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## Role of New Appetite Hormones Ghrelin, Orexin-A and Obestatin in the Mechanism of Healing of Chronic Gastric Ulcers

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### 1. Introduction

The regulation of appetite is regulated by complex mechanism involving brain-gut axis and hormonal and non-hormonal factors acting through the activity of satiety and hunger centers located in the hypothalamus [Wren & Bloom, 2007]. Among hormones controlling appetite are gastrointestinal hormones such as cholecystikinin, PYY, pancreatic polypeptide, glucagon-like peptide 1, oxyntomedulin, the endocrine pancreatic hormones including insulin and glucagon and endocannabinoids [Wren & Bloom, 2007]. Recent discoveries of ghrelin and orexin-A gave a new insight into the understanding of mechanism of appetite control, satiety and obesity. Ghrelin is a novel 28-amino acid peptide that has recently been discovered in rat and human gastrointestinal tract, particularly in gastric mucosa, as an endogenous ligand for growth hormone (GH) 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 [Kojima et al., 1999; Tomasetto et al., 2000]. This peptide was also shown to enhance the gastric motility and gastric secretion [Tschop et al., 2000; Date et al., 2001]. Ghrelin is considered as an orexigenic peptide produced by the stomach, acting as a meal initiator [Masuda et al., 2000; Ariyasu et al., 2001]. Little is known about other factors that might affect ghrelin secretion in the stomach and its influence on gastrointestinal integrity has not been fully elucidated. A recent study in humans revealed that stomach is a major source of circulating ghrelin and that gastrectomy produced dramatic fall in the plasma ghrelin levels, whereas fasting and *anorexia nervosa* was accompanied by elevated plasma ghrelin levels [Ariyasu et al., 2001].

The orexins also called hypocretins, are neuropeptides novel originally discovered in a small group of neurons in the hypothalamic area (LHA) [Sakurai et al., 1998; de Lecea et al., 1998]. identified two peptides that were endogenous ligands to orphan receptor HFGAN72 [Sakurai et al., 1998]. They found that these peptides after i.c.v. injection stimulated food

intake and their expression was upregulated upon fasting. Therefore, the group of these peptides has been named "orexins" after the Greek word "orexis" meaning appetite [Komaki et al., 2001]. The orexin group of peptides was originally identified as regulators of food intake and of sleep behavior and act at one or the other of 2 G protein-coupled receptors, the orexin-1 (OX-R1) and orexin-2 (OX-R2) receptors [Sakurai et al., 1998; Mondal et al., 1999]. OX-A is a 33-residue peptide possessing the identical sequence in humans and rodents, whereas human and rat OX-B differ by two residues. OX-A and OX-B activate two G protein-coupled receptors known as OX-1 and OX-2, the OX-1 receptor having a greater affinity for OX-A over OX-B, whereas the OX-2 receptor has similar affinity for both ligands [Sakurai, 2003]. Besides the presence in the brain, orexin-like immunoreactivity was found in the ENS of different species including guinea pig, rat, mouse and humans [Kirchgessner & Liu, 1999]. Interestingly, the prepro-orexin, orexin A and orexin receptor mRNA expression were demonstrated in the myenteric plexus and afferent nerves co-localizing with VIP-ergic nerves and in the gastrin-producing cells of the rat stomach [Kirchgessner & Liu, 1999]. Orexin-A has been linked with the peripheral energy balance and central nervous system mechanisms that coordinate sleep-wakefulness and motivated behaviors such as food seeking, especially in physiological state of fasting stress [Mondal et al., 1999; Sakurai, 2003]. Recent studies in humans revealed that the plasma orexin-A concentrations are increased during fasting in humans [Komaki et al., 2001].

The question remains as to whether these newly discovered peptides could play a role in the mechanism of gastric integrity and gastric mucosal defense has not been fully explored. Both, ghrelin and orexin-A were implicated in the mechanism of gastroprotection but their role in the healing process of chronic gastric ulcers has been little investigated. Previous studies by [Sibilia et al., 2003] and our group [Brzozowski et al., 2004; Brzozowski et al., 2006 b.; Konturek et al., 2004] revealed that that central and peripheral administration of ghrelin reduced the formation of acute gastric lesions induced by ethanol and cold stress. It was proposed that NO and sensory neuropeptides mediate these gastroprotective effects of ghrelin because the blockade of NO-synthase (NOS) activity with L-NAME and the functional ablation of sensory afferent nerves with capsaicin attenuated the protection and gastric hyperemia induced by ghrelin [Brzozowski et al., 2004; Brzozowski et al., 2006a.; Dimitrova et al., 2010]. In contrast to prevention of acute damage to the mucosal structure, the healing of chronic gastric ulcers lasts days and weeks and may involve restoration of gland architecture, angiogenesis and scar formation and it remains unknown whether appetite hormones may influence these processes.

Recently, endogenous prostaglandins (PG) have been implicated in the control of food intake and appetite [Lugarini et al., 2002; Scholz, 2003] but the possibility that these arachidonate metabolites may play any important role in the mechanism of ghrelin or orexin-A affecting ulcer healing has not been explored. This prompted our interest in endogenous PG because these arachidonate metabolites play an essential role in the mechanism of gastric defense and ulcer healing [Brzozowski et al., 2001] but the importance of PG derived from cyclooxygenase (COX-1) and COX-2 in the possible ulcer healing effects of orexigenic peptides has not been so far determined.

This study was designed to compare the effects of daily intraperitoneal (i.p.) administration of vehicle, ghrelin, orexin-A and obestatin on healing of preexisting gastric ulcers and accompanying changes in the gastric blood flow (GBF) at ulcer margin and the generation of PGE<sub>2</sub> in the gastric mucosa. An attempt was made to examine the effect of antagonism of

GHS-R1a and OX-A1 receptors with D-lys<sup>3</sup>GHRP-6 and SB334867 [Peeters, 2005; Smart et al., 2001] respectively, on spontaneous ulcer healing and the alteration in ulcer size induced by ghrelin and orexin-A. We also determined the effects of PG inhibition with a non-selective (indomethacin) and selective cyclooxygenase (COX)-1 (SC-560) and COX-2 (rofecoxib) inhibitors and blockade with L-NNA, the NO-synthase inhibitor on healing of these ulcers and accompanying changes in the GBF in rats without and with ghrelin or orexin-A administration. In addition, the expression of ghrelin mRNA at the ulcer margin and that in non-ulcerated gastric mucosa was assessed in rats with gastric ulcer. Finally, ghrelin rats were concomitantly treated with obestatin to check whether obestatin, recently considered as physiological opponent of ghrelin regarding food intake and appetite control [Zhang et al., 2005], could influence the ulcer healing itself and affect the healing and hyperemic effects of ghrelin.

## 2. Material and methods

Male Wistar rats, weighing 200-250 g and fasted for 24 h were used in all studies. All experimental procedures were run according to Helsinki Declaration and approved by the Jagiellonian University Institutional Animal Care and Use Committee.

### 2.1 Production of gastric ulcers

Gastric ulcers were produced in rats using our modification (19) of acetic acid method originally proposed by Okabe et al. (1971) with our group modification [Konturek et al., 1987]. Animals were anesthetized with ether, the stomach was exposed and 75 µl of acetic acid was poured through the plastic mold (6 mm diameter) onto serosal surface of anterior wall of the stomach just proximal to the antral gland area for 25 s. This produced an immediate necrosis of the entire mucosa and submucosa (but not serosa) within the area where the acetic acid was applied, i.e., about 28 mm<sup>2</sup>. The excess of acetic acid was then removed and the serosa was gently washed out with saline. Our previous studies documented that these ulcers became chronic within 2-3 days and healed completely within 2-3 weeks without perforation or penetration to the surrounding organs as described in original technique [Konturek et al., 1987; Okabe et al., 1971]. After the application of acetic acid the animals were allowed to recover from anesthesia and received only water at the day of operation (day 0). Then, they were divided into various groups and received normal chow and water *ad libitum* for the next 9 days and then were sacrificed.

### 2.2 Effect of orexin-A, ghrelin without and with the antagonists of ghrelin and orexin-A receptors on ulcer healing and the alterations in GBF at ulcer margin

Several groups of rats with gastric ulcers, each consisting of 6-8 animals, were treated daily either with 1) vehicle (saline), 2) ghrelin or orexin-A (2.5-30 µg/kg-d i.p.), 3) D-Lys<sup>3</sup>-GHRP-6 (200 µg/kg i.p.), the ghrelin receptor antagonist (Peeters, 2005), or SB 334867 which is a selective non-peptide OX-R1 receptor antagonist [Smart, et al., 2001; Holland, et al., 2006] administered alone or in the combination with ghrelin and orexin-A (standard dose of 30 µg/kg-d i.p.), respectively. SB 334867 was dissolved in DMSO and kept in a 20 mM solution as recommended by manufacturer [Tocris Cookson Ltd., Bristol, UK]. The dose of OX-R1 antagonist used in this study was selected on the basis of our earlier studies (unpublished

observation) with dose-dependent inhibition of orexin-A protection against WRS damage obtained with this orexin-A antagonist.

In another group of animals with gastric ulcers and treated with COX-1 and COX-2 inhibitors with or without ghrelin, orexin-A and obestatin administration, the effect of prostaglandin replacement therapy using 16,16 dimethyl PGE<sub>2</sub> [Upjohn, Kalamazoo, MI, USA] applied i.g. in a dose of 5 µg/kg-d was examined. This dose of dimethyl PGE<sub>2</sub> was found in our preliminary study to be without any influence on ulcer healing and accompanying fall in GBF at ulcer margin. For this reason, synthetic PGE<sub>2</sub> analog was administered together with each COX-1 or COX-2 inhibitor with or without ghrelin, orexin-A and obestatin administration.

Upon the termination of experiment, 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 H<sub>2</sub>-gas clearance technique as described previously [Brzozowski et al., 2000 a]. 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 at ulcer margin 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., 2000 b] by a researcher blind to the experimental grouping.

Samples of the oxyntic gland area from the ulcer margin were taken by biopsy (about 100 mg) immediately after the animals were sacrificed to determine the mucosal generation of PGE<sub>2</sub> by specific radioimmunoassay (RIA) as described previously [Konturek et al., 1995; Brzozowski et al., 2000]. PGE<sub>2</sub> was measured in duplicate using RIA kits (New England Nuclear, Munich, Germany). The mucosal generation of PGE<sub>2</sub> was expressed in nanograms per gram of wet tissue weight.

### **2.3 Effect of ghrelin and orexin-A on the healing of acetic acid ulcers and accompanying changes in the GBF at ulcer margin. Involvement of the COX-PG and NO-NOS systems in ghrelin-induced acceleration of ulcer healing**

The involvement of the COX-PG system in the ulcer healing effects of ghrelin was studied in rats treated 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 [Brzozowski et al., 1999]. In the experimental protocol, the following groups of rats, each consisting of 6-8 animals, were used: 1) vehicle (saline 1 ml i.p.); 2) ghrelin, orexin-A or obestatin, each applied in a standard dose of 30 µg/kg-d i.p.; 3) indomethacin (5 mg/kg i.p.), SC-560 (5 mg/kg-d i.g.) and rofecoxib (10 mg/kg-d i.g.), each applied alone or co-administered with ghrelin, orexin-A or obestatin (30 µg/kg-d i.p.). The doses of SC-560 and rofecoxib were selected on the basis of previous studies showing that these agents almost completely suppress PGE<sub>2</sub> generation in exudates of air-pouch inflammation and inhibit gastric PGE<sub>2</sub> production in mucosa with preexisting gastric ulcer [Futaki et al., 1993; Lesch et al., 1998]. 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. At the dose used in the present study, indomethacin has been shown previously to inhibit gastric PGE<sub>2</sub> generation by ~ 90 % without itself causing any mucosal damage [Konturek et al., 1987].

In another group of animals with gastric ulcers and treated with COX-1 and COX-2 inhibitors with or without ghrelin, orexin-A and obestatin administration, the effect of prostaglandin replacement therapy using 16, 16 dimethyl PGE<sub>2</sub> (Upjohn, Kalamazoo, MI, USA) applied i.g. in a dose of 5 µg/kg-d was examined. This dose of dimethyl PGE<sub>2</sub> was found in our preliminary study to be without any influence on ulcer healing and accompanying fall in GBF at ulcer margin. For this reason, synthetic PGE<sub>2</sub> analog was administered together with each COX-1 or COX-2 inhibitor with or without ghrelin, orexin-A and obestatin administration.

Upon the termination of experiment, 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 H<sub>2</sub>-gas clearance technique as described previously [Brzozowski et al., 2000 a]. 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 at ulcer margin 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., 2000b] by a researcher blind to the experimental grouping.

Samples of the oxyntic gland area from the ulcer margin were taken by biopsy (about 100 mg) immediately after the animals were sacrificed to determine the mucosal generation of PGE<sub>2</sub> by specific radioimmunoassay (RIA) as described previously [Konturek et al., 1995]. PGE<sub>2</sub> was measured in duplicate using RIA kits (New England Nuclear, Munich, Germany). The mucosal generation of PGE<sub>2</sub> was expressed in nanograms per gram of wet tissue weight.

#### **2.4 Determination of plasma ghrelin, orexin-A and obestatin levels by radioimmunoassay**

At the termination of some experiments with obestatin applied i.p. alone or co-administered with ghrelin, the rats were anesthetized with pentobarbital and blood samples (about 3 ml) taken from the *vena cava* for the measurement of plasma ghrelin, orexin-A and obestatin levels by RIA. Intact rats fasted overnight and given only vehicle (saline) i.p. were measured similarly in order to determine control values for plasma ghrelin, orexin-A and obestatin 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, orexin-A and obestatin levels using an RIA-kit for rat ghrelin, orexin-A and obestatin (Phoenix Peptide, Belmont, CA, USA) [Brzozowski et al., 2004; Brzozowski et al., 2006 b]. Briefly, the ghrelin, orexin-A and obestatin RIA involved the competition of each rat peptide sample with <sup>125</sup>I-rat ghrelin, orexin-A and obestatin tracers for binding to a specific rabbit anti-ghrelin-, anti-orexin-A- and anti-obestatin polyclonal antibody. The limit of assay sensitivity for ghrelin, orexin-A and

obestatin were 3 pg, 6pg and 5 pg per tube, the intra-assay variation was less than 8%,7% and 9%, and the interassay variation less than 5%, 4% and 7%, respectively.

### **2.5 Reverse-transcriptase-polymerase chain reaction (RT-PCR) for detection of messenger RNA (mRNA) for ghrelin and proinflammatory cytokines IL-1 $\beta$ and TNF- $\alpha$ in rats without and with gastric ulcers**

The stomachs were removed from vehicle (control) rats for the determination of ghrelin 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  $\mu$ g 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  $\beta$ -actin were selected on the basis of the published cDNA encoding ghrelin and  $\beta$ -actin, respectively [Konturek et al., 2004; Brzozowski et al., 2006 b]. The sense primer for ghrelin was 5'-TTGAGCCCAGAGCACCAGAAA-3', and the antisense primer was 5'-AGTTGCAGAGGAGGCAGAAGCT-3'. The IL-1 $\beta$  primer sequences were designed according to the published cDNA sequence for primer sequences and were as follows: up-stream, 5' GCT ACC TAT GTC TTG CCC GT; downstream, 3' GAC CAT TGC TGT TTC CTA GG. The expected length product was 543 bp. The TNF- $\alpha$  primer sequences were as follows: up-stream, 5'TAC TGA ACT TCG GGG TGA TTG GTC C; downstream, 3' CAG CCT TGT CCC TTG AAG AGA ACC. The expected length product was 295 bp [Konturek et al., 2010]. The oligonucleotide sequences for  $\beta$ -actin were TTG TAA CCA ACT GGG ACG ATA TGG (sense) and GAT CTT GAT CTT CAT GGT GCT AGG (antisense). The primers were synthesized by GIBCO BRL/Life Technologies, Eggenstein, Germany. The signals for ghrelin mRNA was standardized against the  $\beta$ -actin signal for each sample and results were expressed as the ratio of ghrelin mRNA to  $\beta$ -actin mRNA.

## **3. Results**

### **3.1 Effect of treatment with orexin-A, ghrelin and obestatin on ulcer healing and the accompanying changes in the GBF at ulcer margin**

The effect of i.p. administration of orexin-A applied in graded doses ranging from 1  $\mu$ g/kg-d up to 30  $\mu$ g/kg-d and ghrelin administered in a dose of 30  $\mu$ g/kg-d (i.p.) on acetic acid-induced gastric ulcers are shown in Fig.1. Orexin-A dose-dependently reduced the ulcer area, the dose inhibiting this area by 50% (ED<sub>50</sub>) was 15  $\mu$ g/kg.

Almost double reduction of the ulcer area was obtained with orexin-A administered at the dose 30  $\mu$ g/kg as compared with control rats injected i.p. with vehicle (saline). This reduction in ulcer area achieved with orexin-A was accompanied by the dose-dependent increase in GBF. Ghrelin administered in a dose of 30  $\mu$ g/kg-d i.p. significantly reduced the ulcer area and significantly increased GBF with the extent similar to that observed with orexin-A applied in the highest dose. Eight days of i.p. administration of obestatin applied in graded doses ranging from 2.5  $\mu$ g/kg up to 30  $\mu$ g/kg-d, dose-dependently reduced the

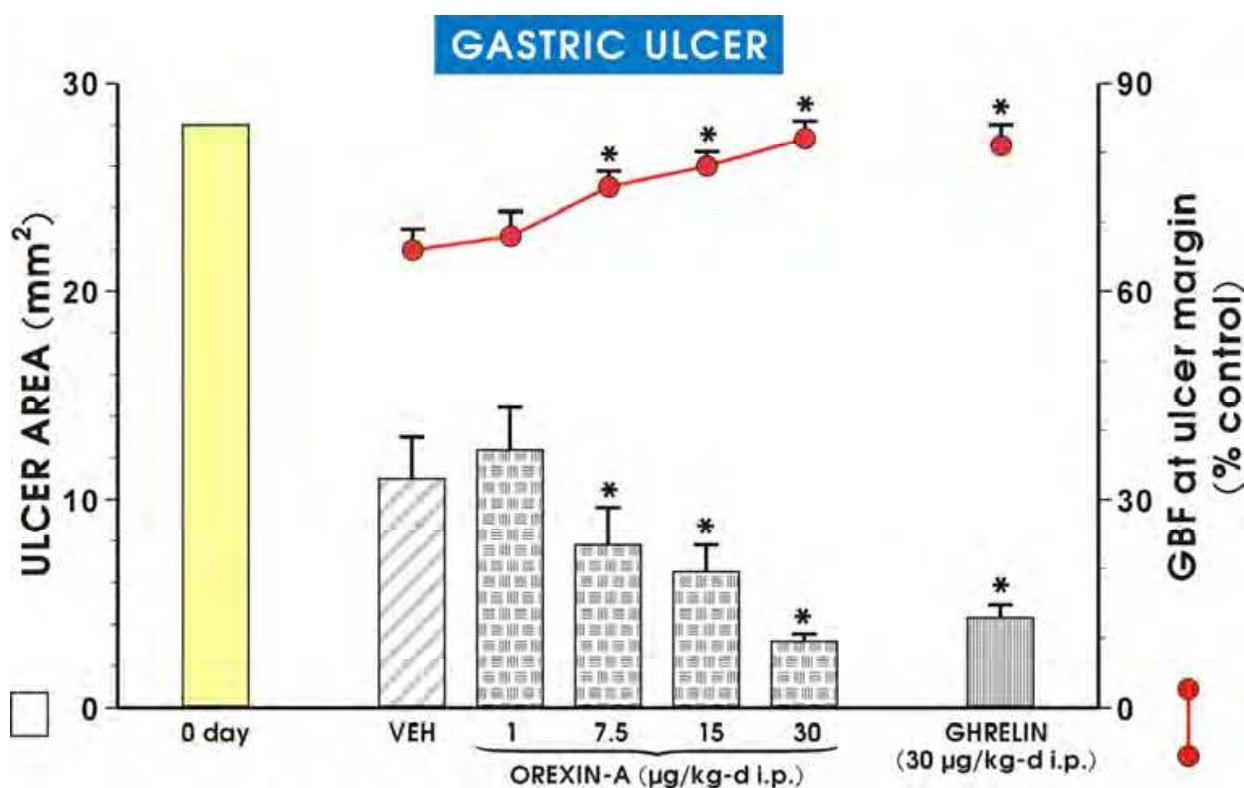


Fig. 1. The area of gastric ulcer induced by acetic acid and gastric blood flow (GBF) at ulcer margin at 8 day upon ulcer induction in rats treated with vehicle (saline) or with various doses of orexin-A (1 - 30 µg/kg-d i.p.) or ghrelin (30 µg/kg-d i.p.). Means ± SEM of 6-8 rats. Asterisk indicates a significant change ( $p < 0.05$ ) as compared to the vehicle-control values.

area of gastric ulcer and significantly increased the GBF at ulcer margin starting from the dose of 5 µg/kg (Fig. 2). The maximal reduction of ulcer area accompanied by a significant rise in the GBF at ulcer margin was recorded at the dose of 30 µg/kg of obestatin where 35% reduction of ulcer area and an increase by about 19% of GBF at ulcer margin were observed as compared to the respective values in vehicle-treated animals.

### 3.2 Role of cyclooxygenase (COX)-1 and COX-2 inhibition and the blockade of nitric oxide (NO) synthase on ulcer healing activity and alterations in the GBF at ulcer margin in rats treated with ghrelin, orexin-A and obestatin

As shown in Fig. 3, ghrelin and orexin-A given i.p. in dose 30 µg/kg-d significantly decreased ulcer area and enhanced GBF as compared to those observed in vehicle. Treatment with D-Lys<sup>3</sup>-GHS-6 (200 µg/kg i.p.), the ghrelin GHS-R1a receptor antagonist, by itself failed to affect the area of gastric ulcers and GBF at ulcer margin at day 8 upon ulcer induction. The decrease in the area of these ulcers and accompanying rise in the GBF at ulcer margin induced by ghrelin and orexin-A were significantly attenuated by concurrent treatment with ghrelin and orexin-A receptor antagonists, D-Lys<sup>3</sup>-GHS-6 and SB334867. The administration of D-lys<sup>3</sup>GHRP, which is a specific ghrelin receptor antagonist or SB334867, an antagonist of orexin-A OXR-1 receptors when applied alone, failed to affect the area of gastric ulcer and the GBF at ulcer margin as compared to those measured in vehicle-treated rats. Treatment with D-lys<sup>3</sup>GHRP significantly attenuated ghrelin-induced acceleration of ulcer healing and the accompanying increase in GBF at ulcer margin.

Concomitant treatment of orexin-A with its receptor antagonist, SB334867 (5 mg/kg-d s.c.), completely abolished the reduction in ulcer area induced by orexin-A. This antagonist of orexin-A receptors reversed also an increase in GBF at ulcer margin caused by orexin-A.

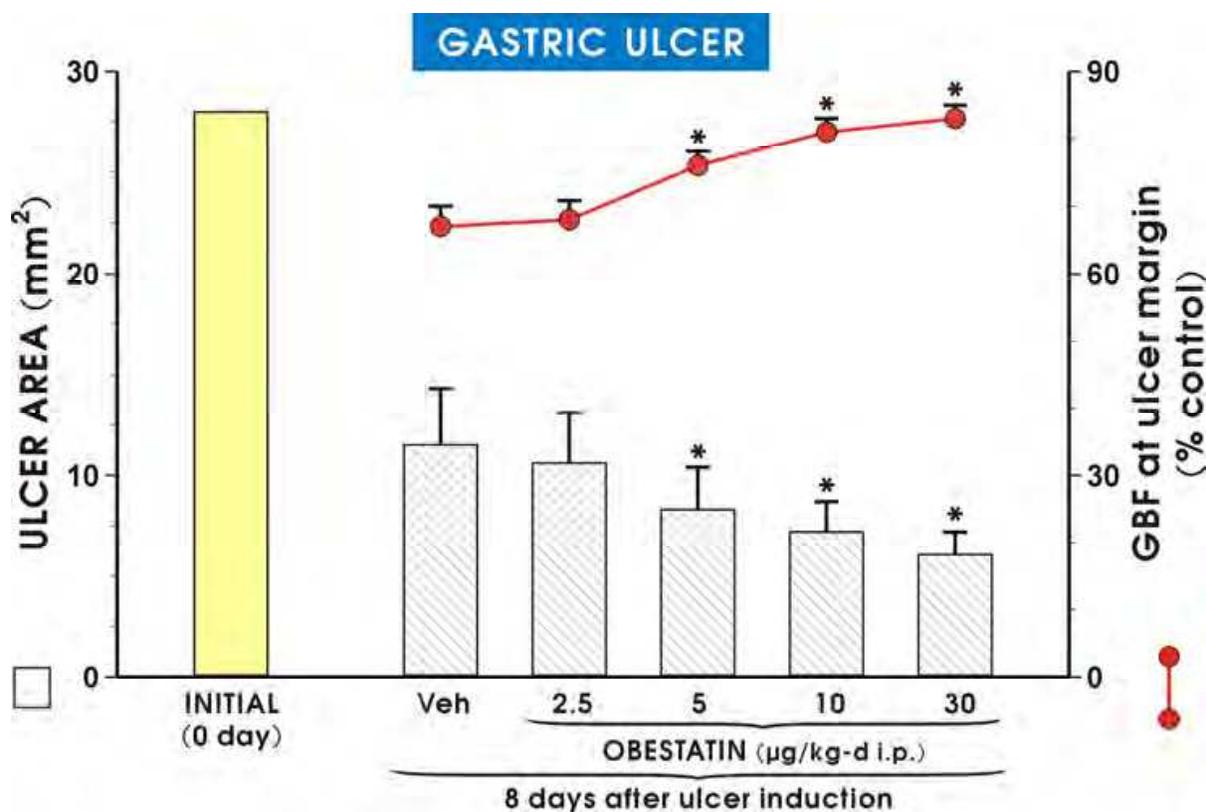


Fig. 2. The area of gastric ulcers and the alterations in the GBF at ulcer margin in rats treated for 8 days with vehicle (Veh; control) or obestatin applied i.p. in graded doses ranging from 2.5  $\mu\text{g}/\text{kg-d}$  up to 30  $\mu\text{g}/\text{kg-d}$ . Mean  $\pm$  SEM of 6-8 rats. Asterisk indicates a significant change ( $p < 0.05$ ) compared to the vehicle-pretreated controls.

As shown in Fig. 4, treatment with ghrelin, orexin-A and obestatin (30  $\mu\text{g}/\text{kg-d}$  i.p.) resulted in a similar attenuation in the area of gastric ulcer and a similar rise in GBF as that shown in Figs. 1 and 2. The mucosal generation of  $\text{PGE}_2$  was significantly increased at ulcer margin as compared to the respective value in the non-ulcerated control ( $128 \pm 9$  vs.  $189 \pm 12$  ng/g wet tissue weight,  $p < 0.05$ ). Ghrelin, orexin-A and obestatin applied i.p. resulted in a significant increase in the  $\text{PGE}_2$  generation at ulcer margin as compared to vehicle-treated animals with gastric ulcer ( $189 \pm 12$  vs  $234 \pm 19$  ng/g,  $189 \pm 12$  vs  $228 \pm 9$  ng/g and  $189 \pm 12$  vs  $219 \pm 8$  ng/g, respectively,  $p < 0.05$ ). Indomethacin (5 mg/kg i.p.), which by itself significantly increased the ulcer area, suppressed the generation of  $\text{PGE}_2$  by about 85% ( $p < 0.02$ ) and produced a significant fall in GBF as compared to vehicle-pretreated animals (Fig. 4). Treatment with this non-selective COX-1 and COX-2 inhibitor completely abolished the reduction in the area of gastric ulcers and the accompanying rise in GBF evoked by ghrelin, orexin-A and obestatin. The decrease in the area of ulcer and accompanying increase in GBF caused by ghrelin, orexin-A and obestatin as well as the rise in the  $\text{PGE}_2$  generation they induced were also significantly attenuated by pretreatment with rofecoxib, the selective COX-2 inhibitor (Fig. 4). SC-560 (5 mg/kg-d i.g.), which by itself significantly reduced the  $\text{PGE}_2$  generation (not shown), significantly attenuated the ghrelin-, orexin-A- and obestatin-induced decrease in ulcer area

and the accompanying rise in GBF at ulcer margin (Fig. 4). Concurrent treatment with a minute amount of synthetic dimethyl analog of PGE<sub>2</sub> (5 µg/kg-d i.g.) in addition to ghrelin, orexin-A and obestatin restored the decrease in the ulcer area and the increase in GBF at ulcer margin in rats treated with indomethacin, SC-560 or rofecoxib (Fig. 4).

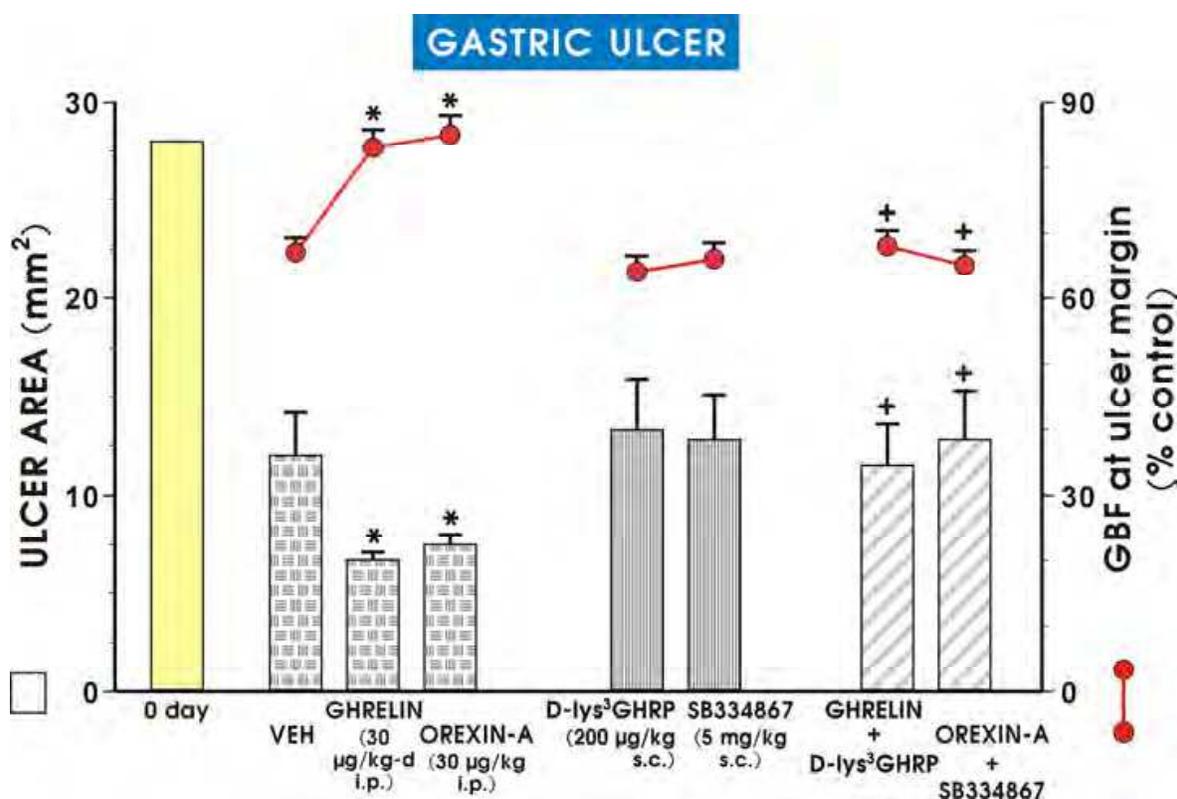


Fig. 3. The area of gastric ulcers and the accompanying changes in the GBF at ulcer margin in rats with or without ghrelin or orexin-A (both at a dose of 30 µg/kg-d i.p.) applied without or with the ghrelin receptor antagonist D-Lys<sup>3</sup>-GHSR-6 (200 µg/kg-d i.p.) or orexin-A receptor antagonist SB334867 (200 µg/kg-d i.p.). Mean ± SEM of 6-8 rats. Asterisk indicates a significant change (p<0.05) compared to the vehicle-pretreated controls. Cross indicates a significant change (p<0.05) compared to the values obtained in rats treated with ghrelin and orexin-A without the concurrent treatment with GHS-R1a receptor or OXR-1 receptor antagonists.

### 3.3 Expression of ghrelin and proinflammatory cytokines in ulcer area during ulcer healing without and with ghrelin treatment

The effect of eight day administration of L-NNA (20 mg/kg-d i.p.), the NO-synthase inhibitor on ulcer area and the changes in GBF at ulcer margin in rats treated without and with vehicle, ghrelin, orexin-A and obestatin, each applied in a dose of 30 µg/kg-d i.p. is presented in Fig. 5. Treatment with ghrelin, orexin-A and obestatin significantly reduced the ulcer area and increased the GBF at ulcer margin as shown in Fig. 4. Treatment with L-NNA, which by itself significantly enhanced ulcer area also significantly decreased GBF at ulcer margin as compared to respective values in vehicle-control. Concurrent treatment with L-NNA together with ghrelin, orexin-A and obestatin reversed the ghrelin-, orexin-A- and obestatin-induced decrease in ulcer area and the accompanying rise in the GBF at ulcer margin.

Fig. 6 shows that ghrelin was expressed in intact non-ulcerated gastric mucosa and that with gastric ulcer. Ghrelin mRNA expression was detected as the strong signal in intact gastric mucosa and vehicle-treated gastric mucosa with gastric ulcer. Ratio of mRNA for ghrelin over  $\beta$ -actin revealed that this ratio was significantly higher in ulcerated gastric mucosa as in case of that in intact animals without an ulcer (Fig. 6, left panel).

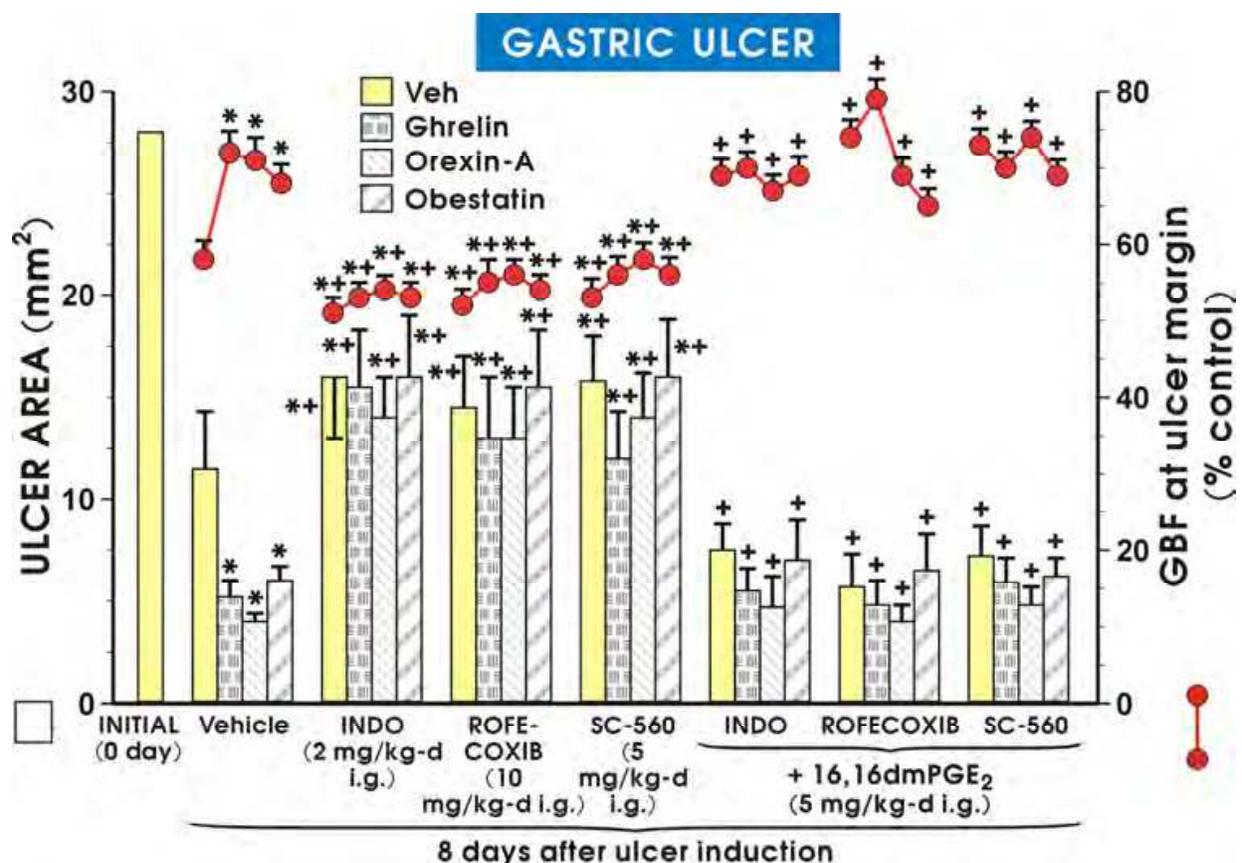


Fig. 4. The area of gastric ulcers and the accompanying changes in the GBF at ulcer margin in rats treated for a period of 8 days with vehicle (saline) and ghrelin, orexin-A and obestatin (each given in a dose of 30  $\mu$ g/kg-d i.p.) with or without the concurrent treatment with indomethacin (5 mg/kg-d i.p.), rofecoxib (10 mg/kg-d i.g.) or SC-560 (5 mg/kg-d i.g.) applied alone or combined with synthetic 16,16 dimethyl (dm) PGE<sub>2</sub> analog (5  $\mu$ g/kg-d i.g.). Mean  $\pm$  SEM of 6-8 rats. Asterisk indicates a significant decrease ( $p < 0.05$ ) as compared to the value obtained in vehicle-controls. Asterisk and cross indicate a significant increase ( $p < 0.05$ ) as compared to vehicle-treated controls administered without or with ghrelin, orexin-A or obestatin. Cross indicate a significant change ( $p < 0.02$ ) as compared to respective values obtained in animals treated with indomethacin, SC-560 or rofecoxib without the concurrent treatment with PGE<sub>2</sub> analog.

As shown in Fig. 7, the signal of mRNA for IL-1 $\beta$  and TNF- $\alpha$  mRNAs was faintly expressed in intact gastric mucosa but it was observed as strong signal in vehicle-control mucosa of animals with gastric ulcer. Ratio of mRNA for ghrelin over  $\beta$ -actin indicated that expression of mRNA for IL-1 $\beta$  and TNF- $\alpha$  was significantly increased in vehicle-treated rats with gastric ulcer over that recorded in intact gastric mucosa. In contrast, in rats treated with ghrelin applied i.p. at the doses of 15  $\mu$ g/kg and 30  $\mu$ g/kg, the signal for IL-1 $\beta$  and TNF- $\alpha$

mRNAs was significantly decreased in gastric mucosa with gastric ulcer as compared to that in vehicle-control animals. Ratio of mRNA for IL-1 $\beta$  and TNF- $\alpha$  mRNA over  $\beta$ -actin mRNA revealed that this ratio was significantly decreased in gastric mucosa of rats treated with ghrelin administered at the dose of 15  $\mu$ g/kg. The ratio of IL-1 $\beta$  and TNF- $\alpha$  was significantly decreased in animals treated with ghrelin applied i.p. in a higher dose of 30  $\mu$ g/kg than in those treated with ghrelin administered in a lower dose of 15  $\mu$ g/kg of this peptide (Fig. 7).

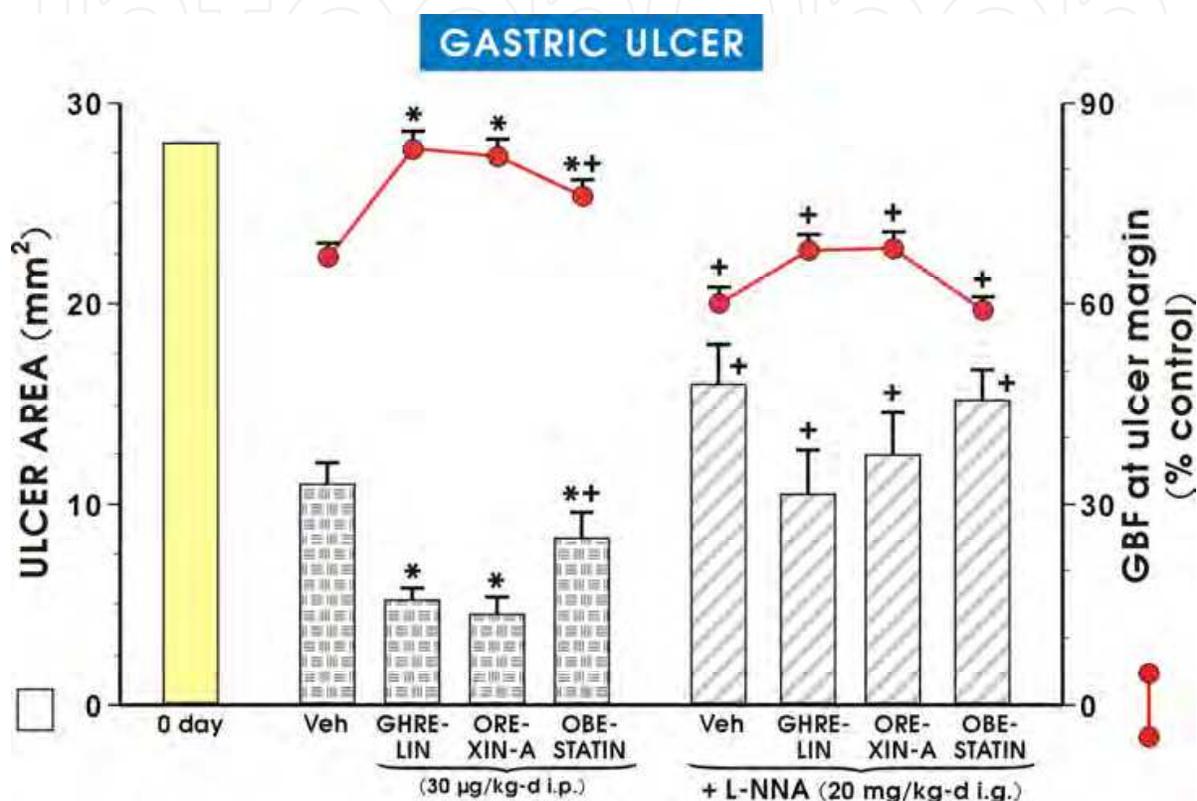


Fig. 5. The area of gastric ulcers and the accompanying changes in the GBF at ulcer margin in rats treated with vehicle (Veh; saline-control), ghrelin, orexin-A and obestatin (each administered in a dose of 30  $\mu$ g/kg-d i.p.) applied without or with the NO-synthase inhibitor L-NNA (20 mg/kg-d i.p.). Mean  $\pm$  SEM of 6-8 rats. Asterisk indicates a significant change ( $p < 0.05$ ) compared to the vehicle-treated controls. Asterisk and cross indicate a significant change ( $p < 0.05$ ) compared to the values obtained in rats treated with vehicle. Cross indicates the significant change ( $p < 0.02$ ) compared to the values obtained in ghrelin, orexin-A and obestatin without the concurrent treatment with L-NNA.

### 3.4 Effect of treatment with obestatin on ulcer healing in rats with of ghrelin administration

Fig. 8 shows the effects of eight day of concomitant administration of ghrelin, the appetite stimulating peptide, and obestatin which is opponent of ghrelin acting as the natural ligand of the GPR39 receptor, on the alterations in area of gastric ulcer and accompanying changes in GBF at ulcer area and plasma ghrelin and obestatin levels. Ghrelin and obestatin given i.p. in the same comparable dose of 30  $\mu$ g/kg-d significantly reduced the area of gastric ulcer, however the significantly greater reduction in ulcer area was observed in ghrelin-

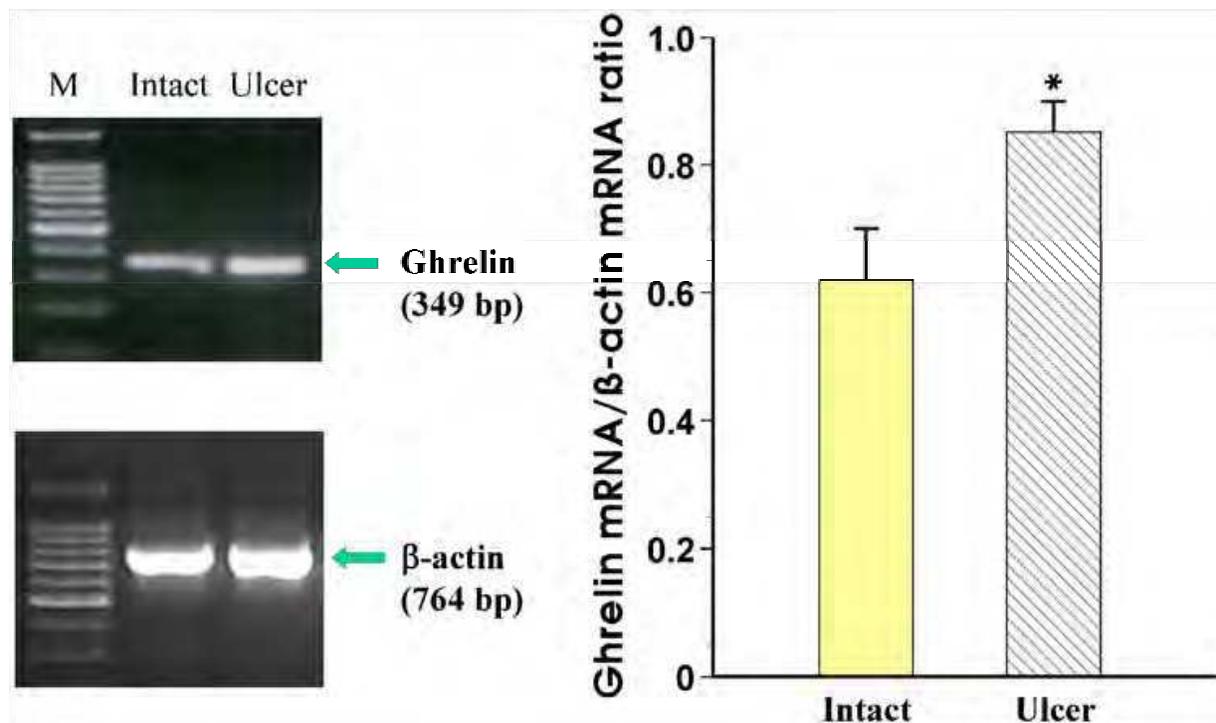


Fig. 6. Determination of expression of  $\beta$ -actin and ghrelin mRNAs (left panel) by RT-PCR and the ratio of ghrelin to  $\beta$ -actin mRNA (right panel) in non-ulcerated (intact) gastric mucosa (lane Intact) and in gastric mucosa at ulcer margin (lane Ulcer), M - DNA size marker. Mean  $\pm$  SEM of 4-6 rats. Asterisk indicates a significant change ( $p < 0.05$ ) as compared to the value obtained in non-ulcerated gastric mucosa.

treated rats as compared with those treated with obestatin. Plasma ghrelin level increased after i.p. administration of exogenous ghrelin but remained unchanged after combined treatment of ghrelin with obestatin as compared with those treated with ghrelin alone. The plasma obestatin concentration which was significantly increased in obestatin-treated rats with gastric ulcer was not significantly affected by the concurrent ghrelin administration as compared to vehicle-treated rats. The combined treatment of obestatin co-administered with ghrelin failed to significantly affect the ghrelin-induced attenuation of ulcer area and the accompanying rise in plasma ghrelin level which reached similar values, as in case of ghrelin administered alone.

#### 4. Discussion

This study shows that appetite hormones such as ghrelin, orexin-A and obestatin besides their well recognized action in the control of food intake and energy expenditure, exhibit ulcer healing and hyperemic activities as documented by an acceleration of ulcer healing by these peptides accompanied by an increase in the GBF at ulcer margin. These healing and hyperemic effects of ghrelin and orexin A seem to be very specific because both hormones induced acceleration of the healing and hyperemia at ulcer margin were reversed by ghrelin receptor antagonist D-Lys<sup>3</sup>-GHRP-6, and orexin-A receptor antagonist SB 334867, respectively, indicating that ghrelin- and orexin A-induced ulcer healing promoting and hyperemic effects are mediated by the functionally active form of GHS-R1a receptor and orexin (OX-R1) receptors. These receptors GHS-R1a and OX-R1 have been shown to bind

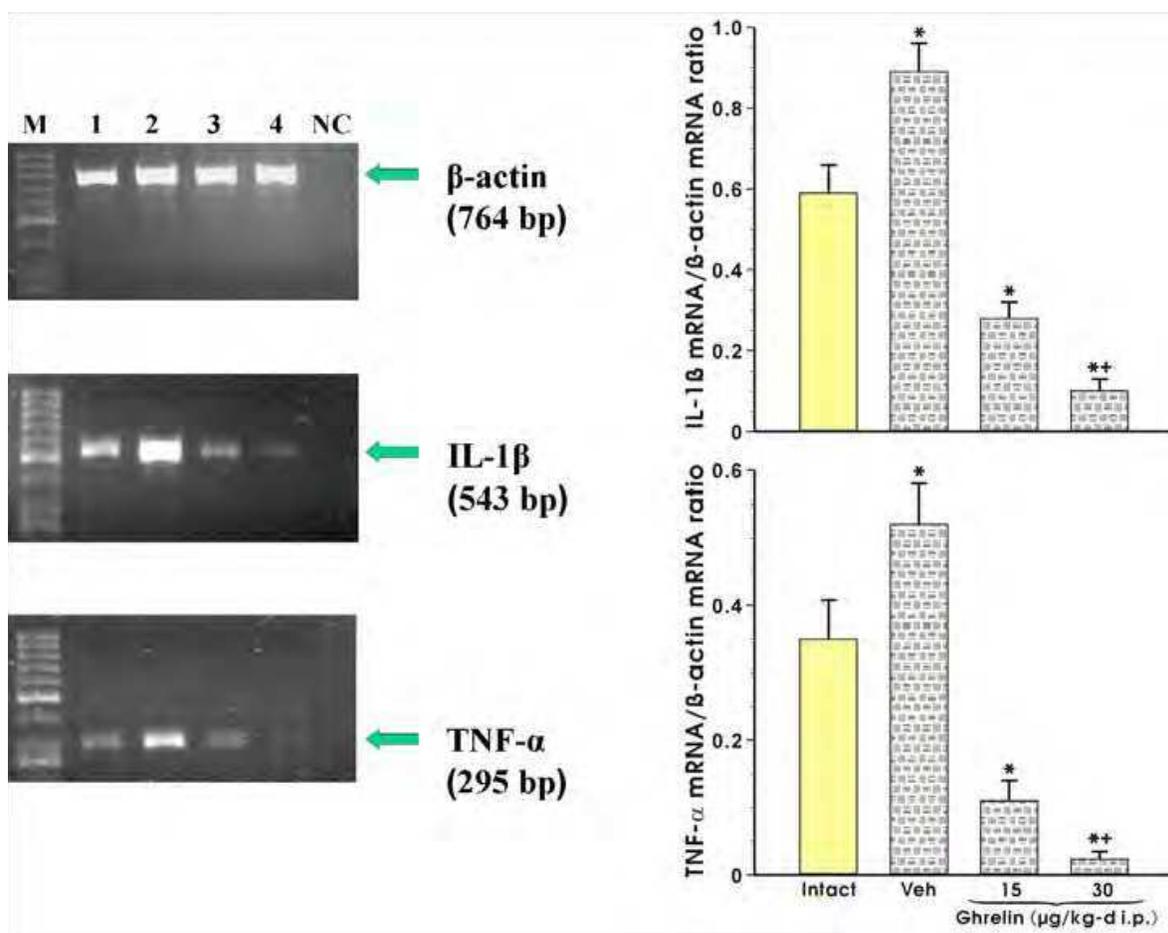


Fig. 7. Determination of expression of  $\beta$ -actin, IL-1 $\beta$  and TNF- $\alpha$  mRNAs (left panel) by RT-PCR and the ratio of mRNA for ghrelin, orexin-A and obestatin (each administered at the dose of 30  $\mu$ g/kg-d i.p.) to  $\beta$ -actin mRNA (right panel) in intact non-ulcerated gastric mucosa and in those with acetic acid-induced gastric ulcer treated with vehicle (saline) or ghrelin applied i.p. in a dose of 15  $\mu$ g/kg or 30  $\mu$ g/kg, M - DNA size marker. Mean  $\pm$  SEM of 4-6 rats. Asterisk indicates a significant change ( $p < 0.05$ ) as compared to the value obtained in intact non-ulcerated gastric mucosa. Asterisk and cross indicate a significant change ( $p < 0.05$ ) as compared to the value obtained in animals treated eight days with ghrelin administered daily i.p. at the dose of 15  $\mu$ g/kg-d.

acylated ghrelin and orexin A, respectively. Moreover, we found that ghrelin, orexin A and to lesser extent also obestatin increased the PGE<sub>2</sub> generation at ulcer margin and that these peptides ulcer healing promoting and hyperemic effects and an increase in PGE<sub>2</sub> generated at ulcer margin were significantly attenuated by non-selective (indomethacin), selective COX-1 (SC-560) and selective COX-2 inhibitors (rofecoxib). These findings indicate that endogenous PG derived from both COX-1 and COX-2 enzymatic pathways are involved in the mechanism of ulcer healing by these hormones. Obestatin which was originally claimed to act as ghrelin opponent in the regulation of appetite behaviour [Zhang et. al., 2005], also accelerated ulcer healing and increased the GBF at ulcer margin in our study though these effects were less pronounced as compared with those of ghrelin and orexin-A.

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

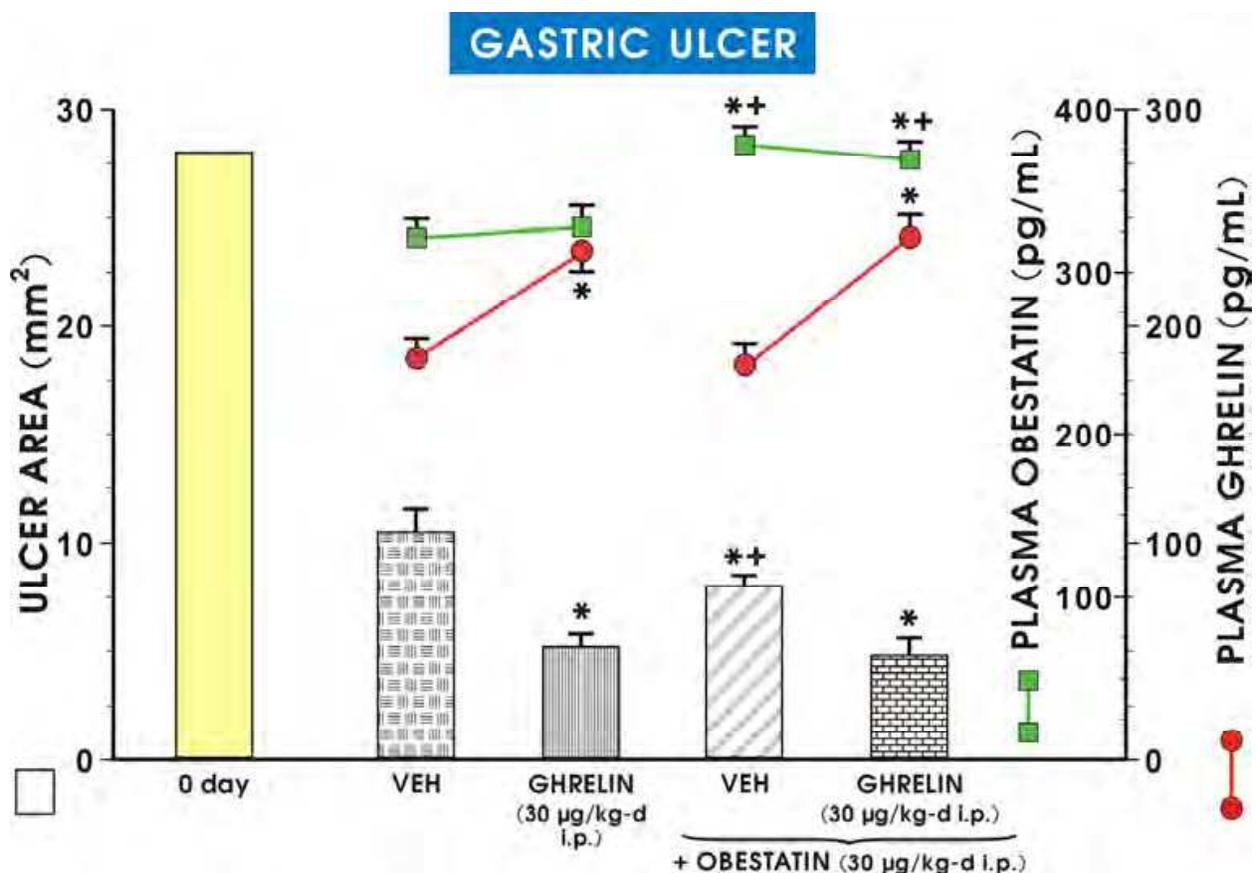


Fig. 8. The area of gastric ulcers and the alterations in the GBF at ulcer margin in rats treated for 8 days with vehicle (Veh, saline) or ghrelin (30 µg/kg-d i.p.) without or with concurrent treatment with obestatin (30 µg/kg-d i.p.). Mean ± SEM of 6-8 rats. Asterisk indicates a significant change ( $p < 0.05$ ) compared to vehicle-control- or obestatin-treated rats. Asterisk and cross indicate a significant change ( $p < 0.05$ ) compared to rats treated with ghrelin.

growth hormone secretagogue receptor (GHS-R) [Kojima et al., 1999, Hosoda et al., 2000]. 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) [Peeters, 2005; Brzozowski et al., 1999]. 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; Fukuhara et al., 2005]. The acylation by n-octanoic acid of the hydroxyl group of their third residue, which is either serine or threonine, is essential for binding of ghrelin to the GHS-R1a [Hosoda et al., 2000]. We attempted in this study to determine whether the expected ulcer healing activity of ghrelin and orexin A is due to a direct activation of GHS-1a and orexin A receptors or involves other mediators or receptors, as yet uncharacterized and distinct from the GHS-R and orexin A. We have found that the acceleration of healing and the functional features of this healing such as an improvement in the GBF at ulcer area, the crucial mechanism involved in the healing of gastric ulcer, were mediated by specific ghrelin and orexin-A receptors.

Ghrelin was originally reported to exhibit gastroprotective activity against mucosal lesions induced by corrosive substances such as ethanol as well as against damage induced by

stress and ischemia-reperfusion [Sibilia et al., 2003; Brzozowski et al., 2004; Konturek et al., 2004; Brzozowski et al., 2006]. These effects were associated with a significant increase in plasma ghrelin concentration as well as marked attenuation of the fall in GBF provoked by stress and I/R but remained independent on gastric acid secretory activity of this peptide [Brzozowski et al., 2006]. This study attempted to determine the effects of graded doses of exogenous orexin-A and obestatin compared with ghrelin on healing of acetic acid ulcers and GBF at ulcer margin and PGE<sub>2</sub> generation in the gastric mucosa. We found that peripherally administered orexin-A dose-dependently accelerated ulcer healing, elevated mucosal generation of PGE<sub>2</sub> and raised GBF at ulcer margin. Ghrelin and obestatin also accelerated ulcer healing with the magnitude similar to orexin-A while causing an increase in GBF and PGE<sub>2</sub> generation in the ulcerated gastric mucosa. The key finding of this study is the demonstration that ghrelin mRNA is upregulated in the margin of gastric ulcer suggesting that endogenous ghrelin might reduce the size of the ulcer and improve the microcirculation around the ulcer bed. Interestingly, ghrelin mRNA was also increased in the gastric mucosa exposed to ischemia-reperfusion suggesting that enhanced expression of transcripts of ghrelin may limit the extent of gastric damage provoked by I/R. Thus, our study is in partial agreement with previous observations that plasma ghrelin is increased following stress-induced gastric lesions, and that its enhanced immunoreactivity is associated with duodenal ulcerations induced by cysteamine in rats [Brzozowski et al., 2006; Fukuhara et al., 2005].

Since endogenous prostaglandins (PGs) have been implicated in the control of food intake and appetite [Eberhart, E.C, 1995; Scholz, H, 2003] we hypothesized that these cytoprotective arachidonate metabolites could play an important role in the healing effect of ghrelin. This prompted our interest in endogenous PGs because their role as well as the importance of expression of cyclooxygenase (COX-1) and COX-2 at ulcer margin in the possible ulcer activity of appetite hormones such as ghrelin, orexin-A and obestatin have not been studied. The present study supports the notion that the ulcer healing and hyperemic activities of ghrelin, orexin-A and obestatin during the time-course of ulcer healing involve an increase in mucosal generation of endogenous PGs. Previous studies have established that PG synthesis depends upon the activity of cyclooxygenase (COX), a rate limiting enzyme in the synthesis of eicosanoids from arachidonic acid [Eberhart CE & Dubois RN, 1995]. In contrast to COX-1, COX-2 is not constitutively expressed in most of tissues, but is dramatically upregulated during inflammation and ulcer healing [Takeuchi et al., 2004; Brzozowski et al., 2006] and following inhibition of mucosal COX-1 activity [Davies et al., 1997; Tanaka et al., 2002; Takeuchi et al., 2004]. First, we found that all hormones tested that is mean ghrelin, orexin A and obestatin enhance the PGE<sub>2</sub> generation at ulcer margin as compared with that of vehicle-control at day 8 upon ulcer induction. Second, indomethacin almost completely abolished, while SC-560, the selective COX-1 inhibitor and rofecoxib, the selective COX-2 inhibitor, greatly attenuated the ulcer healing and hyperemic effects of ghrelin, orexin A and obestatin, 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 on ulcer healing. It is of interest that COX-1 and COX-2 inhibitors by themselves delayed ulcer healing and abolished the ulcer healing promoting activity of ghrelin, orexin A and obestatin and counteracted their accompanying rise in GBF at ulcer margin suggesting that endogenous PG might be considered as primary mediators involved in the mechanism of ulcer healing by these peptides.

It is not excluded that the beneficial effect of these hormones depend also upon the activation of NO/NOS system by these hormones, which is an important metabolic pathway involved in the mechanism of mucosal defense and ulcer healing. The involvement of cNOS/iNOS-NO system in ulcer healing and the possible role of ghrelin in ulcer healing are not quite clear from this and other studies [Sibillia et al., 2009]. Although the non-specific suppression of cNOS/iNOS-NO system by L-NNA was found in our study to delay ulcer healing, little is known whether appetite hormones could affect the expression and activity of cNOS or iNOS or both. Indeed, the concurrent treatment with L-NNA not only markedly reduced the acceleration of ulcer healing but also the accompanying hyperemia at ulcer margin caused by these hormones. This finding suggests that NO could be considered as one of the essential mediator of both, the ulcer healing and the hyperemic activity of ghrelin, orexin A and obestatin. Thus we propose that NO possibly derived from constitutive cNOS rather than inducible iNOS expression and activity, plays an important role in the ghrelin-, orexin A and obestatin induced acceleration of the ulcer healing process. However, factors other than endogenous PGs and NO, possibly also sensory neuropeptides such as CGRP could also contribute to this effect, and therefore, their involvement in ulcer healing activity of ghrelin, orexin A and obestatin should be addressed in future studies. The finding that COX-1 and COX-2 inhibitors greatly attenuated the ulcer healing activity of ghrelin, is in keeping with the observation that ghrelin is ineffective in the protection against indomethacin-induced gastric ulcers [Sibillia et al., 2004], where the mucosal generation of PGE<sub>2</sub> is greatly suppressed.

Interestingly, the overexpression of TNF- $\alpha$  and IL-1 $\beta$  was detected in vehicle treated control rats with gastric ulcer comparing to intact gastric mucosa, and this effect was probably caused by severe tissue ischemia, resulting from the application of acetic acid. This overexpression of proinflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$ , likely contributed to early tissue damage and formation of ulceration. As reported by our group recently [Brzozowski et al., 2006] vascular injury leading to ischemia is the major factor associated with early induction of acute mucosal damage and also involved in the time-course of chronic ulceration in the stomach, where the acetic acid was applied. [Guo et al., 2003] reported an early rise in the expression of iNOS, suggesting that this expression accompanied by excessive generation of NO possibly forming peroxynitrate, was probably responsible for the enlargement of ulcer crater observed at early first days upon ulcer induction by acetic acid in rats. Our results seem to support this notion by overexpression of mRNA for proinflammatory cytokines, TNF- $\alpha$  and IL-1 $\beta$ , that could be responsible for the formation of focal tissue damage caused by acetic acid application. Treatment with ghrelin markedly attenuated the expression of IL-1 $\beta$  and TNF- $\alpha$  in gastric mucosa around the ulcer suggesting that the acceleration of ulcer healing by ghrelin could be, at least in part, due to the potent anti-inflammatory activity of this hormone.

We reported before that the induction of gastric ulcer by serosal application of acetic acid is accompanied by the co-expression of gastroprotective COX-2-PG system with the increased expression and release of vasoactive, proliferative and trophic gastric hormones such as gastrin and ghrelin [Konturek et al., 2008]. Ghrelin was shown to exhibit gastroprotective activity and our present study confirming that this hormone can accelerate ulcer healing, suggests that the upregulation in the ghrelin gene expression could play the central event in the mechanism of gastric ulcer healing. These protective and trophic hormonal responses of gastric mucosa to ulceration, as manifested by increased expression of ghrelin co-expressed

with COX-2 [Konturek et al., 2008] possibly triggered by an overexpression of IL-1 $\beta$  and TNF- $\alpha$ , seem to play a crucial role in the restoration cellular and glandular structure of the mucosa and the quality of ulcer healing.

Interestingly, obestatin, which is encoded by the same gene as ghrelin has recently been reported to counteract physiological effects of ghrelin [Zhang et al., 2005]. Intraperitoneal and intracerebroventricular treatment with obestatin suppressed food intake in a time-dependent and dose-dependent manner. This was supported by observation that obestatin effectively blunted the hunger caused by short-term starvation and inhibited feeding or body weight in rats [Sibillia et al., 2009] and food consumption in mice [Lagaut et al., 2007]. However, the vast majority of studies indicates that obestatin exerts little or no effects on food intake and body weight and failed to modify the CCK-induced decrease in food intake [Moechrs et al., 2006; Nogueiras et al., 2007; Tremblay et al., 2007]. Our present findings are in keeping with these latter observations because obestatin by itself accelerated ulcer healing but when administered together with ghrelin, remained without influence on ghrelin induced promotion of the ulcer healing and an increase in the GBF at ulcer margin and plasma ghrelin levels. This suggests that obestatin might not be considered as physiological opponent of ghrelin with the respect to ulcer healing activity which clearly was enhanced by this peptide.

## 5. Conclusion

In summary, these results demonstrate for the first time, that administration of exogenous ghrelin dose-dependently accelerates healing of chronic gastric ulcers and that other hormones involved in the control of appetite and food intake such as orexin-A and obestatin can also exert beneficial effect on the speed of ulcer healing. An evidence was provided that these ulcer healing effects of orexin-A, ghrelin and obestatin may depend upon endogenous PGs derived from COX-1 and COX-2 activity and an enhancement of gastric microcirculation around the ulcer area, possibly mediated by NO. Our finding that ghrelin is expressed in gastric mucosa, particularly at ulcer margin during ulcer healing suggests that this hormone may act locally by paracrine pathway to activate the vasoactive compounds such as PG and NO that may lead to an acceleration of the ulcer healing and the suppression of the mucosal expression and the release of proinflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$ .

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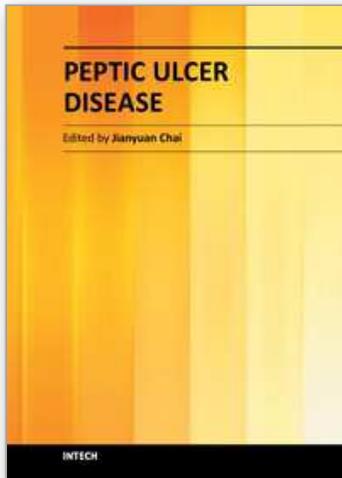
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## **Peptic Ulcer Disease**

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Peptic ulcer disease is one of the most common chronic infections in human population. Despite centuries of study, it still troubles a lot of people, especially in the third world countries, and it can lead to other more serious complications such as cancers or even to death sometimes. This book is a snapshot of the current view of peptic ulcer disease. It includes 5 sections and 25 chapters contributed by researchers from 15 countries spread out in Africa, Asia, Europe, North America and South America. It covers the causes of the disease, epidemiology, pathophysiology, molecular-cellular mechanisms, clinical care, and alternative medicine. Each chapter provides a unique view. The book is not only for professionals, but also suitable for regular readers at all levels.

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