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## ENHANCED EXPRESSION OF LEPTIN FOLLOWING ACUTE GASTRIC INJURY IN RAT

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Leptin, a product of *ob*-gene plays an important role in the regulation of food intake. Recently, leptin expression has been detected in gastric epithelium, but the physiologic role of gastric leptin remains unknown. The purpose of this study was: 1) to determine the effect of gastric injury by ethanol and aspirin on the expression of leptin in gastric mucosa and 2) to investigate whether exogenous leptin affects the integrity of gastric mucosa exposed to noxious agents such as ethanol or aspirin. In Wistar rats the acute gastric lesions were induced by intragastric application of 1.5 ml of 75% ethanol or acidified aspirin (100 mg/kg in 0.2 N HCl). Rats were divided into two groups and pretreated either with leptin (1–10 µg/kg i.p.) or vehicle (saline). Rats were anesthetized 1 h after i.g. induction of acute gastric lesions and the gastric blood flow (GBF) was measured by H<sub>2</sub> gas clearance method. Then the rats were sacrificed, the stomach was excised and the mean lesions area was assessed by planimetry. In addition, mRNA and protein expression for leptin was analyzed in the gastric mucosa by reverse transcription polymerase chain reaction (RT-PCR) and Western blot, respectively. Both ethanol and acidified aspirin induced acute gastric lesions and led to significant reduction in GBF. In the intact gastric mucosa, the mRNA and protein expression for leptin was small but detectable. The exposure of gastric mucosa to noxious agents such as ethanol and aspirin was associated with markedly increased expression for gastric leptin at mRNA and protein level. Application of 75% ethanol or acidified aspirin caused wide-spread mucosal lesions. The pretreatment with exogenous leptin reduced dose-dependently these ethanol or aspirin-induced gastric lesions. The protective effects of exogenous leptin were accompanied by a significant attenuation of the fall of GBF. We conclude that: 1) Exogenous leptin exerts potent gastroprotective and hyperemic actions on gastric mucosa, and 2) Acute injury of gastric mucosa is associated with increased expression of leptin suggesting a possible role of this peptide in mediating of repair process in injured gastric mucosa.

**Key words:** *leptin, Western blot, reverse transcriptase-polymerase chain reaction (RT-PCR), gastric blood flow (GBF), aspirin, ethanol*

### INTRODUCTION

Leptin is a 16-kilodalton product of *ob* gene which plays a pivotal role in the regulation of body weight and energy expenditure (1). Leptin is predominantly expressed and secreted by differentiated adipocytes and the

major determinant of circulating leptin concentration in both rodents and humans is fat body mass (2, 3).

Recent study revealed that leptin is also present in the rat gastric epithelium, suggesting that the stomach might be an important source of this circulating hormone (4). Our previous study demonstrated that exogenous leptin and that released endogenously by cholecystokinin and meal exert a potent gastroprotective action depending upon vagal activity, sensory nerves and gastric hyperemia probably mediated by nitric oxide (5).

To our knowledge no study assessed the expression of leptin in gastric mucosa following acute gastric injury. The purpose of the present study was to analyze the mRNA and protein expression for leptin in the gastric mucosa after exposure to two different injurious substances such as ethanol and acidified aspirin. Furthermore, we have studied whether the pretreatment with exogenous leptin can attenuate the lesions induced by acid-dependent ulcerogens such as acidified aspirin and acid-independent irritants such as ethanol.

## MATERIAL AND METHODS

Male Wistar rats, weighing 180–220 g and fasted for 24 h, were used in all studies. All experimental procedures were approved by the Jagiellonian University Institutional Animal Care and Use Committee.

### *Induction of acute gastric lesions and effect of exogenous leptin on the ethanol- and aspirin induced gastric lesions*

Acute gastric lesions were induced in male Wistar rats weighing 180–220 g by intragastric (i.g.) application of topical irritants such as 75% ethanol or acidified aspirin (ASA) as described previously (6,7). Briefly, 75% ethanol or acidified ASA (100 mg/kg dispersed in 0.2 N HCl) was administered i.g. through the metal orogastric tube in the volume of 1.5 ml.

To study the effects of exogenous leptin given i.p. on the mucosal lesions induced by 75% ethanol or acidified ASA, the following series of experiments were carried out: 1) vehicle (1 ml saline i.p.) followed 30 min later by 75% ethanol or acidified ASA and 2) leptin (1 and 10 µg/kg i.p.) followed 30 min later by 75% ethanol or acidified ASA.

### *Measurement of gastric blood flow (GBF)*

After 60 min following i.g. application of ethanol or aspirin, the animals were lightly anesthetized with ether, their abdomen was opened by a midline incision and stomach was exposed for the measurement of GBF by means of the hydrogen (H<sub>2</sub>)-gas clearance technique as described previously (8). For this purpose double electrodes of an electrolytic regional blood flowmeter (Biotechnical Science, Model RGF-2, Osaka, Japan) were inserted into the gastric mucosa. One of these electrodes was used for the local generation of gaseous H<sub>2</sub> and another for the measurement of tissue H<sub>2</sub>. With this method, the H<sub>2</sub> generated locally is carried out by the blood flow, while the

polarographic current detector shows the decreasing  $H_2$ . The tissue  $H_2$  clearance curve was used to calculate an absolute flow rate (ml/100g/min.) in the oxyntic area as described previously (8). The measurement of GBF were made in three areas of the gastric mucosa and the mean values of measurements were calculated and expressed as percent changes from the values recorded in the vehicle (saline) treated animals.

After measurement of the GBF, the stomach was removed, rinsed with water and the area of acute gastric lesions in the oxyntic mucosa was determined by computerized planimetry by a person who did not know to which experimental group the animals belonged.

### *Determination of mRNA transcripts for leptin in gastric mucosa by RT-PCR*

Immediately after the measurement of the surface of gastric mucosal lesions mucosal specimens (about 200 mg) were scraped off using a slide glass and immediately snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis. 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 (9). Following precipitation, RNA was resuspended in RNase-free TE buffer and its concentration was estimated by absorbance at 260 nm wavelength. Furthermore, the quality of each RNA sample was determined by running the agarose-formaldehyde electrophoresis. RNA samples were stored at  $-80^{\circ}\text{C}$  until analysis.

Single stranded cDNA was generated from 5  $\mu\text{g}$  of total cellular RNA using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Stratagene, Heidelberg, Germany) and oligo-(dT)-primers (Stratagene, Heidelberg, Germany). Briefly, 5  $\mu\text{g}$  of total RNA was uncoiled by heating ( $65^{\circ}\text{C}$  for 5 min.) and then reverse transcribed into complementary DNA (cDNA) in a 50  $\mu\text{l}$  reaction mixture that contained 50 U MMLV-RT, 0.3  $\mu\text{g}$  oligo-(dT)-primer, 1  $\mu\text{l}$  RNase Block Ribonuclease Inhibitor (40U/ $\mu\text{l}$ ), 2  $\mu\text{l}$  of a 100 mM/l mixture of deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP), 10 mM/l Tris-HCl (pH = 8.3), 50 mM KCl, 5 mM  $\text{MgCl}_2$ ). The resultant cDNA (2  $\mu\text{l}$ ) was amplified in a 50  $\mu\text{l}$  reaction volume containing 2 U Taq polymerase, dNTP (200  $\mu\text{M}$  each) (Pharmacia, Germany), 1.5 mM  $\text{MgCl}_2$ , 5  $\mu\text{l}$  10 $\times$  polymerase chain reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH = 8.3) and specific primers for GAPDH or leptin used at final concentration of 1 mM (all reagents from Takara, Shiga, Japan). The mixture was overlaid with 25  $\mu\text{l}$  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  $94^{\circ}\text{C}$  for 1 min., annealing at  $60^{\circ}\text{C}$  for 45 sec and extension  $72^{\circ}\text{C}$  for 2 min. The number of cycles was 35 for leptin and 30 for GAPDH. The nucleotide sequence of the primers were as follows: GAPDH, sense 5'-TGA AGG TCG GTG TCA ACG GAT TTG GC-3'; antisense 5'-CAT GTA GGC CAT GAGGTC CAC CAC-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'. The primer sequences for leptin were based on the sequences of the published cDNAs (4) and were synthesized by GIBCO BRL/Life Technologies (Eggenstein, Germany). The primer for GAPDH were purchased by Clontech (Heidelberg, Germany).

Polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Location of a predicted products was confirmed by using 100-bp ladder (Takara, Shiga, Japan) 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) as described earlier (8). The signal for leptin mRNA was standardized against that of the  $\beta$ -actin mRNA from each sample and the results were expressed as leptin/ $\beta$ -actin mRNA ratio.

## *Protein extraction and analysis of leptin expression in the gastric mucosa by Western blot*

Shock frozen tissue from rat stomach was homogenized in lysis buffer (100 mmol Tris-HCl, pH 7.4, 15% glycerol, 2 mmol EDTA, 2% SDS, 100 mmol DDT) by the addition of 1:20 dilution of aprotinin and 1:50 dilution of 100 mmol PMSF. Insoluble material was removed by centrifugation at 12000g for 15 min. Approximately 100 µg of cellular protein extract were loaded into a well, separated electrophoretically through a 13.5% SDS-polyacrylamide gel and transferred onto Sequi-Blot™ PVDF membrane (BioRad, USA) by electroblotting. Skim fast milk powder (5%w/v) in 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 hr at room temperature. 1:500 dilution of specific primary rabbit polyclonal antibody against leptin (A-20) (Santa Cruz, USA) or 1:1000 dilution of rabbit polyclonal anti-β actin (Sigma Aldrich, Germany) antibody was added to the membrane, followed by an anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:2000, Santa Cruz, USA). Incubation of primary antibody was followed by 3 washes with TBS-Tween-20 buffer for 10 min. Incubation of the secondary antibody was followed by 4 washes for 10 min. Non-isotopic visualization of immunocomplexes was achieved by chemiluminescence using BM Chemiluminescence Blotting Substrate (Boehringer, Mannheim, Germany). Thereafter, the developed membrane was exposed to an X-ray film (Kodak, Wiesbaden, Germany). Comparisons between different treatment groups were made by determining the leptin/β-actin ratio of the immunoreactive area by densitometry.

## *Statistical analysis*

The results are expressed as means ± SEM. Statistical analysis was performed by using an analysis of variance and nonpaired Student's *t* test. Differences with  $p < 0.05$  were considered as significant.

## RESULTS

### *Effect of exogenous leptin on the ethanol and aspirin-induced gastric lesions and the GBF*

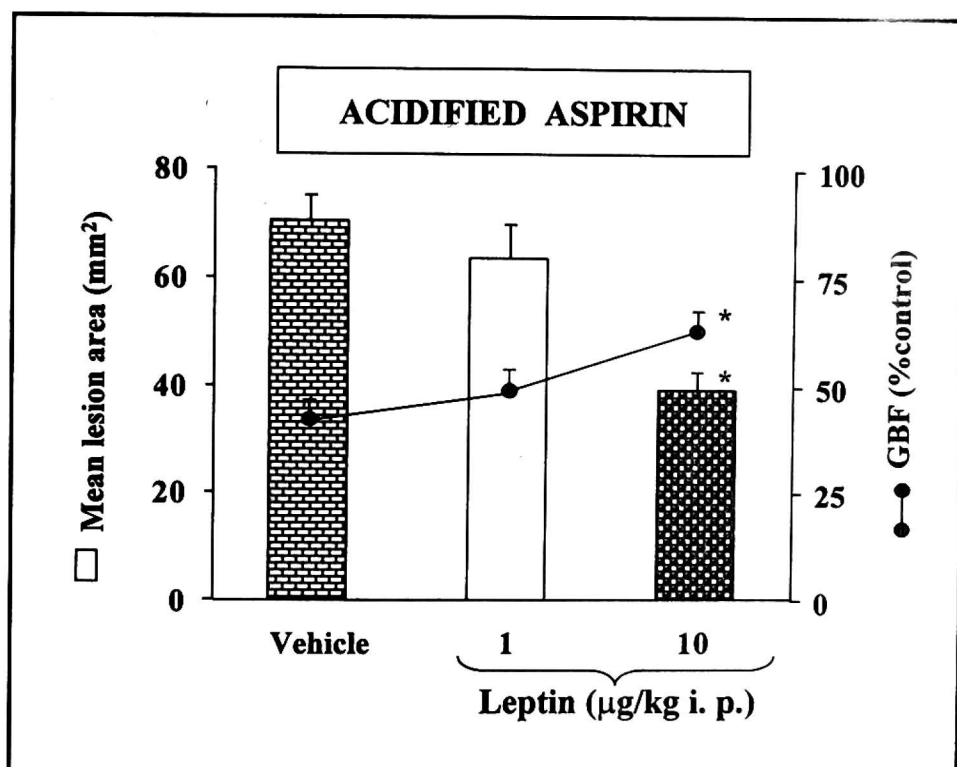
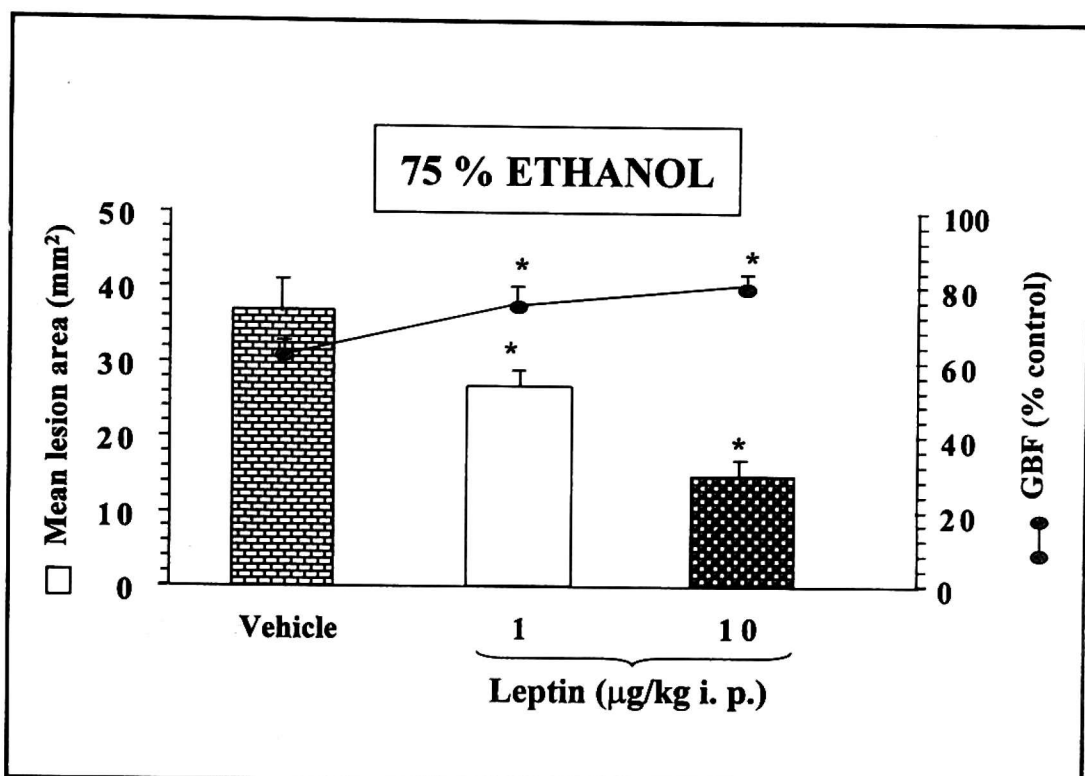
The exposure of gastric mucosa to 75% ethanol, resulted in acute erosions with a mean area of about 38 mm<sup>2</sup>. Acidified ASA, given i.g. at a dose of 100 mg/kg, induced acute gastric erosions with a mean area about 68 mm<sup>2</sup> (Figs 1 and 2).

As shown on Figs 1 and 2, the pretreatment with leptin given i.p. reduced dose-dependently the lesion area caused by 75% ethanol or acidified aspirin. The protective effect of leptin was accompanied by a significant and dose-dependent rise in GBF as compared with respective values in gastric mucosa exposed to ethanol or ASA applied alone.

The GBF in the intact gastric mucosa averaged  $49 \pm 5$  ml/min/100g and this value was not significantly affected following i.p. administration of vehicle (saline). When ethanol or acidified aspirin were applied i.g. to vehicle-pretreated rats, a significant reduction in GBF by about 30% and 60% respectively, was observed as compared to that in vehicle-control gastric mucosa.



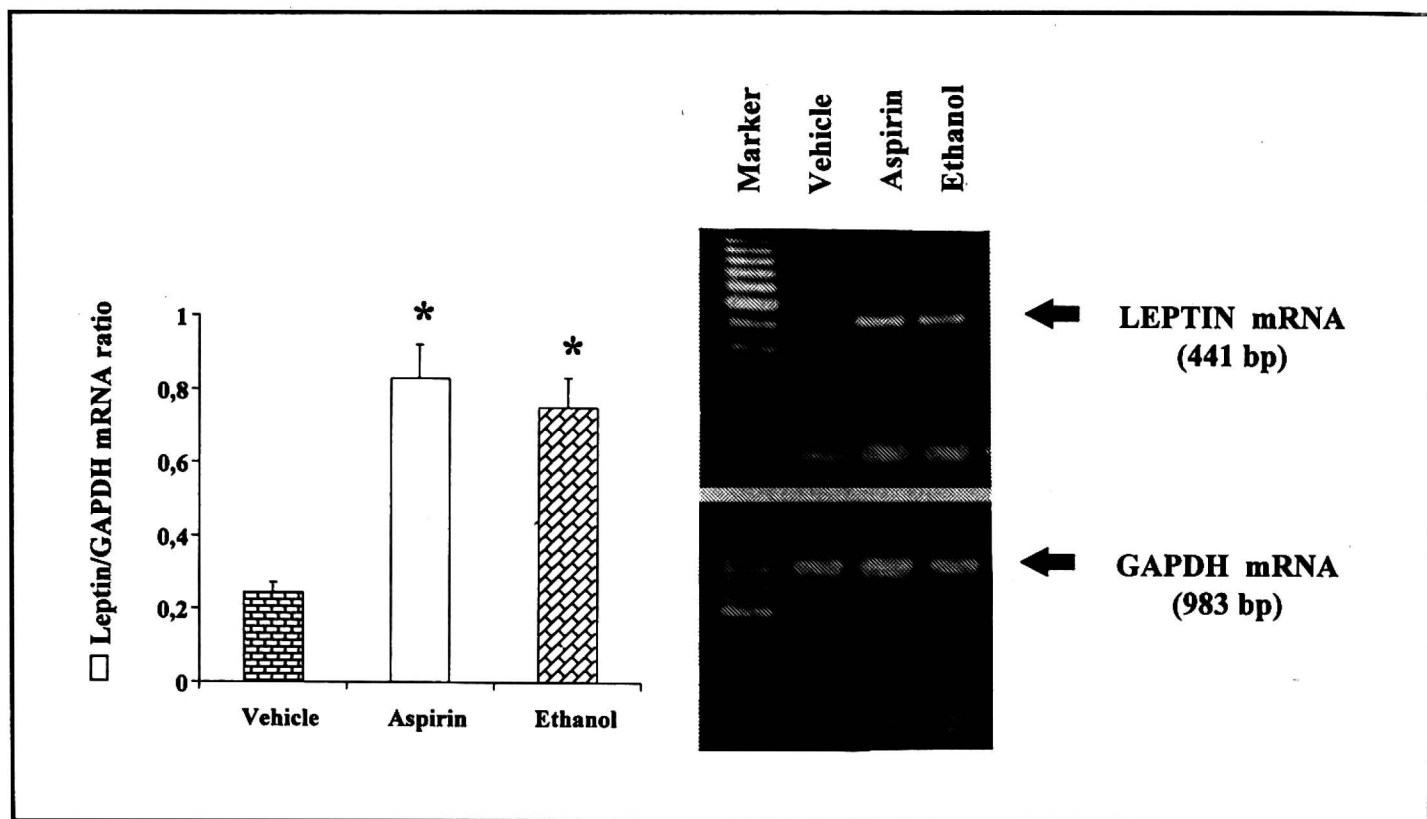
*Fig. 1.* Mean area of ethanol-induced gastric lesions and GBF in rats treated with vehicle (saline) or with two different doses of leptin (1 and 10  $\mu\text{g}/\text{kg}$  i.p.). Means  $\pm$  S.E.M. for 6–8 rats. Asterisk indicates a significant change as compared to the vehicle control values.



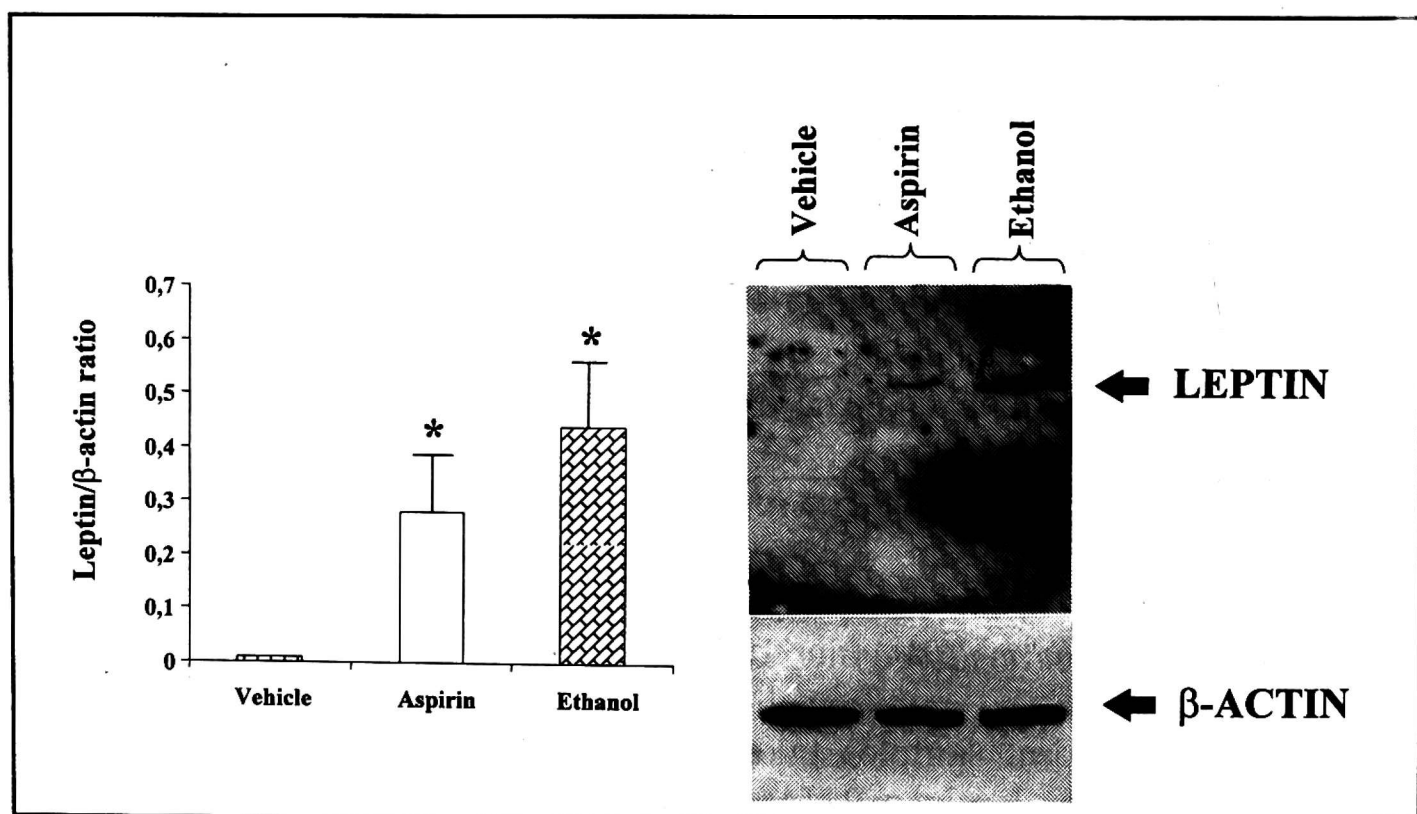
*Fig. 2.* The area of aspirin-induced gastric lesions and GBF in rats treated with vehicle (saline) or with two different doses of leptin (1 and 10  $\mu\text{g}/\text{kg}$  i.p.). Means  $\pm$  S.E.M. for 6–8 rats. Asterisk indicates a significant change as compared to the vehicle control values.

### *Expression of leptin at mRNA and protein level in the gastric mucosa following acute injury with 75% ethanol or acidified ASA*

In gastric mucosal samples taken from vehicle treated rats a weak but detectable signal for leptin mRNA was observed. In contrast, in the gastric mucosa of rats exposed to 75% ethanol or acidified aspirin, the expression of leptin mRNA increased significantly. The expression of mRNA for GAPDH was well preserved in all mucosal samples taken from rats treated with vehicle or exposed to 75% ethanol or acidified ASA (*Fig. 3*).



*Fig. 3.* RT-PCR analysis of leptin mRNA expression in the gastric mucosa treated with vehicle (lane 1) and in those treated with acidified aspirin (lane 2) and 75% ethanol (lane 3). M- DNA size marker. Comparison between the groups were made by determining the leptin/GAPDH ratio by densitometry. Asterisk indicates a significant change as compared to the value obtained in the vehicle (saline) treated group.



*Fig. 4.* Immunoblot analysis of leptin expression in the gastric mucosa treated with vehicle and in those treated with acidified aspirin and 75% ethanol. Comparison between the groups were made by determining the leptin/β-actin ratio by densitometry. Asterisk indicates a significant change as compared to the value obtained in the vehicle (saline) treated group.

In the gastric mucosa of rats treated with vehicle, very low, almost no detectable level of leptin protein was detected. In contrast, in the gastric mucosa of rats exposed to 75% ethanol or acidified aspirin, strong signal for the expected ~19 kD leptin protein was observed. The expression of  $\beta$ -actin was well preserved in the mucosal samples taken from rats treated with vehicle or exposed to 75% ethanol or acidified ASA (*Fig. 4*).

## DISCUSSION

In accordance with our previous report (5) leptin attenuated dose-dependently acute gastric lesions induced by 75% ethanol or acidified aspirin. The fact that this protection occurs without alterations in gastric secretion (5) confirms that leptin is a truly gastroprotective substance.

In this report we showed for the first time that acute gastric damage with 75% ethanol or acidified aspirin is associated with an up-regulation of leptin expression at mRNA and protein level in the gastric mucosa. This observation supports further our proposal that the protective effects of leptin are at least partly due to increased production of this peptide in the gastric mucosa.

The mechanisms responsible for the increase of leptin expression in the gastric mucosa after acute injury with ethanol or aspirin remain unclear. It is likely that multiple factors are involved in stimulation of leptin expression in acutely injured gastric mucosa. Previous studies have shown that some proinflammatory cytokines such as  $\text{TNF}\alpha$  or IL-1 increase the level of leptin mRNA in adipose tissue (10). Because ethanol as well as aspirin-induced gastropathy is associated with increased expression of these cytokines (11, 12), we suggest that both these cytokines could mediate this increased synthesis of leptin after ethanol- or aspirin-induced gastric damage.

The mechanisms underlying the protective effects of leptin on ethanol- and aspirin-induced gastropathy remains unclear. We have demonstrated recently that the protective effect of leptin is due, at least in part, to increased generation of nitric oxide which is involved in gastric mucosal defense. Moreover, this vasodilator was shown to reduce gastric damage induced by ethanol and non steroidal anti-inflammatory drugs (13—15). This protection induced by leptin probably does not involve endogenous prostaglandins because the suppression of their biosynthesis by indomethacin failed to influence this gastroprotection (5).

Exogenous leptin dose-dependently reduced gastric lesions induced by 75% ethanol or acidified aspirin and this effect was accompanied by a significant and dose-dependent rise in the mucosal GBF which plays a critical role in mucosal defense against gastric injury by topical irritants (15). The hyperemic effect of leptin could depend, at least in part, upon an increase of the formation

of nitric oxide which is considered as an important factor modulating gastric mucosal blood flow (5).

Recently, leptin has been also shown to be overexpressed in the gastric mucosa of patients with *Helicobacter pylori* infection as well *in vitro* in gastric epithelial cells incubated with *Helicobacter pylori* suggesting that this peptide may be involved in the protection of gastric mucosa against this bacterium (16).

The results of our study are in keeping with this observation by demonstration that leptin is overexpressed in gastric mucosa following acute mucosal injury and, therefore, it may contribute to the mechanism of gastric mucosal defense.

In conclusion, this study indicates that: 1) acute injury of gastric mucosa with ethanol or aspirin is associated with an enhanced gene and protein expression of leptin in gastric mucosa and 2) exogenous leptin exerts a potent gastroprotective action through the hyperemic effect probably mediated through the upregulation of nitric oxide synthesis in the gastric mucosa. Thus, leptin seems to play an important role in mucosal defense and the maintenance of gastric mucosal integrity.

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