GASTROENTEROLOGY

In vivo and in vitro antioxidant activity of ghrelin: Attenuation of gastric ischemic injury in the rat

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Abstract

Background and Aim: Gherlin, an endogenous ligand for the growth hormone secretagogue receptor (GHS-R), is produced by stomach cells. It regulates food intake, gastric secretion and motility. However, its role as a protective agent in gastric ischemia/reperfusion (I/R) injury has not yet been investigated. Therefore, the objectives of the present study were to: (i) test the in vivo effect of peripherally administered ghrelin on gastric I/R-induced lesions in rats; and (ii) investigate in vitro the effect of ghrelin on reactive oxygen species (ROS) production by human polymorphonuclear (PMN) cells.

Methods: The present study was carried out on three groups of rats (six per group): control (sham–operated), I/R (clamping of celiac artery for 30 min and reperfusion for 1 h), and I/R + ghrelin (200 ng/kg i.v., 15 min before ischemia and before reperfusion, respectively). Histological assessment of hematoxylin and eosin stained sections was performed and immunostaining with inducible nitric oxide (iNOS) antibody were performed on a gastric paraffin embedded section. Oxidative stress markers thiobarbituric acid reactive substance (TBARS) and glutathione (GSH) were measured in gastric tissue homogenates. Serum lactic acid dehydrogenase (LDH) was determined. Tumor necrosis factor-alpha (TNF-α) was assayed in gastric tissue homogenate. Gastric permeability was assessed calorimetrically using Evans blue dye. In vitro studies were carried out on isolated human PMN cells incubated with ghrelin and tested for ROS generation as measured by chemiluminescence (CL).

Results: Peripheral administration of ghrelin attenuated gastric injury by reducing ulceration, tissue congestion, cellular infiltration and vascular permeability. Serum level of LDH and tissue content of TNF-α were markedly reduced. A decrement in TBARS and an increment in GSH were observed. Ghrelin treatment attenuated iNOS protein expression which was upregulated by gastric ischemic injury. In vitro studies showed for the first time that ghrelin inhibited ROS generation by human PMN in a dose-dependent manner.

Conclusions: These results provide evidence that peripherally administered ghrelin protects against gastric I/R injury. We also demonstrated that this protection is possibly accomplished through the antioxidant activity of ghrelin observed in vivo and in vitro.

Key words
chemiluminescence, gastroprotection, ghrelin, ischemia-reperfusion, reactive oxygen species.

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Introduction

Ghrelin is a 28-amino acid acylated peptide, mainly produced by the rat and human stomach, and recognized as the main endogenous ligand for growth hormone secretagogue receptors (GHS-R). Gastric cells producing ghrelin have been located in oxyntic glands as demonstrated by in situ hybridization, northern blot analysis and immunocytochemistry. These cells represent about 20% of endocrine gastric cells. Gastric ghrelin represents the main part of circulating ghrelin; after gastrectomy or fundectomy plasma circulating levels of ghrelin were reduced by 80%. The implication of ghrelin in gastric function so far investigated includes gastric motility and secretion. In order to exert its functions, circulating ghrelin needs to activate a 7-transmembrane G protein-coupled receptor (GPCR) called GHS-R. Ghrelin and/or its receptors are widely distributed in other tissues including: the hypothalamus; pituitary; cardiovascular system; and human immune cells, specifically T cells, B cells and neutrophils, suggesting an endocrine as well as extra-endocrine action of ghrelin.

Acute gastric mucosal injury has been reported to result from decreased blood flow and alterations in gastric microvasculatization, leading to ischemia reperfusion injury (I/R). The pathophysiology of I/R-induced injury is associated with inflammatory responses which elicit tissue damage. The acute inflammatory
response is characterized by induction of inflammatory cytokines, neutrophil infiltration and generation of oxygen free radicals. Recruitment of neutrophils to the sites of inflammation has been shown to be associated with the production of cytokines such as tumor necrosis factor-alpha (TNF-α), a major proinflammatory cytokine playing an important role in inflammation. Moreover, reactive oxygen species (ROS) readily react with cellular macromolecules, causing damage to proteins, lipids and nucleic acids. Malondialdehyde (MDA), which arises from the breakdown of lipid peroxyl radicals, is one of the indicators of oxidative stress. Conversely, glutathione systems, such as intracellular glutathione (GSH) or sulfhydryl (SH) groups of proteins in extravascular areas are considered to represent one of the major mechanisms of reducing oxidative stress. Thus an imbalance between gastro-toxic and protective agents can lead to acute inflammation and impairment of mucosal integrity.

Despite the fact that ghrelin is a gastric hormone, its role in mucosal defense and gastroprotection has been scarcely investigated. Sibilia et al. and Brzozowski et al. have recently demonstrated the gastroprotective effect of centrally and peripherally administered ghrelin, respectively, in an ethanol model of gastric ulceration. They suggested that ghrelin’s protective effect is mediated by endogenous nitric oxide (NO) release as well as vagal activity. NO is a free radical that regulates a variety of processes in the gastrointestinal tract and is largely produced by three isoforms of NO synthase (NOS): neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). Interestingly, iNOS is markedly upregulated by numerous agents including TNF-α. Nevertheless, the role of ghrelin in situations characterized by inflammation, oxidative stress and impairment of microvascular circulation which occurs in I/R injury needs to be elucidated. Therefore, the present study was designed to investigate the ability of ghrelin in abrogating oxidative stress in vivo in a model of gastric I/R injury. Because ghrelin receptors are found on neutrophils and I/R-induced injury is characterized by neutrophil infiltration, we conducted in vitro studies to elucidate the possible mechanisms, if any, involved in ghrelin’s protective effect, by incubating isolated human polymorphonuclear (PMN) cells with ghrelin to test for its effect on ROS generation.

Methods

In vivo study

Animals

Male Wistar rats weighing 170–210 g were obtained from the College of Medicine Animal House at King Saud University. Rats were maintained on standard rat chow and tap water ad libitum. Rats were kept in an air-conditioned room with a 12 h day/night cycle. Animals were fasted 12 h prior to the experimental procedure. All studies were approved by the Ethics Committee of King Saud University.

Experimental design

Rats were divided into three experimental groups (six rats/group): (i) control sham-operated group; (ii) I/R group; and (iii) I/R + ghrelin group. Rat ghrelin peptide was purchased from Phoenix (Phoenix Pharmaceuticals, Belmont, CA, USA).

Rats were anesthetized by intraperitoneal injection of urethane at a dose of 125 mg/100 g bodyweight (BW). I/R lesions were produced as described by Yoshikawa et al. Briefly, the stomach was exposed and the esophagus and the pylorus were occluded using bulldog clamps. The celiac artery was clamped and 100 mM HCL (1 mL/100 g BW) was placed in the stomach to maintain acid levels during ischemia. The acid was then removed 25 min after ischemia and clamps were removed 30 min after ischemia. The tissues were allowed to reperfuse for 60 min and then the stomach was removed and examined.

Ghrelin was applied intravenously via a cannula inserted into the femoral vein, 15 min before ischemia and before reperfusion. Ghrelin was injected at two doses: 20 ng and 200 ng per kg. A dose of 200 ng/kg was found to be effective depending on histological examination and Evans blue (EB) dye extravasation.

By the end of the experiment serum and gastric tissues were collected. Gastric samples were snap-frozen in liquid nitrogen and stored at −80°C for subsequent assays of glutathione (GSH), thiobarbituric acid reactive substance (TBARS) and TNF-α. Sera were stored at 20°C for the assay of lactate dehydrogenase (LDH). A piece of each stomach was fixed in 4% phosphate-buffered formalin, embedded in paraffin and cut. Paraffin sections were hydrated and stained with hematoxylin and eosin (HE) for mucosal damage assessment or stained with sera specific for iNOS (Serotec, Oxford, UK).

Determination of vascular permeability

EB dye, an azo dye, is widely used as an indicator of increased capillary permeability. Systemic administration of EB leads to the formation of a dissociable complex with serum albumin, and when there is microvascular tissue damage, EB extravasates. In another set including the three experimental groups, 1 mL of EB (0.5% v/w) was injected intravenously after reperfusion or sham operation. The amount of EB that accumulated in the stomach within the reperfusion period was measured. Briefly, the animals were killed and the stomach was removed. After collecting the gastric content carefully by lavaging with 5 mL of cold distilled water, the stomach was opened along the greater curvature and the corpus mucosa was scraped off and put into a tube containing 5 mL of distilled water. The EB was extracted by a modified method of Lange et al. and its concentration was spectrophotometrically quantified. The EB present in gastric contents and mucosa was extracted by adding 5 mL of formamide to each tube and kept in a shaking water bath at a temperature of 50°C for 24 h. This was followed by centrifugation at 3000 g for 10 min and the absorbance of supernatant was measured at 612 nm (Lambda 5 Perkin-Elmer, Pomona, CA, USA). The amount of EB was calculated from a previously made standard curve and expressed as micrograms per stomach.

Determination of gastric thiobarbituric acid reactive substance

TBARS was measured as an indicator of lipid peroxidation. The amount of gastric TBARS was measured by the thiobarbituric acid...
Determination of gastric glutathione content

Gastric GSH was determined as previously described by Ellman and modified by Nagi et al. Briefly, GHS in gastric tissue homogenate was allowed to react with Ellman’s reagent (5,5-dithio-2-nitrobenzoic acid) in phosphate buffer (pH 8.0) and the absorbance was measured at 412 nm. GSH concentration was calculated using a standard solution of GSH. The results were expressed as nmol/g wet tissue weight.

Determination of serum lactic acid dehydrogenase

Serum LDH was measured using commercially available kits (Human, Wiesbaden, Germany).

Determination of gastric tumor necrosis factor-α content

Gastric tissue homogenate was prepared in four parts of homogenate buffer (0.1 M sodium phosphate [pH 7.5], 2.0 mM EGTA, 1.0 mM benzamidine, 1.0 mM phenyl-methylsulfonyl fluoride). Centrifugation was carried out at 4°C, 1000 g for 15 min. TNF-α in the supernatant was measured by a rat ELISA kit purchased from R & D Systems (R & D Systems Europe, Abingdon, UK). Protein concentration in the sample was quantified with a protein assay kit from Bio-Rad (Bio-Rad Laboratories, Hertfordshire, UK). Results were presented in picograms of TNF-α per milligram of protein.

Immunohistochemistry

Immunohistochemistry was performed using formalin fixed, paraffin-embedded sections (4 μm) after dewaxing and rehydration. Endogenous peroxidase was quenched with 3% H2O2 for 30 min and sections were blocked with 10% normal goat serum (Sigma). Sections were incubated with rabbit antimouse iNOS (Serotec) at a concentration of 1:500 and were kept at room temperature for 2 h. Sections were then washed and incubated with secondary antibody (antirabbit IgG antibody) and immunoperoxidase staining was carried out using the Vectastain ABC Elite reagent kit (Vector Laboratories, CA, USA). Di-aminobenzidine was used as a chromogen. All slides were counterstained with hematoxylin.

In vitro study

Isolation of human polymorphonuclear cells

Human venous blood samples were obtained from non-smoking healthy male donors with 0.1% sodium heparin. PMN were separated by using neutrophil’s isolation medium (NIM) (Cardinal Associates, Santa Fe, NM, USA). In a 15 mL tube, 5–7 mL of heparinized blood was layered over 4 mL of NIM and then centrifuged at 400 g for 10 min at room temperature. After centrifugation, the blood was separated into layers according to the weight of the cells. Carefully, the leukocyte–rich plasma was removed and transferred to a 15 mL conical centrifuge tube. The tube was then filled with phosphate buffered saline (PBS) (pH 7.4) and centrifuged at 350 g for 10 min. The residual erythrocytes were lysed using 2 mL of lysis buffer (E-lyze; Cardinal Associates), vortexed then centrifuged at 250 g for 10 min. The supernatant was discarded and the sediment was suspended in 1 mL of 5% fetal calf serum (FCS). The cells were then counted and adjusted to 5 million/mL concentration.

Preparation of reagents

Luminol (LKB-Wallac) was dissolved in dimethyl sulfoxide (DMSO) to give a concentration of 1.77 mg/mL, which was further diluted in PBS to 17.7 μg/mL prior to use. Phorbol myristat acetate (PMA) was dissolved in DMSO and then diluted with PBS to give a final concentration of 20 ng/mL.

Assay for reactive oxygen species generation in human neutrophils

ROS generation in the presence or absence of ghrelin (1–1500 ng/mL) was determined by chemiluminescence (CL), as described by Van Dyke et al. Briefly, control cuvettes contained 500 μL PBS, 200 μL luminol, 100 μL PMN and 200 μL PMA (20 ng/mL). Cuvettes to which ghrelin was added contained 400 μL PBS, 200 μL luminol, 100 μL PMN, 100 μL ghrelin (of different concentrations) and 200 μL PMA. The cells were incubated for 60 min with different concentrations of ghrelin (1–1500 ng/mL). The contents were mixed gently and measurements of light emission were recorded as mV using LKB-Wallac 1251 Luminometer (Turkv, Finland). Each sample had been measured over a period of 30 min and the results were plotted using a computer as a graph with the Y-axis representing the light intensity in mV, and the X-axis representing the time in minutes. The assay temperature was kept constant at 37°C.

Cell-free xanthine–xanthine oxidase system

The cell-free xanthine-xanthine oxidase generating system was used as a superoxide generating system. Briefly, the production of O2– radicals generated by the catalyzed reaction of xanthine oxidase upon xanthine was induced by incubating 0.05 U/mL of xanthine oxidase in PBS, pH 7.4, containing 0.1 mM/L EDTA, 50 mM/L xanthine and 10-4 mol/L luminol. Ghrelin was incubated at different concentrations with xanthine and luminol for 5 min before the addition of xanthine oxidase.

The generation of superoxide radicals was measured by monitoring the luminol-dependent CL in a luminometer (LKB 1251; Wallac) at 37°C.

Chemicals and reagents

All chemicals were purchased from Sigma unless otherwise specified.
**Statistical analysis**

All values are expressed as the mean ± SEM. Statistical significance of differences was determined using analysis of variance (one-way ANOVA). Further statistical analysis for post-hoc comparisons was carried out using the Tukey-Kramer multiple comparison test. A level of $P < 0.05$ was considered statistically significant.

**Results**

**In vivo studies**

**Vascular permeability changes**

Thirty minutes of ischemia followed by 60 min of reperfusion resulted in a significant leakage of EB in the stomachs compared with the control sham-operated group (17.52 ± 0.96 μg/stomach vs 0.486 ± 0.024 μg/stomach, respectively; $P < 0.005$). Pretreatment with ghrelin significantly ameliorated EB leakage (3.58 ± 0.55 μg/stomach), suggesting its impact on reducing vascular permeability in a compromised tissue (Fig. 1). However, extravasation of EB in the ghrelin treated group was still higher than in the control ($P < 0.05$).

**Effect of ghrelin pretreatment on markers of oxidative stress**

*Glutathione tissue content.* I/R injury produced a reduction in gastric GSH content (253 ± 16 nmol/g wet tissue, $P < 0.05$) as compared to the sham-operated control group (783 ± 41 nmol/g wet tissue) (Table 1). Treatment with ghrelin abrogated I/R injury induced GSH depletion as compared to the I/R group (752 ± 23 nmol/g wet tissue vs 253 ± 16 nmol/g wet tissue; $P < 0.05$). Besides, ghrelin treatment resumed GSH content to the control level (752 ± 56 vs 783 ± 101 nmol/g wet tissue; $P > 0.05$, i.e. not significant).

*Thiobarbituric acid reactive substance tissue content.* Gastric TBARS showed a high concentration in the I/R group that was statistically significant (3.45 ± 0.264 μmol/g wet tissue) when compared with the control group (1.10 ± 0.136 μmol/g wet tissue, $P < 0.05$). Ghrelin treatment produced a reduction in gastric TBARS content down to 1.17 ± 0.051 μmol/g wet tissue, which was markedly lower than in the non-treated I/R group ($P < 0.05$) and was similar to the control group ($P > 0.05$, i.e. not significant) (Table 1).

**Effect of ghrelin pretreatment on serum lactic acid dehydrogenase level**

Levels of LDH in serum showed a significant elevation after I/R injury with respect to that of sham-operated rats (3695 ± 261 U/L vs 742 ± 53 U/L, respectively; $P < 0.05$). Treatment with ghrelin reduced serum LDH as compared to the I/R group (1240 ± 109 U/L vs 3695 ± 261 U/L; $P < 0.05$) (Table 1). Administration of ghrelin reduced serum LDH to the control level.

**Effect of ghrelin pretreatment on inflammatory cytokine tumor necrosis factor-α**

As illustrated in Figure 2, I/R gastric injury caused an increment in TNF-α from the control level of 15.2 ± 0.608 to 44.7 ± 4.38 pg/mg protein ($P < 0.05$). Ghrelin administration (200 ng/kg i.v.) significantly reduced TNF-α release to a level of 22.8 ± 3.8 pg/mg protein, which was nearly 50% lower than the I/R untreated group ($P < 0.05$) and close to the control level.

**Histological studies**

As demonstrated in Figure 3a the stomachs of the control rats showed normal intact mucosa and submucosa. I/R injury induced hemorrhages, ulcerations of the mucosa and inflammatory cell

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**Table 1** Effect of ghrelin (200 ng/kg i.v.) given 15 min before ischemia and reperfusion on biochemical markers of oxidative stress and tissue damage

<table>
<thead>
<tr>
<th></th>
<th>Sham-operated</th>
<th>I/R</th>
<th>Ghrelin + I/R</th>
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</thead>
<tbody>
<tr>
<td>Glutathione (nmol/g wet tissue)</td>
<td>783 ± 41</td>
<td>253 ± 16$^a$</td>
<td>752 ± 23$^a$</td>
</tr>
<tr>
<td>TBARS (μmol/g wet tissue)</td>
<td>1.10 ± 0.136</td>
<td>3.45 ± 0.264$^a$</td>
<td>1.17 ± 0.051$^a$</td>
</tr>
<tr>
<td>Serum LDH level (U/L)</td>
<td>742 ± 53</td>
<td>3695 ± 261$^a$</td>
<td>1240 ± 109$^a$</td>
</tr>
</tbody>
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1$^a$/R versus sham-operated controls; 2ghrelin treated group versus I/R. I/R, ischemia-reperfusion; LDH, lactic acid dehydrogenase; TBARS, thiobarbituric acid reactive substance.
infiltration (Fig. 3b). The distribution of lesions was patchy throughout the mucosa. These structural changes were almost abolished with prior ghrelin administration (200 ng/kg i.v.) (Fig. 3c).

**Inducible nitric oxide immunohistochemistry**

Immunohistochemical study revealed that iNOS was upregulated following I/R injury and was localized to the mucosa, with the highest expression present in areas of significant ulceration and inflammation (Fig. 4c). More specifically, the majority of iNOS expression was present within the inflammatory cell infiltrate in the mucosa and desquamated epithelial cells. Ghrelin treatment markedly downregulated iNOS expression in rats subjected to I/R which was associated with preservation of mucosal integrity (Fig. 4d). Normal gastric mucosa did not show expression of iNOS (Fig. 4b). A sero-negative staining of gastric sections from the I/R group was performed to confirm the specificity of immunostaining (Fig. 4a).

**In vitro studies**

**Effect of ghrelin on reactive oxygen species production by human polymorphonuclear cells**

When PMNs were challenged with PMA in the presence of different concentrations of ghrelin (1-1500 ng/mL), a dose-dependent inhibition of the CL response was observed (Fig. 5a). The inhibition was calculated as a percent of the baseline. The highest inhibitory effect was achieved at a concentration of 1000 ng/mL and then a plateau was obtained, indicating no more inhibition could be produced by increasing the concentration of ghrelin above that level. The concentration of ghrelin required to inhibit maximal peak CL by 50% was 200 ng/mL. Thus ghrelin after incubation with PMN inhibited ROS generation in response to PMA stimulation.
Effect of ghrelin on superoxide production in the cell-free system

The effect of ghrelin was tested on O$_2^-$ generation from the hypoxanthine-xanthine oxidase system. As demonstrated in Figure 5b, ghrelin at different concentrations did not prove to have an inhibitory effect on ROS generation in this system, suggesting that ghrelin is probably lacking scavenging activity. Superoxide dismutase (SOD) significantly reduced ROS generation. The control represents PBS.

Figure 4 Inducible nitric oxide (iNOS) immunostaining of gastric mucosa after ischemia-reperfusion (I/R) challenge in rats treated with (c) saline (d) or ghrelin (200 ng/kg i.v.) administered 15 min before ischemia and before reperfusion, respectively. (b) Normal gastric mucosa. iNOS protein was markedly expressed in gastric mucosa subjected to reperfusion injury and ameliorated by ghrelin treatment (magnification x20). Arrows point to the cytoplasmic brownish staining of iNOS. (a) A serumnegative staining of gastric section from the I/R group was used as a control for the specificity of staining.

Figure 5 (a) Effect of various concentrations of ghrelin (1–1500 ng/mL) on the chemiluminescence (CL) response (mV) of isolated human polymorphonuclear (PMN) leukocytes stimulated with phorbol myristate acetate (PMA). Ghrelin caused a dose–response dependent inhibition of reactive oxygen species (ROS) production by PMN. (b) Effect of ghrelin (1–1000 ng/mL) on ROS production in a cell-free medium was compared with superoxide dismutase (SOD). Results show lack of scavenging effect of ghrelin. ###SOD versus all groups (P < 0.001).
**Discussion**

The present work demonstrates that peripheral administration of ghrelin produced a gastroprotective effect against I/R injury in the rat. Furthermore, our data show that the protective effect of ghrelin is mediated by its antioxidant activity in vivo as determined by a decrement in the tissue level of TBARS, an indicator of lipid peroxidation, and serum level of LDH, while preserving tissue GSH content. Ghrelin reduced vascular mucosal permeability and iNOS protein expression. The in vitro study provided evidence that ghrelin inhibited ROS production by isolated human PMN in a dose-dependent manner, suggesting the antioxidant activity of ghrelin as a potential mechanism for its gastroprotective effect.

ROS are major contributors to tissue damage and are involved in a variety of biological phenomena such as I/R injury. ROS, including superoxide anion, hydrogen peroxide and hydroxyl radical, constitute a major part of biologically important free radicals. The most prominent characteristic of ROS is their high reactivity with other molecules. ROS produced inside or outside the cell induce reactive stress unless the prepared antioxidant mechanism completely compensates for ROS load. In the present work, the involvement of ROS in tissue damage was strongly suggested by a marked increase in lipid peroxidation products (TBA-reactive substances) following gastric I/R injury and this was ameliorated by ghrelin treatment. GSH is a physiological reductant that maintains the intracellular environment in a mildly reductive condition. This tripeptide is present within the cytosol of cells and is the major intracellular non-protein thiol compound (TBA-reactive substances) following gastric I/R injury and was resumed back to control level by ghrelin, suggesting an in vivo antioxidant activity of this peptide. These data suggest that ghrelin directs the redox reactions toward the reduction state thus reducing the reactivity of oxygen free radicals with other molecules.

Ghrelin has been shown to be not only produced in the stomach, but also found to be expressed together with its receptor (GHS-R) in the immune cells, namely, T lymphocytes, B lymphocytes and neutrophils, suggesting that ghrelin and its receptor may function as a signal modulator between the endocrine and immune systems. However, little information is available regarding the function of ghrelin in the immune cells. Because neutrophil infiltration and activation has been implicated in ischemic injury, we found it tempting to investigate in vitro the effect of ghrelin on ROS generation by human PMN. An important finding of the present study was the demonstration that ghrelin inhibited ROS production from isolated human PMN when stimulated with PMA in a dose-dependent manner. These in vitro studies support our in vivo data that ghrelin, via its antioxidant activity, provides tissue protection. When incubating ghrelin in the xanthine-xanthine oxidase reaction, inhibition of ROS was not observed, suggesting lack of scavenging activity of ghrelin. The mechanism by which ghrelin inhibited ROS is probably through blocking of some of the enzymes needed for their production. This speculation is supported by the design of the in vitro experiment that allowed us to incubate the cells with ghrelin followed by the addition of PMA. The latter was found to activate PMN nicotinic-amin di-nucleotide phosphate oxidase (NADPH-oxidase) through redistribution of protein kinase C (PKC) and phosphorylation of serial proteins including NADPH-oxidase, not only in the plasma membrane but also in the granular membrane of PMN. Hence PMA is considered a good tool to investigate the signal transduction pathways leading to activation of NADPH-oxidase in the two membranes. With these data in mind it could be suggested that ghrelin inhibits ROS production by PMN through modulating intracellular enzyme activity.

On the other hand, controversial results have been reported by Li et al., who demonstrated that ghrelin did not reduce the level of intracellular ROS in human endothelial cells treated with H₂O₂. This controversy may be explained by differences in the experimental design regarding cell type, concentration of ghrelin and incubation time, and the number of receptors activated. In the present study we incubated human PMN with different concentrations of ghrelin (1 ng/mL–1500 ng/mL) and 50% of CL inhibition was produced at 200 ng/mL. Our system allowed the measurement of total ROS by CL, while the one used by Li et al. measured H₂O₂ using the oxidant sensitive dichlorodihydrofluorescein diacetate fluorescence probe (H₂DCFDA).

The role of iNOS in gastric lesions induced by I/R injury in the presence and absence of ghrelin was investigated using iNOS antibody immunostaining of gastric sections. We observed that I/R injury produced intense iNOS protein expression which was prominent in mucosal areas but not in normal intact mucosa or submucosa. Theoretically, NOS upregulation would be protective, injurious, or simply a marker of inflammation and tissue injury. Interestingly, we demonstrated that iNOS protein expression (Fig. 4c) was associated with extravasations of EB dye (Fig. 1), a measure of tissue vascular permeability, as well as with neutrophil infiltration seen in histological sections (Fig. 3b) from rats subjected to gastric I/R. Similarly, recent reports showed that I/R tissue injury was associated with an increase in iNOS expression in various tissues including liver and brain. Therefore, the increased iNOS activity is considered cytotoxic to the microvascular endothelium and to promote neutrophil infiltration in the gastric mucosa.

In the present study, peripheral administration of ghrelin decreased the expression of gastric iNOS protein as observed from the immunostaining, suggesting that the gastroprotective effect of ghrelin is at least partially attributed to inhibition of iNOS. This inhibition is not transcriptional but is also probably translational as it has been recently demonstrated that ghrelin suppressed iNOS mRNA expression in gastric mucosa of rats subjected to ethanol or water stress. The consequences of iNOS activation is NO formation which proved to have proinflammatory actions, and therefore its inhibition is beneficial in several settings including I/R injury and lipopolysaccharide induced intestinal injury. The mechanisms involved in the proinflammatory actions of NO are unclear but may involve the function of NO as a free radical and/or conversion of NO to more reactive nitrogen species that can induce both cell damage and apoptosis. Hence, our results suggest that the gastroprotective effect of ghrelin is at least partially mediated by inhibition of iNOS. In contrast, it was reported that nitro-L-arginine methyl ester (L-NAME) blunted the gastroprotective effect of ghrelin. This discrepancy is clarified by three important points: (i) L-NAME inhibited all NOS isoforms; (ii) selective
inhibition of iNOS induced tissue protection; and (iii) reduction of NO produced by iNOS but neither neuronal (nNOS) nor endothelial (eNOS) forms, induced gastroprotection. Moreover, eNOS and nNOS affect gastric mucosal integrity via an action on the microcirculation and on the surface mucous–bicarbonate barrier. Therefore, inhibition of iNOS and maintenance of nNOS and eNOS activities would provide gastroprotection. This suggestion is further supported by the observation that the constitutive form of NOS was upregulated in the gastric mucosa of rats treated with ghrelin. The possibility of involvement of sensory nerves in mediating gastric protection by ghrelin cannot be excluded. Another factor that might be involved in ghrelin-induced gastroprotection is its anti-inflammatory effect. In the present study we reported that pretreatment of ghrelin in gastric I/R model was accompanied by a decrease in TNF-α. This anti-inflammatory effect of ghrelin has been recently documented in various cells including T lymphocytes and endothelial cells. Moreover, Li et al. reported that ghrelin inhibited basal and TNF-α-induced activation of nuclear factor-kB in human endothelial cells, suggesting a potential anti-inflammatory mechanism of ghrelin in tissue protection. Recall that iNOS is markedly upregulated by TNF-α so it could be speculated that ghrelin inhibits the TNF-α-iNOS axis.

Finally, a central mechanism for the gastroprotective effect of peripherally administered ghrelin cannot be excluded, as the activation of hypothalamic arcuate nucleus has been demonstrated when the peptide was administered intravenously in the rat. In conclusion, the present study provides the first evidence that peripheral administration of ghrelin exerts a potent gastroprotective effect against I/R induced lesions and provides a potential mechanism for this protection via the novel antioxidant activity of ghrelin observed in vivo and in vitro. These findings may help to explain the beneficial effects of ghrelin administration in various pathophysiological conditions associated with oxidative stress including I/R injury and encourage the use of ghrelin as an approach in the prevention and/or treatment of I/R injury.

References


