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Administration of obestatin accelerates the healing of chronic gastric ulcers in rats

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

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Summary

Background:

Previous studies have shown that administration of obestatin exhibits a protective effect in the pancreas, attenuating the development of acute pancreatitis. The aim of the present study was to investigate the influence of obestatin administration on the healing of chronic gastric ulcers.

Material/Methods:

Chronic gastric ulcers were induced in rats by 100% acetic acid applied to the serosal surface of the gastric wall. Obestatin was given twice a day intraperitoneally at the dose of 4, 8 or 16 nmol/kg/dose for 6 days. Six days after induction of ulcers, rats were anesthetized and the stomach was exposed for measurement of gastric blood flow and ulcer area. Biopsy samples from the gastric mucosa were taken for determination of mucosal DNA synthesis and for measurement of gastric expression of mRNA for interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α).

Results:

Induction of gastric ulcers alone increased mucosal blood flow and tissue expression of mRNA for TNF- α and IL-1 β , whereas gastric mucosal DNA synthesis was reduced. In rats with gastric ulcers, administration of obestatin increased gastric mucosal blood flow, accelerated the healing rate of these ulcers and partly reversed the gastric ulcer-induced reduction in gastric mucosal DNA synthesis. These results were associated with a reduction in gastric mucosal expression of pro-inflammatory IL-1 β and TNF- α .

Conclusions:

Treatment with obestatin increases gastric mucosal blood flow and cell proliferation, leading to acceleration of healing of gastric ulcers. These effects are associated with a reduction in mucosal expression of pro-inflammatory IL-1 β and TNF- α .

key words:

obestatin • gastric ulcer • mucosal blood flow • cell proliferation

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BACKGROUND

Obestatin is a 23-amino acid peptide derived from proghrelin, a common prohormone for ghrelin and obestatin [1]. Obestatin, like ghrelin, was originally extracted from rat stomach, and the stomach seems to be a major source of circulating obestatin [1,2]. Secretion of obestatin is pulsatile, and plasma obestatin level exhibits an ultradian pulsatility with a frequency slightly lower than octanoylated ghrelin and growth hormone [3].

Synthesis and release of ghrelin in the stomach is related to gastric condition. In patients with *Helicobacter pylori*-evoked antral gastritis, plasma concentration of ghrelin and gastric expression of mRNA for ghrelin are increased; whereas in the next stage of *Helicobacter pylori* infection, in chronic atrophic gastritis, level of ghrelin is reduced [4]. Influence of *Helicobacter pylori* infection on the level of obestatin is unclear [5].

Previous studies have shown that administration of ghrelin protects various organs such as the heart [6], kidney [7] and brain [8] against ischemic injury and attenuates sepsis-induced lung injury and mortality [9]. Moreover, ghrelin affects the bone metabolism, stimulating osteogenesis and improving the repair of bone defects [10]. In the gut, pretreatment with ghrelin reduces gastric mucosal damage induced by noxious agents [11–13] and inhibits the development of acute experimental pancreatitis [14,15]. Moreover, apart from its protective effect, ghrelin accelerates the healing of gastric ulcers [16] and exhibits a therapeutic effect in the course of acute pancreatitis [17] and colonic inflammation [18].

Recent studies have shown to that also obestatin exhibits some protective effects. Obestatin promotes survival of pancreatic islets [19], and pretreatment with obestatin inhibits the development of cerulein-induced acute pancreatitis [20].

Tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) are cytokines involved in systemic inflammation and are members of a group of cytokines that stimulate acute phase reaction. TNF- α and IL-1 β are produced mainly by macrophages, but a broad variety of other cell types is also involved in their production [21].

The role of obestatin in the healing of gastric ulcers is unknown. Therefore, the aim of our present investigation was to examine whether obestatin administration exhibits any effect on the healing of chronic gastric ulcers and whether obestatin affects mucosal expression of pro-inflammatory cytokines.

MATERIAL AND METHODS

Animals and treatment

Studies were performed on 80 male Wistar rats weighing 180-200 g and divided randomly into 8 groups. Experiments were conducted following the experimental protocol approved by the Local Commission of Ethics for the Care and Use of Laboratory Animals. Animals were housed in cages with wire mesh bottoms, in normal room temperature (22 \pm 1°C) and a 12-h light-dark cycle.

After fasting for 16 h, rats were anesthetized with pentobarbital (30 mg/kg i.p., Vetbutal, Biowet, Puławy, Poland) and chronic gastric ulcers were induced using our modification [22] of a method originally described by Okabe et al [23]. Briefly, the abdomen was opened and the stomach was exposed. A plastic tube of 4.2 mm inner diameter was applied tightly to the serosal surface of the anterior wall of the distal portion of the stomach, proximal to the pylorus. About 70 μ l of 100% acetic acid was applied through this tube for 20 s. After removal of the acetic acid, the abdomen was closed by sutures. This method was found to result in the formation of chronic ulceration of mucosa and submucosa within the area of acetic acid application. All rats were fasted with unlimited access to water at day 0 and then had free access to food and water.

After induction of ulcers or sham operation, rats were treated with saline (0.9% solution of NaCl) or obestatin given intraperitoneally twice a day for 6 days (the first injection at the day of ulcer induction, the last injection 1 h before the end of experiment). Obestatin was administered at the dose of 4, 8 or 16 nmol/kg/dose. Experiments were repeated to obtain 10 observations in each experimental group.

Rat obestatin was synthesized at the Yanaihara Institute by a solid phase methodology with Fmoc strategy using an automated peptide synthesizer (Applied Biosystem 9030 Pioneer, Foster, CA, USA). Analytical HPLC and MALDI-TOF MS confirmed the homology of the product.

Determination of gastric blood flow and mucosal lesions

Six days after induction of chronic gastric ulcers, rats were anesthetized again with pentobarbital and the abdomen was opened by a midline incision. The stomach was exposed and the gastric mucosal blood flow was measured using laser Doppler flowmeter (PeriFlux 4001 Master monitor, Perimed AB, Järfälla, Sweden). Blood flow was measured in 5 areas of gastric mucosa and mean value of 5 recordings was presented as percent of mucosal blood flow recorded in saline-treated control rats without induction of ulcers. After measurement of mucosal blood flow the area of ulcerated mucosa was measured using a computerized planimeter (Morphomat, Carl Zeiss, Berlin, Germany) as described previously [24]. The measurement was made by a person blinded to the origin of coded specimens.

Biochemical analysis

Expression of mRNA for interleukin-1 β and TNF- α was determined using reverse transcriptase-polymerase chain reaction (RT-PCR) as described previously [25]. Samples of gastric mucosa were snap frozen in liquid nitrogen and stored at -80°C until RNA extraction. The interleukin-1 β primer sequences were: sense, GCTACCTATGTCTTGCCCGT, and antisense, GACCATTGCTGTTTCTTAGG; the expected length of the product was 543 bp. The TNF- α primer sequences were: sense, TACTGAACCTCGGGGTGATTGGTCC, and antisense, CAGCCTTGCCCTTGAAGAGAACC; the expected length of the product was 295 bp. The β -actin primer sequences were: sense, TTGTAACCAACTGGGACGATATGG, and antisense, GATCTTGATCTTCATGGTGCTAGG; the expected length of the product was 764 bp. Polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gel

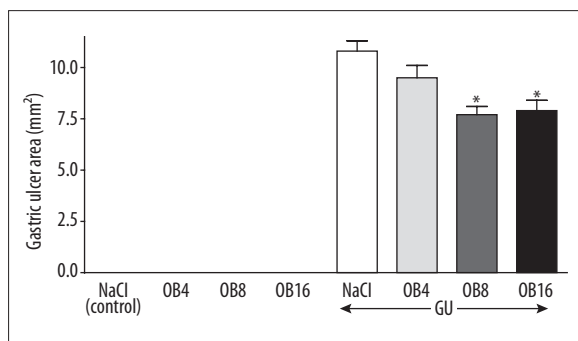


Figure 1. Effect of saline (NaCl) or obestatin given at the dose of 4 (OB4), 8 (OB8) or 16 nmol/kg/dose (OB16) and induction of gastric ulcers (GU) on the area of gastric mucosal damage. Mean \pm SEM. $N=10$ in each group of animals. * $P<0.05$ compared to saline-treated rats with ulcers.

containing ethidium bromide. Location of predicted products was confirmed by using 100-bp ladder (Takara, Shiga, Japan) as a standard size marker. The gel was then photographed under UV trans-illumination. The intensity of PCR products was measured using a video image analysis system (Kodak Digital Science). The signal for investigated mRNA was standardized against that of the β -actin mRNA from each sample and the results were expressed as analyzed mRNA/ β -actin mRNA ratio as described earlier [26].

DNA synthesis in gastric mucosa was determined by measurement of [3 H]thymidine incorporation ([6- 3 H]-thymidine, 20–30 Ci/mmol, Institute for Research, Production and Application of Radioisotopes, Prague, Czech Republic) into mucosal DNA as described previously [27]. The incorporation of labeled thymidine into DNA was determined by counting 0.5 ml DNA-containing supernatant in a liquid scintillation system. DNA synthesis was expressed as disintegrations of tritium per minute per μ g DNA (dpm/ μ g DNA).

Statistical analysis

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

RESULTS

Figure 1 shows the influence of obestatin administration on the healing of chronic gastric ulcers. In saline- or obestatin-treated rats without induction of ulcer, no ulcers were observed. In saline-treated rats, 6 days after induction of ulcers, ulcer area was 10.8 ± 0.5 mm 2 . Treatment with obestatin given at the dose of 4, 8 and 16 nmol/kg/dose reduced ulcer area by 12%, 29% and 27%, respectively. Effect of obestatin given at the doses of 8 and 16 nmol/kg/dose was statistically significant.

In rats without induction of ulcers, administration of obestatin failed to affect gastric mucosal blood flow (Figure 2). Induction of ulcers significantly increased gastric mucosal blood flow by 23% and this effect was additionally enhanced by treatment with obestatin. Obestatin given

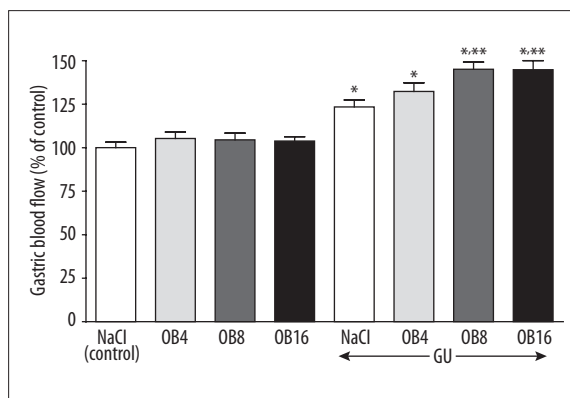


Figure 2. Effect of saline (NaCl) or obestatin given at the dose of 4 (OB4), 8 (OB8) or 16 nmol/kg/dose (OB16) and induction of gastric ulcers (GU) on gastric mucosal blood flow. Mean \pm SEM. $N=10$ in each group of animals. * $P<0.05$ compared to saline-treated rats without induction of ulcers (control); ** $P<0.05$ compared to saline-treated rats with ulcers.

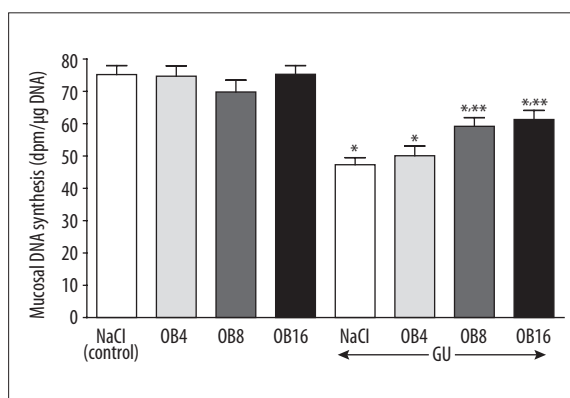


Figure 3. Effect of saline (NaCl) or obestatin given at the dose of 4 (OB4), 8 (OB8) or 16 nmol/kg/dose (OB16) and induction of gastric ulcers (GU) on mucosal DNA synthesis in the stomach. Mean \pm SEM. $N=10$ in each group of animals. * $P<0.05$ compared to saline-treated rats without induction of ulcers (control); ** $P<0.05$ compared to saline-treated rats with ulcers.

at the dose of 8 and 16 nmol/kg/dose exhibited similar and statistically significant effect on gastric mucosal blood flow in rats with ulcers; whereas effect of obestatin given at the dose of 4 nmol/kg was statistically insignificant.

In control saline-treated animals, gastric mucosal DNA synthesis reached a value of 75.2 ± 2.8 dpm/ μ g DNA (Figure 3). Administration of obestatin failed to affect DNA synthesis in gastric mucosa in rats without induction of ulcers. In saline-treated rats with ulcers, gastric mucosal DNA synthesis was reduced by 42%. Treatment with obestatin partly reversed the ulcer-evoked reduction in DNA synthesis in gastric mucosa. This effect was statistically significant after obestatin was administered at the dose of 8 or 16 nmol/kg/dose.

In rats with intact gastric mucosa, administration of obestatin at the dose of 8 nmol/kg/dose was without effect on mucosal expression of mRNA for pro-inflammatory IL-1 β in the stomach (Figure 4). Induction of gastric ulcer significantly

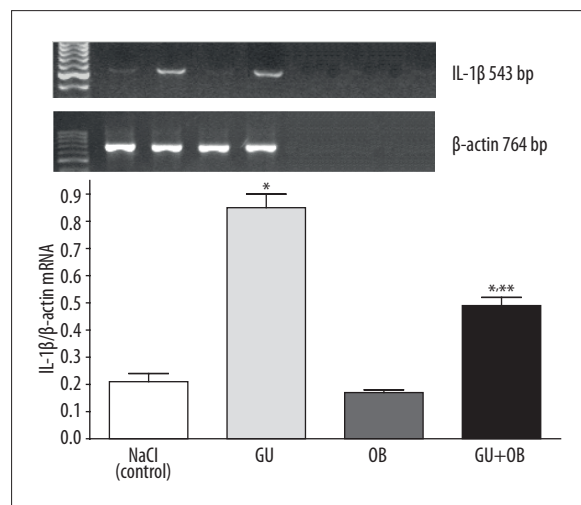


Figure 4. Effect of saline (NaCl), induction of gastric ulcers (GU) or administration of obestatin (OB) at the dose of 8 nmol/kg/dose on gastric mucosal expression of mRNA for interleukin-1 β (IL-1 β) and β -actin, and densitometric analysis of IL-1 β / β -actin ratio. Mean \pm SEM. $N=10$ in each group of animals. * $P<0.05$ compared to saline-treated rats without induction of ulcers (control); ** $P<0.05$ compared to saline-treated rats with ulcers.

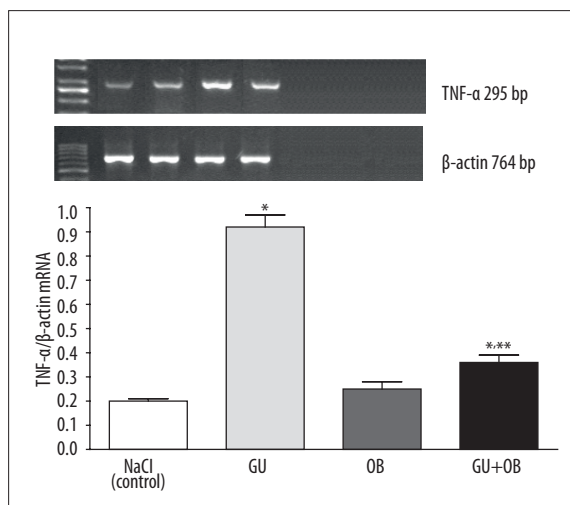


Figure 5. Effect of saline (NaCl), induction of gastric ulcers (GU) or administration of obestatin (OB) at the dose of 8 nmol/kg/dose on gastric mucosal expression of mRNA for TNF- α and β -actin, and densitometric analysis of TNF- α / β -actin ratio. Mean \pm SEM. $N=10$ in each group of animals. * $P<0.05$ compared to saline-treated rats without induction of ulcers (control); ** $P<0.05$ compared to saline-treated rats with ulcers.

increased mucosal expression of mRNA for IL-1 β by 304%. Administration of obestatin at the dose of 8 nmol/kg/dose partly, but significantly, reversed the ulcer-evoked increase in mucosal expression of mRNA for interleukin-1 β .

Administration of obestatin at the dose of 8 nmol/kg/dose was without effect on the ratio of mRNA for TNF- α to mRNA for β -actin in gastric mucosa in rats without induction of ulcers (Figure 5). Induction of ulcers increased expression of mRNA for TNF- α by 360%. Treatment with obestatin at the dose 8 nmol/kg/dose reduced the ulcer-evoked increase in expression of TNF- α mRNA by 61%, but this value was still higher than that observed in control rats with intact mucosa.

DISCUSSION

Our present study has shown for the first time that obestatin exhibits therapeutic effect in the course of chronic gastric ulcers. This effect was related to a decrease in local inflammatory process, improvement of mucosal blood flow and enhancement of mucosal cell proliferation, leading to acceleration of gastric ulcer healing.

Direct mechanism of obestatin's therapeutic effect is not clear because the cellular target of this peptide is uncertain. Initially, Zhang et al reported that obestatin binds and activates the orphan G protein-coupled receptor GPR39 [1]. On the other hand, numerous researchers have obtained opposite results. Chartrel et al. [28] found no specific binding of I^{25} -obestatin to GRP39 receptor, and they observed no effects of obestatin on GPR39-transfected cells in various functional assays, using the same experimental conditions as Zhang et al. [1], therefore they concluded that obestatin is not the cognate ligand for GPR39 receptor. Similar negative results were also observed by Lauwers et al. [29] and Holst [30]. Facing the problem of inconsistent result, Zhang et al. have

performed a new, precise study [31], investigating target cells for obestatin based on induction of an early-response gene *c-fos* in different tissues. After administration of obestatin, *c-fos* staining was found in the nuclei of gastric and intestinal mucosa, white adipose tissues, hepatic cords, and kidney tubules. Immunohistochemical analysis using GPR39 antibodies revealed cytoplasmic staining in these tissues. Binding studies, using jejunum homogenates and recombinant GPR39, revealed obestatin-specific displacement curves. Furthermore, Zhang et al. [31] demonstrated that treatment with obestatin induces *c-fos* expression in gastric mucosa of wild-type, but not GPR39 null, mice, indicating a mediating role of this receptor in obestatin actions. Finally, Zhang et al. concluded that obestatin is a metabolic hormone capable of binding to GPR39 receptor to regulate the functions of diverse gastrointestinal and adipose tissues. However, future studies are needed to confirm results obtained by Zhang et al., as well as to investigate physiological and pathophysiological role of obestatin.

Circulation and perfusion of organs is a basic physiological process necessary to sustain oxygenation and nutrition at a cellular level. Microvascular impairment of the abdominal organs has been implicated in the pathogenesis of a variety of disorders, including peptic ulcer disease and inflammatory bowel disease [32]. Blood flow plays an important role in protection of normal gastric mucosa and in the healing of damaged mucosa [33]. Gastric hypoxia resulting in accumulation of H $^+$ within gastric mucosa leads to mucosal acidification. Low mucosal blood flow predisposes to injury, whereas high blood flow protects against injurious agents. [33]. Development of peptic ulcer is associated with damage to blood vessels and reduction in mucosal blood flow. During ulcer healing mucosal blood flow returns to normal level [33]. These data are in agreement with results of our present study and indicates the role of blood flow in the therapeutic effect of obestatin in the course of chronic

gastric ulcer. Treatment with obestatin has increased mucosal blood flow in the stomach; this effect has been associated with reduction in gastric ulcer area. This obestatin-evoked influence on gastric blood flow seems to be an indirect effect because administration of obestatin has failed to affect gastric blood flow in animals with intact gastric mucosa.

Healing of gastric ulcers needs cell proliferation; this process is required for complete regeneration of damaged mucosa [34]. DNA synthesis precedes cell division and the rate of mucosal DNA synthesis is an index of mucosal cell proliferation. In our present study, obestatin failed to affect DNA synthesis in gastric mucosa in rats without ulcers, but significantly reversed the ulcer-evoked reduction in this parameter. These findings indicate that the obestatin-evoked acceleration of gastric ulcer healing involves promotion of mucosal cell proliferation, but it seems to be the secondary, indirect effect.

Our present study has shown that induction of ulcers increases mucosal expression of mRNA for IL-1 β and TNF- α . Both of these factors are cytokines involved in local and systemic inflammation, and act synergistically. They are inducers of endothelial adhesion molecules, which are essential for the adhesion of leukocytes to the endothelial surface prior to their migration into the tissues. IL-1 β and TNF- α play a crucial role in the stimulation of acute phase reaction [21,35]. These data, together with our observation that treatment with obestatin reverses the ulcer-induced increase in mucosal expression of mRNA for IL-1 β and TNF- α , indicate that the therapeutic effect of obestatin involves inhibition of mucosal inflammation.

CONCLUSIONS

Our study has demonstrated that treatment with obestatin accelerates healing of chronic gastric ulcers and this effect is related to improvement of mucosal blood flow, increase in mucosal cell proliferation and reduction in local expression of pro-inflammatory cytokines.

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