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Role of leptin in ulcer healing

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Abstract

Leptin was shown to exhibit similar to cholecystokinin (CCK) cytoprotective activity against acute gastric lesions, but its role in ulcer healing has not been examined. The aims of this study were: (1) to compare the effects of exogenous leptin to those of CCK on the course of healing of chronic gastric ulcers; (2) to study the gene and protein expression of leptin at the ulcer margin during ulcer healing; and (3) to assess the effects of leptin administration on the mucosal gene expression of main growth factor such as transforming growth factor alpha (TGFα). Gastric ulcers were produced in rats by the acetic acid method. Rats with ulcers were divided in following treatment groups: (1) vehicle; (2) leptin (10 μg/kg i.p.); (3) CCK (10 μg/kg s.c.); and (4) leptin or CCK with or without tyrphostin A46 (200 μg/kg i.p.), an inhibitor of epidermal growth factor (EGF)-receptor tyrosine kinase or N^G-nitro-L-arginine (20 mg/kg i.g.), a blocker of nitric oxide synthase. Animals were euthanized 9 days after ulcer induction. The area of gastric ulcers and the gastric blood flow at the ulcer area were determined. In addition, mucosal biopsy samples were taken from the ulcer area for histological evaluation as well as for the determination of mRNA and protein expression for leptin and constitutive nitric oxide synthase (cNOS) and inducibile nitric oxide synthase (iNOS) by reverse-transcriptase polymerase chain reaction (RT-PCR) and Western blot, respectively. In addition, the gene expression for TGFα was analyzed by RT-PCR. Both leptin and CCK reduced significantly the ulcer area as compared to vehicle-treated group by ~50%. The treatment with tyrphostin or N^G-nitro-L-arginine reversed in part the acceleration of ulcer healing by leptin and CCK. The expression of leptin mRNA and protein was significantly increased at the ulcer edge. The leptin-induced acceleration of ulcer healing was associated with increased expression of transcripts for TGFα as well as increased mRNA and protein expression for cNOS and iNOS at the ulcer margin. We conclude that leptin accelerates ulcer healing by mechanisms involving the up-regulation of TGFα and increased production of nitric oxide due to up-regulation of cNOS and iNOS in the ulcer area.

Keywords: Leptin; Ulcer healing; Western blot; RT-PCR (reverse transcriptase polymerase chain reaction); Gastric blood flow; Nitric oxide (NO)

1. Introduction

Leptin, the 16-kDa product of *ob* gene, is a cytokine-like molecule which plays a key role in the homeostasis of body weight and energy expenditure. Leptin is produced and secreted mainly by adipose tissue (Zhang et al., 1998). Increasing number of evidences suggest that leptin, apart from the regulation of food intake and energy homeostasis, participates in many physiological functions including regulation of neuroendocrine and immune systems, reproduction, angiogenesis and lipolysis (Rosenbaum and Leibel, 1999; Lord et al., 1998; Sierra-Honigmann et al., 1998). Leptin has been also proposed to participate in acute phase response to inflammation (Sarraf et al., 1997; Grunfeld et al., 1996). The role of leptin in the inflammatory response is further strengthened by the findings that in interleukin-1β deficient mice the increase in leptin production is absent after an inflammatory stimulus (Faggioni et al., 1998).

Recently, the presence of leptin as well as the expression of its receptors was detected in rat and human stomach (Bado et al., 1998; Breidert et al., 1999; Mix et al., 2000; Sobhani et al., 2000) but little is known about the role of leptin produced in the gastric mucosa. Our previous studies demonstrated that exogenous as well as endogenous leptin released by cholecystokinin (CCK), gastrin and meal exerted a potent gastroprotective action depending upon vagal activity, intact sensory nerves and increased

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gastric microcirculation probably mediated by nitric oxide (Brzozowski et al., 1999a). We also demonstrated that mRNA and protein expression of leptin in gastric mucosa increased after acute damage induced by ethanol or aspirin (Konturek et al., 1999).

Previous studies showed that the leptin stores in the gastric mucosa are mobilized by CCK in rats (Bado et al., 1998) and by pentagastrin in humans (Sobhani et al., 2000). CCK is also involved in the control of gastric emptying, gastric secretion, gastrointestinal motility and food intake (Brand and Schmidt, 1995; Walsh, 1994). Recently, the gastroprotective effects of CCK and its beneficial effect on ulcer healing have been recognized (Brzozowski et al., 1999b; Evangelista and Maggi, 1991) and attributed, at least in part, to local release of leptin (Brzozowski et al., 1999a). In view of these data we assessed whether leptin, similar to CCK, affects the process of healing of chronic gastric ulcerations.

The healing process of peptic ulcer is very complex and involves epithelial cell migration and proliferation, reconstruction of gastric glands, angiogenesis and production of granulation tissue (Tarnawski, 2000). Various endogenous factors are known to contribute to ulcer healing including prostaglandins (Arakawa et al., 1998), nitric oxide (Brzozowski et al., 1997) as well as several growth factors including basic fibroblast growth factor (bFGF) (Pohle et al., 1999), transforming growth factor alpha (TGFα) and epidermal growth factor (EGF) (Konturek et al., 1995). The latter growth factors act via the binding to the common EGF/TGF receptor followed by receptor dimerization and autophosphorylation. This triggers signal transduction pathway, in which the activation of an enzyme called the mitogen–activated protein kinase (MAP kinase) plays a central role (Pai et al., 1998).

The present study was designed: (1) to determine the messenger RNA and protein expression of leptin at the margin of chronic gastric ulcer; (2) to compare the effects of exogenous CCK and leptin on ulcer healing and gastric blood flow at the ulcer margin; (3) to assess the effects of leptin administration on gene expression for TGFα in gastric mucosa; and (4) to study the effect of blockade of EGF-receptor tyrosine kinase by tyrphostin A46 and nitric oxide synthase (NOS) inhibition by N^G-nitro-L-arginine (L-NNA) on the course of ulcer healing.

2. Materials and methods

Male Wistar rats, weighing 200–250 g and fasted for 24 h, were used in all studies.

2.1. Production of gastric ulcers

Gastric ulcers were produced in 72 rats using our modification (Brzozowski et al., 1995) of the acetic method originally proposed by Okabe et al. (1971). Animals were anesthetized with ether, the stomach was exposed and a round plastic mold (6 mm in diameter) was placed tightly on the anterior serosal surface of the stomach at the antro-oxyntic border. Seventy-five microliters of 100% acetic acid was poured into the mold and allowed to remain on the gastric wall for 25 s. This produced immediate necrosis of the entire mucosa and submucosa (but not serosa) within the area where the acetic acid was applied, i.e. 28 mm^2. The excess of acetic acid was then removed and the serosa was gently washed out with saline. Our previous studies (Konturek et al., 1988; Brzozowski et al., 1999a) on numerous rats documented that these ulcers achieved the area of 28 mm^2 at day 0 (initial value) and then became chronic within 2–3 days and healed completely within 2–3 weeks. After the application of acetic ulcers the animals were allowed to recover from anesthesia and received only water at the day of operation.

2.2. Effect of exogenous leptin and CCK with or without blocker of MAP kinase or nitric oxide synthase on the ulcer healing and gastric blood flow in the ulcer area

Three major series (A, B and C) of experiments were carried out in rats with chronic gastric ulcers. In each series of experiments 24 rats (eight rats per group) were included. Series A was used to determine the effects of subcutaneous (s.c.) injections of recombinant human leptin (Chemicon, Hofheim, Germany; 10 μg/kg), CCK-8 (10 μg/kg) or vehicle (saline) given twice daily for 9 days on the rate of ulcer healing and the gastric blood flow at the ulcer edge. Series B received subcutaneous doses of leptin or CCK-8 (each at the same dose of 10 μg/kg s.c.) twice daily for 9 days in combination with vehicle (saline) or the MAP kinase inhibitor (tyrphostin A46) applied at a dose 200 mg/kg i.p. Finally, in the rats of series C, we studied the implication of NO in the ulcer healing effects of leptin and CCK. Rats received (throughout 9 days upon ulcer induction) twice daily either leptin or CCK-8 (in a dose of 10 μg/kg s.c.) alone or in combination with vehicle (saline) or L-NNA applied i.g. at a dose of 20 mg/kg. The doses of leptin and CCK or N^G-nitro-L-arginine used in this study were similar to those producing gastroprotective (Brzozowski et al., 1999a; Arakawa et al., 1998) or ulcer healing (Brzozowski et al., 1999b) effects as described before.

To evaluate the effects of leptin, CCK or vehicle on gastric blood flow, the animals were anesthetized with ether, the abdomen was opened and the stomach exposed to assess the blood flow at the ulcer margin and the intact contralateral mucosa using H_2 gas clearance technique as described before (Konturek et al., 1992; Brzozowski et al., 1999a). Briefly, the double needle electrodes were inserted through the serosa into the mucosa just around the ulcer (ulcer margin) or into the intact mucosa. With this method one electrode was used for local generation of H_2 gas and the other for the measurement of tissue H_2 using polaro-

graphic current detector. The tissue H_2 clearance curve was used to calculate absolute flow rate that was expressed as percent of control flow in the intact mucosa. Using this technique the gastric blood flow was measured immediately after ulcer induction and then 1 day (day 0) and 9 days thereafter. The stomach was then removed and pinned open for the quantitative determination of the area of gastric ulcers by planimetry (Morphomat, Carl Zeiss, Berlin, Germany) by two investigators under blinded conditions as described (Brzozowski et al., 1999a).

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) for detection of messenger RNA (mRNA) for leptin TGF_2 and nitric oxide synthases

The extraction of total RNA from gastric ulcerated tissues and control tissues was carried out as previously described (Konturek et al., 1997a). Briefly, total RNA was extracted from mucosal samples using a guanidium isothiocyanate/phenol chloroform single step extraction kit from Stratagene (Heidelberg, Germany) based on the method described by Chomczynski and Sacchi (1987). Following precipitation, RNA was resuspended in RNAse-free Tris–EDTA (TE) buffer and its concentration was estimated by absorbance at 260-nm wavelength. Furthermore, the quality of each RNA sample was determined by running an agarose-formaldehyde electrophoresis. RNA samples were stored at −80°C until analysis.

Single stranded cDNA was generated from 5 μg of total cellular RNA using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and oligo-(dT)-primers. Briefly, 5 μg of total RNA was uncoiled by heating (65°C for 5 min) and then reverse transcribed (at 37°C for 1 h) into complementary DNA (cDNA) in a 50 μl reaction mixture that contained 50 U MMLV-RT, 0.3 μg oligo-(dT)-primer, 40 U RNase Block Ribonuclease Inhibitor, 2 μl of a 100-mM mixture of deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP), 5 μl of 10× first-strand buffer (all reagents provided by Stratagene, Heidelberg, Germany). The resultant cDNA (2 μl) was amplified in a 50-μl reaction volume containing 2 U Taq polymerase, dNTP (200 μM each) (Pharmacia, Germany), 1.5 mM $MgCl_2$, 5 μl 10× polymerase chain reaction buffer (100 mM KCl, 20 mM Tris–HCl, pH = 8.3) and specific primers used at final concentration of 1 mM (all reagents were obtained from Takara, Shiga, Japan). 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) 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 number of cycles was 30 for β-actin, 35 for leptin, 33 for TGF_2, 35 for inducible nitric oxide synthase (iNOS), and 33 for constitutive nitric oxide synthase (cNOS). The nucleotide sequence of the primers were as follows: β-actin, sense 5′-GAT CTT GAT CTT CAT GGT GCT AGG-3′; antisense 5′-TTG TAA CCA ACT GGG ACG ATA TGG-3′, leptin (*ob* gene), sense 5′-CGA GAA GAT GAC CCA GATCAT G-3′; antisense 5′-AGT GAT CTC CTT CTG CAT CCT G-3′; cNOS sense 5′-TAC TTG AGG ATG TGG CTG-3′; antisense 5′-GTC TTC TTC CTG GTG ATG-3′; inducibile nitric oxide synthase (iNOS) sense 5′-CAG TGG CAA CAT CAG GTC-3′; antisense 5′-GGT CTC GGA CTC CAA TCT-3′ and TGFα sense 5′-ATG GTC CCC GCG GCC GGA CA 3′; TGFα antisense 5′-ATG GTC CCC GCG GCC GGA CA-3′. The primer sequences for β-actin, leptin (Bado et al., 1998), cNOS, iNOS (Griffiths et al., 1995) and TGFα (Fan et al., 1995) were based on the sequences of the published cDNAs and 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 a predicted product was confirmed by using 100-bp ladder (Takara) as a standard size marker. The gel was then photographed under UV transillumination. The intensity of PCR products was measured using video image analysis system (Kodak Digital Science). The signal for leptin TGF_2, iNOS and cNOS mRNA was standardized against that of the β-actin mRNA from each sample and the results were expressed as leptin or cNOS/β-actin mRNA ratio.

2.4. Western blot analysis for leptin, cNOS and iNOS

Shock frozen tissue from rat stomach was homogenized in lysis buffer (100 mmol Tris–HCl, pH 7.4, 15% glycerol, 2 mmol disodium ethylenediaminetetraacetate (EDTA), 2% sodium dodecyl sulfate (SDS), 100 mmol dithiothreitol (DTT) by the addition of 1:20 dilution of aprotinin and 1:50 dilution of 100 mmol phenylmethylsulfonyl fluoride (PMSF). Insoluble material was removed by centrifugation at $12{,}000 \times g$ for 15 min. Approximately 100 μg of cellular protein extract were loaded into a well, separated electrophoretically through a 10% SDS-polyacrylamide gel and transferred onto Sequi-Blot™ PVDF membrane (Bio-Rad, USA) by electroblotting. 0.4% I-Block (Tropix, Bedford, USA) in Tris-buffered saline (TBS)/Tween-20 buffer (137 mmol NaCl, 20 mmol Tris–HCl, pH 7.4, 0.1% Tween-20) was used to block filters for at least 1 h at room temperature. Specific primary antibody against leptin (rabbit polyclonal, A-20; dilution 1:500; Santa Cruz, USA) or iNOS (mouse monoclonal, N32020; dilution 1:200; Transduction Laboratories USA) or cNOS (mouse monoclonal, N30020; dilution 1:1000; Transduction Laboratories USA) or β-actin (mouse monoclonal, dilution 1:1000; Sigma Aldrich, Germany) was added to the membrane, followed by an anti-rabbit G horseradish peroxidase-conjugated secondary antibody (1:2000, Santa Cruz). Incuba-

tion of primary antibody was followed by three washes with TBS/Tween-20 buffer for 10 min. Incubation of the secondary antibody was followed by four washes for 10 min. Non-isotopic visualization of immunocomplexes was achieved by chemiluminescence using chemiluminescence blotting substrate (Western-Star-System from Tropix). Thereafter, the developed membrane was exposed to an X-ray film (Kodak, Wiesbaden, Germany). Comparison between different treatment groups was made by determining the leptin- or NOS/β-actin ratio of the immunoreactive area by densitometry.

2.5. Immunohistochemical staining

Some intact stomachs and those with chronic gastric ulcers were fixed in 10% formalin and embedded in paraffin. Sections (5 μm) obtained from paraffin blocks were dewaxed and rehydrated. Endogenous peroxidase was blocked by immersing sections in 0.3% H_2O_2–methanol for 15 min. Sections were then incubated for 60 min with a polyclonal rabbit primary antibody raised against leptin (A20, Santa Cruz), washed and then incubated with secondary biotynylated goat anti-rabbit antibody. After 60-min incubation, sections were rinsed in phosphate-buffered saline (PBS) and incubated for 30 min with an avidin–biotin complex made up according to the manufacturer instructions (Unitect Rabbit Immunohistochemistry System; Dianova, Germany). Sections were rinsed again in PBS before substrate development in a 0.05% solution of diamonobenzidine (Sigma) in PBS containing 0.01% hydrogen peroxide.

2.6. Statistical analysis

All values are expressed as mean ± S.E.M. Statistical analysis was determined by two way ANOVA followed by non-parametric Mann–Whitney test. Significance was set at $P < 0.05$.

3. Results

3.1. Effect of exogenous leptin and CCK on the rate of ulcer healing and gastric blood flow in the ulcer area

As shown in Fig. 1, rats treated with vehicle (saline) throughout the 9-day period upon ulcer induction, showed a significant [$F(1,14) = 65.8$, $P < 0.001$] reduction in the area of the ulcers from their initial size of 28 mm^2 to about 10.57 ± 2.7 mm^2. The treatment with CCK-8 or leptin (each at 10 μg/kg s.c.) was accompanied by a significant reduction in ulcer area to 4.44 ± 0.73 mm^2 in CCK-8 group [$F(1,42) = 19.11$, $P < 0.001$] and 5.98 ± 1.18 mm^2 in leptin group [$F(1,42) = 12.79$, $P < 0.001$], compared to vehicle control. The gastric blood flow in the intact antro-oxyntic mucosa of rats treated with vehicle averaged 48 ± 7 ml/min per 100 g and this was taken as 100%. Immediately after ulcer induction the blood flow at the margin of acute lesion was significantly [$F(1,14) = 64.42$, $P < 0.001$] reduced by about 68% compared with the intact mucosa. At day 9 after ulcer induction, the GBF at the margin of the chronic ulcer was still significantly reduced by about 20% when compared to the value recorded in the intact mucosa (Fig. 1). In rats treated with CCK-8 or leptin and

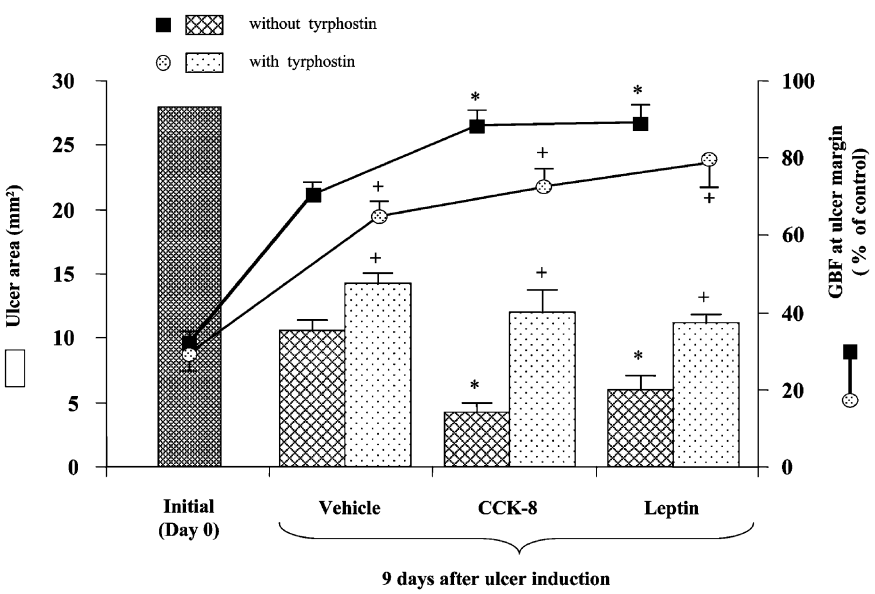

Fig. 1. Mean area of gastric ulcers and the gastric blood flow (GBF) at ulcer margin in rats treated throughout 9 days with vehicle, CCK (10 μg/kg s.c.) or leptin (10 μg/kg s.c.) without or with addition of tyrphostin (200 μg/kg i.p.). Mean ± S.E.M of 6–8 rats. Asterisk indicates a significant change as compared to the value obtained in vehicle-treated controls. Cross indicates a significant change as compared to the value obtained in rats treated with vehicle, CCK or leptin without tyrphostin.

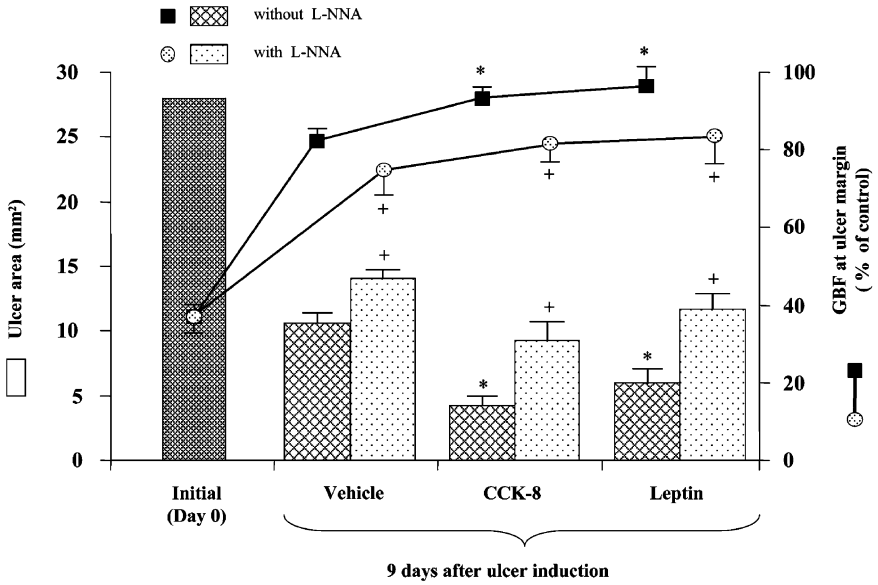

Fig. 2. Mean area of gastric ulcers and the gastric blood flow (GBF) at ulcer edge in rats treated throughout 9 days with vehicle, CCK (10 μg/kg s.c.) or leptin (10 μg/kg s.c.) without or with L-NNA (20 mg/kg i.p.). Mean ± S.E.M of 6–8 rats. Asterisk indicates a significant change as compared to the value obtained in vehicle-treated animals. Cross indicates a significant change as compared to the value obtained in rats treated with vehicle, CCK or leptin without L-NNA.

showing significant reduction in the ulcer area, a significant increase in the gastric blood flow at the ulcer margin was also recorded in tests with CCK [$F(1,42) = 184.1$, $P < 0.001$] or leptin [$F(1,42) = 112.4$, $P < 0.001$] when compared to that recorded in vehicle-treated animals.

3.2. Effect of tyrphostin and L-NNA on CCK- or leptin-induced acceleration of ulcer healing

The effects of the treatment with vehicle, CCK or leptin without or with combination with tyrphostin on the ulcer size and gastric blood flow at the ulcer margin are described in Fig. 1. Tyrphostin given at a standard dose (Pai et al., 1998) of 200 μg/kg i.p. significantly attenuated the acceleration of ulcer healing induced by CCK [$F(1,42) = 57.0$, $P < 0.001$] or leptin [$F(1,42) = 24.02$, $P < 0.001$], and reduced the rise in gastric blood flow at the ulcer margin produced by CCK [$F(1,42) = 184.41$, $P < 0.001$] or leptin [$F(1,42) = 131.47$, $P < 0.001$]. The increment in ulcer area and the decrease of gastric blood flow caused by pretreatment with tyrphostin in vehicle group was not

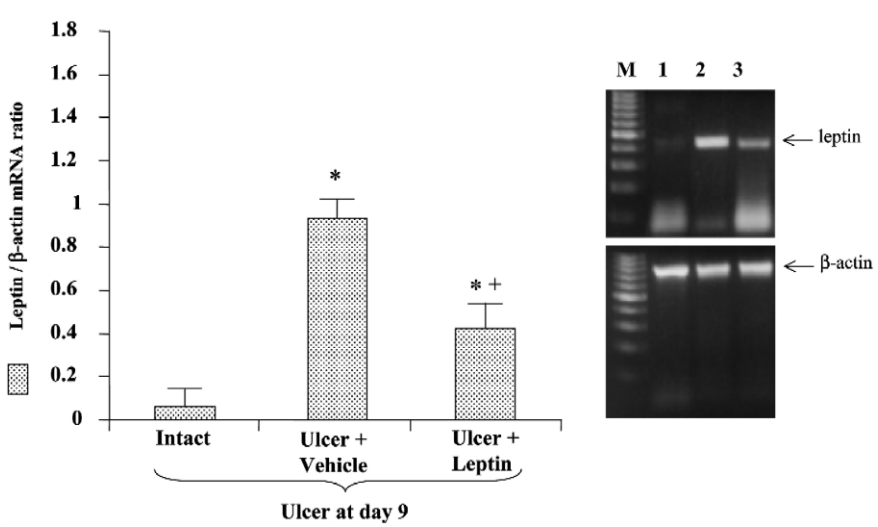

Fig. 3. Expression of leptin mRNA in the normal gastric mucosa (lane 1) and in the mucosa around the gastric ulcer in rats treated with vehicle (lane 2) and with leptin (lane 3). The arrow indicates PCR product size for leptin (415 bp) and β-actin (983 bp) (left panel). The index of mRNA expression determined as ratio of leptin mRNA over β-actin mRNA (right panel). Mean ± S.E.M of 6–8 rats. Asterisk indicates significant change compared to value in intact rats. Cross indicates significant decrease below the value obtained at the ulcer margin in vehicle-treated rats.

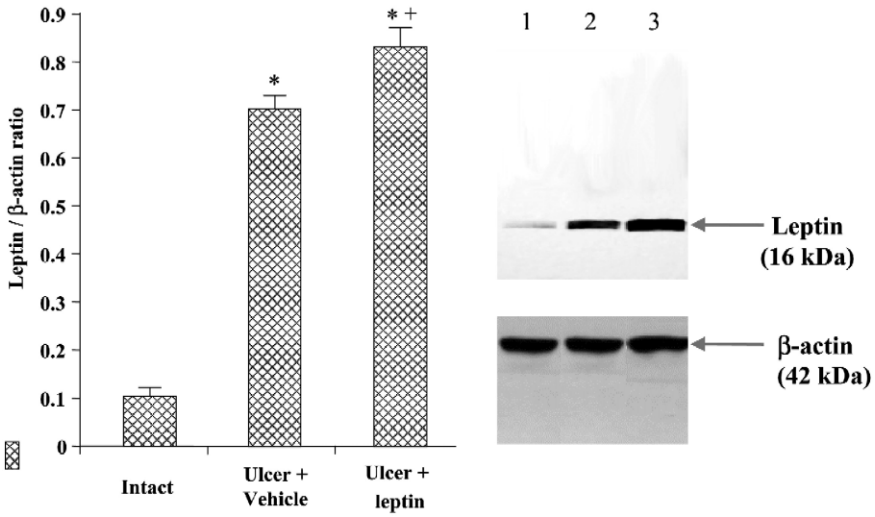

Fig. 4. Western blot analysis of leptin expression in normal gastric mucosa (lane 1) or around ulcer treated with vehicle (lane 2) or treated with exogenous leptin (lane 3). The index of leptin expression over β-actin protein expression in intact mucosa and that treated with vehicle or leptin (left panel). Mean ± S.E.M of 6–8 rats. Asterisk indicates significant increase above the value in intact mucosa. Cross indicates significant increase above the value recorded in vehicle-treated rats.

significantly different from those with tyrphostin in CCK- or leptin-treated group.

As shown in Fig. 2, a significant acceleration of ulcer healing was observed again after the treatment with CCK [$F(1,42) = 85.0$, $P < 0.001$] or leptin [$F(1,42) = 61.5$, $P < 0.001$], compared to vehicle. This was accompanied by significant increase of gastric blood flow at the ulcer margin after CCK [$F(1,42) = 84.9$, $P < 0.001$] or leptin [$F(1,42) = 61.5$, $P < 0.001$]. N^G-nitro-L-arginine (L-NNA) applied intraperitonneally in a dose of 20 mg/kg per day significantly increased the ulcer size in vehicle-treated rats [$F(1,42) = 18.6$, $P < 0.001$], as well as those treated with

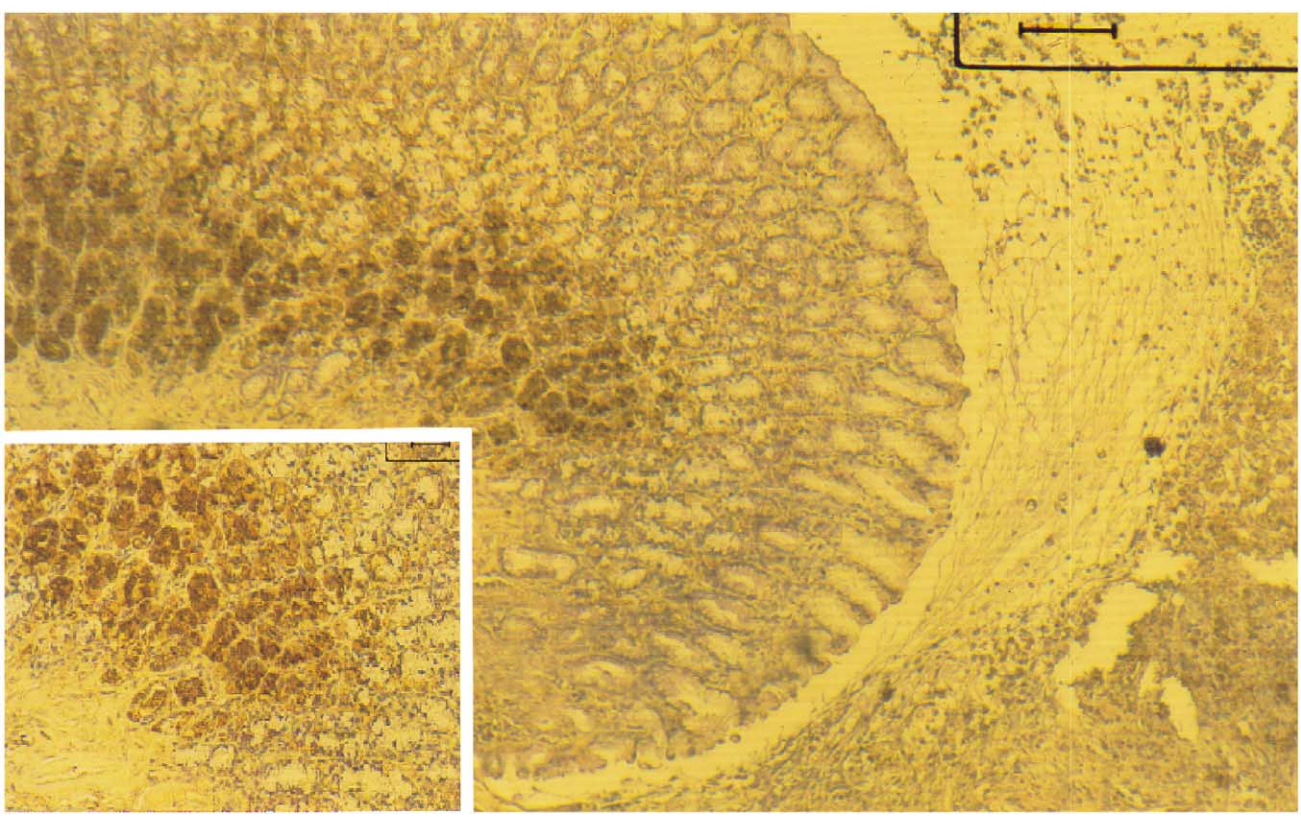

Fig. 5. Immunohistochemical expression of leptin at the ulcer edge (magnification: ×63), a small inset at the left shows the positive staining for leptin detected in the lower half of the gastric glands (magnification: ×250).

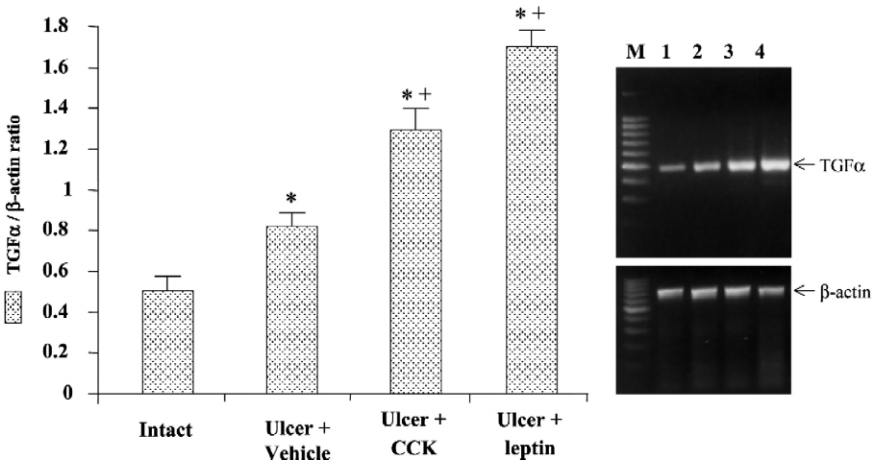

Fig. 6. The index of mRNA expression of TGFα determined as ratio of TGFα mRNA over β-actin mRNA in intact gastric mucosa, or in gastric mucosa around the ulcer of rats treated with vehicle, CCK (10 μg/kg s.c.) or leptin (10 μg/kg s.c.). Mean ± S.E.M of 6–8 rats. Asterisk indicates a significant change as compared to the value obtained in intact gastric mucosa. Cross indicates a significant change to the value obtained in rats with ulcers and treated with vehicle (left panel). Expression of TGFα mRNA assessed by RT-PCR in experiments as described above: intact mucosa (lane 1), ulcer (lane 2), ulcer + CCK (lane 3), ulcer + leptin (lane 4) (right panel).

CCK [$F(1,42) = 44.5$, $P < 0.001$] or leptin [$F(1,42) = 61.5$, $P < 0.001$], there was also a significant decrease in blood flow caused by L-NNA in rats receiving vehicle [$F(1,42) = 18.6$, $P < 0.001$], as well as those treated with CCK [$F(1,42) = 44.5$, $P < 0.001$] or leptin [$F(1,42) = 61.5$, $P < 0.001$]. There was no significant difference in the rise of ulcer area and in the fall of gastric blood flow between vehicle-treated rats and those treated with CCK or leptin.

3.3. The mRNA and protein expression of leptin at the ulcer margin

In intact fasted rats without gastric ulcers, the expression of leptin mRNA in the gastric mucosa was detected only as a weak signal (Fig. 3). Compared with the intact mucosa, the ratio of leptin over β-actin mRNA levels in rats with gastric ulcers was significantly increased [$F(1,21) = 217.0$, $P < 0.001$] at the ulcer margin and this ratio at this margin reached 0.95 ± 0.12 compared to 0.07 ± 0.005 in the intact mucosa. In rats with gastric ulcers treated with leptin, the mRNA expression for this peptide at the ulcer margin was significantly decreased [$F(1,21) = 50.3$, $P < 0.001$] as compared to that in rats with ulcers treated with vehicle (leptin/β-actin ratio 0.43 ± 0.08 vs. 0.95 ± 0.12) (Fig. 3). Using Western blot analysis for protein expression, a weak signal for leptin was detected in the intact oxyntic rat gastric mucosa. At the ulcer margin a significant increase in protein expression for leptin [$F(1,21) = 175.4$, $P < 0.001$] was found in vehicle-treated

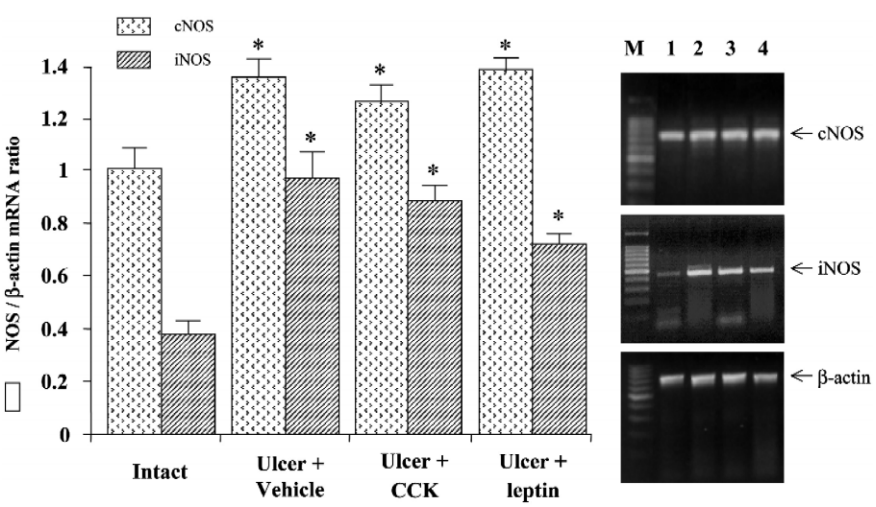

Fig. 7. The index of mRNA expression of cNOS and iNOS determined as ratio of cNOS or iNOS mRNA over β-actin mRNA in intact gastric mucosa, or in gastric mucosa around the ulcer of rats treated with vehicle, CCK (10 μg/kg s.c.) or leptin (10 μg/kg s.c.). Mean ± S.E.M of 6–8 rats. Asterisk indicates a significant change as compared to the value obtained in intact animals (left panel). Expression of cNOS and iNOS mRNA assessed by RT-PCR in experiments as described above: intact mucosa (lane 1), ulcer (lane 2), ulcer + CCK (lane 3), ulcer + leptin (lane 4) (right panel).

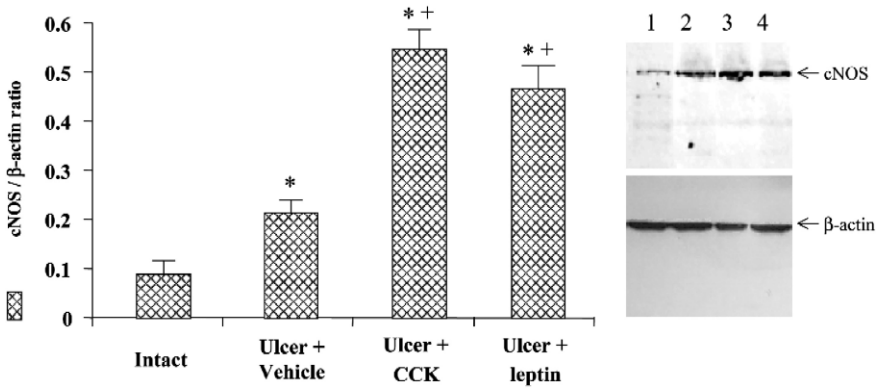

Fig. 8. Densitometric analysis of cNOS protein expression assessed by Western blot and expressed as cNOS/β-actin ratio in normal gastric mucosa (lane 1), in mucosa around ulcer treated with vehicle (lane 2), CCK (lane 3) or leptin (lane 3) (left panel). Mean ± S.E.M of 6–8 rats. Asterisks indicate a significant change as compared to intact gastric mucosa. Cross indicates a significant change as compared to the value obtained in vehicle-treated gastric mucosa. Representative Western blot analysis of cNOS and β-actin is presented in the right panel.

rats. In rats with gastric ulcers treated with leptin further significant [$F(1,21) = 47.2,\ P < 0.001$] increment in the protein expression of leptin in gastric mucosa was observed (Fig. 4).

3.4. Immunohistochemistry

In normal gastric mucosa staining for leptin was detected in the lower half of the fundic glands. In rats with gastric ulcers no expression of leptin was observed directly at the ulcer bottom or margin. In contrast, in gastric mucosa adjacent to the ulcer the staining for leptin was detected (Fig. 5).

3.5. mRNA expression of TGFα, cNOS and iNOS as analysed by RT-PCR at the ulcer margin in gastric mucosa of rats treated with vehicle, CCK-8 or leptin

The mRNA expression for TGFα was detected by RT-PCR in intact gastric mucosa. In rats with gastric ulcers a significant upregulation of TGFα in the ulcerated mucosa was observed [$F(1,28) = 124.4,\ P < 0.001$], as compared to that in the intact mucosa. As shown in Fig. 6, further significant increase in the ratio of TGFα over β-actin was observed in rats with ulcers after the treatment with CCK-8 [$F(1,28) = 194.2,\ P < 0.001$] or leptin [$F(1,28) = 250.0,\ P < 0.001$].

Using RT-PCR we detected strong mRNA signal for cNOS and weak but detectable signal for iNOS mRNA in intact gastric mucosa (Fig. 7). In contrast to intact mucosa, the ratio of cNOS mRNA over β-actin mRNA was significantly increased [$F(1,56) = 30.7,\ P < 0.001$] in the ulcer margin and it remained similarly increased at this margin after the administration of CCK [$F(1,56) = 30.1,\ P < 0.001$ or leptin [$F(1,56) = 39.7,\ P < 0.001$]. Also the ratio of iNOS mRNA over β-actin mRNA was significantly increased at the ulcer margin [$F(1,56) = 144.8,\ P < 0.001$], as compared intact gastric mucosa but it was not further significantly altered following administration of CCK or leptin (Fig. 7).

β-Actin, which was used as an internal standard to verify successful RNA extraction and reverse transcription, was detected as a strong signal in all probes.

3.6. Western blot analysis of protein expression of cNOS and iNOS at the ulcer edge in gastric mucosa of rats treated with vehicle, CCK-8 or leptin

At the protein level cNOS was detected as a weak signal and no signal was found for iNOS in the intact gastric mucosa (Fig. 8). In rats with gastric ulcers an increased expression of cNOS was detected by Western blot at the ulcer margin [$F(1,28) = 12.5,\ P < 0.05$] when compared to that in the intact gastric mucosa. The densitometric evaluation revealed about twofold overexpression of cNOS protein in the ulcer margin after treatment with CCK [$F(1,28) = 156.9,\ P < 0.001$)] or leptin [$F(1,28) = 105.0,\ P < 0.001$]. The protein expression of iNOS was below the level of detection and these data are omitted for the sake of clarity.

4. Discussion

The results of this study show for the first time that exogenous recombinant human leptin promotes, similarly to CCK-8, the healing of gastric ulcer and this is accompanied by enhanced gastric mucosal blood flow and an enhanced expression of TGFα and nitric oxide synthase in the margin of gastric ulcers.

The effect of leptin on ulcer healing was similar to that described previously for CCK-8 (Brzozowski et al., 1999b). The fact that exogenous CCK increased plasma level of

leptin and decreased the leptin stores in the gastric mucosa (Bado et al., 1998) suggests that the beneficial effects of CCK on ulcer healing might be mediated, at least in part, by leptin originating from the stomach.

On the other hand, the recent findings that the rise in plasma leptin following refeeding can be significantly lowered by the pretreatment with specific gastrin/CCK_2 receptor antagonist suggests that under physiological conditions such as feeding the rise in plasma leptin may originate from the action of gastrin rather than CCK on body leptin stores (Attoub et al., 1999). The observation that adipocytes express the CCK_1 rather than CCK_2 receptors suggests that the postprandial rise in leptin release may originate also from adipocytes. This does not exclude the role of locally generated leptin in the gastric mucosa in the healing of gastric ulcers and our present study supports this notion by finding that leptin is strongly expressed at the ulcer margin. Furthermore, we recently reported that leptin-induced acceleration of stress-induced gastric damage can be significantly attenuated by the suppression of CCK_2 receptors, but remained unaffected by the blockade of specific CCK_1 receptors (Pierzchalski et al., 2000). This supports the notion that although CCK enhances ulcer healing, leptin released by this CCK or gastrin requires the presence of active CCK_2 receptors to accelerate ulcer healing. Additional studies with measurement of plasma gastrin and blocking of CCK_2 receptors are needed to elucidate the involvement of gastrin and its receptors in leptin-induced ulcer healing.

Our previous studies demonstrated that leptin plays an important role in gastric mucosal integrity and gastroprotection. The pretreatment with this protein prevented the formation of acute gastric lesions induced by strong irritants such as alcohol or aspirin (Brzozowski et al., 1999a; Konturek et al., 1999). Since the protective effects of leptin were abolished by the blockade of nitric oxide synthase, vagotomy or capsaicin-induced denervation of sensory nerves, we concluded that the mechanism of the gastroprotective effects of leptin involves a variety of endogenous protectors except prostaglandins because the blockade of prostaglandin synthesis by indomethacin failed to affect the gastroprotection afforded by leptin. These findings, together with the observation that the acute injury of gastric mucosa in rats is accompanied by an increased expression of leptin in gastric mucosa (Konturek et al., 1999), indicate that local leptin may play an important role in the mediation of the repair process in injured gastric mucosa.

Indeed, we found in this study an enhanced expression of leptin at the margin of gastric ulcers at both mRNA and protein levels. However, following administration of exogenous leptin, the mRNA expression was significantly reduced at the ulcer margin (see Fig. 3) possibly due to negative feedback mechanism triggered by exogenous leptin. The fact that the expression of leptin protein was increased at ulcer margin in rats treated with leptin may simply reflect the influence of exogenous administration of this leptin on its protein expression.

Despite of the documented gastroprotection afforded by leptin, no information is available regarding the involvement of leptin in the healing process of chronic gastric ulcer. This study provides first evidence that leptin is expressed both at mRNA and protein levels in the ulcerated gastric mucosa. Furthermore, exogenous leptin was observed in this report to accelerate the rate of healing process and to raise gastric blood flow at the ulcer margin. Since these effects that were attenuated by the inhibition of nitric oxide synthase, we postulate that the ulcer healing activity of leptin is mediated, at least in part, by endogenous nitric oxide. This notion is supported by the finding that the administration of leptin (and CCK-8) is accompanied by increased gene expression of cNOS and iNOS as detected by RT-PCR. These findings suggest an important role of nitric oxide in mediating of the mucosal repair and healing process of leptin and CCK-8. Several previous studies have showed that nitric oxide is a critical defensive factor for the gastrointestinal mucosa by increasing gastric blood flow and mucus secretion, by inhibiting the gastric acid secretion and by accelerating ulcer healing (Brzozowski et al., 1997; Elliot and Wallace, 1998).

To further identify the underlying mechanism by which leptin and CCK-8 accelerates the ulcer healing, we analyzed the mRNA expression of TGFα, an important growth promoting factor in the gastric mucosa involved also in the protection and repair of injured gastric mucosa (Polk et al., 1992; Konturek et al., 1992). Previous studies have demonstrated that the increased synthesis of TGFα is accompanied by increased cell migration and proliferation at the ulcer edge (Konturek et al., 1997b). All these cellular events are triggered by the binding of TGFα to EGF receptor and are followed by activation of the tyrosine kinase which in turn triggers the intracellular signal transduction pathways leading to cell migration and proliferation (Pai et al., 1998; Relan et al., 1995). Our analyses have shown that the treatment of gastric ulcers with leptin or CCK-8 resulted in the upregulation of TGFα mRNA in the ulcerated mucosa suggesting that the acceleration of ulcer healing by leptin could be attributed, at least in part, to the increased expression of this important mucosal integrity peptide. The importance of TGFα in the acceleration of healing by leptin and CCK-8 is further strengthened by the fact that the administration of tyrphostin, a specific EGF-receptor tyrosine kinase inhibitor, attenuated the beneficial effects of leptin and CCK-8 on ulcer healing. Our results concerning the effect of tyrphostin on ulcer healing are in agreement with the previous studies by Pai et al. (1998), who showed that tyrphostin A46 significantly inhibits the mitogen-activated protein kinase signal transduction pathway and ulcer healing. Our results with the attenuation by tyrphostin of ulcer healing by CCK or leptin should be assessed with caution because this EGF-receptor tyrosine kinase inhibitor also significantly increased the

gastric ulcer and delayed its healing in vehicle-treated rats without administration of CCK or leptin. This ulcerogenic action of tyrphostin could be simply attributed to the interference by this inhibitor or ulcer healing action of endogenous TGFα upregulated at the ulcer margin.

Finally, leptin may also accelerate the ulcer healing through the stimulation of angiogenesis, which plays an essential role in ulcer healing (Jones et al., 2000; Szabo et al., 1998). The previous studies have shown that leptin interacts with endothelial cell via specific leptin receptors (Ob-R) and promotes angiogenesis (Sierra-Honigmann et al., 1998; Bouloumie et al., 1998). Further studies are needed to clarify the role of leptin as an angiogenic factor in ulcer healing. Our findings indicate that leptin accelerates ulcer healing in a manner similar to CCK and this effect depends, at least in part, upon an upregulation of TGFα and increased NO production at the ulcer margin. Leptin may be considered as a new factor participating in the process of ulcer healing via local production of TGFα and NO.

References

Arakawa, T., Higuchi, K., Fukuda, T., Fujiwara, Y., Kobayashi, K., Kuroki, T., 1998. Prostaglandins in the stomach: an update. J. Clin. Gastroenterol. 27 (Suppl. 1), S1–S11.

Attoub, S., Levasser, S., Buyse, M., Gioit, H., Laigneau, J.P., Moiso, J.P., Hervatin, F., Le-Marchand-Brusiel, Y., Lewin, J.M., Bado, A., 1999. Physiological role of cholecystokinin B gastrin receptor in leptin secretion. Endocrinology 140, 4406–4410.

Bado, A., Lavasseur, S., Attoub, S., Kermorgant, S., Laigneau, J.P., Bortoluzzi, M.N., Moizo, L., Lehy, T., Guerre Millos, M., Le Marchand-Brustel, Y., Lewin, M.J.M., 1998. The stomach is a source of leptin. Nature 394, 790–793.

Bouloumie, A., Drexler, H.C.A., Lafontan, M., Busse, R., 1998. Leptin, the product of *Ob* gene, promotes angiogenesis. Circ. Res. 83, 1059–1066.

Brand, S.J., Schmidt, W.E., 1995. Gastrointestinal hormones, Textbook of Gastroenterology. Lippincott Company, Philadelphia.

Breidert, M., Miehlke, S., Glasow, A., Orban, Z., Stolte, M., Ehninger, G., Bayerdorffer, E., Nettesheim, O., Halm, U., Haidan, A., Bornstein, S.R., 1999. Leptin and its receptors in normal human gastric mucosa and in *Helicobacter pylori*-associated gastritis. Scand. J. Gastroenterol. 34 (10), 954–961.

Brzozowski, T., Konturek, S.J., Drozdowicz, D., Dembinski, A., Stachura, J., 1995. Healing of chronic gastric ulcerations by L-arginine. Role of nitric oxide, prostaglandins, gastrin and polyamines. Digestion 56, 463–471.

Brzozowski, T., Konturek, S.J., Sliwowski, Z., Drozdowicz, D., Zaczek, M., Kedra, D., 1997. Role of L-arginine, a substrate for nitric oxide-synthase, in gastroprotection and ulcer healing. J. Gastroenterol. 32 (4), 442–452.

Brzozowski, T., Konturek, P.C., Konturek, S.J., Pajdo, R., Duda, A., Pierzchalski, P., Bielanski, W., Hahn, E.G., 1999a. Leptin in gastroprotection induced by cholecystokinin or by a meal. Role of vagal and sensory nerves and nitric oxide. Eur. J. Pharmacol. 374, 263–276.

Brzozowski, T., Konturek, P.C., Konturek, S.J., Pajdo, R., Drozdowicz, D., Kwiecien, S., Hahn, E.G., 1999b. Acceleration of ulcer healing by cholecystokinin (CCK): role of CCK-A receptors, somatostatin, nitric oxide and sensory nerves. Regul. Pept. 82, 19–33.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Elliot, S.N., Wallace, J.L., 1998. Nitric oxide: a regulator of mucosal defense and injury. J. Gastroenterol. 33, 792–803.

Evangelista, S., Maggi, C.A., 1991. Protection induced by cholecystokinin-8 (CCK-8) in ethanol-induced gastric lesions is mediated via vagal capsaicin-sensitive fibers and CCK receptors. Br. J. Pharmacol. 102, 119–124.

Faggioni, R., Fantuzzi, G., Fuller, J., Dinarello, C.A., Feingold, K.R., Grunfeld, C., 1998. IL-1beta mediates leptin induction during inflammation. Am. J. Physiol. 274, R204–R208.

Fan, X., Nagle, G.T., Collins, T.J., Childs, G.V., 1995. Differential regulation of epidermal growth factor and transforming growth factor-alpha messenger ribonucleic acid in the rat anterior pituitary and hypothalamus induced by stress. Endocrinology 136, 873–880.

Griffiths, M.J.D., Liu, S., Curzen, N.P., Messent, M., Evans, T.W., 1995. In vivo treatment with endotoxin induces nitric oxide synthase in rat main pulmonary artery. Am. J. Physiol. 268, L509–518.

Grunfeld, C., Zhao, C., Fuller, J., Pollack, A., Moser, A., Friedman, J., Feingold, K.R., 1996. Endotoxin and cytokines induce expression of leptin, the *ob* gene product, in hamsters. J. Clin. Invest. 97, 2152–2157.

Jones, M.K., Wang, H., Peskar, B.M., Levin, E., Itani, R.M., Sarfeh, I.J., Tarnawski, A.S., 2000. Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing. Nat. Med. 5 (12), 1418–1423.

Konturek, S.J., Brzozowski, T., Dembinski, A., Warzecha, Z., Konturek, P.C., Yanaihara, N., 1988. Interaction of growth hormone releasing factor and somatostatin on ulcer healing and mucosal growth in rats. Role of gastrin and epidermal growth factor. Digestion 41, 121–128.

Konturek, S.J., Brzozowski, T., Majka, J., Dembinski, A., Slomiany, A., Slomiany, B.L., 1992. Transforming growth factor alpha and epidermal growth factor in protection and healing of gastric mucosal injury. Scand. J. Gastroenterol. 27 (8), 649–655.

Konturek, P.C., Konturek, S.J., Brzozowski, T., Ernst, H., 1995. Epidermal growth factor and transforming growth factor-alpha: role in protection and healing of gastric mucosal lesions. Eur. J. Gastroenterol. Hepatol. 7 (10), 933–937.

Konturek, P.C., Brzozowski, T., Konturek, S.J., Elia, G., Wright, N., Sliwowski, Z., Thim, L., Hahn, E.G., 1997a. Role of spasmolytic peptide in healing of stress-induced gastric lesions in rats. Regul. Pept. 68, 71–79.

Konturek, P.C., Brzozowski, T., Konturek, S.J., Ernst, H., Drozdowicz, D., Pajdo, R., Hahn, E.G., 1997b. Expression of epidermal growth factor and transforming growth factor alpha during ulcer healing. Time Sequence Study 32 (1), 6–15.

Konturek, P.C., Brzozowski, T., Sulekova, Z., Meixner, H., Hahn, E.G., Konturek, S.J., 1999. Enhanced expression of leptin following acute gastric injury in rat. J. Physiol. Pharmacol. 50, 587–595.

Lord, G.M., Matarese, G., Howard, J.K., Baker, R.J., Bloom, S.R., Lechler, R.I., 1998. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. Nature 394, 897–901.

Mix, H., Widjaja, A., Jandl, O., Cornberg, M., Kaul, A., Goke, M., Beil, W., Kuske, M., Brabant, G., Manns, P., Wagner, S., 2000. Expression of leptin and leptin receptor isoforms in the human stomach. Gut 47, 481–486.

Okabe, S., Pfeifer, C.J., Roth, I.L.A., 1971. A method for experimental penetrating ulcers in rats. Am. J. Dig. Dis. 916, 277–284.

Pai, R., Ohta, M., Itani, R.M., Sarfeh, I.J., Tarnawski, A.S., 1998. Induction of mitogen-activated protein kinase signal transduction pathway during gastric ulcer healing in rats. Gastroenterology 114 (4), 706–713.

Pierzchalski, P., Brzozowski, T., Sliwowski, Z., Drozdowicz, D., Szlachcic, A., Konturek, S.J., 2000. Role of epidermal growth factor (EGF)

and EGF receptors in healing of stress lesions induced by leptin and cholecystoninin. Gastroenterology 118, A552.

Pohle, T., Shahin, M., Domschke, W., Konturek, J.W., 1999. Effect of basic fibroblast growth factor on gastric ulcer healing and its own mRNA expression. Aliment. Pharmacol. Ther. 13, 1543–1551.

Polk Jr., W.H., Dempsey, P.J., Russel, W.E., Brown, P.I., Beauchamp, D., Barnard, J.A., Coffey Jr., R.J., 1992. Increased production of transforming growth factor alpha following acute gastric injury. Gastroenterology 102, 1467–1474.

Relan, N.K., Fligiel, S.E., Dutta, S., Tureaud, J., Chauhan, D.P., Majumdar, A.P., 1995. Induction of EGF-receptor tyrosine kinase during early reparative phase of gastric mucosa and effects of aging. Lab. Invest. 73 (5), 717–726.

Rosenbaum, M., Leibel, R.L., 1999. The role of leptin in human physiology. Nature 341, 913–915.

Sarraf, P., Frederich, R.C., Turner, E.M., Ma, G., Jaskowiak, N.T., Rivet, D.J., Flier, J.S., Lowell, B.B., Fraker, D.L., Alexander, H.R., 1997. Multiple cytokines and acute inflammation raise mouse leptin levels: potential role in inflammatory anorexia. J. Exp. Med. 185, 171–175.

Sierra-Honigmann, M.R., Nath, A.K., Murakami, C., Garcia-Cardena, G., Papapetropoulos, A., Sessa, W.C., Madge, L.A., Schechner, J.S., Schwabb, M.B., Polverini, P.J., Flores-Riveros, J.R., 1998. Biological action of leptin as an angiogenic factor. Science 281, 1683–1686.

Sobhani, I., Bado, A., Vissuzaine, C., Buyse, M., Kermorgant, S., Laigneau, J.P., Attroub, S., Lehy, T., Henin, D., Mignon, M., Lewin, M.J., 2000. Leptin secretion and leptin receptor in human stomach. Gut 47, 178–183.

Szabo, S., Vincze, A., Sandor, Z., Jadus, M., Gombos, Z., Pedram, B.S., Levin, E., Hagar, J., Iaquinto, G., 1998. Vascular approach to gastroduodenal ulceration: new studies with endothelins and VEGF. Dig. Dis. Sci. 43 (Suppl. 9), 40S–45S.

Tarnawski, A., 2000. Cellular mechanisms of gastric ulcer healing. In: Domschke, W., Konturek, S.J. (Eds.), The Stomach. Springer Verlag, Berlin, pp. 177–192.

Walsh, J.H., 1994. Gastrointestinal hormones. In: Johnson, L.R., Alpers, D.H., Christensen, J., Jacobson, E.D., Walsh, J.H. (Eds.), Physiology of the Gastrointestinal Tract. Raven Press, New York, NY, pp. 1–128.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, l., Friedman, J.M., 1998. Leptin and the regulation of body weight in mammals. Nature 395, 763–770.