© Med Sci Monit, 2012; 18(5): BR181-187
PMID: 22534700

Received: 2011.05.13
Accepted: 2011.12.21
Published: 2012.05.01

Ghrelin accelerates the healing of cysteamine-induced duodenal ulcers in rats

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Source of support: This study was supported by a grant from the Polish Ministry of Science and Higher Education (Project N N401 000635) and a grant from Jagiellonian University

Summary

Background: Previous studies have shown that administration of ghrelin exhibits protective and therapeutic effects in the gut. The aim of the present investigation was to examine the influence of ghrelin administration on the course of cysteamine-induced duodenal ulcers, as well as effects on mucosal production of oxygen free radicals and duodenal antioxidant defense.

Material/Methods: Duodenal ulcers were induced in male Wistar rats by cysteamine administered intragastrically at the dose of 200 mg/kg in 1 ml of saline, 3 times at 4-h intervals. Starting 24 h after the first dose of cysteamine, rats were treated intraperitoneally twice a day with saline or ghrelin given at the dose of 4, 8 or 16 nmol/kg/dose. Seven days after administration of the first dose of cysteamine, the study was terminated.

Results: Induction of ulcers by cysteamine was accompanied by a reduction in duodenal blood flow, mucosal DNA synthesis and mucosal activity of superoxide dismutase (SOD); whereas mucosal concentration of interleukin-1β and malonyldialdehyde (MDA – an index of lipid peroxidation) were increased. Treatment with ghrelin increased healing rate of duodenal ulcers and enhanced duodenal blood flow, mucosal DNA synthesis and mucosal activity of SOD, and reduced mucosal concentration of interleukin-1β and MDA.

Conclusions: Treatment with ghrelin increases the healing rate of duodenal ulcers and this effect is related, at least in part, to improvement of duodenal mucosal blood flow, mucosal cell proliferation and antioxidant defense, as well as being related to reduction in mucosal oxidative stress and inflammatory response.

key words: ghrelin • duodenal ulcer • mucosal blood flow • cell proliferation • oxidative stress

Full-text PDF: http://www.medscimonit.com/fulltxt.php?ICID=882727

Word count: 3333
Tables: –
Figures: 6
References: 74

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Background

Ghrelin is a circulating growth hormone-releasing peptide primarily isolated from human and rat stomachs [1,2]. The stomach is a main source of ghrelin, but this peptide has been also detected in other organs [1,3,4]. Ghrelin is a natural ligand for growth hormone secretagogue receptor (GHS-R) [1]. Ghrelin strongly and dose dependently stimulates release of growth hormone from the anterior pituitary and promotes release of adrenocorticotropic hormone, corticosterone, and prolactin [1,5,6].

Ghrelin stimulates the appetite and fat deposition in rats and humans and fasting plasma level of ghrelin is negatively correlated with body mass index [7–10].

Previous studies have shown that administration of ghrelin exhibits a protective effect in the gut. Pretreatment with ghrelin inhibits the development of cerulein- or ischemia/reperfusion-induced acute pancreatitis and accelerates pancreatic recovery in the course of cerulein-induced pancreatitis [11–14]. In the stomach, pretreatment with ghrelin reduces gastric mucosal damage induced by ethanol, stress or alendronate and accelerates healing of acetic acid-induced gastric and duodenal ulcers. Moreover, animal and clinical studies suggest that ghrelin reduces colonic inflammation [15–20].

The protective effect of ghrelin seems to be dependent on different mechanisms. Sibilia et al. reported that the gastroprotective effect of ghrelin is mediated by release of endogenous nitric oxide and requires activity of capsaicin-sensitive sensory nerves [15]. Other studies have shown that pretreatment with ghrelin dose-dependently reduces the area of the ethanol-induced gastric lesions, improves gastric blood flow and reduces mucosal expression of pro-inflammatory tumor necrosis factor-α (TNF-α) [16]. The anti-inflammatory effect of ghrelin was also found in our previous studies [11,12].

Recent research has also shown a relationship between plasma level of endogenous ghrelin and clinical gastric pathology. Presence of *Helicobacter pylori* in the stomach of patients with peptic ulcers increases plasma ghrelin level; whereas gastric cancer and atrophic gastritis are accompanied by a marked decrease in plasma concentration of ghrelin [21].

It is very interesting that obestatin, a 23-amino acid peptide derived from the same prohormone as ghrelin, exhibits similar protective and therapeutic effects as ghrelin. Recent studies have indicated that obestatin promotes survival of pancreatic islets, inhibits development of experimental acute pancreatitis and accelerates the healing of chronic gastric ulcers [21–24].

Cysteamine (β-mercaptoethylamine) is the chemical compound with the formula $HSCH_2CH_2NH_2$. It is the simplest stable aminothiol and is a degradation product of the amino acid cysteine. Cysteamine is used in the body to form the essential biochemical coenzyme A (CoA) by combining with pantothenate (vitamin B 5) and adenosine triphosphate [25]. CoA may act as an acyl group carrier to form acetyl-CoA and other related compounds; this is a way to transport carbon atoms within the cell. CoA is critical in the metabolism and synthesis of carbohydrates, proteins and fats [26,27]. Clinically, cysteamine has been used in the treatment of cystinosis and cystinuria [28,29]. Cysteamine and its analog amifostine have been also used for treatment of radiation sickness [30]. Moreover, cysteamine, as a potent antioxidant, improves the maturation of oocytes and development of embryos in *in vitro* culture [31].

On the other hand, numerous experimental studies have shown that administration of cysteamine at high doses leads to induction of duodenal ulcers [32–34]. Pathogenesis of ulcerogenic activity of cysteamine is not fully understood. Previous studies have indicated that cysteamine increases gastric acid secretion and decreases neutralization of acid in the proximal duodenum. These effects seem to be related to somatostatin depletion in gastric mucosa and elevation of serum level of gastrin [35–37]. Some studies have suggested that transcription factors such as hypoxia-inducible factor-1α and early growth response factor-1 and their target genes are involved in pathogenesis of cysteamine-induced ulcers [38,39]. Moreover, a recent study performed by Khomenko et al. has produced strong evidence that cysteamine disrupts regulation of mucosal iron transport, leading to increased mucosal susceptibility to oxidative stress [40].

Fukuhara et al reported a relationship between cysteamine and endogenous ghrelin [41]. Induction of duodenal ulcer by cysteamine increases plasma level of ghrelin and reduces gastric level of this peptide [41]. However, the effect of ghrelin on the healing of ulcers induced by cysteamine is unknown. The aim of the present study was to examine the influence of ghrelin administration on the course of cysteamine-induced duodenal ulcers and on mucosal production of oxygen free radicals, duodenal antioxidant defense and release of pro-inflammatory interleukin-1β.

Material and Methods

Animals and treatment

Studies were performed on male Wistar rats weighing 200–220 g and were conducted following the experimental protocol approved by the Local Commission of Ethics for the Care and Use of Laboratory Animals. Experiments were performed in the Animal Laboratory of the Department of Physiology, Jagiellonian University Medical College. Animals were housed in cages with wire mesh bottoms, at normal room temperature (22±1°C) and a 12-h light-dark cycle. Rats had unlimited access to food and water.

Rats were divided into the following 6 experimental groups: (1) control rats without induction of ulcers and treated with saline; (2) rats without induction of ulcers and treated with ghrelin at the dose of 8 nmol/kg/dose; (3) rats with ulcers treated with saline; (4–6) rats with ulcers treated with ghrelin at the dose 4, 8 or 16 nmol/kg/dose, respectively. Experiments were repeated to obtain 10 observations in each experimental group.

Duodenal ulcers were induced by cysteamine (cysteamine-HCl, Sigma-Aldrich, St. Louis, MO, USA) administered intragastrically at the dose of 200 mg/kg 3 times at 4-h intervals. Each dose of cysteamine was dissolved in 1 ml of saline.

After intragastric administration of cysteamine (groups 3–6) or saline (groups 1–2), rats were treated with saline (groups

1 and 3) or ghrelin (groups 2 and 4–6) given intraperitoneally twice a day for 6 days at the doses shown above. The first dose of intraperitoneal saline or ghrelin was administered 24 h after the first dose of intragastric cysteamine or saline.

Active N-octanoyl rat ghrelin was synthesized at Yanaihara Institute by a solid phase methodology with Fmoc-strategy using automated peptide synthesizer (Applied Biosystem 9030 Pioneer, Foster, CA, USA) as described previously [12].

Determination of duodenal blood flow and mucosal lesions

Seven days after the first dose of cysteamine, rats were anesthetized with pentobarbital (30 mg/kg i.p., Vetbutal, Biowet, Puławy, Poland) and the abdomen was opened by a midline incision. The duodenum was exposed and the duodenal mucosal blood flow was measured using a laser Doppler flowmeter (PeriFlux 4001 Master monitor, Perimed AB, Järfälla, Sweden). Blood flow was measured in 5 areas of duodenal mucosa, and the mean value of 5 recordings was presented as percent of mucosal blood flow found in saline-treated control rats. After measurement of mucosal blood flow, the area of ulcerated mucosa was measured using a computerized planimeter (Morphomat, Carl Zeiss, Berlin, Germany) as described previously [42].

Biochemical analysis

After measurement of duodenal blood flow, biopsy samples from the duodenal mucosa were taken for determination of mucosal DNA synthesis (an index of mucosal cell proliferation), mucosal concentration of pro-inflammatory interleukin-1β, mucosal concentration of malonyldialdehyde (MDA) (an index of lipid peroxidation) and mucosal activity of superoxide dismutase (SOD).

DNA synthesis was determined by measurement of [^{3}H]thymidine incorporation ([6-^{3}H]-thymidine, 20–30 Ci/mmol (Institute for Research, Production and Application of Radioisotopes, Prague, Czech Republic) into mucosal DNA as described previously [43]. The incorporation of labeled thymidine into DNA was determined by counting 0.5 ml DNA-containing supernatant in a liquid scintillation system. DNA synthesis was expressed as tritium disintegrations per minute per ìg of DNA (dpm/µg DNA).

Lipid peroxidation was determined by measurement of malondialdehyde (MDA) using the commercial kit Bioxytech® LPO-586™ (OxisResearch™, OXIS Health Products, Inc., Portland, OR, USA), as described previously [44]. Prior to homogenization of tissue samples, 10 µl 0.5 M butylated hydroxytoluene in acetonitrate was added to prevent sample oxidation during homogenization. Mucosa was homogenized in ice-cold Tris buffer (20 mM, pH 7.4), centrifuged (3000 g at 4°C for 10 min) and supernatant was used for the assay. The Bioxytech® LPO-586™ is a colorimetric assay based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA and HAE at 45°C. One molecule of either MDA or 4-hydroxyalkens (HAE) may react with 2 molecules of N-methyl-2-phenylindole to yield a stable chromophore with maximal absorbance at 586 nm. We performed the assay using hydrochloric acid as the acid solvent. For this reason we only detected MDA content without detection of HAE. Results are expressed in nmol per g of duodenal mucosa.

To determine mucosal activity of SOD, tissue was homogenized in 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and sucrose. Homogenate was centrifuged at 1500 g for 5 min at 4°C. Activity of SOD in the supernatant was measured using the Superoxide Dismutase Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). Results are expressed in units per g of duodenal mucosa.

Samples for determination of interleukin-1β in duodenal mucosa were homogenized in ice-cold phosphate-buffered saline (PBS, 20 mM, pH 7.4). Homogenate was centrifuged at 1500 g for 10 min at 4°C. Content of interleukin-1β in the supernatant was measured using the BioSource Cytoscreen rat IL-1β kit (BioSource International, Camarillo, California, USA) based on ELISA. Concentration of interleukin-1β in duodenal mucosa is expressed as ng per g of protein.

Statistical analysis

Results are expressed as mean ±SEM. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using GraphPadPrism (GraphPad Software, San Diego, CA, USA). Differences were considered to be statistically significant when P was less than 0.05.

Results

In control saline-treated rats, no lesions of duodenal mucosa were observed (Figure 1) and ghrelin given alone without cysteamine administration did not induce duodenal ulcers. Administration of cysteamine resulted in induction of duodenal ulcers; 7 days after the first dose of cysteamine, the mean ulcer area reached 6.5±0.5 mm^2. Treatment with ghrelin reduced the ulcer area in the duodenum. Ghrelin given at the dose of 4 nmol/kg/dose tended to decrease the area of ulcers, but this effect was statistically insignificant. In contrast to that, higher doses of ghrelin (8 and 16 nmol/kg/dose) caused significant and similar reduction of the duodenal ulcer area by 34% and 37%, respectively (Figure 1).

Seven days after administration of the first dose of cysteamine, duodenal blood flow was significantly reduced by 28% (Figure 2). Ghrelin given alone without induction of duodenal ulcers markedly increased duodenal blood flow by 25.5%. In rats with duodenal ulcers, treatment with ghrelin partly reversed the cysteamine-induced decrease in duodenal blood flow; this effect was statistically significant after administration of ghrelin at the dose of 8 or 16 nmol/kg/dose (Figure 2). Value of duodenal blood flow in rats with ulcers treated with ghrelin at the dose of 8 or 16 nmol/kg almost reached the control level and no significant differences were observed between these groups and control group (Figure 2).

DNA synthesis, an index of cell proliferation, reached a value 54.2±2.1 dpm/µg DNA in duodenal mucosa of control saline-treated rats (Figure 3). In saline-treated rats with cysteamine-induced ulcers, DNA synthesis in duodenal

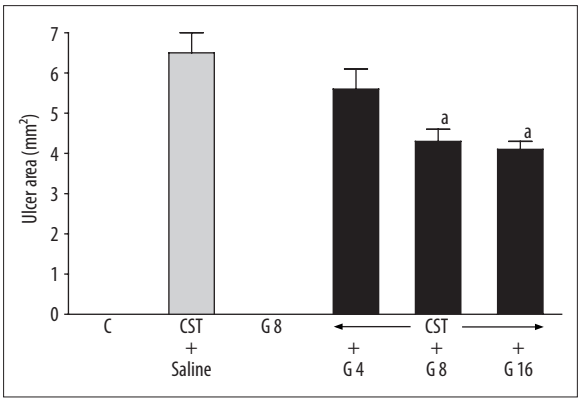

Figure 1. Influence of treatment with ghrelin given at the dose of 4, 8 or 16 nmol/kg (G 4, G 8 or G 16) on the area of cysteamine (CST)-induced duodenal ulcers. Mean ±SEM. *N*=10 in each group of animals. [a] *P*<0.05 compared to CST + saline.

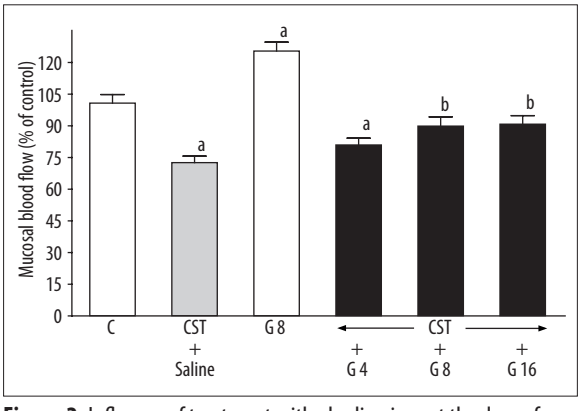

Figure 2. Influence of treatment with ghrelin given at the dose of 4, 8 or 16 nmol/kg (G 4, G 8 or G 16) on duodenal mucosal blood flow in rats with cysteamine (CST)-induced duodenal ulcers. Mean ±SEM. *N*=10 in each group of animals. [a] *P*<0.05 compared to control (C); [b] *P*<0.05 compared to CST + saline.

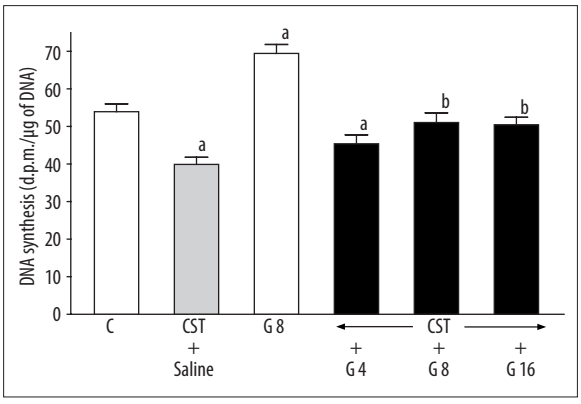

Figure 3. Influence of treatment with ghrelin given at the dose of 4, 8 or 16 nmol/kg (G 4, G 8 or G 16) on duodenal mucosal DNA synthesis in rats with cysteamine (CST)-induced duodenal ulcers. Mean ±SEM. *N*=10 in each group of animals. [a] *P*<0.05 compared to control (C); [b] *P*<0.05 compared to CST + saline.

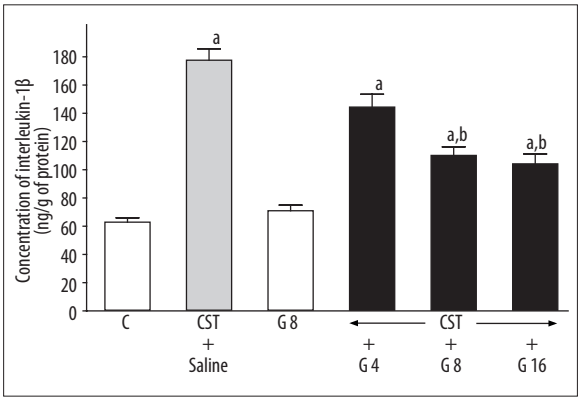

Figure 4. Influence of treatment with ghrelin given at the dose of 4, 8 or 16 nmol/kg (G 4, G 8 or G 16) on concentration of interleukin-1β in duodenal mucosa in rats with cysteamine (CST)-induced duodenal ulcers. Mean ±SEM. *N*=10 in each group of animals. [a] *P*<0.05 compared to control (C); [b] *P*<0.05 compared to CST + saline.

mucosa was reduced by 26% as compared to control rats. In rats without induction of ulcers, administration of ghrelin increased mucosal DNA synthesis in the duodenum by 29%. In rats with duodenal ulcers, treatment with ghrelin partly reversed the cysteamine-induced decrease in DNA synthesis in duodenal mucosa. This effect reached statistical significance when ghrelin was given at the dose of 8 or 16 nmol/kg/dose; no statistical difference was observed between these groups and the control group (Figure 3).

In saline-treated control rats, concentration of interleukin-1β in duodenal mucosa was 62.0±3.0 ng/g of protein (Figure 4). In saline-treated rats with cysteamine-induced ulcers, concentration of interleukin-1β in duodenal mucosa reached a value 176±8 ng/g of protein. Administration of ghrelin had no effect on interleukin-1β concentration in duodenal mucosa in saline-treated rats without induction of ulcers. In contrast, administration of ghrelin reduced the cysteamine-induced increase in mucosal interleukin-1β content in rats with duodenal ulcers; this effect was statistically significant after ghrelin given at the dose of 8 or 16 nmol/kg/dose (Figure 4).

In duodenal mucosa of control saline-treated rats, concentration of MDA was 4.1±0.2 nmol/g of tissue (Figure 5). Induction of duodenal ulcers by administration of cysteamine caused a more than 3-fold increase in concentration of MDA in duodenal mucosa. Administration of ghrelin in rats without induction of ulcers failed to affect MDA concentration in the duodenum. In rats with ulcers, treatment with ghrelin significantly reduced the cysteamine-induced increase in mucosal concentration of MDA. This effect was the strongest and was similar to the effect of ghrelin at the dose of 8 or 16 nmol/kg/dose (Figure 5).

In rats with cysteamine-induced ulcers, mucosal activity of SOD was reduced by 32% (Figure 6). Administration of ghrelin failed to affect mucosal activity of SOD in rats treated with saline and without ulcers, but reversed the cysteamine-induced decrease in SOD activity in rats with ulcers. This last effect reached statistical significance after ghrelin given at the dose of 8 or 16 nmol/kg/dose (Figure 6).

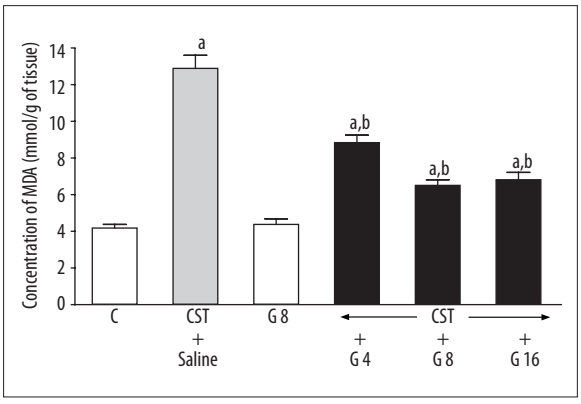

Figure 5. Influence of treatment with ghrelin given at the dose of 4, 8 or 16 nmol/kg (G 4, G 8 or G 16) on concentration of MDA in duodenal mucosa in rats with cysteamine (CST)-induced duodenal ulcers. Mean ±SEM. *N*=10 in each group of animals. [a] *P*<0.05 compared to control (C); [b] *P*<0.05 compared to CST + saline.

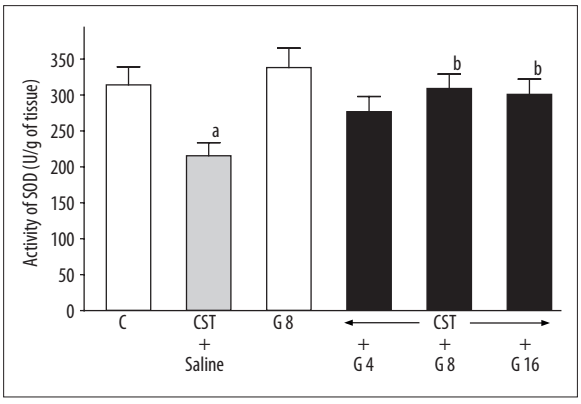

Figure 6. Influence of treatment with ghrelin given at the dose of 4, 8 or 16 nmol/kg (G 4, G 8 or G 16) on SOD activity in duodenal mucosa in rats with cysteamine (CST)-induced duodenal ulcers. Mean ±SEM. *N*=10 in each group of animals. [a] *P*<0.05 compared to control (C); [b] *P*<0.05 compared to CST + saline.

Discussion

Previous studies have shown that pretreatment with ghrelin inhibits the development of gastric ulcers [15–18] and accelerates healing of acetic acid-induced gastric and duodenal ulcers [19]. The present study confirms and extends these observations. Treatment with ghrelin increased the healing rate of the cysteamine-induced duodenal ulcers and this effect was accompanied with increased mucosal blood flow, DNA synthesis and activity of SOD. Mucosal concentrations of interleukin-1β and MDA were reduced.

Mucosal blood flow plays an important role in the protection and healing of gastro-duodenal mucosa. [43,45–47]. Numerous experimental studies have shown that exposure of gastric mucosa to potentially noxious factors results in little or no damage as long as adequate blood flow is maintained, whereas reduction in mucosal blood flow leads to severe gastric injury [45]. Blood flow contributes to protection by supplying the mucosa with oxygen, bicarbonate and nutritious substances, and by removal of carbon dioxide, hydrogen ions and other toxic agents diffusing from the gastric lumen [45]. Hypoxia, resulting in accumulation of H^+ within gastric mucosa leads to mucosal acidification and subsequently to the development of gastric ulcers [48]. On the other hand, improvement of mucosal blood flow reduces mucosal damage in the stomach [43,45–47]. The same influence of mucosal blood flow alterations on induction of mucosal damage and mucosal healing has been also shown in the duodenum [19,49]. These data are in agreement with our present observation indicating that improvement of mucosal blood flow plays an essential role in the healing effect of ghrelin on duodenal cysteamine-induced ulcers. Moreover, Ahluwalia et al. has shown that ghrelin stimulates sprouting of new capillary blood vessels and that deficiency of ghrelin is a major cause of the aging-related impairment of angiogenesis [50].

Peptic ulcer has been attributed to imbalance between the aggressive factors and mucosal resistance [51]. Mucosal resistance is related to, among other factors, mucosal blood flow, mucus and prostaglandin production, and rapid mucosal cell turnover. Inhibition of mucosal cell proliferation or excessive apoptosis results in the development of ulcers [52,53]. On the other hand, the increase in cell proliferation leads to reduction of mucosal damage and accelerates healing of mucosal ulcers [54–57]. Rate of DNA synthesis is an index of cell proliferation. Our present study has shown that administration of cysteamine leads to induction of ulcers and this effect is associated with inhibition of DNA synthesis in duodenal mucosa. Also, we have found that administration of ghrelin alone increases mucosal cell proliferation and treatment with ghrelin of rats with ulcers partly, but significantly, reverses the cysteamine-induced decrease in mucosal DNA synthesis. These findings indicate that ghrelin's growth-promoting effect is involved in healing of duodenal ulcers induced by cysteamine.

Another finding of our present study is the observation that ghrelin reduces mucosal concentration of interleukin-1β in the duodenum of rats with cysteamine-induced ulcers. Interleukin-1β is a well known mediator of acute inflammation and plays a crucial role in the induction of systemic acute phase response and in the release of other members of the pro-inflammatory cytokine cascade [58]. This interleukin stimulates production and release of mediators of inflammation such as tumor necrosis factor, platelet activating factor, prostaglandins and pro-inflammatory interleukins [58]. These data taken together indicate that ghrelin reduces local inflammatory response in cysteamine-induced duodenal ulcers, but the mechanism of ghrelin's anti-inflammatory effect is still not clear.

Numerous immune cells, including human leukemic B, T and myeloid cell lines, human peripheral lymphocytes and neutrophils, as well as mouse splenic T cells, exhibit the presence of receptors for ghrelin [59,60]. Biological action of ghrelin on the immune system includes attenuation of septic shock, promotion of thymopoiesis during aging in mice and inhibition of expression of pro-inflammatory cytokines by human monocytes and T lymphocytes [61–64]. Moreover, administration of ghrelin reduces phagocytic activity of peritoneal macrophages in rats exposed to cold-restraint stress [65].

Reactive oxygen species (ROS) are produced by inflammatory cells, particularly activated leukocytes, to attack and destroy pathogens. On the other hand, ROS can impair proteins, nucleic acid, lipids and other molecular species. ROS production and lipid peroxidation are well-established mechanisms of cellular and tissue injury, including the development of gastric and duodenal ulcers [66–68], as well as intestinal inflammation [69]. Moreover, oxidative stress and lipid peroxidation lead to fibroblast dysfunction and impairment of wound healing [70].

In normal conditions, cells and tissues are protected from the ROS-induced injury by antioxidant defense, involving oxygen scavenger enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase, as well as vitamins E and C [69,71].

Conclusions

In agreement with previous data, our present study has shown that administration of cysteamine leads to the development of duodenal ulcers and that this effect is associated with an increase in mucosal concentration of MDA and reduction in mucosal activity of SOD [72]. Moreover, we have found that treatment with ghrelin partly reduces the cysteamine-induced mucosal oxidative stress. In rats with ulcers, treatment with ghrelin decreased mucosal concentration of MDA and increased mucosal activity of SOD. These findings indicate the therapeutic effect of ghrelin in the cysteamine-induced duodenal ulcers involves the reduction of cellular lipid peroxidation and the increase in mucosal antioxidant defense. This conclusion is in agreement with data obtained by Zhang et al. [73], who found that ghrelin reduces apoptosis and ROS level in rat aortic endothelial cells exposed to palmitate.

Therapeutic and anti-inflammatory effects of ghrelin in the treatment of cysteamine-induced duodenal ulcers are most likely based on 2 different mechanisms – direct influence on cells and tissues through ghrelin's receptor (GHS-R), and indirect action by a release of growth hormone and IGF-1. For example, activation of GHS-R has been shown to attenuate the palmitate-induced apoptosis in pancreatic β cell line and this effect was related to a rapid stimulation of protein kinase B and inhibition of C-Jun N-terminal kinase, and mitochondrial pathway of apoptosis [74]. On the other hand, our previous studies have shown that protective and therapeutic effects of ghrelin in acute pancreatitis are mediated by a release of growth hormone and IGF-1 [12,14]. Hypophysectomy, performed prior to induction of acute pancreatitis, has eliminated serum growth hormone, reduced serum IGF-1 concentration by 90% and increased serum level of endogenous ghrelin, and effects were associated with aggravation of acute pancreatitis severity. Moreover, hypophysectomy has abolished the protective and therapeutic effect of ghrelin administration in the course of acute pancreatitis. In contrast to that, administration of IGF-1 reduced the severity of acute pancreatitis and accelerated healing in this disease to the same degree as ghrelin in pituitary-intact rats [12,14]. These observations indicate that the protective and therapeutic effects of ghrelin in the course of acute pancreatitis are based on an indirect mechanism related to release of endogenous growth hormone and IGF-1. A similar indirect therapeutic effect of ghrelin has been also found in the healing of acetic acid-induced gastric and duodenal ulcers [19].

Finally, the present study demonstrated that treatment with ghrelin increases healing rate of cysteamine-induced duodenal ulcers. This effect is related, at least in part, to an improvement of duodenal mucosal blood flow and mucosal cell proliferation, as well as to a reduction in mucosal oxidative stress and inflammatory response.

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