INTRODUCTION

Ghrelin, an acylated 28-amino acid polypeptide was primarily isolated from the human and rat stomach (1, 2), and circulating ghrelin is produced predominantly in gastric oxyntic mucosa (2). Ghrelin is an endogenous ligand for the growth hormone secretagogue receptor (GHS-R). GHS-Rs are predominantly expressed in the pituitary and hypothalamus; however their presence has also been shown in other central and peripheral tissues, but at much lower levels (3). Acting on GHS-R, ghrelin strongly and dose dependently stimulates release of growth hormone from the anterior pituitary (1). Beside a release of growth hormone, administration of ghrelin generates a positive energy balance. Peripheral or intracerebroventricular treatment with ghrelin increases food intake and fat deposition in rats (4). Also human studies indicate that intravenous administration of ghrelin stimulates food intake from a free-choice buffet (5). The orexigenic effect of ghrelin is mediated by activation of hypothalamic neurons expressing neuropeptide Y, agouti-related protein (AgRP) (6) and orexin (7), as well as by inhibition of hypothalamic proopiomelanocortin (POMC) neurons (8). There are also papers showing that orexigenic effect of ghrelin involves the influence of this peptide on vagal nerve activity (9).

Apart from an increase in appetite, the ghrelin-induced positive energy balance involves a decrease in energy expenditure and a reduction in cellular fat oxidation (10).

Previous studies have shown that ghrelin exhibits protective and therapeutic effects in the gut. Aim of our present investigation was to examine the influence of ghrelin administration on the healing of ethanol-induced gastric ulcers and determine the role of cyclooxygenase-1 and cyclooxygenase-2 in this effect. Our studies were performed on male Wistar rats. Gastric ulcers were induced by intragastric administration of 75% ethanol. Ghrelin alone or in combination with cyclooxygenase inhibitors was administered twice, 1 and 13 hours after ethanol application. Cyclooxygenase-1 (COX-1) inhibitor (SC-560, 10 mg/kg/dose) or COX-2 inhibitor (celecoxib, 10 mg/kg/dose) were given 30 min prior to ghrelin. Twelve or 24 hours after administration of ethanol, rats were anesthetized and experiments were terminated. The study revealed that administration of ethanol induced gastric ulcers in all animals and this effect was accompanied by the reduction in gastric blood flow and mucosal DNA synthesis. Moreover induction of gastric ulcer by ethanol significantly increased mucosal expression of mRNA for COX-2, IL-1β and TNF-α. Treatment with ghrelin significantly accelerated gastric ulcer healing. Therapeutic effect of ghrelin was associated with significant reversion of the ulcer-evoked decrease in mucosal blood flow and DNA synthesis. Ghrelin administration also caused the reduction in mucosal expression of mRNA for IL-1β and TNF-α. Addition of SC-560 slightly reduced the therapeutic effect of ghrelin in the healing of ethanol-induced ulcer and the ulcer area in rats treated SC-560 plus ghrelin was significantly smaller than that observed in rats treated with saline or SC-560 alone. Pretreatment with celecoxib, a COX-2 inhibitor, abolished therapeutic effect of ghrelin. We concluded that treatment with ghrelin increases healing rate of gastric ulcers evoked by ethanol and this effect is related to improvement in mucosal blood flow, an increase in mucosal cell proliferation, and reduction in mucosal expression of proinflammatory cytokines. Ghrelin is able to reverse a deleterious effect of COX-1 inhibitor on healing of ethanol-induced gastric ulcers. Activity of COX-2 is necessary for the therapeutic effect of ghrelin in healing of ethanol-induced gastric ulcers.

Key words: ghrelin, gastric ulcer, cyclooxygenase, cell proliferation, mucosal blood flow, interleukin-1beta, tumor necrosis factor-alpha

INTEGRATION OF CYCLOOXYGENASE-1 AND CYCLOOXYGENASE-2 ACTIVITY IN THE THERAPEUTIC EFFECT OF GHRELIN IN THE COURSE OF ETHANOL-INDUCED GASTRIC ULCERS IN RATS

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Previous studies have shown that treatment with ghrelin exhibits protective and therapeutic effects in the gut. Aim of our present investigation was to examine the influence of ghrelin administration on the healing of ethanol-induced gastric ulcers and determine the role of cyclooxygenase-1 and cyclooxygenase-2 in this effect. Our studies were performed on male Wistar rats. Gastric ulcers were induced by intragastric administration of 75% ethanol. Ghrelin alone or in combination with cyclooxygenase inhibitors was administered twice, 1 and 13 hours after ethanol application. Cyclooxygenase-1 (COX-1) inhibitor (SC-560, 10 mg/kg/dose) or COX-2 inhibitor (celecoxib, 10 mg/kg/dose) were given 30 min prior to ghrelin. Twelve or 24 hours after administration of ethanol, rats were anesthetized and experiments were terminated. The study revealed that administration of ethanol induced gastric ulcers in all animals and this effect was accompanied by the reduction in gastric blood flow and mucosal DNA synthesis. Moreover induction of gastric ulcer by ethanol significantly increased mucosal expression of mRNA for COX-2, IL-1β and TNF-α. Treatment with ghrelin significantly accelerated gastric ulcer healing. Therapeutic effect of ghrelin was associated with significant reversion of the ulcer-evoked decrease in mucosal blood flow and DNA synthesis. Ghrelin administration also caused the reduction in mucosal expression of mRNA for IL-1β and TNF-α. Addition of SC-560 slightly reduced the therapeutic effect of ghrelin in the healing of ethanol-induced ulcer and the ulcer area in rats treated SC-560 plus ghrelin was significantly smaller than that observed in rats treated with saline or SC-560 alone. Pretreatment with celecoxib, a COX-2 inhibitor, abolished therapeutic effect of ghrelin. We concluded that treatment with ghrelin increases healing rate of gastric ulcers evoked by ethanol and this effect is related to improvement in mucosal blood flow, an increase in mucosal cell proliferation, and reduction in mucosal expression of proinflammatory cytokines. Ghrelin is able to reverse a deleterious effect of COX-1 inhibitor on healing of ethanol-induced gastric ulcers. Activity of COX-2 is necessary for the therapeutic effect of ghrelin in healing of ethanol-induced gastric ulcers.
acetic acid or cysteamine (19, 21). Ghrelin accelerates the healing of oral ulcers (22). Moreover, animal and clinical studies suggest that ghrelin reduces colonic inflammation (23-25).

Prostaglandins play an essential role in gastric mucosal defense. This effect is dependent on the prostaglandin-induced stimulation of bicarbonate and mucus secretion, inhibition of gastric acid secretion, and regulation of maintaining epithelial cell restitution and mucosal blood flow (26). Cyclooxygenase, the key enzyme in prostaglandin synthesis, exists in two isozymes, cyclooxygenase-1 (COX-1) and COX-2 (27). COX-1 is constitutively expressed in most tissues and has been suggested to mediate the synthesis of prostaglandins required for physiological functions and the maintenance of organ integrity. COX-2 is undetectable in most tissues under normal conditions; however it is highly inducible by cytokines, mitogens and endotoxins, and is responsible for the increased production of prostaglandins during inflammation (27, 28). Early studies have shown that inhibition of COXs activity by nonselective nonsteroidal anti-inflammatory drugs (NSAIDs) leads to gastric ulcers and delays healing of the gastric mucosa (26, 29). Maintenance of gastric mucosal integrity was thus considered to be exclusively dependent on cyclooxygenase-1 activity without a contribution of COX-2 (30). More recently, experimental data indicate that both COXs contribute to gastric mucosal defense. Overexpression of COX-2 has been shown at the ulcer edge during the healing of experimental gastric ulcers, and inhibition of COX-2 activity delays ulcer healing (31, 32).

The aim of present study was to determine whether ghrelin exhibits any effect on the healing of ethanol-induced gastric ulcers and investigate the role of cyclooxygenase-1 and cyclooxygenase-2 activity in this effect.

MATERIALS AND METHODS

Animals

Studies were performed on male Wistar rats weighing 200–220 g and were conducted following the experimental protocol approved by the Committee for Research and Animal Ethics of Jagiellonian University. Animals were fasted with free access to water for 18 hours prior to induction of gastric ulcers. After induction of ulcers, animals had free access to food and water.

Chemicals

Active N-octanoyl rat ghrelin was obtained from Yanaihara Institute, Shizuoka, Japan. Selective inhibitor of cyclooxygenase-1 (iCOX-1), SC-560 was obtained from Cayman Chemical, Ann Arbor, MI, USA. Selective inhibitor of COX-2 (iCOX-2), celecoxib was obtained from Pfizer, New York, NY, USA. Ethanol (EtOH) was produced by Polmos Białystok, Białystok, Poland.

Treatment

Studies were performed in two separate series. In both series of studies, gastric ulcers were induced by intragastric administration of 1.5 ml of 75% ethanol given through orogastric intubation as described previously (32).

In the first series, the following groups of rats were used (ten animals in each group and each time of observation):

1. control (C) (NaCl, i.p.);
2. iCOX-1 (SC-560, i.g.);
3. iCOX-2 (celecoxib, i.g.);
4. EtOH (i.g.) + NaCl (i.p.);
5. EtOH (i.g.) + COX-1 (SC-560, i.g.);
6. EtOH (i.g.) + COX-2 (celecoxib, i.g.);
7. EtOH (i.g.) + ghrelin (G) (i.p.);
8. EtOH (i.g.) + COX-1 (SC-560, i.g.) + ghrelin (G) (i.p.);
9. EtOH (i.g.) + COX-2 (celecoxib, i.g.) + ghrelin (G) (i.p.);

In the second series of studies, ghrelin was administered at the dose of 8 nmol/kg/dose because ghrelin at the dose 8 and 16 nmol/kg/dose exhibited similar and strong therapeutic effect in the first series of studies. As in the first series of studies, ghrelin was given once (1 hour after ethanol application in animals killed 12 hours after induction of ulcers) or twice (1 and 13 hours after ethanol application in animals killed 24 hours after ulcer induction). In group treated with combination of COX inhibitor plus ghrelin, SC-560 or celecoxib were given i.g. 30 min prior to each ghrelin administration. In the 2nd, 3rd, 5th and 6th group, SC-560 or celecoxib were given at the same time as in the 8th or 9th group. SC-560 and celecoxib were administered at the dose of 10 mg/kg/dose, because this dose has been shown to be effective in inhibition of COX-1 and COX-2, respectively (33, 34).

Determination of gastric blood flow and mucosal lesions

One, 12 or 24 hours after administration of ethanol, rats were anesthetized with ketamine (50 mg/kg i.p., Bioketan, Vetosquinol Biowet, Gorzow Wielkopolski, Poland) 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, Jarfalla, Sweden). Blood flow was measured in five areas of gastric mucosa, and mean value of five recordings was presented as percent of mucosal blood flow found in saline-treated control rats. After measurement of mucosal blood flow, the area of ulcerated mucosa was measured, using computerized planimeter (Morphomat, Carl Zeiss, Berlin, Germany) as described previously (35).

Biochemical analysis

Following measurement of gastric blood flow, biopsies samples from the gastric mucosa were taken for determination of mucosal DNA synthesis (an index of mucosal cell vitality and proliferation) and expression of mRNA for COX-1, COX-2, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). DNA synthesis was determined by measurement of [3H]thymidine incorporation ([6-3H]-thymidine, 20–30 Ci/mmol, Institute for Research, Production and Application of Radioisotopes, Prague, Czech Republic) into mucosal DNA as described previously (36). The incorporation of labeled thymidine into DNA was determined by counting 0.5 ml DNA-
containing supernatant in a liquid scintillation system. DNA synthesis was expressed as tritium disintegrations per minute per µg of DNA (dpm/µg DNA).

Expression of mRNA for COX-1, COX-2, IL-1β and TNF-α was determined using reverse transcriptase-polymerase chain reaction (RT-PCR) as described previously (37). Samples of gastric mucosa were snap frozen in liquid nitrogen and stored at −80°C until RNA extraction. The COX-1 primer sequences were as follows: upstream: 5'-AGC CCC TCA TTC ACC CAT CAT TT-3', downstream: 5'-CAG GGA CGC CTG TTC TAC GG-3'. The expected length of this PCR product was 561 bp. The COX-2 primer sequences were as follows: upstream: 5'-ACA ACA TTC CCT TCC TTC-3', downstream: 5'-CCT TAT TTC CTT TCA CAC C-3'. The expected length of this PCR product was 201 bp. The interleukin-1β primer sequences were: upstream: 5'-GCT ACC TAT GTC TTG CCC GT-3', downstream: 5'-GAC CAT TGC TGT TTC CTA GG-3'; the expected length of the product was 543 bp. The TNF-α primer sequences were: upstream: 5'-TAC TGA ACT TCG GGG TGA TTG GTC C-3', downstream: 5'-CAG CCT TGT CAT GGT CTT AGG AAC C-3'; the expected length of the product was 295 bp. The β-actin primer sequences were: upstream: 5'-TTG TAA CCA ACT GGG ACG ATA TGG-3', downstream: 5'-GAT CTT GAT CTT CAT GGT GCT AGG-3'; the expected length of the product was 764 bp. Polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide and then visualized under UV light. Localization of predicted products was confirmed by using of 100-base pair ladder (Gibco BRL/life Technologies, Eggenstein, Germany), as standard size marker. The intensity of bands was quantified in semi-quantitative manner, using densitometry (LKB, Ultrascan, Pharmacia, Sweden). The gel was photographed under UV transillumination. The intensity of PCR products was measured by means of 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 according to previously described method (38).

Statistical analysis

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

RESULTS

The first series of studies

Fig. 1 shows the influence of ghrelin administration on the healing of ethanol-induced gastric ulcers. In saline-treated rats, 1 hour after application of ethanol, the ulcer area was 103.0 ±4.0 mm². Eleven hours later, the ulcer area in those rats was significantly reduced to 90.8 ±3.2 mm² (Fig. 1). Treatment with ghrelin accelerated the healing of ethanol-induced gastric ulcers. Twelve hours after application of ethanol, the gastric ulcer area in rats treated with ghrelin given at the dose of 4, 8 or 16 nmol/kg/dose was reduced by 13, 36 or 38%, respectively, when compared to saline-treated rats at the same time after induction of ulcers. This effect was statistically significant for ghrelin given at the dose of 8 and 16 nmol/kg/dose. Moreover, the ulcer area in rats treated with ghrelin at the dose of 8 or 16 nmol/kg/dose was significantly smaller than that observed in rats treated with ghrelin at the dose of 4 nmol/kg/dose (Fig. 1).

Twenty-four hours after induction of ulcers, the ulcer area in saline-treated rats was 76.0 ±1.9 mm² and this area was significantly smaller then area observed 1 or 12 hours after induction of ulcers. This effect was statistically significant for ghrelin given at the dose of 8 and 16 nmol/kg/dose. Moreover, the ulcer area in rats treated with ghrelin at the dose of 8 or 16 nmol/kg/dose was significantly smaller than that observed in rats treated with ghrelin at the dose of 4 nmol/kg/dose (Fig. 2).

![Fig. 1. Effect of saline (NaCl) or ghrelin administered at the dose of 4, 8 or 16 nmol/kg/dose (G4, G8 or G16) on the area of gastric lesions 1 or 12 hours after induction of gastric ulcers by ethanol. Mean ± S.E.M. N=10 in each group of animals. P<0.05 compared to control (C); P<0.05 compared to ethanol + NaCl 1 hour after induction of ulcers; P<0.05 compared to ethanol + NaCl 12 hours after induction of ulcers; P<0.05 compared to ethanol + G4 12 hours after induction of ulcers.](image-url)
In saline-treated rats with ulcers, gastric mucosal blood flow was significantly decreased when compared to values observed in rats without ulcers. One, 12 and 24 hours after induction of ulcers, gastric mucosal blood flow was reduced by 51, 41 and 34%, respectively (Figs. 3 and 4). Treatment with ghrelin, after induction of gastric ulcers, improved mucosal blood flow. Ghrelin given at the dose of 8 or 16 nmol/kg/dose caused the similar and statistically significant increase in gastric mucosal blood flow and this effect was observed 12 and 24 hours after induction of ulcers (Figs. 3 and 4). Administration of ghrelin at the dose of 4 nmol/kg tended to improve gastric mucosal blood flow; however, this effect was not statistically significant.

In control saline-treated rats, gastric mucosal DNA synthesis reached a value of 58.3 ±1.7 dpm/μg DNA (Fig. 5). One and 12 hours after induction of ulcers, gastric mucosal DNA synthesis was significantly reduced by 61 and 23%, respectively. Difference between those both groups was statistically significant (Fig. 5). Administration of ghrelin improved DNA synthesis in gastric mucosa. Twelve hours after induction of ulcers, treatment with ghrelin at the dose of 4 nmol/kg/dose reversed the ulcer-evoked reduction in DNA synthesis.

**Fig. 2.** Effect of saline (NaCl) or ghrelin administered at the dose of 4, 8 or 16 nmol/kg/dose (G4, G8 or G16) on the area of gastric lesions 1, 12 or 24 hours after induction of gastric ulcers by ethanol. Mean ± S.E.M. N=10 in each group of animals. aP<0.05 compared to control (C); bP<0.05 compared to ethanol + NaCl 1 hours after induction of ulcers; cP<0.05 compared to ethanol + NaCl 12 hours after induction of ulcers; dP<0.05 compared to ethanol + NaCl 24 hours after induction of ulcers.

**Fig. 3.** Effect of saline (NaCl) or ghrelin administered at the dose of 4, 8 or 16 nmol/kg/dose (G4, G8 or G16) on gastric mucosal blood flow 1 or 12 hours after induction of gastric ulcers by ethanol. Mean ± S.E.M. N=10 in each group of animals. aP<0.05 compared to control (C); bP<0.05 compared to ethanol + NaCl 12 hours after induction of ulcers.
synthesis in gastric mucosa; whereas ghrelin administered at the dose of 8 or 16 nmol/kg/dose, significantly increased gastric mucosal DNA synthesis above the value observed in control rats (Fig. 5).

Twenty four hours after induction of ulcers in rats treated with saline, the spontaneous reversion of the ethanol-induced decrease in DNA synthesis in gastric mucosa was observed and DNA synthesis was significantly increased by 21% above a value observed in control rats without induction of ulcers (Fig. 6). Treatment with ghrelin additionally increased gastric mucosal DNA synthesis and 24 hours after induction of ulcers, this effect was statistically significant after each ghrelin dose used (Fig. 6).

Induction of gastric ulcers by ethanol and treatment with ghrelin had no effect in expression of mRNA for COX-1 in gastric mucosa (Fig. 7F). This lack of effect was found 12 and 24 hours after induction of ulcers. In contrast, expression of mRNA for COX-2 was significantly increased following the induction of ulcers and remained unchanged despite the treatment with ghrelin (Fig. 7G).

Induction of gastric ulcer by ethanol significantly increased mucosal expression of mRNA for IL-1β and TNF-α (Fig. 7H).
Fig. 6. Effect of saline (NaCl) or ghrelin administered at the dose of 4, 8 or 16 nmol/kg/dose (G4, G8 or G16) on gastric mucosal DNA synthesis 1 or 24 hours after induction of gastric ulcers by ethanol. Mean ± S.E.M. N=10 in each group of animals. aP<0.05 compared to control (C); bP<0.05 compared to ethanol + NaCl 1 hour after induction of ulcers cP<0.05 compared to ethanol + NaCl 24 hours after induction of ulcers.

Fig. 7. Expression of mRNA for β-actin (A), COX-1 (B), COX-2 (C), IL-1β (D) and TNF-α (E), and densitometric analysis of COX-1/β-actin ratio (F), COX-2/β-actin ratio (G), IL-1β/β-actin ratio (H) and TNF-α/β-actin ratio in gastric mucosa of control rats (C, line 1), rats treated with saline and killed 12 hours after induction of ulcers (U 12 h, line 2), rats treated with ghrelin at the dose 8 nmol/kg/dose and killed 12 hours after induction of ulcers (U 12 h + G8, line 3), rats treated with saline and killed 24 hours after induction of ulcers (U 24 h, line 4) rats treated with ghrelin at the dose 8 nmol/kg/dose and killed 24 hours after induction of ulcers (U 24 h + G8, line 5). Mean ± S.E.M. N=7 in each group of animals. aP<0.05 compared to saline-treated rats without induction of ulcers (control); bP<0.05 compared to saline-treated rats with ulcers at the same time of observation.
Administration of ghrelin given at the dose of 8 nmol/kg/dose significantly reduced the ulcer-evoked increase in mucosal expression of mRNA for IL-1β and TNF-α and these effects were observed 12 and 24 hour after ulcer induction (Fig. 7H and 7I).

The second series of studies

The selective COX-1 inhibitor (SC-560) or COX-2 inhibitor (celecoxib) given alone, without administration of ethanol, caused small but significant ulceration of gastric mucosa and these ulcers have been found in both periods of observation (Fig. 8 and 9). In rats with ethanol-induced ulcers, administration of SC-560 additionally and significantly increased the area of mucosal damage and inhibited spontaneous ulcer healing (Fig. 8 and 9). Also administration of celecoxib increased the area of gastric mucosa damage in rats with ethanol-induced gastric ulcers, but this effect was less pronounced than that evoked by SC-560 and statistically significant only at the time of 12 hours after induction of ulcers (Fig. 8 and 9). In the first series of studies, treatment with ghrelin significantly accelerated the healing of ethanol-induced gastric ulcers. Addition of SC-560 slightly reduced the therapeutic effect of ghrelin in the healing of ethanol-induced ulcer, but this effect was statistically significant only at the 24th h after ethanol application (Fig. 9). On the other hand, the ulcer area in rats treated SC-560 plus ghrelin after application of ethanol was significantly smaller than that observed in rats treated with saline or SC-560 alone after induction of ulcers. This effect was found 12 and 24 hours after induction of ulcers (Fig. 8 and 9).

Administration of celecoxib in rats with ethanol-induced ulcers and treated with ghrelin, abolished therapeutic effect of ghrelin and increased the area of gastric damage to the value observed in rats treated with saline alone after induction of ulcers, but this effect was less pronounced than that evoked by SC-560 and statistically significant only at the time of 12 hours after induction of ulcers (Fig. 8 and 9). As in the first series of studies, treatment with ghrelin significantly accelerated the healing of ethanol-induced gastric ulcers. Addition of SC-560 slightly reduced the therapeutic effect of ghrelin in the healing of ethanol-induced ulcer, but this effect was statistically significant only at the 24th h after ethanol application (Fig. 9). On the other hand, the ulcer area in rats treated SC-560 plus ghrelin after application of ethanol was significantly smaller than that observed in rats treated with saline or SC-560 alone after induction of ulcers. This effect was found 12 and 24 hours after induction of ulcers (Fig. 8 and 9).
ulcers (Fig. 8 and 9). However, 12 hours after induction of ulcers, the ulcer area in rats treated with colecexib plus ghrelin after application of ethanol was significantly smaller than that observed in rats treated with colecexib alone after induction of ulcers (Fig. 8). Twelve hours later, this effect was statistically insignificant (Fig. 9).

In rats without induction of ulcers, administration of SC-560 significantly reduced gastric mucosal blood flow and this effect was observed 11.5 h after single dose of SC-560 (Fig. 10). Eleven and half h after single dose of colecexib gastric mucosal blood flow was significantly reduced (Fig. 10). Twelve hours later this effect was statistically insignificant, but tendency to reduction of gastric mucosal blood flow by colecexib was still observed (Fig. 11). As in the first series of studies, induction of ulcers by ethanol significantly reduced gastric mucosal blood flow and this effect was observed 12 and 24 hours after ethanol application (Fig. 10 and 11). Neither SC-560 nor colecexib administered alone significantly affected gastric mucosal blood flow in rats with ethanol-induced gastric ulcers (Fig. 10 and 11). Treatment with ghrelin significantly increased gastric mucosal blood flow in rats with ethanol-induced gastric ulcers (Fig. 10 and 11). Twelve hours after induction of gastric ulcers by ethanol, administration of SC-560 partly reversed the ghrelin-evoked improvement of mucosal blood flow; whereas colecexib completely abolished the ghrelin-evoked beneficial effect on mucosal blood flow (Fig. 10). After next 12 hours these effects were statistically insignificant (Fig. 11).

Neither SC-560 nor colecexib given alone affected DNA synthesis in gastric mucosa (Fig. 12 and 13). Twelve hours after induction of ulcers, in rats treated with saline, mucosal DNA synthesis was reduced to 42.1 ±1.6 dpm/µg DNA v. 56.4 ±2.2
dpm/µg DNA in control group without induction of ulcers (Fig. 12). In this time of observation, administration of SC-560 additionally and significantly reduced mucosal DNA synthesis in rats with ethanol-induced ulcers; whereas colecobix was without effect on mucosal synthesis of DNA in rats with ulcers (Fig. 12). Opposite effects of COX inhibitors administration were observed in rats treated with ghrelin after induction of ulcers. Ghrelin given alone significantly increased DNA synthesis in gastric mucosa in rats with ulcers above a value observed in saline-treated rats with ulcers, as well as above a value found in control rats without induction of ulcers (Fig. 12). Addition of SC-560 tended to reduce DNA synthesis in gastric mucosa in rats with ulcers and treated with ghrelin, but this effect was insignificant 12 hours after induction of ulcers (Fig. 12). On the other hand, addition of colecobix completely reversed the ghrelin-evoked increase in mucosal DNA synthesis in rats with ulcers (Fig. 12).

Twenty four hours after induction of ulcers in rats treated with saline alone, DNA synthesis in gastric mucosa was significantly elevated above a value observed in control rats without induction of ulcers (Fig. 13). Administration of SC-560 or colecobix totally abolished the ulcer-induced increase in mucosal DNA synthesis (Fig. 13). Twenty four hours after induction of ulcers, treatment with ghrelin still enhanced DNA synthesis in gastric mucosa above a observed in control rats without induction of ulcer, as well as above a value found in saline-treated rats with induction of ulcers (Fig. 13). This effect was partly and insignificantly reduced by SC-560. Mucosal DNA synthesis in rats with ulcers treated with SC-560 plus ghrelin was significantly higher than that observed in rats with ulcers and treated with SC-560 alone. Treatment with colecobix completely abolished the ghrelin induced increase in gastric mucosal DNA synthesis (Fig. 13).
flow can result from increased perfusion through existing vitality followed by stimulation of epithelial cell proliferation. 

Aim of our present investigation was to examine the influence of ghrelin administration on the healing of ethanol-induced gastric ulcers and to determine the role of cyclooxygenase-1 and cyclooxygenase-2 in this effect. In saline-treated rats, induction of ulcers by ethanol was accompanied by the reduction in gastric mucosal blood flow and DNA synthesis. This effect has been observed 1 and 12 hours after induction of ulcers. The ethanol-induced gastric ulcers have undergone a spontaneous healing. Twenty four hours after induction of ulcers, the ulcer area in rats treated with saline was significantly reduced in comparison with the ulcer area observed 23 or 12 hours earlier. For this reason, 24 hours after induction of ulcers, gastric mucosal blood flow was only partly reduced, whereas mucosal DNA synthesis was greater than in control rats without induction of ulcers.

Mucosal blood flow plays an important role in the protection and healing of mucosa in the gut (39-41). Experimental studies have shown that exposure of gastric mucosa to potentially noxious factors results in little or no damage, as long as adequate blood flow is maintained, whereas reduction in mucosal blood flow leads to severe gastric injury (39). Blood flow contributes to protection by supplying the mucosa with oxygen, bicarbonate and nutritious substances, and also by removing carbon dioxide, hydrogen ions and other toxic agents diffusing from the gastric lumen (39). Gastric hypoxia leads to mucosal acidification and subsequently to the development of gastric ulcers (42).

The most important finding of our present study is the observation that treatment with ghrelin accelerated the healing of ethanol-induced gastric ulcers. This finding is in agreement with our previous observation that ghrelin demonstrates the healing effect in gastric ulcers evoked by acetic acid (19). Moreover previous studies, performed by other authors, have shown that ghrelin given peripherally (15-18) or centrally (48) protects the gastric mucosa against various noxious agents (43, 45-47).

Our present data indicate that reduction in blood flow and cell proliferation in gastric mucosa is involved in induction of gastric ulcers by ethanol. On the other hand, an increase in DNA synthesis in gastric mucosa 24 hours after induction of ulcer, plays an essential role in restitution of gastric mucosa.

The most important finding of our present study is the observation that treatment with ghrelin accelerated the healing of ethanol-induced gastric ulcers. This finding is in agreement with our previous observation that ghrelin demonstrates the healing effect in gastric ulcers evoked by acetic acid (19). Moreover previous studies, performed by other authors, have shown that ghrelin given peripherally (15-18) or centrally (48) protects the gastric mucosa against various noxious agents. These observations taken together suggest that therapeutic effect of ghrelin in gastric ulcers have the universal nature and is not dependent on a primary cause of gastric damage.

Spontaneous healing of gastric mucosa damage involves rapid mucosal restitution and reepithelialization by migration of surrounding epithelial cells from the ulcer margin to cover the denuded area (49), which is subsequently enhanced by an increase in mucosal cell proliferation. In our present study, 12 hours after induction of ulcers, treatment with ghrelin has reversed the ethanol-induced decrease in mucosal DNA synthesis in the stomach. Twelve hours later, gastric mucosal DNA synthesis in rats treated with ghrelin was above a value observed in control rats without induction of ulcers, as well as above a value observed in rats with ulcers treated with saline. This observation indicates that therapeutic effect of ghrelin administration in the healing of gastric ulcers in biphasic and involves improvement mucosal cell vitality followed by and stimulation of epithelial cell proliferation.

In rats treated with ghrelin, improvement of mucosal blood flow can result from increased perfusion through existing vessels, and/or may be due the stimulation of angiogenesis. Study performed by Ahluwalia et al. (50) has shown that ghrelin stimulates sprouting the new capillary blood vessels and deficiency of ghrelin leads to impairment of angiogenesis.

Our present study has shown that intragastric application of ethanol induces gastric mucosal damage and increases mucosal expression of mRNA for IL-1β and TNF-α. Both these cytokines are well known mediators of acute inflammation. IL-1β plays a crucial role in the induction of systemic acute phase response and in the release of other members of the pro-inflammatory cytokine cascade such as TNF-α, platelet activating factor (PAF), prostaglandins and pro-inflammatory interleukins (51). TNF-α and IL-1β are produced mainly by macrophages, but several other cell types also are involved in their production (52). These data taken together with our observation that treatment with ghrelin reverses the ulcer-induced increase in mucosal expression of mRNA for IL-1β and TNF-α, indicate that inhibition of local inflammatory process is one of the mechanisms involved in the therapeutic effect of ghrelin in ethanol-induced gastric ulcers. This finding is consistent with the observations that ghrelin attenuates septic shock (53), inhibits the expression of pro-inflammatory cytokines by human monocytes and T lymphocytes (54) and reduces phagocytic activity of macrophages (55). Moreover, previous studies have shown that administration of ghrelin inhibits sodium metabisulphite-induced apoptosis in rat gastric mucosa (56), hypoxia/reoxygenation-induced apoptosis in human gastric epithelial cell line (57) and TNF-α-induced apoptosis and autophagy in human visceral adipocytes (58).

In the second series of the present study, we have tested the influence of COX-1 and COX-2 inhibitors on the healing of gastric ulcers and therapeutic effects of ghrelin. COX inhibitors induce prostaglandin-dependent and prostaglandin-independent injury of the gastric mucosa (26). Prostaglandins play a key role in gastric mucosa protection by enhancing defense mechanisms at the pre-epithelial, epithelial and post-epithelial levels. Prostaglandins stimulate bicarbonate and mucous secretion by gastric mucosa, inhibit acid secretion by oxyntic cells and are responsible for maintaining epithelial cell restitution and mucosal blood flow (26). The prostaglandin-independent mechanism of the NSAID-induced stomach injury is most likely related to the disruption of mitochondrial function and liberation of reactive oxygen species (ROS) (26).

Our present study has shown that administration of selective COX-1 or COX-2 inhibitors induce gastric ulcers in rats unexposed to ethanol. These findings are in contrast to the observation by Wallace et al. (59) that both cyclooxygenases, COX-1 and COX-2, must be inhibited to induce gastric ulcers. The inconsistency between our findings and those reported by Wallace et al. seems to be as result of the differences in the route of administration of COX inhibitors. We have administered SC-560 or celecoxib intragastrically through orogastric intubation in immobilized rats. Moreover, 1 hour later, rats were immobilized again and treated intragastrically with saline. This procedure is likely to be highly stressful for the animals. It is well known that stress can trigger gastric ulcers in clinical setting (60), as well as in laboratory animals (37, 40). Stress can also synergize with others pathogenic factors such as Helicobacter pylori or NSAIDs (61). This explanation of differences between our results and findings of Wallace et al. (59) can be additionally supported by an observation that administration of COX-1 or COX-2 inhibitors given alone significantly increases the number of ulcers induced by exposing rats to water immersion and restraint associated stress (37).

Early studies have shown the important role of COX-1 and COX-2 in healing of gastric ulcers (31, 32). Expression of COX-2 is markedly upregulated around the margins of gastric ulcers and inhibition of COX-2 activity leads to a delay of ulcer healing.
(31, 32, 62-64). Our preset study has shown that pretreatment with celecoxib abolishes therapeutic effect of ghrelin in ethanol-induced gastric ulcers. The influence of celecoxib was associated with full reversion of the ghrelin-evoked improvement of gastric mucosal blood flow and DNA synthesis. This observation indicates that mechanism of ghrelin’s therapeutic effect in gastric ethanol-induced ulcers involves activity of COX-2.

Pretreatment with SC-560 tended to reduce the healing promoting effect of ghrelin in ethanol-induced ulcers, but this influence was weak and mainly statistically insignificant. On the other hand, the ulcer area in rats treated SC-560 plus ghrelin was significantly smaller than that observed in rats treated with saline or SC-560 alone. This finding indicates that ghrelin may reverse noxious effects of COX-1 inhibitors on gastric mucosa.

Finally, we conclude that ghrelin exhibits therapeutic effect in the healing of experimental gastric ulcers suggesting its application in patients. This effect seems to be related to the improvement of mucosal blood flow and mucosal cell viability, as well as to an increase in mucosal cell proliferation, and a reduction in local inflammation. Activity of COX-2 is necessary for the therapeutic effect of ghrelin in the stomach whereas treatment with ghrelin can reverse a deleterious effect of COX-1 inhibitor on healing of ethanol-induced gastric ulcers. This last observation suggests that ghrelin could be useful in the treatment of gastric mucosal damage induced by COX-1 inhibitors.

Conflict of interests: None declared.

REFERENCES