Prostaglandin/cyclooxygenase pathway in ghrelin-induced gastroprotection against ischemia-reperfusion injury

Tomasz Brzozowski, Peter C. Konturek, Zbigniew Sliwowski, Robert Pajdo, Danuta Drozdowicz, Sławomir Kwiecien, Grzegorz Burnat, Stanislaw J. Konturek, Wieslaw W. Pawlik

Department of Physiology Jagiellonian University Medical College, Cracow, Poland (T.B., Z.S., R.P., D.D., S.K., S.J.K., W.W.P.) & *Department of Medicine I, University of Erlangen-Nuremberg, Erlangen, Germany (P.C.K., G.B.)
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Address correspondence to:

Prof. dr Tomasz Brzozowski
Department of Physiology Jagiellonian University Medical College
16 Grzegorzecka Str., 31-531-Cracow, Poland
Phone: (+4812) 424-7231; Fax: (+4812) 421-1578
e-mail: mpbrzozo@cyf-kr.edu.pl

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Abbreviations:
GH, Growth hormone; GHS-R, growth hormone secretagogue receptor; PG, Prostaglandin; COX, Cyclooxygenase; I/R, ischemia/reperfusion; GBF, gastric blood flow; GF, gastric fistula; NSAID, nonsteroidal antiinflammatory drugs; MPO, myeloperoxidase; MDA, malonyldialdehyde; RT-PCR, reverse transcriptase polymerase chain reaction; NO, nitric oxide; NOS, NO-synthase; L-NAME, N^G^-nitro-L-arginine methylester; CGRP, Calcitonin gene related peptide
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 A) inhibition of ghrelin growth hormone secretagouge type 1a receptor (GHS-R1a) by using ghrelin antagonist, D-Lys^3^-GHRP-6; B) blockade of cyclooxygenase (COX)-1 (indomethacin, SC-560) and COX-2 (rofecoxib) and C) bilateral vagotomy or capsaicin denervation. I/R produced typical gastric erosions, a significant fall in the GBF, an increase in gastric MPO activity and MDA content and the upregulation of mucosal ghrelin mRNA. Ghrelin dose-dependently increased gastric acid secretion and significantly reduced I/R-induced gastric erosions, while producing a significant rise in the GBF and mucosal PGE_2_ 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-Lys^3^-GHRP-6 and capsaicin denervation and completely abolished by vagotomy. Indomethacin, SC-560 and rofecoxib, selective COX-1 and COX-2 inhibitors, attenuated ghrelin-induced protection that was restored by supplying methyl analog of PGE_2_. 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 upregulated 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.
Introduction

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 through afferent nerves and directly on hypothalamic feeding centers (Tschop et al., 2000). This peptide was also shown to enhance the gastric motility and gastric secretion (Date et al., 2001; Masuda et al., 2000).

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 is 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-R type 1b (GHS-R1b) (McKee et al., 1997). The GHS-R1a is the functionally active, signal transducing form of the GHS-R, while 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-n-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 NO-synthase (NOS) activity with L-NAME 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 (Lugarini et al., 2002; Scholz, 2003) but the possibility that these cytoprotective arachidonate metabolites could play an important role in the gastroprotective effect of ghrelin has not been explored. Moreover, the question remains whether ghrelin contributes to gastroprotection against gastric lesions caused not only by artificial irritants such as ethanol, but also can protect against those caused by vascular disturbances resulting from gastric ischemia-reperfusion that leads to widespread mucosal damage and decrease in the gastric blood flow. This prompted our interest in endogenous PGs because their role as well as the importance of expression of cyclooxygenase (COX-1) and COX-2 in the possible gastroprotective activity of ghrelin against I/R erosions have not been studied.

This study was designed to compare the effects of intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) administration of ghrelin on gastric acid secretion in rats equipped with chronic gastric fistula (GF) and gastric lesions induced by ischemia-reperfusion (I/R). Accompanying changes in the gastric blood flow (GBF), plasma ghrelin levels and the generation of PGE₂ in the gastric mucosa were measured. Since oxidative stress and lipid peroxidation were implicated in the pathogenesis of I/R injury, we examined the myeloperoxidase (MPO) activity and malonyldialdehyde (MDA), an index of lipid peroxidation in the gastric mucosa and we also determined the effects of inhibition of GHS-1a
receptors by ghrelin antagonist D-Lys³-GHSR-6, vagotomy and sensory denervation with large doses of capsaicin on ghrelin-induced gastroprotection and changes in the GBF in I/R injury. An attempt was made to elucidate the effects of treatment with non-selective (indomethacin) and selective COX-1 (SC-560) and COX-2 (rofecoxib) inhibitors on I/R-induced gastric lesions and accompanying changes in the GBF and gastric mucosal generation of PGE₂. In addition, we evaluated the expression of ghrelin-, COX-1- and COX-2 mRNA transcripts and COX-1 and COX-2 proteins in the gastric mucosa of intact rats and those exposed to I/R injury with or without ghrelin administration.
Material and Methods

Animals

Male Wistar rats, weighing 190-230 g and fasted for 24 h, were used in gastric secretory tests and in studies on gastroprotection against I/R lesions. This study was approved by the Institutional Animal Care and Use Committee of Jagiellonian University Medical College in Cracow and run in accordance with the statements of the Helsinki Declaration regarding handling of experimental animals.

Gastric secretory studies

The effects of acylated ghrelin (Bachem AG, Bubendorf, Switzerland), on gastric acid secretion were examined in 50 conscious rats weighing 200-230 g and equipped about 1 month earlier with a Thomas-type gastric fistula (GF) as described previously (Konturek et al., 1995). The animals were fasted overnight but had free access to water 24 h before the experiment and they were placed in individual Bollman-type cages to maintain the minimum restraint necessary. For the i.c.v. injection of vehicle or ghrelin, the GF rats had undergone surgery 48 h before the secretory studies according to the method published elsewhere (Brzozowski et al., 2000b). Briefly, under pentobarbital anesthesia, an incision was made along with the mid-line of the skull, the skull bones were cleaned of connective tissue and the point of intersection between the sagittal and coronary sutures was visualized. The point at the distance of approximately 2.5 mm from either sagittal and coronary suture was defined and in this place a small hole in the skull was made, using a needle with a very sharp end. The hole was made by rotary movement of the needle and the wound of the head was closed by a clip. The GF was opened and the stomach rinsed gently with about 5 ml of tap water at 37°C. The basal gastric secretion was collected for 60 min and vehicle or ghrelin was injected in various doses i.c.v. in a volume of 5 μl using a 10 μl Hamilton microsyringe. Vehicle (saline applied in a volume of 1 ml i.g or 5 μl i.c.v.) or ghrelin was injected i.p. or
i.c.v. in gradually increasing doses ranging from 2.5 - 40 µg/kg and 150-2400 ng/rat respectively, each dose being administered on a separate test day. The effectiveness of i.c.v. administration was verified by injecting 10 µl of dye (0.1 % toluidine blue). The visualization of dye on the walls of lateral ventricle indicated the exact location of i.c.v. injection. The collection of gastric juice was continued for the final 2 h after i.p. or i.c.v. injections of ghrelin or vehicle (control). The volume and acid concentration of each collected sample of gastric juice were measured and acid outputs (expressed in term of micromoles of acid per 30 min) were determined as described previously (Brzozowski et al., 1996).

**Gastroprotection studies and measurement of gastric blood flow (GBF)**

Acute gastric lesions were induced in 120 rats by exposing of their stomach to 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). Briefly, under pentobarbital anesthesia (50 mg/kg i.p.), the abdomen was opened, the celiac artery identified and clamped with a small device for 30 min followed by removal of the clamp to obtain reperfusion. Since our previous studies (Brzozowski et al., 2000a; Konturek et al., 2000) documented that 30 min of I fails to induce gastric lesions, the area of gastric erosions were determined after the end of 3 h of R (I/R). In particular groups of rats, an attempt was made to determine whether gastroprotection by ghrelin is affected by ghrelin receptor antagonist, D-Lys3-GHRP-6 (Bachem, Bubendorf, Switzerland) and non-selective and selective COX-1 and COX-2 inhibitors.

Several groups of rats, each consisting of 6-8 animals, were pretreated 30 min before the I/R either with 1) vehicle (saline), 2) ghrelin (standard dose 20 µg/kg i.p.), 3) D-Lys3-GHRP-6 (100 µg/kg i.p.), the ghrelin receptor antagonist (Peeters TL, 2005), 4) SC-560 (5 mg/kg i.g.), a selective COX-1 inhibitor (Brzozowski et al., 2004b; Takeuchi et al., 2004); 5) rofecoxib (10 mg/kg i.g.), the highly selective COX-2 inhibitor (Ehrich et al., 1999;
Brzozowski et al., 2001) and 6) indomethacin (5 mg/kg i.p.), a non-selective COX inhibitor (Konturek et al., 1995). The dose of ghrelin receptor antagonist, D-Lys3-GHRP-6, was selected on the basis of our preliminary studies in rats pretreated with this antagonist 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 µg/kg up to 200 µg/kg i.p. dose-dependently inhibited food intake stimulated by ghrelin in rats with GF.

At the dose used in the present study, indomethacin has been shown previously to inhibit gastric PGE2 generation by ~ 90 % without itself causing any mucosal damage (Konturek et al., 1987). The doses of SC-560 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). SC-560 (Cayman Chemical Co., Ann Arbor, Michigan, USA) 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 & Dhome, Warsaw, Poland) was first dissolved in methanol to obtain a stock solution 75 mg/ml and then diluted to the desired concentration with isotonic saline. Control rats received the corresponding vehicle. Our preliminary studies (data not included) 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 dimethyl PGE2 (Upjohn, Kalamazoo, MI, USA) 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, synthetic PGE2 analog was administered together with each COX-1 or COX-2 inhibitor with or without ghrelin administration starting 30 min prior to I/R.
Upon the termination of reperfusion after 3 h, the animals were anesthetized with pentobarbital, their abdomen was opened by midline incision and the stomach 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 flow meter (Biotechnical Science, Model RBF-2, 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, FRG) (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 non-selective COX-1 and COX-2 inhibitor, and with SC-560, a selective COX-1 inhibitor or rofecoxib, a specific COX-2 inhibitor. In the gastroprotection study, the following groups of rats, each consisting of 6-8 animals, were used: 1) vehicle (saline 1 ml i.p.) followed 30 min later by I/R; 2) ghrelin (2.5-40 µg/kg i.p.) followed 30 min later by I/R; 3) ghrelin (150-2400 ng/rat i.c.v.) followed 30 min later by I/R; 4) ghrelin (standard dose; 20 µg/kg i.p. or 1200 ng/rat) followed 30 min later by I/R; 4) 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; 5) 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 (about 100 mg) immediately after the animals were sacrificed to determine the mucosal generation of PGE₂ by specific
radioimmunoassay (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**

Since lipid peroxidation 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, USA). 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 resulted 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 radioimmunoassay**

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 (about 3 ml) taken from the vena cava for the measurement of plasma ghrelin levels by RIA as described previously (Brzozowski et al., 2004a; Date et al., 2001). Intact rats fasted overnight and given only vehicle (saline) i.p. were measured similarly in order to determine control values for plasma ghrelin concentration. Blood samples were collected in
heparin coated polypropylene tubes and centrifuged at 3000 rpm for 20 minutes 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, Bubendorf, Switzerland). Briefly, the ghrelin RIA involved the competition of a rat ghrelin sample with $^{125}$I-rat ghrelin tracer for binding to a specific rabbit antighrelin polyclonal antibody. The limit of assay sensitivity was 4 pg per tube, the intra-assay variation was less than 9%, and the interassay variation less than 6%.

**Effect of vagotomy and sensory denervation on gastroprotection 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). About 30 min before the experiment rats were anesthetized with ether and the abdomen 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 performed 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 s.c. 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 injections of capsaicin were performed under ether anesthesia to counteract 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 previously described (Holzer et al., 1991). Control rats received vehicle injections. 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 denervated rats were sacrificed and area of gastric lesions and GBF were measured as above.

**Reverse-transcriptase-polymerase chain reaction (RT-PCR) for detection of messenger RNA (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 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, Heidelberg, Germany). The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT) in an area set aside for performing the PCR reaction. The nucleotide sequences of the primers for ghrelin and β-actin were selected on the basis of the published cDNA encoding ghrelin and β-actin, respectively (Konturek et al., 2004a). The sense primer for ghrelin was 5′-TTGAGCCCAGAGCACCAGAAA-3′, and the antisense primer was 5′-AGTTGCAGAGGAGGCAGAAGCT-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; Brzozowski et al., 2004b). The primers were synthesized by GIBCO BRL/Life Technologies, Eggenstein, Germany. The signals for ghrelin mRNA was
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, 100 mmol DDT) by the addition of 1:20 dilution of aprotinin and 1:50 dilution of 100 mmol PMSF as described in detail previously (Konturek et al., 2004a). Insoluble material was removed by centrifugation at 12 000 g 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 Sequi-Blot TMPVDF membrane (BioRad, USA) by electroblotting. The specific primary rabbit polyclonal antibody against COX-1 and COX-2 (dilution 1:500, Santa Cruz, USA) 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, USA). Non-isotopic visualization of immuno-complexes was achieved by chemiluminescence using BM Chemiluminescence Blotting Substrate (Boehringer, Mannheim, Germany) and the developed membrane 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 ± SEM. Statistical analysis was done using analysis of variance and two way ANOVA 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 µmol/30 min to 162 ± 6 µmol/30 min at the highest dose of i.p. 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, while producing gastric lesions, caused a significant increase in the plasma ghrelin levels (121±12 pM in sham-control animals without I/R vs 246±18 pM in rats vehicle-control animals exposed to I/R). 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 about 8 µg/kg of 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 about 35% was recorded as compared to 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 at a dose of 5 µg/kg of 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, and 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 ID$_{50}$, being about 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 as compared with those obtained in vehicle-treated rats. Treatment with D-Lys$_3$-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 GHS-R1a antagonist, D-Lys$_3$-GHRS-6. Similarly, 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-Lys$_3$-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 as compared to 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 as compared to 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 PGE\(_2\) as compared to the respective value in the vehicle-control animals not exposed to I/R (105±8 vs 165±12 ng/g wet tissue weight) (Table 3). Ghrelin applied 30 min prior to I/R resulted in a significant increase in the PGE\(_2\) generation as compared to vehicle-pretreated animals exposed to I/R (Table 3).

Indomethacin (5 mg/kg i.p.), which by itself significantly aggravated gastric lesions induced I/R, suppressed the generation of PGE\(_2\) by about 85% and produced a significant fall in GBF as compared to vehicle-pretreated animals (Table 3, Fig. 3). It completely abolished the reduction in the area of the lesions and the 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 PGE\(_2\) 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). SC-560 (5 mg/kg i.g.), which by itself significantly reduced the PGE\(_2\) generation, significantly attenuated the ghrelin-induced protection and accompanying gastric hyperemia (Table 3, Fig. 3). Concurrent treatment with a minute amount of dimethyl PGE\(_2\) (1 µg/kg i.g.) in addition to ghrelin restored the protective and hyperemic activity of this peptide in rats treated with indomethacin, SC-560 or rofecoxib and then exposed to I/R (Fig. 3).

Effect of vagotomy and functional ablation of sensory afferent nerves by capsaicin on gastric lesions induced by I/R with or without ghrelin administration

As shown in Fig. 4, exposure to I/R resulted in gastric lesions whose area was significantly reduced by the administration of ghrelin at a dose of 20 µg/kg i.p. or 1200 ng/rat
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 three days) of capsaicin to induce a functional ablation of sensory afferent endings on ghrelin-induced protection and increase in the GBF. Such a capsaicin denervation significantly aggravated gastric lesions induced by I/R and significantly decreased GBF as compared to 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 panel) 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 upregulated in rats exposed to I/R as compared to intact gastric mucosa (Fig. 5, left panel). As shown in Fig. 6 (left panel), 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 as compared to 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 panel). Signal intensity for COX-2 mRNA in animals exposed to I/R was significantly stronger in gastric mucosa of ghrelin-pretreated animals as compared to control animals.
exposed to I/R (Fig. 6, left panel). 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 as compared to that in rats exposed to I/R without ghrelin administration (Fig. 6, right panel).

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 which was significantly enhanced by pretreatment with ghrelin (Fig. 7, left panel). The ratio of COX-2 protein to β-actin was significantly higher in gastric mucosa of ghrelin-pretreated animals compared to vehicle-control animals exposed to I/R (Fig. 7, right panel). As shown in Fig. 8, the COX-2 protein expression was significantly decreased in the gastric mucosa of capsaicin-denervated rats exposed to I/R as compared to those with intact sensory nerves. The enhancement in the COX-2 protein expression in ghrelin-treated animals was also significantly attenuated by capsaicin denervation (Fig. 8, upper 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 to that in I/R rats with intact sensory nerves with or without ghrelin administration (Fig. 8, lower panel).
Discussion

This study demonstrates that exogenous ghrelin administered parenterally or intracerebroventricularly exhibits dose-dependent gastroprotective 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 hyperemia were reversed by ghrelin receptor antagonist D-Lys³-GHRP-6, indicating that ghrelin-induced protective and hyperemic effects are mediated by the functionally active form of GHS-R1a receptor which 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 oxygen metabolites under I/R conditions. Both, PG and NO which contribute to ghrelin-induced protection, are known to inhibit neutrophils 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) as well as against damage induced by stress (Brzozowski et al., 2004a; Konturek et al., 2004a). 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 A. Robert (1979). As 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 which is localized in several organs of the CNS including hypothalamus and pituitary as well as in peripheral visceral organs such as stomach, intestine and pancreas (Peeters TL, 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 PGE₂ generation in the gastric mucosa. The key finding of this study is the demonstration that ghrelin mRNA is upregulated 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 (Nakazato et al., 2001; Konturek et al., 2001b; Brzozowski et al., 2004a; Peeters TL, 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., 2004a), the protective and hyperemic activities of ghrelin involve an increase in mucosal generation of endogenous PGs. Cyclooxygenase (COX)-1 and COX-2 isoforms were originally implicated in the mechanism of gastrointestinal mucosal integrity by observations that NSAID-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 upregulated during inflammation (Lesch et al., 1998; Brzozowski et al., 2001) and following 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 PGE2 generation in the gastric mucosa despite, of overexpression of COX-2, and that this was counteracted by ghrelin. Moreover, indomethacin almost completely abolished, while SC-560 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/PG 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, Brzozowski et al. 2004) that suppression of COX-1 activity by non-selective and selective COX-1 inhibitors aggravates I/R lesions in rat gastric mucosa, but in study by Hiratsuka et al., (2005), treatment with SC-560 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 SC-560 chosen, duration of both, ischemia and reperfusion periods and experimental conditions employed.

It is of interest that while 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 to
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 over-expressed in ghrelin-injected animals. This suggests that the upregulation 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 TL, 2005). Vagotomy and capsaicin denervation significantly attenuated the gastroprotection afforded by ghrelin and the accompanying rise in GBF, 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 NOS-NO systems that enhance the integrity of the mucosa (Konturek et al., 2004b). This notion is supported by our present observation that ghrelin-induced upregulation 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 whether capsaicin, a selective sensitizer of afferent nerves or CGRP and NO released from these neurons can upregulate mRNA expression for COX-2 in the gastric mucosa need to be addressed in further studies.
References


Footnotes

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Figure Legends

Fig. 1. The mean area of gastric erosions induced by ischemia/reperfusion (I/R), gastric blood flow (GBF) and plasma immunoreactivity of ghrelin in rats treated with vehicle (saline) or with various doses of ghrelin (5 – 40 µg/kg i.p.). Means ± SEM of 6-8 rats. Asterisk indicates a significant change as compared to the vehicle control values.

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 ghrelin applied 20 µg/kg (i.p.) with or without D-Lys3-GHSR-6 (100 µg/kg i.p.). Mean ± SEM of 6-8 rats. Asterisk indicates a significant change compared to the vehicle-pretreated controls. Cross indicates a significant change compared to rats treated with GHS-R1a antagonist.

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.), SC-560 (5 mg/kg i.g.) and rofecoxib (10 mg/kg i.g.) applied alone or combined with 16,16 dm PGE2 (1 µg/kg i.g.). Mean ± SEM of 6-8 rats. Asterisk indicates a significant decrease as compared to the value obtained in vehicle-controls. Double asterisks indicate a significant increase as compared to vehicle-pretreated controls. Cross indicates a significant increase as compared to the value in animals pretreated with ghrelin applied without COX-inhibitors. Double crosses indicate a significant change as compared to animals treated with indomethacin, SC-560 or rofecoxib.

Fig. 4. The mean area of I/R-induced gastric lesions and the alterations in the GBF in rats with intact vagal nerves or with vagotomy with or without pretreatment with vehicle (Veh; control), ghrelin applied 20 µg/kg (i.p.) or ghrelin injected i.c.v. in a dose 1200 ng/rat. Mean ± SEM of 6-8 rats. Asterisk indicates a significant change compared to control vehicle-treated rats. Cross indicates a significant change compared to rats without vagotomy.

Fig. 5. Determination of β-actin and ghrelin mRNA (left panel) by RT-PCR and the ratio of
ghrelin to β-actin mRNA (right panel) in intact gastric mucosa treated with vehicle (saline) and not exposed to I/R (lane 1); and in those treated with vehicle (saline) and exposed to I/R (lane 2), M - DNA size marker. Mean ± SEM of 4-6 rats. Asterisk indicates a significant change as compared to control.

**Fig 6.** Determination of COX-1 mRNA and COX-2 mRNA by RT-PCR (left panel) and ratio of COX-1 mRNA and COX-2 mRNA (right panel) to β-actin mRNA in control animals (lane 1) and in those treated with vehicle and exposed to 3 h of I/R (lane 2), and those given ghrelin i.p. at a standard dose of 20 µg/kg prior to I/R (lane 3), M - DNA size marker. Mean ± SEM of 4-6 rats. Asterisk indicates a significant change as compared to the value obtained in the intact gastric mucosa. Cross and asterisk indicate a significant change as compared to vehicle-pretreated animals exposed to I/R.

**Fig. 7.** Representative Western Blot analysis of COX-2 and β-actin protein (left panel) and ratio of COX-2 to β-actin protein (left panel) in intact rats and those pretreated with vehicle or ghrelin (20 µg/kg i.p.) with or without subsequent exposure to I/R. Comparisons between the groups were made by determining the COX-2 to β-actin ratio by densitometry. Asterisk indicates a significant change as compared with the value obtained in intact gastric mucosa. Asterisk and cross indicate a significant change as compared to vehicle-pretreated animals.

**Fig. 8.** Western Blot analysis of COX-2 and β-actin protein (upper panel) and ratio of COX-2 to β-actin protein (lower panel) in rats with intact and functionally ablated sensory afferent nerves by capsaicin treated with vehicle or ghrelin (20 µg/kg i.p.) and exposed to I/R. Comparisons between the groups were made by determining the COX-2 to β-actin ratio by densitometry. Asterisk indicates a significant change as compared to the value obtained in intact gastric mucosa. Asterisk and cross indicate a significant change as compared to vehicle-pretreated animals. Cross indicates a significant change as compared to respective values in rats without capsaicin treatment.
Table 1

The effect of vehicle (saline) and ghrelin applied i.c.v. in graded doses ranging from 150 ng/rat up to 2400 ng/rat on the mean area of I/R-induced gastric erosions and accompanying alterations in GBF and plasma ghrelin levels. Mean ± SEM of 8 rats. Asterisk indicates significant change as compared to the value recorded in the vehicle-treated control rats.

<table>
<thead>
<tr>
<th>Type of test</th>
<th>Mean lesion area (mm²)</th>
<th>GBF  (% 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>
Table 2

The effect of vehicle (saline) and ghrelin applied i.p. in a dose of 20 µg/kg on the MPO activity and MDA + 4HNE concentration in the gastric mucosa of intact rats and those subjected to I/R. Mean ± SEM of 6 - 8 rats. Asterisk indicates a significant change as compared to the value in the intact gastric mucosa. Asterisk and cross indicate a significant change as compared to the value recorded in the vehicle-treated control rats.

<table>
<thead>
<tr>
<th>Type of test</th>
<th>MPO activity (ng/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 ± 0.16*</td>
<td>13.2 ± 2.8*</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>2.9 ± 0.7**</td>
<td>5.9 ± 1.1**</td>
</tr>
</tbody>
</table>
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 non-selective (indomethacin 5 mg/kg i.p.) and selective COX-1 (SC-560 5 mg/kg i.g.) or COX-2 (rofecoxib 10 mg/kg i.g.) inhibitors and then exposed to 3 h of I/R. Mean ± SEM of 8 rats. Asterisk indicates significant change as compared to vehicle-treated controls. Cross indicates significant change as compared to groups without I/R. Double cross indicates a significant change as compared to groups pretreated with ghrelin but without COX-1 or COX-2 inhibition.

<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>
<tr>
<td>Type of test</td>
<td>Mean lesion area (mm²)</td>
</tr>
<tr>
<td>---------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td><strong>Without capsaicin denervation</strong></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>65 ± 5</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>11 ± 2*</td>
</tr>
<tr>
<td><strong>With capsaicin denervation</strong></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>74 ± 4+</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>52 ± 2*</td>
</tr>
</tbody>
</table>
Fig. 1

Mean Lesion Area (mm²)

Veh

+ Ghrelin (µg/kg i.p.)

2.5

5

10

20

40

0 15 30 45

Plasma Ghrelin (pg/ml)

GBF (% control)

ISCHEMIA-REPERFUSION

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Fig. 2

ISCHEMIA-REPERFUSION

MEAN LESION AREA (mm²)

Veh

CHREIN (20 µg/kg i.p.)

GHRP-6 (100 µg/kg i.p.)

GBF (% control)

0 15 30 45

0 30 60 90

* *
Fig. 4

Mean Lesion Area (mm²)

Veh (20 µg/kg i.p.)

Ghrelin (1200 ng/rat i.c.v.)

+ Vagotomy

Ghrelin (20 µg/kg i.p.)

(1200 ng/rat i.c.v.)

Ischemia-Reperfusion

GBF (% control)
Fig. 5

- **Ghrelin mRNA/β-actin mRNA ratio**
  - Ve h
  - Ve h + I/R

- **Band Analysis**
  - M 1 2
  - Ghrelin: 349 bp
  - β-actin: 764 bp
Fig. 6

- COX-1: 561 bp
- COX-2: 201 bp
- β-actin: 764 bp

Bar graphs showing COX-1 and COX-2 mRNA ratios under different conditions:
- COX-1 mRNA ratio:
  - Veh: 0.4
  - Veh + I/R: 0.8
  - GHREIN (20 µg/kg i.p.): 1.0
- COX-2 mRNA ratio:
  - Veh: 0.6
  - Veh + I/R: 0.8
  - GHREIN (20 µg/kg i.p.): 1.0

* indicates significant difference.
Fig. 7

Veh  Veh  GHRELIN (20 µg/kg i.p.)
+ I/R

COX-2 (72 kDa)

β-actin (42 kDa)

COX-2 protein/β-actin protein ratio

Veh  Veh  GHRELIN (20 µg/kg i.p.)
+ I/R

*  **
Fig. 8

COX-2/β-actin protein ratio

Intact

Veh

Ghrelin

+ CAPSAICIN DENERVATION

Veh

Ghrelin

+ I/R

β-actin

42 kDa

COX-2

72 kDa

0

0.2

0.4

0.6

0.8