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EXPERIMENTAL HEALING OF PREEXISTING GASTRIC ULCERS INDUCED BY HORMONES CONTROLLING FOOD INTAKE GHRELIN, OREXIN-A AND NESFATIN-1 IS IMPAIRED UNDER DIABETIC CONDITIONS. A KEY TO UNDERSTANDING THE DIABETIC GASTROPATHY?

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Hormonal peptides like ghrelin, orexin A (OXA) or nesfatin-1 not only regulate appetite, which is their basic biological function, but also contribute to mechanisms responsible for maintaining integrity of the gastric mucosa. Previous studies including those from our laboratory have revealed that their gastroprotective effect results from cooperation with other factors responsible for protection of the gastric mucosa, including prostaglandin (PG) synthesis pathway, nitric oxide (NO) and the sensory afferent fibres releasing the vasoactive neurotransmitters. The aim of the present study was to determine whether ghrelin, orexin-A (OX-A) or nesfatin-1 with their protective effect on the gastric mucosa, also can modify the healing of chronic gastric ulcers. Furthermore, an attempt was made to explain participation of these peptides in healing processes of chronic gastric ulcers with comorbid conditions for the human beings resulted from *diabetes mellitus*. In our study, a model of gastric ulcers caused by concentrated acetic acid to induce the chronic gastric ulcers was used, while the clinical condition corresponding to diabetes was induced by single injection of streptozotocin (STZ). We found that ghrelin, OX-A and nesfatin-1 accelerate dynamics of the acetic acid ulcers healing, confirmed by a reduction in the ulcer area and this effect was accompanied by an increase in gastric blood flow at the ulcer margin. Destruction of sensory afferent fibres with capsaicin or blocking of vanilloid receptors with capsazepine resulted in a significant reduction of ghrelin, OX-A and nesfatin-1-induced acceleration of ulcer healing. Similar results were obtained when an NO-synthase blocker, L-NNA was used in a combination with these peptides. Moreover, it was found that OX-A and nesfatin-1 failed to accelerate the healing process under diabetic condition because both these hormones induced reduction in the ulcer area and the increase in blood flow in normal, non-diabetic rats were completely lost in the group of animals with diabetes. Treatment with OX-A and nesfatin-1 increased superoxide dismutase (SOD) mRNA expression even in acetic acid ulcers concurrent with diabetes. However, the treatment with OX-A and nesfatin-1 failed to alter the increase in gastric mucosal mRNA expression for ghrelin and hypoxia-inducible factor 1-alpha (HIF-1α), this latter effect that had been strongly pronounced in diabetic animals. We conclude that the hormonal peptides involved in the regulation of satiety and hunger such as ghrelin, OX-A and nesfatin-1 contribute to the process of chronic gastric ulcers healing cooperating with NO and sensory afferent nerve endings releasing vasoactive neuropeptide CGRP. Furthermore, OX-A and nesfatin-1, the two relatively unrecognized peptides, play an essential role in healing process of chronic gastric ulcers activating the gastric blood flow at ulcer margin and the mucosal regeneration and both ulcer healing and accompanying hyperemia at ulcer margin are greatly impaired during diabetes. Possibly, loss of the healing effect of these peptides during diabetes results from an interaction with radical generation processes as reflected by an increase of mRNA expression for SOD as well as the failure of their attenuating activity on proinflammatory factors such as HIF-1α.

Key words: *gastric ulcers, appetite hormones, ulcer healing, ghrelin, orexin-A, nesfatin-1, diabetes mellitus, gastric blood flow, prostaglandins, nitric oxide, calcitonin gene related peptide*

INTRODUCTION

The gastric mucosa is a region of active cell divisions occurring in gastric fundic glands. This enables a proper regeneration and maintaining integrity of the gastric mucosa, both acting as an essential component enabling presence of highly concentrated hydrochloric acid in the stomach without damaging its wall (1). Numerous factors contribute to protection of the gastric mucosa against damage and to its regeneration including prostaglandins (PG); growth factors such as epidermal growth factor (EGF), transforming growth factor alpha (TGFα), polyamines (spermine, putrescine), the vasoactive neuropeptides released from afferent sensory nerve endings such as calcitonin gene related peptide (CGRP), nitric oxide (NO) released from the vascular endothelium, gastric epithelium and/or the afferent sensory fibres or gastrointestinal hormones produced by endocrine cells including cholecystokinin (CCK) and gastrin (2-4). These factors have a direct protective effect by activating

natural defence mechanism necessary to maintain integrity of the gastric mucosa, mediating in a gastroprotective effect of other mechanisms, as well as cooperating in that area (5).

The question remains whether appetite hormones regulating food intake which were found to exhibit the protective effects on the gastric mucosa could contribute to the ulcer healing. The central or intraperitoneal administration of leptin, an anorexigenic peptide produced by adipocytes and gastric epithelial cells, was found to protect the gastric mucosa against damage caused by 75% ethanol, as well as damage resulting from ischemia and reperfusion (6-9). We proposed that leptin can mimic the gastroprotective effects of CCK, for example causing an increase in gastric blood flow similar to that of CCK and releasing CCK known to exert gastroprotection (8). Therefore, leptin is possibly one of factors enabling CCK to maintain integrity of the gastric mucosa, especially, as CCK, similarly to food or feeding, increases the plasma leptin levels (8, 9). Furthermore, the leptin protective effect on the gastric mucosa could be attributed to an increase in NO production and the release of vasoactive neuropeptide CGRP from afferent sensory fibres (8, 9). It was also demonstrated that leptin stimulates proliferation of the gastric mucosa cells and this seems to confirm its protective and ulcer healing effects (10). Also peptide YY (PYY), belonging to appetite inhibiting peptides, similarly to leptin, participates in regulation of physiological function of the gastrointestinal tract. PYY was indentified in numerous hormone secreting cells in the intestine and the pancreas and in neurones of the gastrointestinal tract (11). When administered intravenously, this peptide inhibits gastric and pancreatic secretion and gastric emptying in humans and inhibits motility in rats and dogs (12, 13). Moreover, it was shown that intravascular administration of PYY at doses corresponding to the increase in the serum PYY levels after meal exerts a gastroprotective effect against the damage caused by concentrated ethanol *via* the central activation of the vagus nerves and on peripheral release of CGRP and NO (12). Since bilateral vagotomy does not affect the gastroprotective effect of intravenously administered PYY, it is assumed that signals transmitted by efferent and afferent fibres do not participate in this effect (12).

Other peptides regulating food intake, including ghrelin, OX-A and especially newly discovered nesfatin-1, similarly to leptin and PYY, are not only involved in the endocrine secretion and regulation of satiety and hunger but also participate in other physiological mechanisms including the gastric and pancreatic secretory and motor activity of the gastrointestinal tract as well as the protection of the gastric mucosa against damaging factors (14-18). Initially, it was thought that the only source of OX-A are neurones of the lateral hypothalamic area, *i.e.*, the region involved in regulation of satiety and hunger (14). Now it is clearly evident that OX-A is also produced in neurones of submucosal and intramuscular plexuses of the gastrointestinal tract, in intestinal secretory cells and in pancreatic islet cells in humans and rodents (15, 16). Apart from stimulating food intake, OX-A which plasma levels rise during hunger, influences the motor and secretory activity of the gastrointestinal tract (17, 18). It was found that OX-A stimulates digestive tract motility in rodents, inhibits MMC, and stimulates gastric emptying and production of gastric juice, acting independently of gastrin (17). Probably, OX-A may act as one of factors triggering the cephalic phase of gastric secretion by modulating vagus nerve activity, as vagotomy or administration of atropine abolished these effects (17, 18).

Ghrelin, a natural ligand for the type 1a growth hormone receptor (GHS-1a), similarly to OX-A, belongs to a group of substances described as hunger peptides, showing an exceptionally high activity potential in this area (19). By acting on the hypothalamic arcuate nucleus it stimulates release of neuropeptide Y (NPY) and agouti related protein (AGRP) having a crucial role in the hunger mechanism and control of the energy balance (19). Most of ghrelin present in the blood plasma is produced by gastric mucosa cells, and this is confirmed by the 65–77% drop in plasma levels of that substance in patients after gastrectomy (20-22). However, the gastrointestinal tract is not its sole source. Ghrelin is also released by cells of visceral organs: pancreas, kidneys, liver, heart muscle, spleen, testicles and adrenals, and by structures of the nervous system including hypothalamus, pituitary gland, cerebellum and hippocampus (23). Doubtlessly, ghrelin has a biological effect on the gastrointestinal tract and on digestive glands, modifying their activity. Systemic or central (intracerebroventricular) administration of ghrelin stimulates secretion of the hydrochloric acid in rats and stimulates gastric motility, gastric emptying and pancreatic secretion in mice (21, 22). Disappearance of the ghrelin activating effect on gastric secretion following atropine administration or vagotomy suggests that ghrelin controls gastric secretory functions through the brain-gut axis. The presence of ghrelin receptors at afferent sensory nerve endings was confirmed, asserting ghrelin participation in the physiological regulation of gastric secretion (21, 22). Furthermore, as ghrelin stimulates gastrin release in rats, it is suspected that it can also control gastric secretion *via* an increase of gastrin release (22). The ghrelin effect on the gastrointestinal tract concerns not only regulation of motility and secretion, but also participation in processes protecting against ulcerogenic factors. Research conducted by Sibilia *et al*. (24) and Brzozowski *et al*. (25, 26) have indicated that ghrelin administered either centrally or peripherally reduces damaging effects of stress resulting from cold and topical hemorrhagic injury induced by ethanol. Furthermore, the protective effect of ghrelin is related to other factors with documented gastroprotective properties, including NO, PG and the neurotransmitters of sensory afferent fibres (27). Moreover, ghrelin which was proposed to act *via* vagal stimulation exerts anti-inflammatory properties as it reduces expression of proinflammatory cytokines IL-1β and TNF-α in the gastric mucosa exposed to stress or ischemia-reperfusion (26, 28, 29).

Contrary to orexigenic peptides ghrelin and OX-A, the recently discovered hormone nesfatin-1 administered peripherally or into cerebral ventricles inhibits food intake in rats (30-32). Nesfatin-1 is produced by posttranslational modification of the nucleobindin2 (NUCB2) molecule in a presence of prohormone convertase (PC)-1/3 (32). Cells immunopositive to NUCB2/nesfatin-1 are found in various brain areas, including groups of neurones in the hypothalamus and the brain stem, including the paraventricular nucleus (PVN), supraoptic nucleus, arcuate nucleus, lateral hypothalamic area, zona incerta and the solitary tract nuclei, as well as in other areas of hypothalamus, mid- and hindbrain (30, 33). Most of peptides participating in regulation of food intake present in brain cells are also of peripheral origin, with their main source being the gastrointestinal tract. This also applies to nesfatin-1 which, as it was proved in numerous studies, can be released from cells other than cerebral neurones (34). Presence of pronesfatin NUCB2 mRNA was recently confirmed in the stomach, cells of pancreatic islets of Langerhans, testicles and the pituitary gland in rats (34, 35). An anatomic location of neurones showing nesfatin-1 expression and its co-localization with other neurotransmitters suggests that a physiological role of nesfatin-1 concerns not only regulation of food intake, but also the neuroendocrine regulation and the autonomic control of internal organs and impulse/emotional behaviour (36). Similarly to ghrelin, OX-A and leptin, the recently discovered

peptide, nesfatin-1 also limits the damaging effects of ulcerogenic factors. Recently we provided evidence that peripherally administered nesfatin-1 exhibits the gastroprotective effect against the stress-induced gastric lesions (37). Additionally, a relationship between nesfatin-1 and endogenous PG system was observed, as the protective effect of nesfatin-1 was significantly reduced by co-administration of cyclooxygenase (COX)-1 and COX-2 inhibitors. Similarly, the reduction in stress-induced gastric lesions was inhibited when NO-synthase blocker was used together with nesfatin-1 (37). Functional studies have revealed a significant inhibition of feeding behaviour and its contribution to glucose homeostasis. These metabolic functions make nesfatin-1/NUCB2 a novel candidate for treatment of obesity and diabetes (38).

Appetite regulating hormones such as ghrelin, orexin-A and nesfatin-1 were shown to contribute to the mechanism of gastric mucosal integrity and protection of gastric mucosa against the formation of gastrointestinal lesions induced by noxious stimuli (39-41) but their effects on healing of chronic gastric ulcers have not been so far recognized. As the physiological effect of ghrelin, OX-A and nesfatin-1 also includes participation in regulation of gastrointestinal protection against acute gastric damage, we have conducted the study to explain a role of these peptides in healing of chronic gastric ulcers induced by acetic acid. Rats with preexisting gastric ulcers were treated for 10 days with ghrelin, OX-A and nesfatin-1 with or without the antagonists of ghrelin and OX-A receptors (18, 26), the COX-1 and COX-2 inhibitors (24, 26) or NO-synthase inhibitor L-NNA (27) to determine the involvement of ghrelin and OX-A receptors, endogenous PG and NO in the possible mechanism of ulcer healing induced by these appetite hormones. Since the orexigenic peptide ghrelin and the anorexigenic peptide nesfatin-1 are expressed by the same endocrine cell of the rat stomach, the X/A-like cell a dual role of this cell type with differential effects on stimulation and inhibition of appetite dependent on the peptide released was proposed (42). Under obese conditions, the expression of these two peptides is differentially regulated with an increase of nesfatin-1 and a decrease of ghrelin indicating negative feedback mechanism preventing further body weight increase (42). That is why we have determined ghrelin mRNA expression in gastric mucosa at ulcer margin of rats with gastric ulcers treated with nesfatin-1 and OX-A.

Another important attempt of our present study was to determine whether the treatment with these appetite hormones could affect the ulcer healing under diabetic conditions (43). The gastric mucosa of diabetic rats is highly susceptible to acute gastric injury (44, 45) but the influence of orexigenic and anorexigenic hormones such as orexin-A and nesfatin-1 on the healing of preexisting gastric ulcers under diabetic conditions has not been so far investigated. Therefore, the effect of OX-A and nesfatin-1 on the rate of ulcer healing and the changes in the gastric blood flow at ulcer margin was determined in separate group of rats with experimental diabetes (46). At the molecular level we determined the effect of these hormones in diabetic animals on the gastric mucosal expression of SOD, proinflammatory factor HIF-1α and ghrelin in the gastric mucosa surrounding gastric ulcer.

MATERIALS AND METHODS

The study was conducted in Wistar rats, of weight ranging from 180 to 220 g. For 24 hours preceding the induction of gastric ulcers the animals were deprived of access to food, but had free access to water. The experimental procedures of this study were approved by the Institutional Animal Care and Ethical Committee of Jagiellonian University Medical College in Cracow. All experiments were run in accordance with statements of the Helsinki Declaration regarding handling of experimental animals.

In the study, an experimental model of chronic gastric ulcers induced with concentrated (100%) acetic acid was used according the method proposed by Okabe *et al*. (47) but modified during previous studies conducted at the Department of Physiology Jagiellonian University Medical College in Cracow and elsewhere (46, 48, 49). According to principle of this method, the concentrated acetic acid applied from the serosa part of the stomach induces a lesion in the gastric mucosa at a border between the stomach body and the pylorus. After 3 days this lesion progresses to a chronic ulcer affecting the whole thickness of the gastric mucosa and submucosa and penetrating down to the lamina muscularis mucosae. The acetic ulcers heal spontaneously within 2 to 3 weeks, thus dynamics of this process can be observed and numerous factors modifying the rate of healing of these ulcers can be studied. In separate group of rats, diabetes was induced by a single dose of streptozotocin at 70 mg/kg, intraperitoneally (i.p.), according to the method described in detail in previous studies (43, 45, 46, 50, 52). After two weeks, when the fasting plasma level of glucose reached the value of about 300 mg/dL indicating diabetes as reported previously (45, 46), the diabetic animals were randomized in the experimental settings.

The animals with gastric acetic acid ulcers were divided essentially into two series of study groups: A) non-diabetic, and B) diabetic. The non-diabetic rats (series A) in which acetic ulcers were induced, were divided into individual study groups consisting of 6–8 animals each, and they received the following substances: 1) 0.9% NaCl solution (vehicle-control group); 2) ghrelin applied daily i.p. at increasing doses ranging from 1 μg/kg-d up to 30 μg/kg-d; 3) OX-A at 30 μg/kg-d; 4) ghrelin at 30 μg/kg-d; i.p. combined with the specific ghrelin receptors antagonist, D-lys^3 GHRP (26) administered at the dose of 200 μg/kg-d; subcutaneously (s.c).

To evaluate interaction between peptides regulating food intake (ghrelin and OX-A) and sensory afferent fibres in the mechanism of ulcer healing in group 25 of animals without diabetes, the functional ablation of sensory afferent nerves was performed using capsaicin administered at the total dose of 125 mg/kg s.c., for 3 consecutive days as reported in our previous studies (25, 27). In another study group, capsazepine was administered at the dose of 2.5 mg/kg intragastrically (i.g.) to inhibit vanilloid receptors (37). In animals of group 5 (capsaicin denervation) and 6 (capsazepine administration), the following treatment was employed: a) 0.9% NaCl solution (vehicle-control group), b) ghrelin, orexin A or nesfatin-1 (both at the dose of 30 μg/kg-d; i.p.); c) OX-A or nesfatin-1 combined with CGRP (10 μg/kg-d; s.c.), a major sensory neuropeptide, and d) ghrelin or OX-A (30 μg/kg-d; i.p.) combined with capsazepine at 2.5 mg/kg-d; i.g.

Since NO plays a crucial role in the gastroprotection and ulcer healing mechanisms, another group of rats with acetic ulcers (group 7) received a co-treatment of OX-A or nesfatin-1 (20 μg/kg-d; i.p) with L-NNA (10 mg/kg-d; i.g.), the NO-synthase inhibitor, with or without the combination with L-arginine (200 mg/kg-d; i.g.), a substrate for that enzyme (25, 27).

The diabetic rats with acetic acid ulcers were administered i.p. for a period of 10 days with the following substances: 1) 0.9% NaCl solution (vehicle-control group), 2) ghrelin, nesfatin-1 or OX-A (each hormone administered at dose of 30 μg/kg-d; i.p), 4) ghrelin and OX-A (30 μg/kg-d; i.p) combined with D-lys^{3}GHRP (200 μg/kg-d; s.c.), the ghrelin receptor antagonist and with SB-334867 (100 μg/kg-d; s.c.) (39), an OX-A receptor inhibitor, respectively.

Measurement of gastric blood flow and assessment of the ulcer size

Before the gastric blood flow (GBF) was measured, the animals received pentobarbital at a dose of 60 mg/kg, i.p. To measure the gastric mucosal blood flow, a laser Doppler flow meter was used (Biotechnical Science, Model RBF-2, Osaka, Japan) (37-40). Measurement was performed in three different areas of the gastric mucosa at ulcer margin, ulcer base and in the intact gastric mucosa not involving gastric ulcer. The blood flow value measured as ml/min/100 g of the tissue was expressed as a percentage of the blood flow measured in the particular area of the stomach, namely in the ulcer margin or ulcer base versus that blood flow in the intact gastric mucosa. Macroscopic evaluation of areas of gastric ulcerations was performed using the planimetry method (Morphomat, Carl Zeiss, Berlin, Germany) as reported in details before (48).

Reverse-transcriptase-polymerase chain reaction for detection of messenger RNA for superoxide dismutase, hypoxia-inducible factor-1α and ghrelin in diabetic rats with chronic gastric ulcers

In biopsies collected from gastric mucosa of diabetic rats with gastric ulcer, the expression of messenger RNA (mRNA) for SOD, HIF-1α and ghrelin was determined by the reverse transcription polymerase chain reaction (RT-PCR) technique according to methods described by Chomczynski and Sacchi as described in details in our previous studies (37, 39, 40). Briefly, the gastric mucosal specimens were scraped off from the 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 kit from Stratagene® (Heidelberg, Germany). According to the method principle, the 1% agarose-formaldehyde gel electrophoresis and ethidium bromide staining determined the total RNA concentration in each sample. Aliquoted RNA samples were stored at –80°C until analysis.

Single stranded cDNA was generated from 5 μg of total cellular RNA using StrataScript reverse transcriptase and oligo-(dT)-primers (Stratagene, Heidelberg, Germany). Briefly, 5 μg of total RNA was uncoiled by heating (65°C for 5 min) and then reversed by transcribing into complementary DNA (cDNA) in a 50 μl reaction mixture that contained 50 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT), 0.3 mg oligo-(dT)-primer, 1 ml RNase Block Ribonuclease Inhibitor (40 U/μl), 2 ml of a 100 mmol/l mixture of deoxyadenosine triphosphate (dATP), deoxyribothymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP), 5 ml 10 × RT buffer (10 mmol/l Tris-HCl, pH=8.3, 50 mM KCl, 5 mM $MgCl_2$). The resultant cDNA (2 μl) was amplified in a 50 μl reaction volume containing 0.3 ml (2.5 U) Taq polymerase, 200 mM (each) dNTP (Pharmacia, Germany), 1.5 mM/l $MgCl_2$, 5 ml 10 × polymerase chain reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH=8.3) and primers used at a final concentration of 0.5 mM. The mixture was overlaid with 25 μl of mineral oil to prevent evaporation. The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT) in the area dedicated for performing PCR reaction. The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT) and the incubation and thermal cycling conditions were as followed: denaturation at 94°C for 1 min, annealing at 60°C for 45 s and extension 72°C for 2 min. The nucleotide sequences of the primers for SOD, HIF-1α, ghrelin and β-actin were as follows: SOD; forward: CAG CCT TGT GTA TTG TCT TC, reverse: GCT TCT CTC GTC TCC TTG CT (201 bp); HIF-1α; forward: TCT GGA CTC TCG CCT CTG, reverse: GCT GCC CTT CTG ACT CTG (510 bp); ghrelin; forward: TTG AGC CCA GAG CAC CAG AA, reverse: AGT TGC AGA GGA GGC AGA AGCT (394 bp), and β-actin; forward: TTG TAA CCA ACT GGG ACG ATA TGG, reverse: GAT CTT GAT CTT CAT GGT GCT AGG (764 bp). The primers were synthesized by GIBCO BRL/Life Technologies (Eggenstein, Germany). Polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Location of predicted products was confirmed by using DNA 100-bp ladder (GIBCO, Eggenstein, Germany) as a standard size marker. The intensity of bands was quantified using densitometry (LKB Ultrascan, Pharmacia, Sweden) as described in details in our previous studies. The signals for SOD, HIF-1α and ghrelin mRNAs were standardized against the β-actin signal for each sample and the results were expressed as SOD, HIF-1α and ghrelin mRNA/β-actin mRNA ratio.

Statistical analysis

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

RESULTS

The serosal application of concentrated acetic acid caused the mucosal necrotic erosion, which within 3 days transformed into a chronic ulcer that healed spontaneously within next 10 days. *Fig. 1* shows the effect of daily treatments with ghrelin administered i.p. in graded doses ranging from 1 μg/kg-d up to 30 μg/kg-d and with OX-A administered in a single dose of 30 μg/kg-d on area of gastric ulcer and the accompanying changes in the GBF at ulcer margin. A blood flow rate in the gastric mucosa is expressed as a percentage of blood flow rate in animals subjected to acetic acid versus vehicle (0.9% NaCl), treated as a control. Ghrelin administration to rats with acetic acid ulcer resulted in dose-dependent reduction ($p<0.05$) in the ulcer area followed by an increase in GBF at the ulcer margin. Moreover, *Fig. 1* presents a comparison between the effect of ghrelin, an orexigenic peptide, and another peptide regulating food intake, OX-A administered i.p. in the same equivalent dose of 30 μg/kg-d, i.p. as ghrelin for 10 days. OX-A at a dose of 30 μg/kg-d exhibited an effect similar to ghrelin, significantly decreasing the area of gastric acetic acid ulcers ($p<0.05$) and significantly increasing the GBF at ulcer margin ($p<0.05$) comparable with those caused by ghrelin.

Fig. 2 shows the effect of ghrelin administered at the dose of 30 μg/kg-d, i.p. with or without D-Lys^3-GHRS-6 (200 μg/kg-d, s.c.), the ghrelin GHR receptor antagonist. The antagonist of ghrelin receptor D-Lys^3-GHRS-6, which by itself failed to influence the area of gastric ulcer and the GBF, almost completely reversed the ghrelin-induced decrease in the ulcer area ($p<0.05$) and the accompanying increase in the GBF at ulcer margin ($p<0.05$) (*Fig. 2*). *Fig. 3* shows results of experiments aiming at verifying whether ghrelin and OX-A can affect the sensory afferent nerve endings in mechanisms responsible for healing of gastric ulcer. Similarly as in *Fig. 1*, ghrelin and OX-A administered at 30 μg/kg-d, i.p. caused reduction in the area of gastric ulcers ($p<0.05$) and significantly raised the GBF at the ulcer margin ($p<0.05$) (*Fig. 3*). When ghrelin and OX-A were administered to rats in which capsaicin denervation of sensory nerves was conducted 10 days earlier, the area of gastric ulcer and the accompanying hyperemia at the ulcer margin were

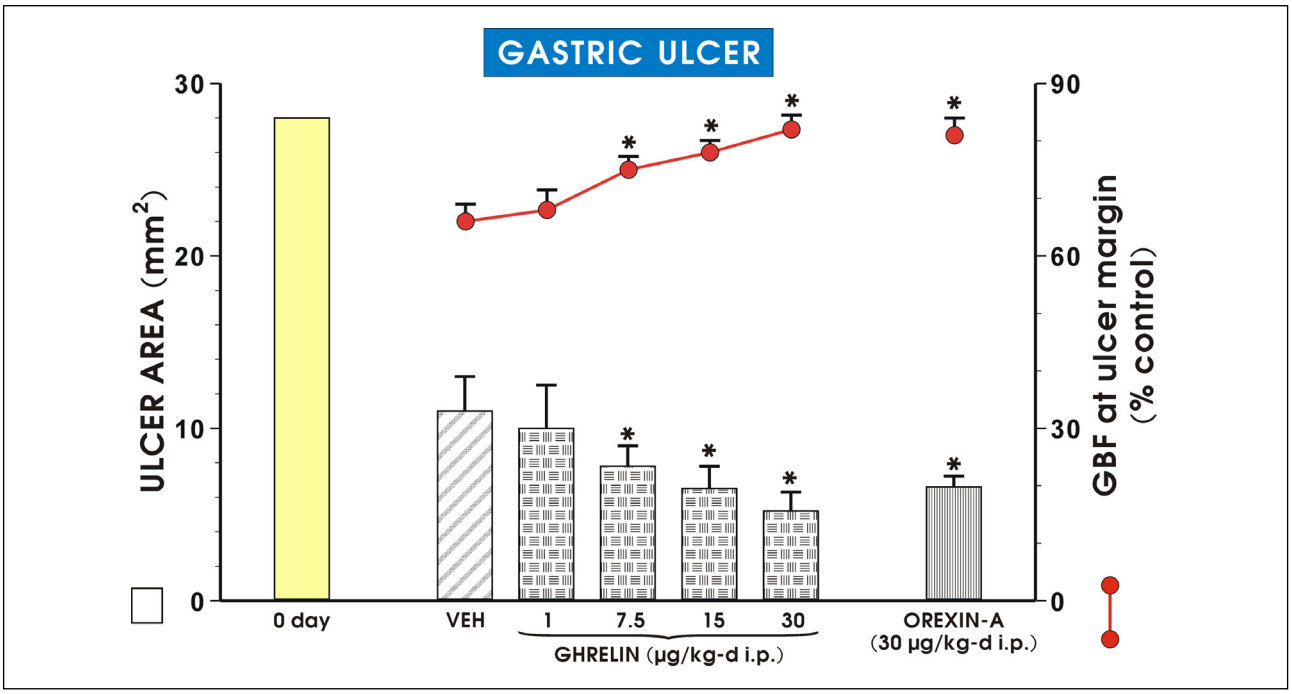

Fig. 1. The effects of 10 days intraperitoneal (i.p.) administration of ghrelin applied in graded doses ranging from 1 µg/kg-d up to 30 µg/kg-d and orexin A (OX-A) administered to rats with acetic acid ulcers in a single dose of 30 µg/kg-d on the changes in the area of gastric ulcer and the alterations in the gastric blood flow (GBF) at ulcer margin. Ghrelin caused a significant reduction in the ulcer area and an increase in GBF at the ulcer margin in a dose-dependent manner. A similar effects on ulcer area and GBF were noticed in rats administered with OX-A. Results are mean ± S.E.M. of 6 rats per each group. * $p<0.05$ vs. vehicle-control treated.

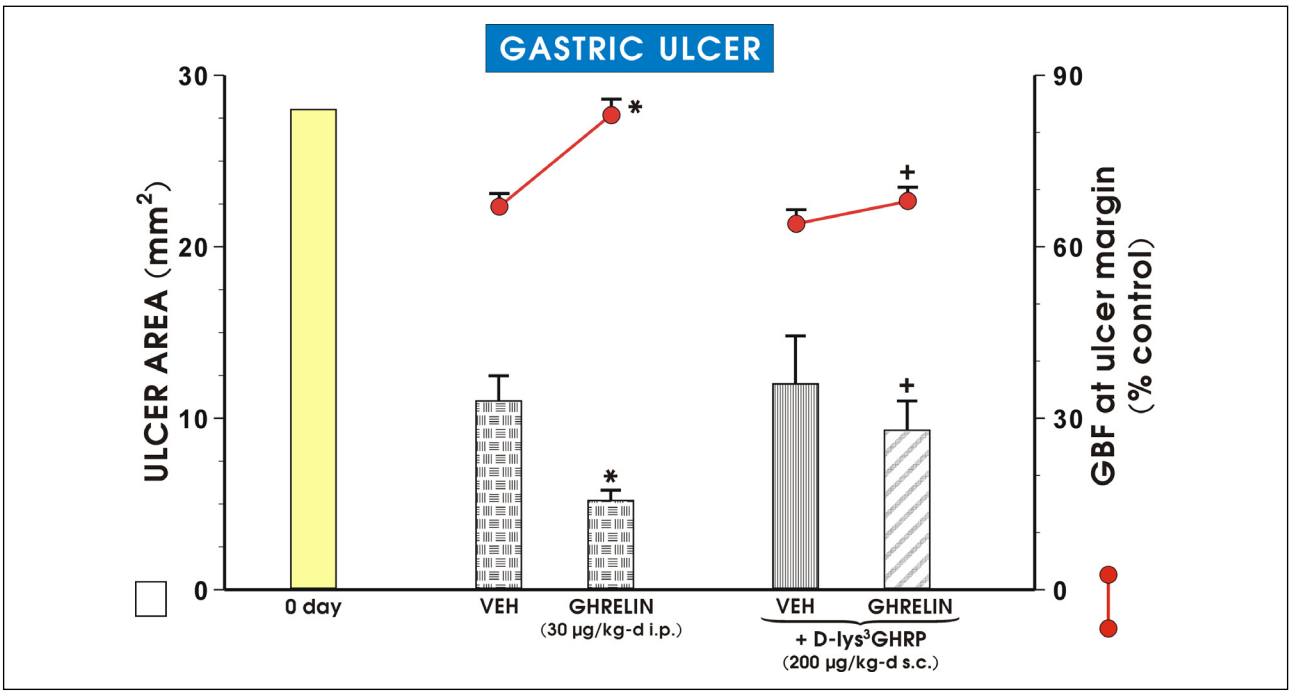

Fig. 2. The effects of 10 days intraperitoneal (i.p.) administration of ghrelin (30 µg/kg-d, i.p.) applied alone or in the combination with its receptor antagonist, D-lys^{3}GHRP (200 µg/kg-d, s.c.) on the area of acetic acid ulcers and the alterations in the gastric blood flow (GBF) at ulcer margin. The administration of D-lys^{3}GHRP significantly reduced healing effects of ghrelin and reversed the rise in GBF at ulcer margin induced by this peptide. Results are mean ± S.E.M. of 7 rats per each group. * $p<0.05$ vs. vehicle-control treated, $^+$ $p<0.05$ vs. ghrelin treated.

significantly reduced as compared to animals with intact sensory afferent nerves treated with ghrelin and OX-A ($p<0.05$) (*Fig. 3*). While ghrelin and OX-A effects on ulcer area and GBF at ulcer margin in animals with intact sensory nerves were comparable, the capsaicin denervation which increased the ulcer area, completely abolished ghrelin and OX-A-induced decrease in area of gastric ulcers, however, these effects of sensory denervation were more intensified in OX-A group of rats than those in case of ghrelin.

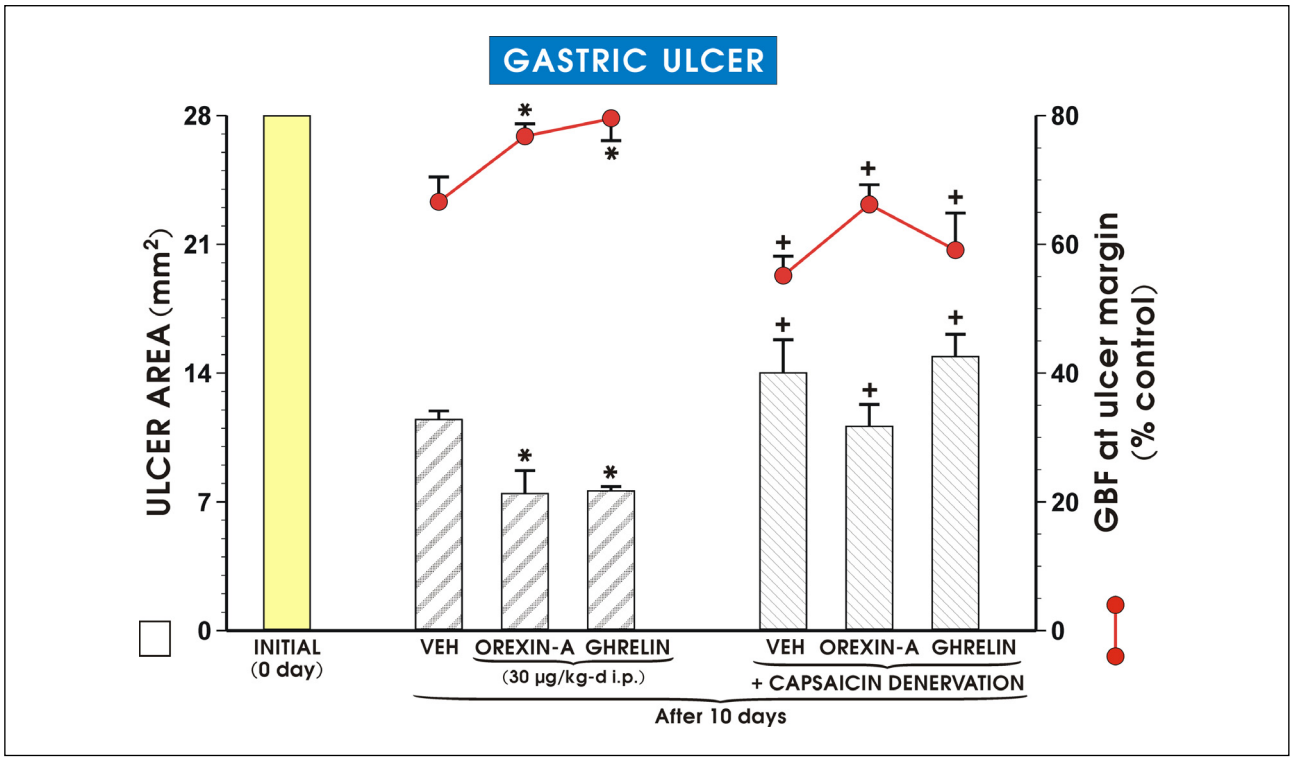

Fig. 3. The effects of intraperitoneal (i.p.) administration of orexin-A (OX-A) and ghrelin (30 µg/kg-d, i.p.) on area of gastric ulcers and the alterations in GBF at ulcer margin in rats with intact sensory nerves and in those with capsaicin denervation. The administration of OX-A and ghrelin significantly reduced the area of gastric ulcers and significantly increased the GBF at ulcer margin and these effects were abolished in capsaicin-denervated rats. Results are mean ± S.E.M. of 7 rats per each group. * $p<0.05$ vs. vehicle-control treated in rats with intact sensory nerves, $^{+}$ $p<0.05$ vs. vehicle, OX-A- and ghrelin-treated rats with intact sensory nerves.

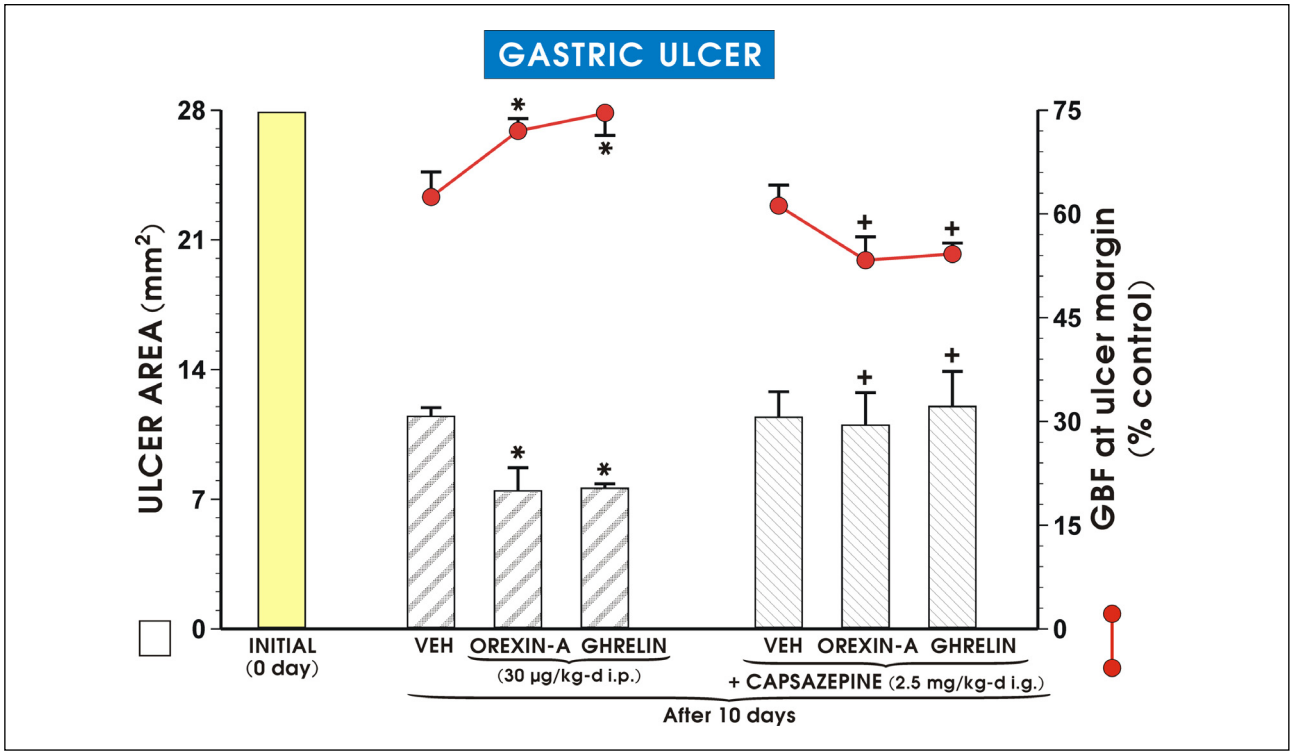

Fig. 4. The effects of intraperitoneal (i.p.) administration of orexin-A (OX-A) and ghrelin (30 µg/kg-d, i.p.) on area of gastric ulcers and the alterations in GBF at ulcer margin in rats with and without concomitant treatment with capsazepine (2.5 mg/kg-d, i.g.), a vanilloid receptor antagonist. The attenuation of ulcer area and the accompanying increase in the GBF observed in OX-A- and ghrelin-treated rats were reversed by the 10 days concurrent treatment with capsazepine. Results are mean ± S.E.M. of 8 rats per each group. * $p<0.05$ vs. vehicle-control treated in rats without capsazepine, * $p<0.05$ vs. OX-A and ghrelin-treated in rats treated without capsazepine.

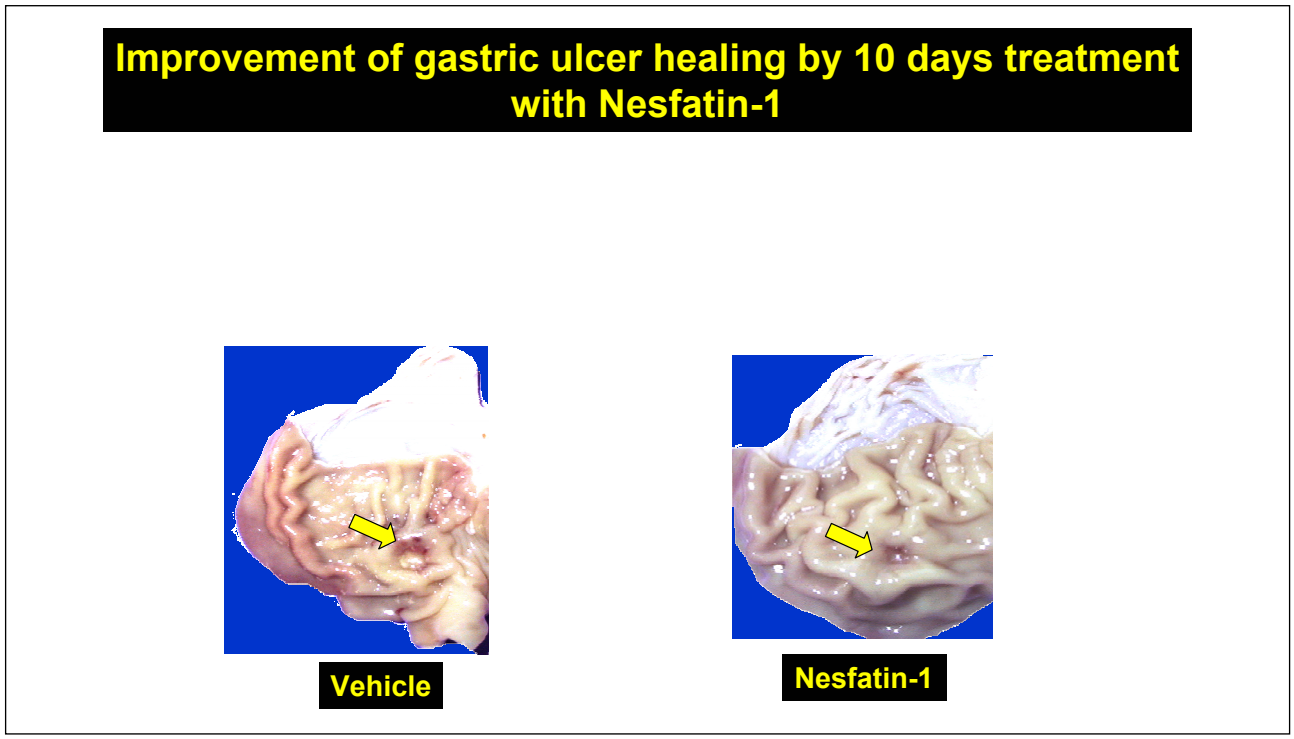

Fig. 5. The representative macroscopical appearance of gastric ulcer in rat treated i.p. for 10 days with vehicle (saline) (left panel) or that treated 10 days with nesfatin-1 (30 µg/kg-d i.p) (right panel). Note the improvement in ulcer healing in nesfatin-1-treated rat as reflected by smaller ulcer size and depth of ulcer as well as restored gastric mucosa at ulcer margin compared to vehicle-control treated.

Administration of the vanilloid receptor antagonist, capsazepine which by itself failed to affect the area of gastric ulcer, in combination with ghrelin and OX-A significantly decreased the ulcer area and the blood flow rate ($p<0.05$) versus the group receiving these orexigenic peptides only (*Fig. 4*). Whereas, when capsazepine was combined with OX-A, smaller than in case of ghrelin, reduction in the size of acetic ulcers was observed, and this effect was accompanied by a significant decrease in the blood flow rate at ulcer margin ($p<0.05$) (*Fig. 4*).

Fig. 5 (left panel) shows the representative macroscopic appearance of gastric ulcer in vehicle (control)-treated rats at day 10 upon ulcer induction. The ulcer rater and the ulcer margin are clearly visible (see arrow) indicating uncompleted healing. In clear contrast, the improvement of the ulcer healing is observed in rat treated 10 days with nesfatin-1 (30 µg/kg-d, i.p.) (*Fig. 5*, right panel) as reflected by smaller size and depth of gastric ulcer as well as restoration of the gastric mucosa at the ulcer margin. As shown in *Fig. 3*, OX-A and sensory afferent neuropeptides support each other in the mechanism of acetic acid ulcer healing, and similar relationships possibly concerns nesfatin-1. In line with this notion, *Fig. 6* shows that capsaicin denervation of sensory nerves limits participation in acetic ulcers healing not only of OX-A, but also of nesfatin-1, as despite nesfatin-1 or OX-A administration, the area of gastric ulcer was larger as compared to corresponding control group with sensory nerves intact and treated with both peptides. Whereas in capsaicin-denervated animals receiving OX-A or nesfatin-1 combined with CGRP (10 µg/kg-d, s.c.), the ulcer area was significantly reduced ($p<0.02$) and the hyperemic effect intensified and comparable to the group with sensory nerves intact (*Fig. 6*). The study results presented in *Fig. 7* indicate that OX-A and nesfatin-1 regulate dynamics of the regeneration processes in the damaged gastric mucosa, cooperating not only with sensory afferent fibres, but also with the pathway for endogenous NO biosynthesis. The daily administration of the NO-synthase (NOS) inhibitor, L-NNA, at the dose of 10 mg/kg-d, i.g., increased the area of gastric ulcer despite the concurrent treatment with OX- A or nesfatin-1 at a dose sufficient to initiate regenerative processes, which was reflected by the rise in the GBF at ulcer margin and a significant reduction in the ulcer area ($p<0.05$) (*Fig. 7)*. Nesfatin-1 and OX-A interaction with the endogenous NO pathway is confirmed by our observation that combined administration of L-NNA and L-arginine, a NOS substrate, in a presence of OX-A or nesfatin-1 restored the protective effect of these peptides on the gastric mucosa *(Fig. 7).* L-arginine was administered at a dose of 200 mg/kg-d, i.g., because in earlier studies (25) we revealed that this dose of L-arginine is sufficient to counteract the inhibitory effect of L-NNA on the NO-synthase.

Fig. 8 shows that the ulcer healing process in animals with experimentally induced diabetes is dramatically delayed as reflected by a significant increase in ulcer size ($p<0.05$) and a significant fall in the GBF at ulcer margin ($p<0.05$) comparing to vehicle (control) group at non-diabetic conditions. Similarly to ghrelin and OX-A, the administration of nesfatin-1 to diabetic animals resulted in an increase in the area of gastric ulcer with the simultaneous significant decrease in GBF at ulcer margin ($p<0.05$) comparing to respective values recorded in non-diabetic animals treated with vehicle (saline) (*Fig. 8*). When ghrelin treatment was combined with its receptor antagonist D-lys^{3}GHRP, the increase in the ulcer size and the fall in the GBF induced by ghrelin, were significantly diminished. Similarly, as in case of ghrelin, the concurrent administration of OX-A with its receptor antagonist, SB-334867, significantly decreased the area of gastric ulcer and significantly increased the GBF at ulcer margin in animals with diabetes compared with OX-A alone. Results shown in *Fig. 8* indicate that anorexigenic and orexigenic peptides, such as nesfatin-1 and OX-A, only tended

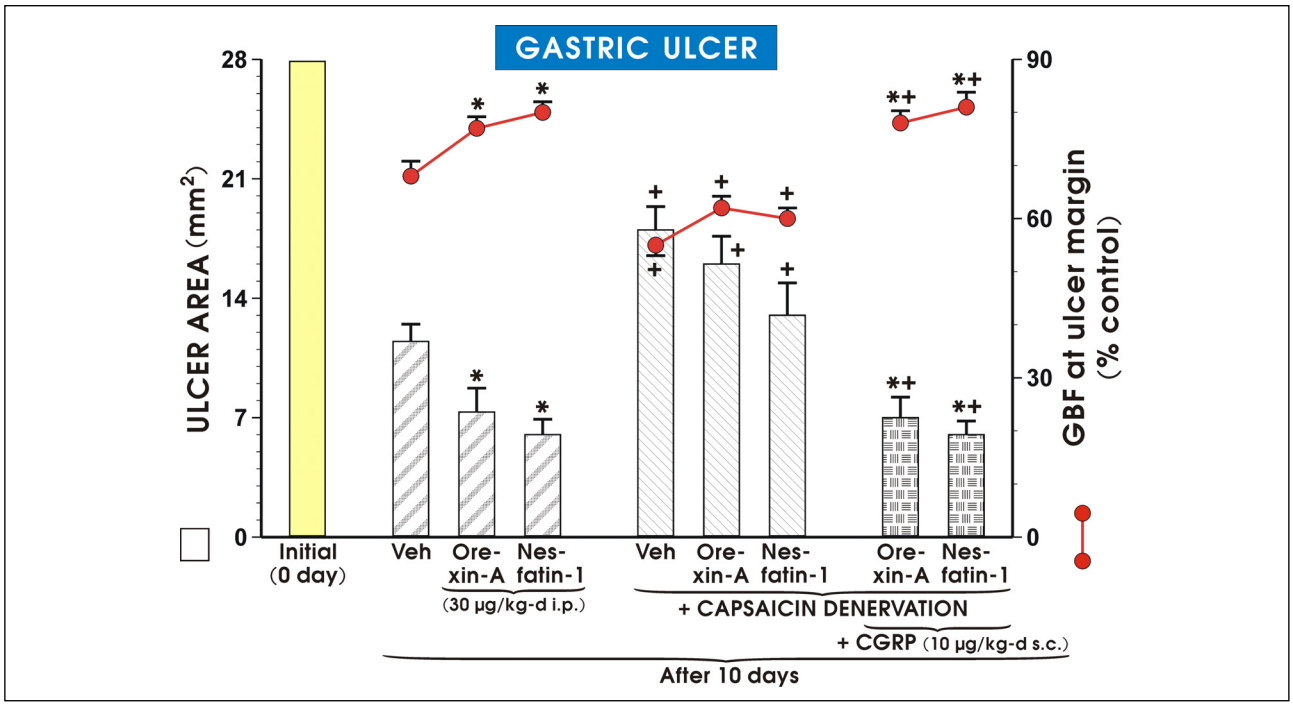

Fig. 6. The effects of intraperitoneal (i.p.) administration of orexin-A (OX-A) and nesfatin-1 (30 μg/kg-d, i.p.) on area of gastric ulcers and the alterations in GBF at ulcer margin in rats with intact sensory nerves and in those with capsaicin denervation with and without the co-treatment with exogenous CGRP (10 μg/kg-d, s.c.). The administration of OX-A and nesfatin-1 significantly reduced the area of gastric ulcers and significantly increased the GBF at ulcer margin and these effects were abolished in rats with capsaicin-denervation. Concurrent treatment with CGRP restored the ulcer healing efficacy of OX-A and nesfatin-1 in rats with capsaicin denervation of sensory nerves. Results are mean ± S.E.M. of 6 rats per each group. * $P<0.05$ vs. vehicle-control treated in rats with intact sensory nerves, $^{+}$ $p<0.05$ vs. vehicle, OX-A- and nesfatin-1-treated rats with intact sensory nerves, $^{*+}$ $p<0.02$ vs. OX-A and nesfatin-1-treated rats with capsaicin denervation without CGRP treatment.

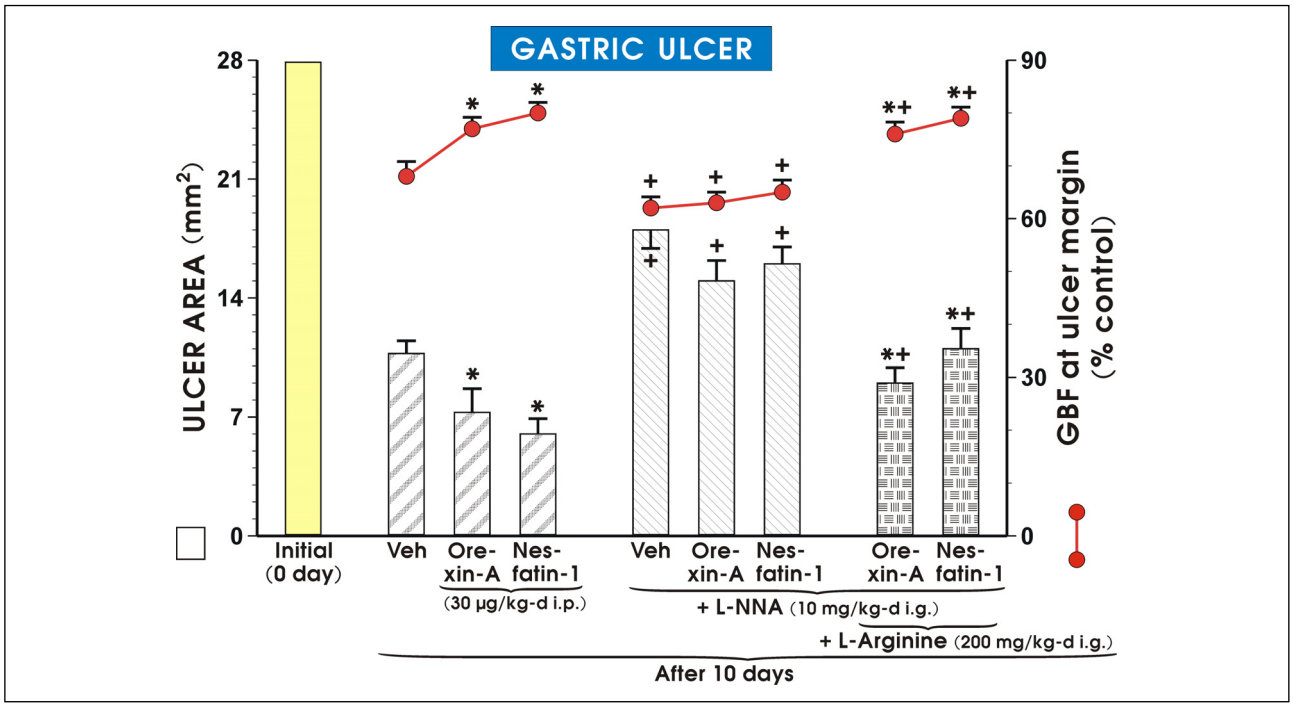

Fig. 7. The effects of intraperitoneal (i.p.) administration of orexin-A (OX-A) and nesfatin-1 (30 μg/kg-d, i.p.) on area of gastric ulcers and the alterations in GBF at ulcer margin in rats with and without NOS blockade by L-NNA (10 mg/kg-d, i.g.) or the combination of L-NNA and L-arginine (200 mg/kg-d, i.g.) treatment. The administration of OX-A and nesfatin-1 significantly reduced the area of gastric ulcers and significantly increased the GBF at ulcer margin and these effects were abolished by L-NNA and further restored when L-arginine was concomitantly administered with OX-A and nesfatin-1 in the presence of L-NNA. Results are mean ± S.E.M. of 7 rats per each group. * $p<0.05$ vs. vehicle-control treated, $^{+}$ $p<0.05$ vs. vehicle, OX-A- and nesfatin-1-treated rats without L-NNA, $^{*+}$ $p<0.05$ vs. OX-A and nesfatin-1 in rats with blockade of NO by treatment with L-NNA.

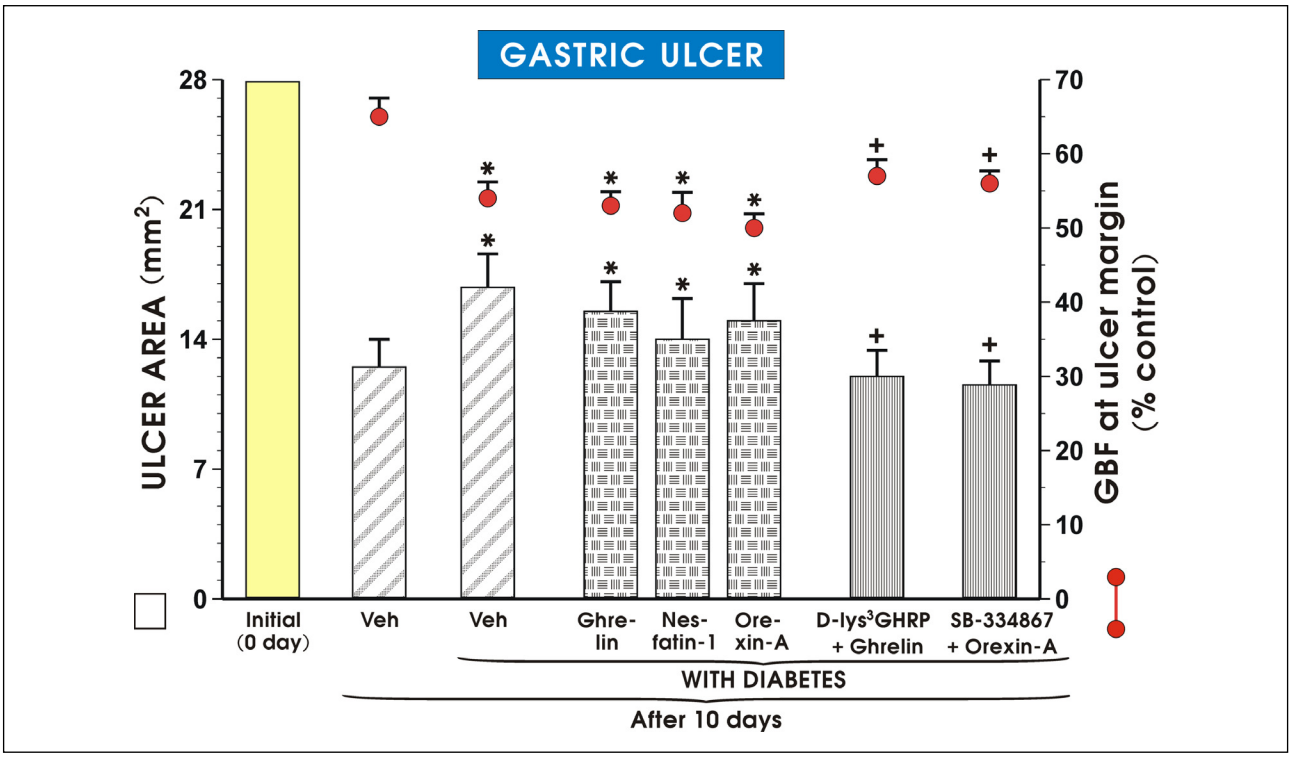

Fig. 8. The effects of intraperitoneal (i.p.) administration of vehicle (saline), ghrelin, nesfatin-1 and orexin-A (OX-A) all applied at the dosage of 30 μg/kg-d alone or the combination of ghrelin and nesfatin-1 with D-lys^{3}GHRP (200 μg/kg-d s.c.) and SB-334867 (100 μg/kg-d s.c.), respectively, on area of gastric ulcers and the alterations in GBF at ulcer margin in rats with experimental streptozotocin (STZ)-induced experimental diabetes. In diabetic animals the reduction in ulcer area and the accompanying increase in the GBF were lost and these effects were partly reversed by treatment with antagonists of ghrelin and OX-A receptors combined with ghrelin and OX-A, respectively. Results are mean ± S.E.M. of 6 rats per each group. * $p<0.05$ vs. vehicle-control treated without diabetes, $^+$ $p<0.05$ vs. vehicle, nesfatin-1- and OX-A-treated rats without the combination with D-lys^{3}GHRP and SB-334867.

to decrease the ulcer area under diabetic conditions, however, these effects were potentiated when both peptides were co-administered with their receptor antagonists. This indicates that under diabetic conditions both, nesfatin-1 and OX-A exhibit a markedly weaker activity to accelerate the ulcer healing versus the control group without diabetes.

Fig. 9 shows the effect of 10 days treatment with vehicle (saline), nesfatin-1 or OX-A applied alone or in the combination with SB-334867 on the expression of mRNA for SOD, HIF-1α and ghrelin in gastric mucosa biopsies excised from margin of gastric ulcers in diabetic animals. In intact gastric mucosa (contralateral to gastric ulcer and not involving any gastric damage) but with diabetes, the strong signals for SOD, HIF-1α and ghrelin mRNAs expression confirmed by the ratio of SOD-, HIF-1α and ghrelin mRNA over β-actin were recorded (*Fig. 9B, C* and *D*, left and right panels). In contrast, the weakly detectable signals for the expression of SOD mRNA were observed in vehicle-control diabetic rats with gastric ulcers. The ratio of SOD mRNA over β-actin confirmed that the expression of SOD mRNA was almost completely inhibited in diabetic animals with gastric ulcers. The treatment with nesfatin-1 and OX-A (both at the dose of 30 μg/kg-d, i.p.) increased the signal of SOD mRNA in diabetic rats with gastric ulcer and this was confirmed by the rise in the ratio of SOD mRNA expression over β-actin expression (*Fig. 9*, left and right panels). The overexpression of mRNA for proinflammatory factor HIF-1α was unchanged in diabetic rats with gastric ulcers treated with vehicle (control) and nesfatin-1 (*Fig. 9C*, left panel). Ratio of HIF-1α over β-actin confirmed that expression of HIF-1α remained at the same level as compared to that in non-ulcerated mucosa and this effect was not significantly influenced by treatment with vehicle, nesfatin-1 or OX-A (*Fig. 9*, right panel). The strong signals for expression of SOD-, HIF-1α and ghrelin were observed in diabetic animals treated with the combination of SB-334867 together with OX-A. As confirmed by ratio of SOD-, HIF-1α and ghrelin over β-actin, this combined administration of SB-334867 (200 μg/kg-d, i.p.), the OX-A receptor antagonist with OX-A had no significant influence on the increase in expression of SOD, HIF-1α and ghrelin caused by OX-A applied alone in diabetic animals (*Fig. 9*, right panel).

DISCUSSION

Our present study was designed to determine the efficacy of hormones controlling food intake, ghrelin, OX-A and nesfatin-1 on healing of chronic gastric ulcers in non-diabetic and diabetic conditions, the subject which has been so far little elucidated. We evaluated the alterations in healing of gastric ulcers and gastric blood flow at ulcer margin in rats with or without diabetes and we assessed the effect of OX-A and nesfatin-1 on gastric mucosal expression of proinflammatory marker HIF-1α, the reactive oxygen metabolite enzyme SOD and another orexigenic hormone ghrelin. We demonstrated for the first time that ghrelin, OX-A and nesfatin-1, all essential peptides controlling satiety and hunger, can participate in mucosal regenerative and healing processes activated in the gastric mucosa under experimental condition of gastric ulcer induction *via* acetic acid technique. Following intraperitoneal administration of these peptides

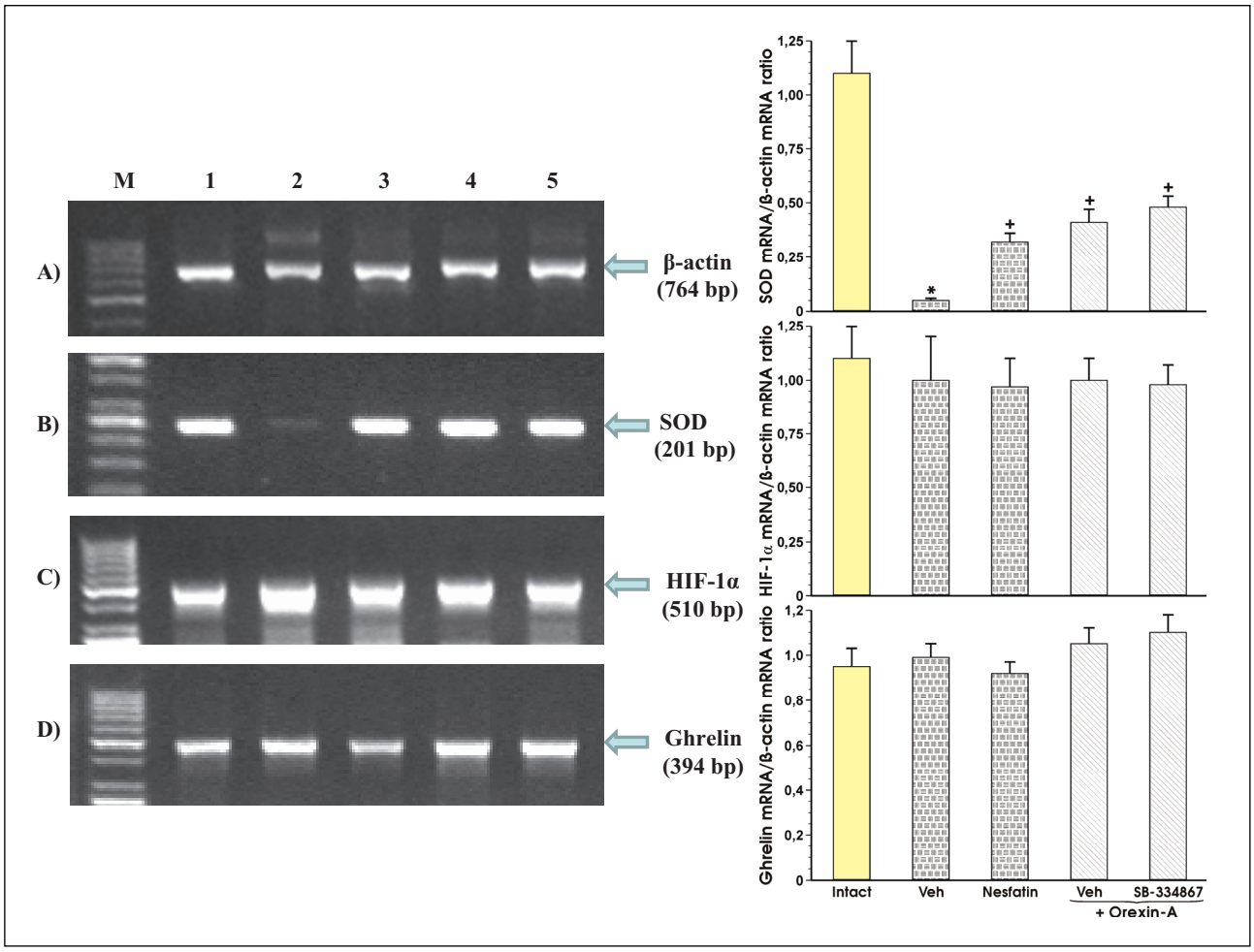

Fig. 9. The effects of intraperitoneal (i.p.) administration of nesfatin-1 (30 μg/kg-d) alone or orexin A (OX-A) (30 μg/kg-d) administered alone or in the combination with the OX-A receptor antagonist SB-334867 (100 μg/kg-d, s.c.), on the expression of mRNA for SOD-, HIF-1α and ghrelin mRNAs in the gastric mucosa of rats with acetic acid gastric ulcers. The downregulation of SOD mRNA was observed in diabetic vehicle-treated control rats with gastric ulcers and this effect was in part, reversed by the treatment with nesfatin-1 alone or OX-A applied alone or in the combination with SB-334867. Results are mean ± S.E.M. of 4 rats per each group. * $p<0.05$ vs. intact rats, $^{+}$ $p<0.05$ vs. vehicle-, OX-A- and ghrelin-treated rats with intact sensory nerves.

throughout the period of 10 days, an increase in the healing rate of these chronic gastric ulcers was observed. Ghrelin, OX-A and nesfatin-1 all caused the reduction in the ulcer area in the gastric mucosa, and an increase in blood flow at the ulcer margin. Ghrelin contribution to the acceleration of ulcer healing, the increased GBF at ulcer margin and the regeneration of the gastric mucosa was further confirmed by the apparent reversal of these changes following administration of D-lys^{3}GHRP, the ghrelin receptor antagonist (26), when co-treated together with this exogenous peptide. We propose that this beneficial healing effect of brain-gut peptides regulating appetite could be mediated by neuropeptides released from sensory nerves such as CGRP and NO because the healing effects and the increase in the GBF at ulcer margin evoked by ghrelin, OX-A and nesfatin-1 were inhibited in rats with functional ablation of sensory nerves by capsaicin and in those treated with either, capsazepine to inhibit TRPV1 receptors and by NOS inhibitor, LNNA. The concurrent treatment with exogenous CGRP, considered as the major sensory neuropeptide or L-arginine, the substrate of NO, restored the healing activity of these peptides and accompanying increase in the GBF at ulcer margin in animals with capsaicin denervation and in those with NOS blockade. For the first time according to our best knowledge, we found that the acceleration of ulcer healing by OX-A and nesfatin-1 is greatly impaired in diabetes possibly due to the fall in the GBF at ulcer margin involving an overexpression of HIF-1α. Interestingly, this impaired healing action of OX-A and another orexigenic peptide, ghrelin was in part, reversed in diabetic animals by concurrent administration of ghrelin and OX-A antagonists together with these peptides, indicating the specificity of effects exerted by these hormones under diabetic conditions. Further studies should confirm whether diabetes may interfere with OX-A- and nesfatin-1-induced stimulation of the afferent nerve activity and TRPV1 receptors thus impairing ulcer healing.

The obtained results are continuation of studies on peptides regulating food intake participation in maintaining integrity of the gastric mucosa performed in our laboratory (25, 26, 28, 39). In earlier studies, we found that both ghrelin and OX-A exhibit protective effects against damage caused by various ulcerogenic agents including ethanol, ischemia-reperfusion, cold-restraint stress or aspirin (25, 26, 39). Herein, the observed reduction in area of gastric ulcer was accompanied by an increase in gastric blood flow at the ulcer margin, which is crucial place for the ulcer to heal. We evidenced before that gastroprotective effect of these peptides could be attributed to activity of sensory afferent fibres and NO (26, 39). In our present chronic study, the

administration of NO-synthase inhibitor, L-NNA, and capsaicin-induced destruction of sensory fibres increased the ulcer area and attenuated the GBF at ulcer margin when ghrelin and OX-A were administered. This remains in agreement with previous observation that ghrelin has a protective effect on the gastric mucosa not only when administered peripherally but also centrally and that this protective activity may involve NO release and sensory neuropeptides (24, 25, 27, 39). The source of NO remains unknown but NO which is synthesized from L-arginine could derive from neurones of plexus of the enteric nervous system, the vascular endothelial cells and surface epithelial cells of the gastric mucosa (5, 27). Thus, it is not known whether ghrelin protects the gastric mucosa *via* reflex or rather local mechanism influencing the mucosal blood flow, production of endogenous PG, and increased secretion of mucus and HCO_3^-. We assume that the centrally administered ghrelin limits the damaging effects of stress with its effect related to the activation of brain-gut axis because capsaicin denervation abolished gastroprotective and hyperemic activity of this peptide (25).

Nesfatin-1 also exerts the protective and hyperemic effect on the gastric mucosa. This notion is supported by observation that nesfatin-1 administration protected the gastric mucosa against stress injury and that the selective or non-selective cyclooxygenase (COX) blockers reduced the blood flow in the gastric mucosa and increased mucosal damage versus the study group administered only with nesfatin-1 alone (28, 37). The protective effect of nesfatin-1 was restored when this peptide was administered together with exogenous PGE_2 in the presence of non-selective and selective COX-1 and COX-2 inhibitors (37). This indicates that nesfatin-1 protects the gastric mucosa against damaging effects of stress probably by activating synthesis of endogenous PG, similarly to orexin A or other appetite controlling peptides. However, it seems that arachidonic acid metabolites are not a sole factor through which nesfatin-1 reduces gastric injury induced by damaging agents. Another protective mechanism could be that release of NO in response to these hormones, as administration of the NO-synthase blocker; L-NNA attenuated these hormones evoked gastroprotective activity against stress-induced gastric lesions (37). In our present study, the concurrent administration of L-NNA with nesfatin-1 or OX-A markedly reduced their ulcer healing efficacy, but when L-arginine, a NOS substrate, was additionally administered together with nesfatin-1 or OX-A, the restoration of ulcer healing and the accompanying increase in the GBF were observed despite the presence of L-NNA. This observation is in keeping with results of our previous study (37) revealing that the luminal levels of NO in the gastric juice were increased in rats treated with nesfatin-1 alone or nesfatin-1 combined with L-arginine. Additionally, rats exposed to onset of stress exhibited decreased expression of constitutive NO synthase (cNOS) and a drastic increase in its inducible isoform of NOS (iNOS) and these effects were reversed by pretreatment with nesfatin-1 (37). Thus, we suggest that both gastroprotective and healing effects of nesfatin-1 may involve endogenous PG and NO.

Doubtlessly, ghrelin, OX-A and nesfatin-1 participation in healing of acetic ulcers can be attributed to these hormones induced activation of sensory afferent fibres. This notion is based on our present observation that the ulcer healing effect and accompanying hyperemia at ulcer margin were ameliorated in rats with functional ablation of sensory nerve endings by capsaicin as well as after blocking vanilloid receptors with capsazepine. Moreover, the concomitant treatment with exogenous CGRP, a major sensory neuropeptide released from sensory fibres under physiological conditions, added to OX-A or nesfatin-1 restored this healing and accompanying hyperemia at ulcer margin in the presence of capsaicin denervation.

A number of mechanisms facilitates the maintenance of gastrointestinal mucosal integrity and contributes to the protection against mucosal damage to this lining. It is of interest that the effectiveness of protective and regenerative agents in the gastric mucosa is reduced during diabetes, and this effect has been already confirmed by studies performed in the experimental model of diabetes induced with streptozocin (43, 50, 51-53). Diabetes was shown to aggravate cold stress, ischemia-reperfusion and nonsteroidal anti-inflammatory drugs (NSAIDs)-induced gastric lesions (45, 52-53). The diabetic gastric mucosa of rodents and humans is more susceptible to damage possibly due to impairment of the antioxidative system in the gastric mucosa, reduction in duodenal HCO_3^- secretion, dysfunction of sensory afferent fibres and the fall in local mucosal synthesis of basic fibroblast growth factor (bFGF) resulting in inhibition of angiogenesis observed under diabetic conditions (51-54). Diabetes not only weakens protective mechanisms in the gastric mucosa, but also reduces dynamics of chronic gastric ulcers healing and causes frequent complications in the ulcer disease in form of gastrointestinal bleeding confirming complexity of these phenomena (54, 56). During diabetes, a decrease in VEGF expression at the mucosa surrounding the ulceration was observed pointing out the importance of this mechanism in regulation of all stages of angiogenesis related to normal gastric ulcers healing (45, 57, 58). Moreover, impaired gastric ulcers healing during diabetes may result from increased production of proinflammatory cytokines IL-1β and TNF-α, both responsible for triggering the inflammatory reaction, forming of lesions and inducing carcinogenesis in many tissues, including the gastric mucosa. Harsch *et al.* (58) reported that IL-1β and TNF-α not only intensify ulcerogenic effects of stress and ischemia-reperfusion, but also reduce the healing rate for ulcers caused by *Helicobacter pylori*. The blood flow rate is extremely important during regeneration of the gastric mucosa under normal conditions, as it ensures adequate supply of oxygen, delivery of antioxidants such as glutathione, the scavengers of reactive oxygen metabolites, nutrients and removes toxic metabolites from the ulcer area during ulcer healing (48, 49). The reduced blood flow in the ulcer area under diabetic conditions is probably related to damage of sensory afferent fibres having a crucial role in defence and more prolonged regenerative mechanisms in the gastric mucosa (46, 52). Another mechanism certainly related to the reduced healing rate during diabetes is impairment of the endogenous PG biosynthesis because the decrease in PGE_2 synthesis at the ulcer margin was observed in diabetic rats with acetic acid ulcers (45, 46). Administration of rofecoxib or aspirin, both inhibiting PGE_2 synthesis in rats with experimental diabetes exacerbated chronic gastric ulcers but effects of COX-2 inhibitor was more pronounced in diabetic versus non-diabetic ones (46, 58).

In this study we found for the first time that ulcer healing action of peptides regulating food intake such as ghrelin, nesfatin-1 and OX-A is impaired in diabetes. In non-diabetic animals with gastric ulcers, the treatment with ghrelin, nesfatin-1 and OX-A accelerated ulcer healing and increased GBF at ulcer margin but these beneficial effects of appetite hormones were lost in animals with diabetes. The concomitant administration of ghrelin- and OX-A receptor antagonists together with ghrelin and OX-A in part, reduced this healing impairment evoked by these hormones in diabetic conditions. The apparent increase in the mRNA expression of an antioxidizing and radical scavenging enzyme SOD, observed in diabetic rats treated with nesfatin-1 and OX-A was counteracted by decreased GBF in the mucosa surrounding gastric ulcer that may result from the increased expression of HIF-1α and possibly other proinflammatory markers such as cytokines IL-1β and TNF-α and upregulation of iNOS expression following the development of diabetes. Additionally, both nesfatin-1 and OX-

A failed to significantly affect the expression of mRNA for proinflammatory factor, HIF-1α, and this increased expression of HIF-1α mRNA was strongly pronounced at diabetic conditions despite the treatment with OX-A and nesfatin-1. Since in the rat gastric mucosa ghrelin and nesfatin-1 are expressed by the same X/A-like cell endocrine cells of the stomach (34, 42), an attempt was made in our study to check whether treatment with nesfatin-1 or OX-A affects ghrelin expression in gastric mucosa of diabetic rats. We found that ghrelin mRNA expression was unaffected by the treatment with nesfatin-1 and OX-A suggesting that the impaired ulcer healing under diabetic conditions is unrelated to expression of "protective" ghrelin.

In summary, we conclude that: 1) ghrelin, OX-A and nesfatin-1 contribute to the healing of chronic acetic gastric ulcers as reflected in the reduced ulcer size and increased blood flow at the ulcer margin; 2) the cooperation between the sensory afferent neuropeptides and NO seems to plays an important role in ulcer healing activity of these hormones; 3) the novel appetite hormones such as OX-A and nesfatin-1 accelerate ulcer healing and evoke an increase in blood flow at ulcer margin but these effects are lost at diabetic gastropathy; and 4) the diabetic gastropathy manifested by delay in ulcer healing could be due to increased expression of proinflammatory cytokine HIF-1α which is unaffected by both peptides despite an enhancement of expression of mRNA for SOD by the treatment with nesfatin-1 and OX-A during course of ulcer healing.

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