS-R1a antagonist D-Lys³-GHRS-6. Likewise, the decrease in the area of I/R lesions and accompanying increase in GBF induced by ghrelin administered i.c.v. (1200 ng/rat) were counteracted in rats pretreated with D-Lys³-GHRS-6 applied in a dose of 100 μg/kg i.p. (data not shown).

Effect of Pretreatment with Vehicle or Ghrelin on MPO Activity and MDA Concentration in Rats Exposed to I/R. Exposure of gastric mucosa to I/R resulted in a significant increase in the gastric mucosal MPO activity and in a significant rise in the mucosal MDA + 4HNE concentration compared with those measured in the intact gastric mucosa (Table 2). Pretreatment with ghrelin (20 μg/kg i.p.) significantly attenuated the increase in the MPO activity and the enhancement in the MDA concentration compared with the respective values recorded in vehicle-pretreated animals exposed to I/R (Table 2).

Effect of COX-PG Suppression on Ghrelin-Induced Gastroprotection against I/R-Induced Gastric Damage and Alteration in GBF. As shown in Fig. 3, pretreatment with ghrelin (20 μg/kg i.p.) resulted in a similar attenuation in the area of gastric lesions induced by I/R and similar rise in GBF as that shown in Fig. 1. Exposure to I/R decreased significantly the mucosal generation of PGE₂ compared with the respective value in the vehicle control animals not ex-

Fig. 2. The mean area of I/R-induced gastric lesions and the alterations in the GBF in rats treated with vehicle (Veh; control) or 20 μg/kg i.p. ghrelin with or without D-Lys³-GHRS-6 (100 μg/kg i.p.). Mean ± S.E.M. of six to eight rats. * significant change compared with the vehicle-pretreated controls. † significant change compared with rats treated with the GHS-R1a antagonist.
posed to I/R (105 ± 8 versus 165 ± 12 ng/g wet tissue weight) (Table 3). Ghrelin applied 30 min before I/R resulted in a significant increase in the PGE₂ generation compared with vehicle-pretreated animals exposed to I/R (Table 3).

Indomethacin (5 mg/kg i.p.), which by itself significantly aggravated gastric lesions induced by I/R, suppressed the generation of endogenous PGE₂ by approximately 85% and produced a significant attenuation of PGE₂ by approximately 85% and produced a significant attenuation of PGE₂ generation, significantly attenuated the reduction in area of the lesions and accompanying rise in GBF evoked by ghrelin. The decrease in the area of I/R lesions and accompanying increase in GBF caused by ghrelin as well as the rise in the PGE₂ generation it induced were also significantly attenuated by pretreatment with rofecoxib (10 mg/kg i.g.), a selective COX-2 inhibitor (Table 3; Fig. 3). SC560 (5 mg/kg i.g.), which by itself significantly reduced the PGE₂ generation, significantly attenuated the ghrelin-induced protection and accompanying gastric hyperemia (Table 3; Fig. 3). Concurrent treatment with a minute amount of dimethyl PGE₂ (1 µg/kg i.g.) in addition to ghrelin restored the protective and hyperemic activity of this peptide in rats treated with indomethacin, SC560, or rofecoxib and then exposed to I/R (Fig. 3).

### TABLE 2

<table>
<thead>
<tr>
<th>Type of Test</th>
<th>MPO Activity mg/ml</th>
<th>MDA + 4HNE Content nmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>0.9 ± 0.06</td>
<td>4.4 ± 0.8</td>
</tr>
<tr>
<td>Vehicle</td>
<td>6.8 ± 0.18*</td>
<td>13.3 ± 2.8*</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>2.9 ± 0.7†</td>
<td>5.9 ± 1.1†</td>
</tr>
</tbody>
</table>

* Significant change compared with the value recorded in the intact gastric mucosa.
† Significant change compared with the value recorded in the vehicle-treated control rats.

### Effect of Vagotomy and Functional Ablation of Sensory Afferent Nerves by Capsaicin on Gastric Lesions Induced by I/R with or without Ghrelin Administration.

Table 3 shows the effects of exposure to I/R resulting in gastric lesions whose areas were significantly reduced by the administration of ghrelin at a dose of 20 µg/kg i.p. or 1200 ng/rat i.c.v. This protective activity of ghrelin was accompanied by a small but significant rise in GBF. Vagotomy alone failed to affect I/R lesions and also failed to influence the fall in GBF in vehicle-pretreated rats exposed to I/R. Such vagotomy significantly attenuated the reduction in area of the lesions and the accompanying rise in the GBF obtained with ghrelin administered i.p. or i.c.v. (Fig. 4).

Table 4 shows the effects of treatment with large doses (total 125 mg/kg in 3 days) of capsaicin to induce a functional ablation of sensory afferent endings on ghrelin-induced protection and increase in the GBF. Such a capsaicin denerva-

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**Fig. 3.** The mean area of gastric erosions induced by I/R and accompanying changes in the GBF in rats treated with vehicle (saline) and ghrelin (20 µg/kg i.p.) with or without the pretreatment with indomethacin (5 mg/kg i.p.), SC560 (5 mg/kg i.g.), and rofecoxib (10 mg/kg i.g.) applied alone or combined with 16,16-dimethyl PGE₂ (1 µg/kg i.g.). Mean ± S.E.M. of six to eight rats. *, significant decrease compared with the value obtained in vehicle controls. **, significant decrease compared with the value in animals pretreated with ghrelin applied without COX inhibitors. ***, significant increase compared with the value in animals pretreated with ghrelin applied with COX inhibitors. ++, significant change compared with animals treated with indomethacin, SC560, or rofecoxib.

---

**TABLE 3**

The effect of vehicle (saline) and ghrelin (20 µg/kg i.p.) on the generation of endogenous PGE₂ in the gastric mucosa of rats treated with vehicle or ghrelin with or without nonselective (5 mg/kg i.p. indomethacin) and selective COX-1 (5 mg/kg i.g. SC-560) or COX-2 (10 mg/kg i.g. rofecoxib) inhibitors and then exposed to 3 h of I/R

<table>
<thead>
<tr>
<th>Type of Test</th>
<th>PGE₂ Generation ng/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>165 ± 12</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>211 ± 15*</td>
</tr>
<tr>
<td>Vehicle + I/R</td>
<td>105 ± 8†</td>
</tr>
<tr>
<td>Ghrelin + I/R</td>
<td>168 ± 10*</td>
</tr>
<tr>
<td>Indo + ghrelin + I/R</td>
<td>12 ± 3†</td>
</tr>
<tr>
<td>SC-560 + ghrelin + I/R</td>
<td>34 ± 4†</td>
</tr>
<tr>
<td>Rofecoxib + ghrelin + I/R</td>
<td>42 ± 6†</td>
</tr>
</tbody>
</table>

* Significant change compared with vehicle-treated controls.
† Significant change compared with groups without I/R.
‡ Significant change compared with groups pretreated with ghrelin but without COX-1 or COX-2 inhibition.
tion significantly aggravated gastric lesions induced by I/R and significantly decreased GBF compared with control animals. The decrease in area of I/R lesions and the accompanying increase in GBF induced by ghrelin (20 μg/kg i.p.) were significantly attenuated in capsaicin-denervated animals.

Mucosal Expression of Ghrelin, COX-1, and COX-2 by RT-PCR and Western Blot in the Gastric Mucosa Exposed to I/R with or without Ghrelin. Fig. 5, left, shows the expression of ghrelin mRNA in intact gastric mucosa and in that exposed to I/R. The signal for ghrelin mRNA was detected in intact gastric mucosa, and this was significantly enhanced in gastric mucosa exposed to I/R. The ratio of ghrelin mRNA to β-actin confirmed that mRNA for ghrelin was significantly up-regulated in rats exposed to I/R compared with intact gastric mucosa (Fig. 5, left). As shown in Fig. 6, left, COX-1 mRNA was detected in the gastric mucosa of all rats including control animals and those exposed to I/R with and without ghrelin administration. The ratio of COX-1 mRNA to β-actin mRNA confirmed that expression of mRNA for COX-1 was similar in ghrelin-treated rats compared with those exposed to I/R only. In contrast, the expression of COX-2 mRNA was not detected in intact gastric mucosa but was detected in rats exposed to I/R alone (Fig. 6, left). Signal intensity for COX-2 mRNA in animals exposed to I/R was significantly stronger in gastric mucosa of ghrelin-pretreated animals compared with control animals exposed to I/R (Fig. 6, left). The ratio of COX-2 mRNA to β-actin mRNA confirmed that COX-2 mRNA was significantly increased in ghrelin-pretreated animals and exposed 30 min later to I/R compared with that in rats exposed to I/R without ghrelin administration (Fig. 6, right).

In the gastric mucosa of intact rats, COX-2 protein was not detected, but when exposed to standard I/R, a marked signal for ~72 kDa of COX-2 protein was observed that was significantly enhanced by pretreatment with ghrelin (Fig. 7, left). The ratio of COX-2 protein to β-actin was significantly higher in gastric mucosa of ghrelin-pretreated animals compared with vehicle control animals exposed to I/R (Fig. 7, right). As shown in Fig. 8, the COX-2 protein expression was significant.

<table>
<thead>
<tr>
<th>Type of Test</th>
<th>Mean Lesion Area (mm²)</th>
<th>GBF % Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without capsaicin denervation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>65 ± 5</td>
<td>67 ± 3</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>11 ± 2*</td>
<td>79 ± 4*</td>
</tr>
<tr>
<td>With capsaicin denervation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>74 ± 4*</td>
<td>55 ± 3*</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>52 ± 2*</td>
<td>58 ± 4*</td>
</tr>
</tbody>
</table>

* Significant change compared with vehicle pretreated with intact sensory nerves.
† Significant change compared with rats without capsaicin denervation.
significantly decreased in the gastric mucosa of capsaicin-dener-
ated rats exposed to I/R compared with those with intact
sensory nerves. The enhancement in the COX-2 protein ex-
pression in ghrelin-treated animals was also significantly
attenuated by capsaicin denervation (Fig. 8, top panel). The
ratio of COX-2 protein to β-actin was significantly lower in
gastric mucosa of capsaicin denervated animals pretreated
with vehicle or ghrelin compared with that in I/R rats with
intact sensory nerves with or without ghrelin administration
(Fig. 8, bottom panel).

Discussion
This study demonstrates that exogenous ghrelin adminis-
tered parenterally or i.c.v. exhibits dose-dependent gastro-
protective activity against I/R-induced gastric lesions and is
accompanied by a dose-dependent rise in the plasma level of
this peptide and GBF. Ghrelin-induced protection and hyper-
emia were reversed by ghrelin receptor antagonist D-Lys³-
GHRP-6, indicating that ghrelin-induced protective and hy-
peremic effects are mediated by the functionally active form
of GHS-R1a receptor that has been shown to bind acylated
ghrelin. Exposure to I/R caused the increase in the gastric
mucosal MPO activity and MDA content, and these effects
were attenuated by ghrelin. These data suggest that ghrelin-
induced protection against I/R injury could be explained, at
least in part, by the suppressive effects exhibited by this
peptide on neutrophil activation and generation of free oxy-
gen metabolites under I/R conditions. Both PG and NO,
which contribute to ghrelin-induced protection, are known to inhibit neutrophil activation and their margination caused by superoxide radicals independently of the increase in the GBF.

Ghrelin was originally reported to exhibit gastroprotective activity against mucosal lesions induced by corrosive substances such as ethanol (Sibilia et al., 2003, 2004) as well as against damage induced by stress (Brzozowski et al., 2004a; Konturek et al., 2004). In the present study, this peptide has been shown to attenuate the lesions caused by I/R, an effect accompanied by an increase in the GBF. The results of secretory studies show that ghrelin applied at gastroprotective doses significantly raises gastric acid secretion and therefore that the protective effects of this peptide occur despite an increase in gastric secretory activity. This represents a specific gastroprotective activity ascribed originally to prostaglandin by Robert (1979). Because the exogenous ghrelin-induced protection was accompanied by a significant and dose-dependent rise in plasma ghrelin concentration as well as marked attenuation of the fall in GBF provoked by I/R, it is reasonable to assume that this effect could be considered as an important mechanism of the gastroprotective effect of this peptide in the rat stomach. However, the mechanism of ghrelin-induced increase in the GBF could be secondary to the primary effect of this hormone such as mucosal protection and increased mucosal integrity due to activation of PG and NO pathways and direct suppression of superoxide radicals and inflammatory response caused by these gastroprotective metabolites.

Ghrelin is a peptide originally extracted from the rat gastric mucosa, in which Ser3 is modified by an n-octanoic acid that is essential for the biological activity of this peptide (Kojima et al., 1999; Date et al., 2001; Kojima and Kangawa, 2002). In its acylated form, it is an endogenous ligand for the GHS-1a receptor that is localized in several organs of the central nervous system including hypothalamus and pituitary as well as in peripheral visceral organs such as stomach, intestine, and pancreas (Peeters, 2005). We found that antagonism of GHSR-1a receptors with D-Lys3-GHRP-6, which by itself failed to influence the lesions evoked by I/R, almost completely reversed the ghrelin-induced gastroprotection against I/R and the accompanying gastric hyperemia, indicating that GHRP-1a receptors are involved in the gastroprotective and hyperemic activities of this peptide.

Using conscious, well conditioned rats equipped with chronic GF for the gastric secretory studies, we found that systemic and intracerebral administration of exogenous ghrelin dose-dependently stimulated gastric acid secretion. This observation is in keeping with the findings of Masuda et al. (2000) and Date et al. (2001), who also demonstrated an increase in the gastric secretion after parenteral administration of ghrelin. In our present study, ghrelin increased gastric secretion and reduced the severity of I/R-induced gastric lesions while raising GBF suggesting that ghrelin-induced protection and hyperemia are independent on secretory activity of this hormone. The mechanism by which ghrelin increases gastric acid secretion and, at the same time, affords protection against I/R damage, may involve an elevation in plasma gastrin concentration, which has been shown to exert gastroprotection against acute gastric lesions and to accelerate healing of I/R injury (Stroff et al., 1995; Brzozowski et al., 2000a).

We found that peripherally administered ghrelin dose-dependently elevated plasma ghrelin levels and attenuated I/R-
induced gastric erosions while causing an increase in PGE$_2$ generation in the gastric mucosa. The key finding of this study is the demonstration that ghrelin mRNA is up-regulated in the gastric mucosa when exposed to I/R and that this is followed by an increase in the plasma ghrelin level, suggesting that endogenous ghrelin might limit the extent of gastric damage provoked by I/R. Thus, ghrelin, similarly to leptin, could be considered a mucosal protector that is also involved in the control of food intake (Konturek et al., 2001; Nakazato et al., 2001; Brzozowski et al., 2004a; Peeters, 2005). Furthermore, plasma ghrelin is increased following stress-induced gastric lesions, and its enhanced immunoreactivity is associated with duodenal ulcerations induced by cysteamine in rats (Brzozowski et al., 2004a; Fukuhara et al., 2005).

The present study supports the notion that, in addition to NO and neuropeptides such as CGRP released from sensory nerves (Sibilia et al., 2003; Brzozowski et al., 2004a; Konturek et al., 2004), the protective and hyperemic activities of ghrelin involve an increase in mucosal generation of endogenous PGs. COX-1 and COX-2 isoforms were originally implicated in the mechanism of gastrointestinal mucosal integrity by observations that nonsteroidal antiinflammatory drug-induced damage resulted from the inhibition of their activities (Wallace et al., 2000; Tanaka et al., 2002; Takeuchi et al., 2004). In contrast to COX-1, COX-2 is not constitutively expressed in most of tissues but is dramatically up-regulated during inflammation (Lesch et al., 1998; Brzozowski et al., 2001) and after inhibition of mucosal COX-1 activity (Davies et al., 1997; Tanaka et al., 2002; Takeuchi et al., 2004).

Although PGs are thought of as the classic mediators of cytoprotection (Robert, 1979), recent studies have shown that they may not contribute to the gastroprotection obtained with peptides such as leptin and CCK against ethanol-induced gastric lesions (Brzozowski et al., 2000b). We found that exposure to I/R produced a significant fall in PGE$_2$ generation in the gastric mucosa despite overexpression of COX-2 and that this was counteracted by ghrelin. Moreover, indomethacin almost completely abolished, whereas SC560 and rofecoxib greatly attenuated, the protective and hyperemic effects of ghrelin, indicating that endogenous PGs, potentially derived from the activities of both COX-1 and COX-2, are responsible for the putative beneficial effects of this peptide in I/R-induced mucosal injury. This remains in agreement with recent report by Hiratsuka et al. (2005) in mice, who demonstrated that the suppression of COX-2 activity by rofecoxib aggravated mucosal damage examined after 90 min of reperfusion indicating that COX-2/PGE$_2$ are involved in inhibition of neutrophil activation and attenuation of the oxidative stress in the gastric mucosa. We confirmed our previous observations (Brzozowski et al., 1999, 2004) that suppression of COX-1 activity by nonselective and selective COX-1 inhibitors aggravates I/R lesions in rat gastric mucosa, but in a study by Hiratsuka et al. (2005), treatment with SC560 reduced the severity of I/R lesions in mouse stomach and attenuated the increase in the gastric blood flow measured after ischemia. The discrepancy between these results could be attributed to the animal species difference, the dose of SC560 chosen, and duration of both ischemia and reperfusion periods and experimental conditions employed.

It is of interest that although COX-1 and COX-2 inhibitors reversed the protective activity of ghrelin, this hormone still provided a small degree of protection even in the presence of COX inhibition. This suggests that factors other than endogenous PGs, possibly NO, or sensory neuropeptides such as CGRP (as shown previously) are implicated in ghrelin-induced protection. Ghrelin failed to interfere with mRNA expression of COX-1 compared with controls. However, COX-2 mRNA and protein, which were not detectable in normal mucosa, were well defined in the mucosa immediately after I/R and further overexpressed in ghrelin-injected animals. This suggests that the up-regulation of COX-2 mRNA with subsequent, probably local production of protective PG, contributes to the gastroprotective activity of ghrelin. Our study does not exclude the possibility that the central parasympathetic outflow to the stomach is modified by peripheral or central ghrelin or that vagal afferent and efferent nerves are involved in gastroprotection it affords, in a manner similar to that proposed in its control of food intake (Peeters, 2005). Vagotomy and capsaicin denervation significantly attenuated the gastroprotection afforded by ghrelin and the accompanying rise in GFB, indicating that vagal pathways and sensory neuropeptides such as CGRP (released from sensory afferent fibers) could in fact account for its protective and hyperemic effects. A possible interpretation is that ghrelin-induced gastroprotection may be due mainly to the stimulation of vagal sensory pathways via brain-gut axis and to the activation of COX-PG and NO synthase-NO systems that enhance the integrity of the mucosa (Konturek et al., 2004). This notion is supported by our present observation that ghrelin-induced up-regulation of COX-2 mRNA under I/R conditions was inhibited in capsaicin-denervated animals, suggesting an important relationship between the vagal afferent sensory pathway and COX-2/PG pathway in gastroprotective activity of this hormone in the stomach. The question is whether capsaicin, a selective sensitizer of afferent nerves, or CGRP and NO released from these neurons can up-regulate mRNA expression for COX-2 in the gastric mucosa need to be addressed in further studies.

Acknowledgments

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References


Ghrelin in Gastroprotection against I/R Injury

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