Original Research

Exogenous ghrelin regulates proliferation and apoptosis in the hypotrophic gut mucosa of the rat

Ignacio A Gómez de Segura[1,2], María Teresa Vallejo-Cremades[3], Jesús Lomas[1], Miriam F Sánchez[1], María Isabel Caballero[1], Carlota Largo[1] and Enrique De Miguel[1]

[1]Department of Experimental Surgery, Hospital Universitario La Paz, Paseo de la Castellana, 261, 28046 Madrid; [2]Department of Medicine and Surgery, Veterinary Faculty, University Complutense of Madrid, Avda. Puerta de Hierro s/n, 28040 Madrid; [3]Research Unit, Biomedical Research Foundation, Hospital Universitario La Paz, Madrid, Paseo de la Castellana, 261, 28046 Madrid, Spain
Corresponding author: Ignacio A Gómez de Segura, Servicio de Cirugía Experimental, Hospital Universitario La Paz, Paseo de la Castellana, 261, 28046 Madrid, Spain. Email: ialvarez@vet.ucm.es

Abstract

Ghrelin is the natural endogenous ligand for growth hormone secretagogue receptors. This peptide regulates energy homeostasis and expenditure and is a potential link between gut absorptive function and growth. We hypothesized that ghrelin may induce a proliferative and antiapoptotic action promoting the recovery of the hypotrophic gut mucosa. Therefore, the aim of the study was to determine the action of exogenous ghrelin following gut mucosal hypotrophia in rats fed an elemental diet. An elemental diet provides readily absorbable simple nutrients and is usually given to patients with absorptive dysfunction. Male Wistar rats ($n = 48$) were fed the elemental diet for one week to induce mucosal hypotrophy and then treated for another week with systemic ghrelin and pair-fed with either a normoproteic or hyperproteic isocaloric liquid diet. Another group received a standard diet instead of the elemental diet and served as control (normotrophy). The elemental diet induced intestinal hypotrophia characterized by decreased proliferation in the ileum and increased apoptosis in jejunum and ileum. Ghrelin administration restored normal levels of proliferation in the ileum and apoptosis in the jejunum, with partial apoptosis restoration in the ileum. Ghrelin levels in plasma and fundus were increased in all groups, although the highest levels were found in rats treated with exogenous ghrelin. Ghrelin administration has a positive effect in the hypotrophic gut, regulating both proliferation and apoptosis towards a physiological balance counteracting the negative changes induced by an elemental diet in the intestines.

Keywords: ghrelin, intestinal trophism, intestinal hypotrophy, proliferation, apoptosis, diet, rat

Experimental Biology and Medicine 2010; **235**: 463–469. DOI: 10.1258/ebm.2009.009247

Introduction

The current knowledge of intestinal physiology strongly supports a key role for endogenous peptides and nutrition in promoting an adequate trophic status in intestinal mucosa to help maintain its absorptive and barrier functions.[1] Situations such as prolonged starvation or chronic fasting, total parenteral nutrition, elemental diet nutrition or intestinal injury like that produced by radiotherapy[2–5] may lead to hypotrophy of the gut mucosa. An elemental diet, a therapeutic diet containing simple readily absorbable nutrients, is usually given to patients with Crohn's disease to facilitate nutrient absorption by the inflamed intestinal mucosal.[6,7]

These factors, peptides and nutrition, have been considered therapeutic tools in gut diseases and some of them, especially those, like growth hormone (GH), that have a trophic action on the intestinal mucosa, have been identified for their potential clinical use.[8] Similarly, selected nutrients and high-quality protein or amino acids, like glutamine, may be given to enrich the diet and promote mucosal growth in diseased intestines.

Ghrelin, a 28-amino acid peptide, is the natural endogenous ligand for GH secretagogue receptors (GHS) originally isolated from rat and human stomach,[9] and subsequently identified in various tissues, including the small bowel.[10]

It is generally assumed that the GHS receptor subtype 1a (GHS-R1a), a G-protein-coupled receptor localized predominantly in the pituitary gland and hypothalamus,[11,12] mediates ghrelin actions. Acylated ghrelin, through ghrelin *O*-acyltransferase,[13] is the biologically active form, since

unacylated ghrelin is unable to bind to GHS-R1a.[13,14] Ghrelin was initially identified as a potent circulating orexigen involved in the control of GH secretion, energy expenditure and adiposity.[9,15,16] Caloric intake and a chronically positive energy balance suppress ghrelin expression and secretion, whereas weight loss and restriction of caloric intake increase ghrelin expression and secretion.[15,17,18] Ghrelin is mainly secreted in the stomach,[19] and also in the intestines,[10] suggesting a link between nutrition and growth. Ghrelin may have a pleiotropic action since it promotes a direct proliferative action,[20,21] which might be favored with a high protein content diet.[22]

Diseases of the intestines, including inflammatory processes, can modify ghrelin levels.[23,24] Ghrelin secretion can be nutritionally manipulated,[25,26] and protein-enriched diets may increase its levels,[27] suggesting a relation between this peptide and the trophic action of a high-protein diet.[2] Since increased levels of ghrelin may be associated with improved gut mucosa following hypotrophy,[2] we hypothesized that exogenous administration of ghrelin might be beneficial to recovery from hypotrophy in the rat intestine.

Methods

Animals

Adult Wistar rats ($n = 48$; Janvier; le Genest, Saint-Isle, France) aged 12 weeks and weighing 237 ± 12 g were used in this study. The animals were allowed one week of acclimatization and placed in individual wire floor cages in a standard environment at 22°C, 50% humidity and 12 h light/dark cycle (European Directive 609/86).

Study design

To determine the effects of exogenous ghrelin in hypotrophic intestines, rats were fed an elemental diet (hypotrophy, $n = 40$) for one week and then treated with ghrelin and fed with either a normoproteic or a hyperproteic diet for another week. One further group was fed a standard rodent chow for one week and served as a normotrophy control group ($n = 8$).

Intestinal hypotrophy (first week)

Before ghrelin administration, animals were randomly fed for one week *ad libitum* with an isocaloric liquid elemental diet giving a daily amount of 100 mL/rat (1 kcal/mL) as described previously.[2] An elemental diet means that the food is given in the simplest formulation as follows: protein as essential and non-essential amino acids; carbohydrate as sugar and dried glucose syrup; fat as fatty acids (canola, coconut and safflower oil); and vitamins, minerals and trace elements.[28] The elemental diet is a nutritionally complete liquid diet prepared daily from powder (Elemental 028, SHS) and served to produce intestinal hypotrophy. Animals had free access to water and were not pair-fed. The total amount of food eaten was not determined. Control normotrophic rats were fed for one week *ad libitum* with a rodent chow (B&K Universal, Barcelona, Spain).

Ghrelin treatment and dietary regimen (second week)

Ghrelin (ghrelin trifluoroacetate lyophilized salt, Cod. G8903; Sigma-Aldrich, St Louis, MO, USA), at a dose (40 μg/kg, intraperitoneally) that has been shown to provide gastroprotection in the rat,[29] or an equal amount of saline were co-administered daily with dietary treatment during 1 week, between 9:00 and 10:00 h. Rats were pair-fed during the second week (305 kcal/kg/d) and received either a standard liquid diet (normoproteic and normocaloric, 1 kcal/mL: Osmolite® HN Plus; Abbott Laboratories SA, Madrid, Spain) or a high-protein liquid diet (hyperproteic and normocaloric, 1 kcal/mL: Promote® HN Plus; Abbott Laboratories SA). Isocaloric diets were given daily for six days at 9:00 h so as to avoid potential circadian changes in hormonal levels. On the seventh day of ghrelin treatment, animals were sacrificed to collect samples.

Groups

According to the variables employed (ghrelin and dietary treatments), rats were randomly allocated to four groups ($n = 8$/group). Additionally, a further group of animals ($n = 8$), given an elemental diet for one week and then sacrificed, served as an intestinal hypotrophy control, and one final group of animals ($n = 8$) fed a standard diet for one week followed by the standard liquid diet for one further week served as a normotrophy control.

Extraction of tissue and plasma samples

Animals were anesthetized with isoflurane (Forane®; Abbott Laboratories SA) for laparotomy. Blood (4 mL) was collected from the abdominal aorta into chilled polypropylene tubes containing disodium ethylenediaminetetraacetic acid (1 mg/mL blood) and aprotinin (500 units/mL blood; Bayer, Leverkusen, Germany) and then centrifuged 15 min at 3000 rpm, and plasma was stored at −80°C. Tissue samples from the gastric fundus were also stored at −80ºC. The tissue was homogenized for the determination of ghrelin levels by radioimmunoassay (RIA). Protein concentration was determined as the reference standard (bicinchoninic acid assay, BCA Protein Assay Kit, Ref. 23227; Pierce, Rockford, IL, USA).

Intestinal morphometry

Since distal bowel is more sensitive to elemental diet-induced hypotrophy,[2] we selected jejunum and ileum to study their morphometric parameters. A 1-cm section of the intestine (jejunum and ileum) was fixed in a 10% buffered formaldehyde solution at 4ºC for 24 h. All samples were sectioned (5 μm) and reoriented by successive slices in the search for the sample with the longest villi. Sections were stained with Harris' hematoxylin and eosin. The lengths of the 10 longest villi in each sample and their

corresponding crypts were determined. The samples were studied in an image analysis system (Image ProPlus; Media Cybernetics Inc, Silver Spring, MD, USA).

Proliferation and apoptosis analyses

Proliferation was studied with a specific antiproliferation antigen antibody (NCL-Ki67-MM1; Novo Castra Laboratories, Newcastle, UK). Counts were carried out in 20 full-length crypts per section. Proliferating nuclei were immunohistochemically stained dark brown by the Ki-67 antigen, whereas non-proliferating nuclei were stained blue. The area covered by both types of nuclei in a full-length section of crypt was determined by means of a computerized system (CAS-200 Image Analyzer) in order to obtain their ratio.

Apoptotic cells were detected using the TUNEL assay (TdT-mediated biotin-dUTP nick end labeling; TdT-FragEL DNA Fragmentation Detection Kit, Oncogene Research Products; Calbiochem, San Diego, CA, USA). Visualization was accomplished using diaminobenzidine. Apoptotic nuclei were counted by means of direct visualization and an apoptotic index (percentage of apoptotic TUNEL-positive cells) was determined in 20 full-length crypts from each sample. Non-apoptotic nuclei were stained with methyl green.

Hormone determinations

Plasma aliquots were prepared to measure active ghrelin with the RIA kit (Linco Research, St Charles, MO, USA). Optimal sample dilution was determined and then samples were assayed in triplicate. The test was repeated when the interassay coefficient of variation (CV) was >10%. The minimum detectable amount of ghrelin was 10 pg/tube, with 13% inter- and 7% intra-assay CV, respectively.

Since the pulsatile changes in GH serum peptide levels could not be detected with this experimental design, we used insulin-like growth factor (IGF)-1 to screen ghrelin-dependent GH plasma levels. Total serum IGF-1 was measured by RIA after acid ethanol extraction with a commercial kit (Diagnostic Systems Laboratories, Webster, TX, USA). Interassay CV was 5.5% and the intra-assay CV was 3%. The sensitivity of this assay is 0.8 μg/mL.

Statistics

Statistical analysis of data was performed with a commercial computer program (Statview 4.5; Abacus Concepts, Berkeley, CA, USA). The one-sample Kolmogorov–Smirnov test was applied to the data to determine the normality of the distribution of the data to be used in the parametric test. One-way analysis of variance and the *post hoc* Fisher least-squares difference test were used to compare the diet groups and a P value <0.05 was considered to indicate statistically significant differences.

Results

Animals fed the elemental diet during one week before ghrelin treatment had a reduced body weight (226 ± 7 g) compared with rats fed a standard diet (326 ± 5 g; $P < 0.05$). One week later, rats given a normoproteic or hyperproteic diet similarly increased their body weight (280 ± 6 g and 280 ± 6 g, respectively; P versus hypotrophic rats fed the elemental diet <0.05). Ghrelin administration further increased body weight (294 ± 5 g and 292 ± 6 g in rats fed a normoproteic and hyperproteic diet, respectively; P versus same groups not treated with ghrelin <0.05).

The administration of an elemental diet reduced the mucosal length (villous and crypt, $P < 0.001$). When rats were fed for another week with a liquid diet containing a normal amount of protein, morphometric figures were significantly increased in the jejunum (villi), but decreased in the ileum ($P < 0.05$). When a high-protein diet was given after the elemental diet, villous and crypt lengths were improved or not modified, i.e. no decrease was detected, in either of the two intestinal segments analyzed (Figures 1 and 2).

The effect of ghrelin was variable and, whereas intestinal parameters appeared to be slightly, although non-significantly, improved in the proximal intestine, mucosal thickness was increased in the ileum of ghrelin-treated rats that were fed a normoproteic diet ($P < 0.05$; Figure 1). However, the largest increase in villous and crypt lengths was observed when ghrelin treatment was associated with a hyperproteic diet and in the ileal segment ($P < 0.05$), suggesting both a selective effect in the distal intestine as well as an additive or synergistic action on mucosal growth. This selective action of ghrelin in the ileum was associated with a significant increase in proliferation ($P < 0.001$), i.e. the proliferative index in the ileum following an elemental diet was two to three times lower than that found in the jejunum and ghrelin administration fully corrected this reduction (Figure 3).

The elemental diet induced a marked increase in apoptosis compared with the normotrophic intestine of rats fed a standard diet. However, one week later differences were found depending on the intestinal segment and the administration of exogenous ghrelin. This peptide reduced the five-fold increase in apoptotic levels observed in elemental diet-induced jejunal hypotrophic rats to baseline values or below ($P < 0.001$). In the ileum, apoptosis levels were lower than those observed in the jejunum and the largest reduction was observed in the intestine of rats fed the high-protein diet. Ghrelin did not provide any further reduction in apoptotic levels in the ileum.

Ghrelin levels in the plasma of rats treated with this peptide averaged two to three times higher than the levels found in rats given either diet, but without the ghrelin treatment ($P < 0.0001$). Feeding rats with the elemental diet doubled the endogenous levels of ghrelin and these were maintained following dietary treatment, although at levels below the levels found with the exogenous administration of ghrelin ($P < 0.001$). A similar pattern was observed in fundic tissue ($P < 0.0001$), although levels for the ghrelin-treated rats were comparatively higher (2–3-fold) compared with non-treated rats ($P < 0.0001$). Finally, IGF-1 levels were reduced in rats fed the elemental diet, regaining levels close

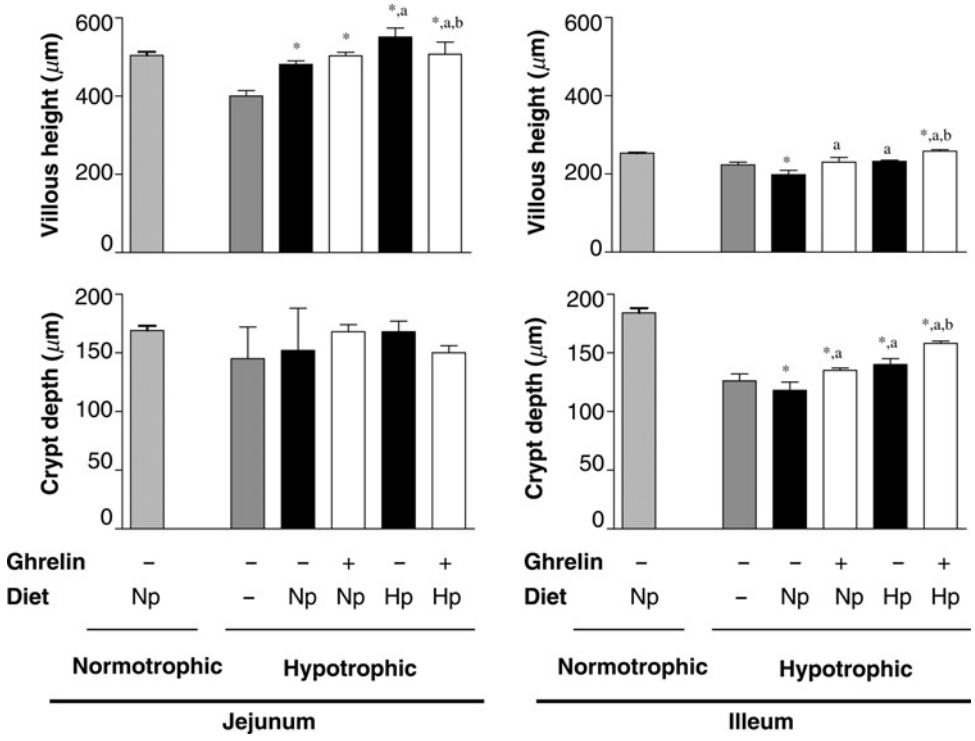

Figure 1 Intestinal mucosal morphometry. Morphometry of jejunal and ileal samples from rats with normal and elemental diet-induced mucosal hypotrophy fed with either a normoproteic (Np) or a hyperproteic (Hp) diet, and then treated with exogenous ghrelin (+) or saline (−). Data are expressed as mean ± SD. Significant differences: [a]versus saline-treated, normoproteic-fed group ($P < 0.05$, Fisher least-squares difference [LSD] test) and [b]versus saline-treated, hyperproteic-fed group ($P < 0.05$, Fisher LSD test); *versus hypotrophy baseline group ($P < 0.05$, Fisher LSD test)

to baseline in all groups given either diet with or without ghrelin administration. However, the highest levels of IGF-1 were found when both high-protein diet and ghrelin were given (Figure 4).

Discussion

Ghrelin administration promotes mucosal proliferation and reduces apoptosis in the elemental diet-induced hypotrophic intestine. Actually, the proliferative and apoptotic mucosal status suggest a differentiated effect of ghrelin on the proximal and distal gut; while apoptosis is clearly decreased in the jejunum, proliferation is promoted in the ileum. A major functional difference between the jejunum and ileum is the physiologically lower proliferative status of the latter, probably as a result of a reduced amount of the nutrients that are absorbed in the proximal gut. This might be considered a physiologically adaptive hypotrophy

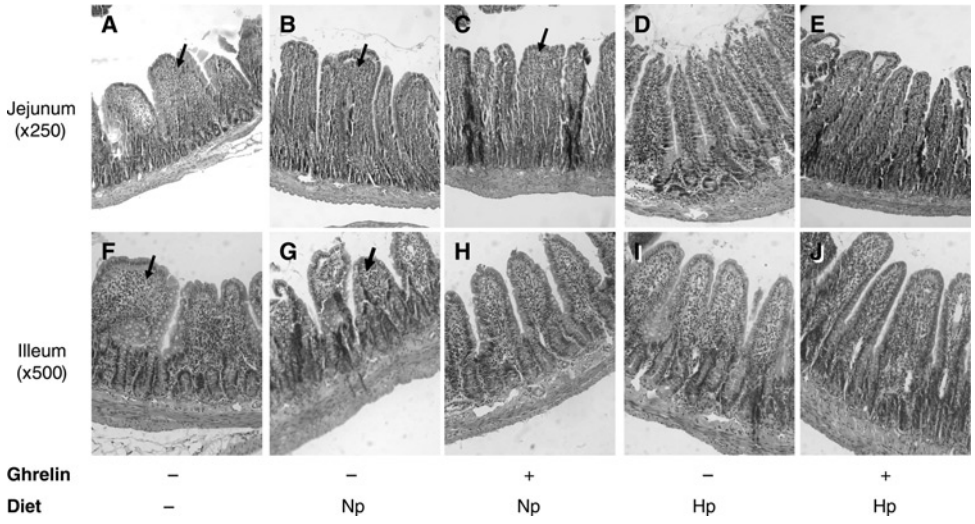

Figure 2 Microscopic sections of hypotrophic intestinal mucosa. Microscopic sections of the jejunum from rats with an elemental diet-induced hypotrophic intestinal mucosa (left) and fed either a normoproteic (Np) or a hyperproteic (Hp) diet, and then treated with exogenous ghrelin (+) or saline (−). Elemental diet-fed rats are indicated with elemental diet. Fusion of adjacent villi is indicated by arrows. Hematoxylin–eosin

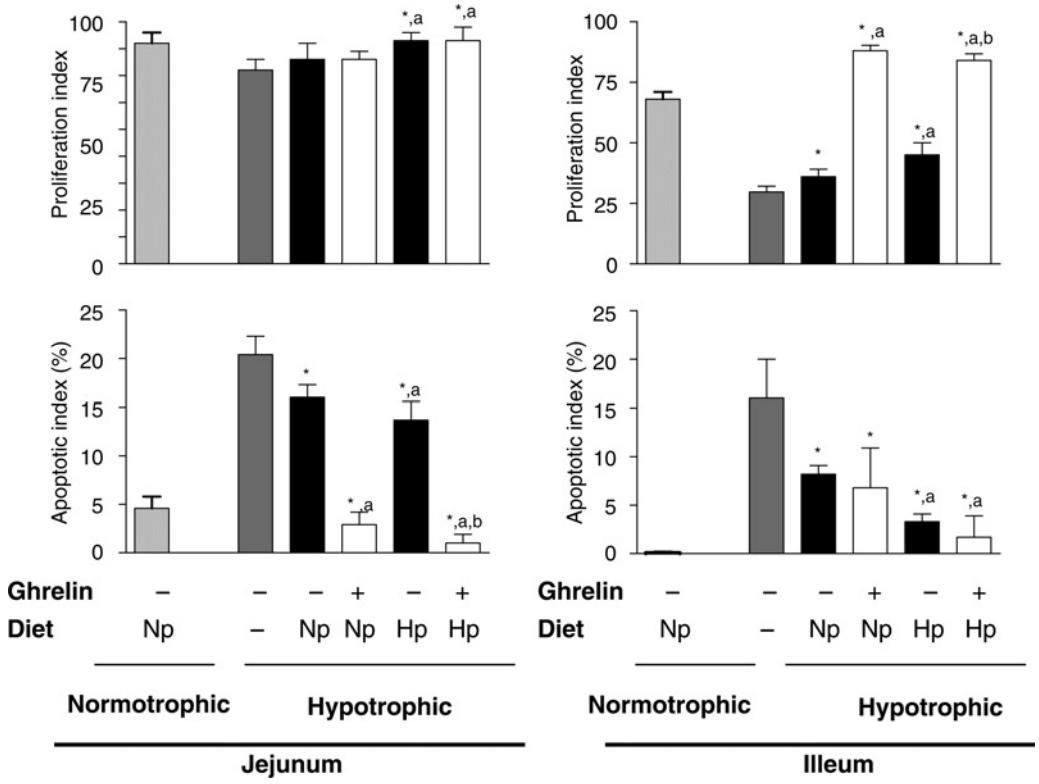

Figure 3 Effects of ghrelin in the hypotrophic intestine. Proliferative and apoptotic index of jejunal (left) and ileal (right) samples from rats with a normotrophic intestinal mucosa and fed either a normoproteic (Np) or a hyperproteic (Hp) diet, and then treated with exogenous ghrelin (+) or saline (−). Elemental diet-fed rats are indicated with elemental diet. Data are expressed as mean ± SD. Significant differences: [a]versus saline-treated, normoproteic-fed group ($P < 0.05$); [b]versus saline-treated, hyperproteic-fed group ($P < 0.05$, Fisher's least-squares difference [LSD] test); *versus hypotrophy baseline (ED) group ($P < 0.05$, Fisher's LSD test)

of the distal bowel corroborated by the fact that surgical transposition of the ileum to a proximal position promotes a higher trophic level.[30]

Nutrients and trophic factors are generally regarded as regulators of intestinal mucosal growth. Among nutrients, high-protein diets promote mucosal growth and recovery from injury, and when such diets are combined with trophic factors, mucosal growth is further improved.[22] Although mucosal morphometric changes were relatively small, as was the moderate hypotrophia induced by the elemental diet, in this study the hyperproteic diet improved mucosal morphometry associated with increased proliferation. However, this effect was observed in the proximal intestine, suggesting a direct effect for the high protein content in the jejunal mucosa. Nevertheless, there was a limited detectable effect of diet on apoptosis, which was mostly decreased as a consequence of ghrelin administration. In the distal bowel, an additive action of both the high protein content in the diet and ghrelin administration was seen on mucosal growth, suggesting a potent proliferative action for ghrelin and an antiapoptotic action of the high-protein diet. The differential effects observed on the proximal and distal intestines seem to promote a trend towards normal baseline proliferation and apoptotic levels, leading to normal mucosal growth. Ghrelin had a potent antiapoptotic action in the proximal intestine of rats fed on the elemental diet where apoptosis was 3–4 times above the baseline values. Similarly, this peptide doubled the proliferation index in the distal bowel. Overall, ghrelin might be considered as an intestinal regulator of the balance between proliferation and apoptosis.

Fasting induces apoptosis and decreases cell proliferation in the intestinal mucosa of rats, an effect prevented by ghrelin administration.[31] Feeding rats with an elemental diet produces a similar effect where the elemental diet model of hypotrophy is based on the lack of a sufficient supply of complex nutrients to the intestinal mucosa. Therefore, small, simple, readily absorbable molecules are given to provide an adequate caloric intake, although the absence of bulky material may induce increased apoptosis.[32] In our study, all rats were pair-fed receiving the same caloric intake during one week following the elemental diet. Therefore, the observed differences are unlikely to be a consequence of changes in caloric intake.

Several factors may have modified ghrelin secretion in the present work and may include the nutritional status, the feeding pattern[33] or exogenous ghrelin administration. Animals fed an elemental diet during the first week weighed 10–15% less than those receiving a standard diet *ad libitum*. This may explain the elevated ghrelin levels observed in rats with elemental diet-induced intestinal hypotrophia, although the caloric intake of the elemental diet given was >305 kcal/kg/d, which has been considered adequate to meet rat energy requirements. Anyway, the highest levels of ghrelin were observed in rats in which supraphysiological doses of this peptide were given daily.

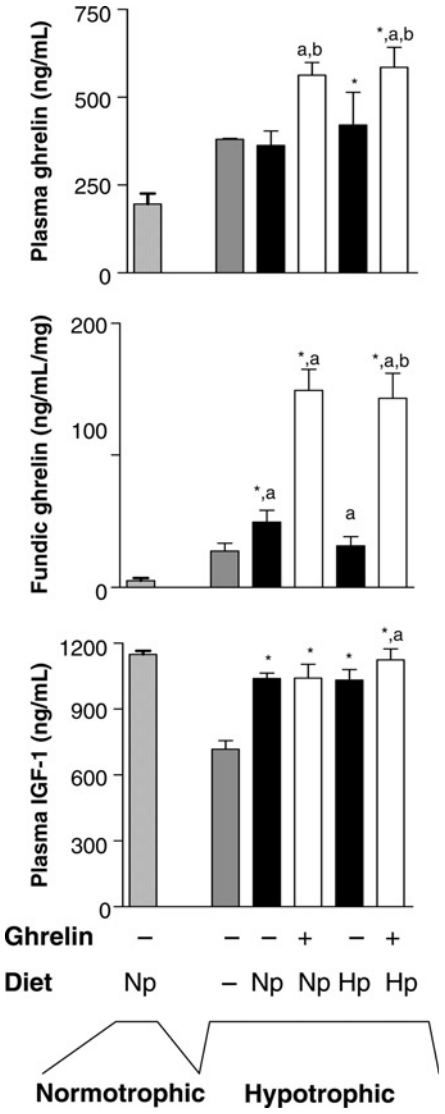

Figure 4 Ghrelin and insulin-like growth factor (IGF)-1 levels. Plasma (ghrelin and IGF-1) and fundic (ghrelin) tissue levels from rats with an elemental diet-induced (ED) mucosal hypotrophy fed with either a normoproteic (Np) or a hyperproteic (Hp) diet, and then treated with exogenous ghrelin (+) or saline (−). One further group was fed a standard diet (normotrophy; shaded bar). Data are expressed as mean ± SD. Significant differences: [a]versus saline-treated, normoproteic-fed group ($P < 0.05$, Fisher's least-squares difference [LSD] test); [b]versus saline-treated, hyperproteic-fed group ($P < 0.05$, Fisher's LSD test); *versus hypotrophy baseline (ED) group ($P < 0.05$, Fisher's LSD test)

Recent data suggest that ghrelin may act both indirectly, e.g. through the stimulation of GH- releasing hormone, as well as directly.[9,34] It has been suggested that exogenous ghrelin is involved in the mechanisms of gastric secretion, mucosal defense and gastroprotection,[29,35] although a ghrelin-regulated somatotrophic hormone action cannot be ruled out, since GH secretion is induced mainly by circulating ghrelin[9] and GH receptor is widely expressed throughout the intestinal mucosa. However, the relative lack of effect of exogenous ghrelin on IGF-1 plasma levels does not suggest a potent ghrelin stimulation of GH levels in hypotrophic intestines. Only a moderate increase in IGF-1 levels was observed in rats given both a high-protein diet and ghrelin, suggesting a synergistic action. Since ghrelin may have a trophic action that is independent of its GH-mediated action, this peptide may have a potential clinical use in conditions of intestinal hypotrophy such as total parenteral nutrition where a tissue-specific resistance to GH action is suggested.[36]

A differentiated action of ghrelin on the intestines might be a consequence of a variable expression of the ghrelin receptor where the nutritional status may be involved.[37] In fact, this may act as an adaptive mechanism governing intestinal growth and function. An increase in ghrelin receptor expression has been observed in rats with trinitrobenzene-induced mucosal damage, a condition that resembles Crohn's disease.[24] Ghrelin and its receptors were detected in immune system cells,[38] suggesting a role as an anti-inflammatory factor. However, some intestinal and non-intestinal inflammatory states induce reduced ghrelin levels,[39,40] whereas other intestinal diseases actually increase ghrelin levels, such as celiac and Crohn's diseases or ulcerative colitis.[24,41,42] It has been suggested that the inhibition of ghrelin secretion during inflammatory processes might potentiate the ongoing inflammatory insult.[24] Therefore, increased ghrelin levels, like those observed with an elemental diet, may reduce an inflammatory response by the gut.

In summary, the present study shows a trophic action produced by exogenous ghrelin on the hypotrophic intestinal mucosa, with selective regulatory actions on the proliferative and apoptotic status. However, further work should be carried out to determine the real physiological relevance of these findings.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. IAGS carried out the studies, data analyses and drafted the manuscript; MTV-C carried out the samples, data analyses and drafted the manuscript; MFS and MIC carried out the samples and data analyses; JL performed the statistical analysis and helped to draft the manuscript; CL carried out the animal model; and EDM conceived the study, co-ordination and helped to draft the manuscript.

ACKNOWLEDGEMENTS

This work is supported by the Spanish National Institute of Health, project FIS PI020724. MTV-C, MFS and JL are supported by a grant from the University Hospital LA PAZ research fund. MIC is supported by a grant from MAPFRE Medicina fund.

REFERENCES

1 Ziegler TR, Estivariz CF, Jonas CR, Gu LH, Jones DP, Leader LM. Interactions between nutrients and peptide growth factors in intestinal growth, repair, and function. *J Parenter Enteral Nutr* 1999;**23**:S174–83
2 Vallejo-Cremades MT, Gomez De Segura IA, Gomez-Garcia L, Perez-Vicente J, De Miguel E. A high-protein dietary treatment to intestinally hypotrophic rats induces ghrelin mRNA content and serum peptide level changes. *Clin Nutr* 2005;**24**:904–12

3 Raguso CA, Leverve X, Pichard C. Protective effects of recombinant growth hormone on intestinal mucosa in rats receiving abdominal radiotherapy. *Clin Nutr* 2002;**21**:487–90
4 Alpers DH. Enteral feeding and gut atrophy. *Curr Opin Clin Nutr Metab Care* 2002;**5**:679–83
5 Chappell VL, Thompson MD, Jeschke MG, Chung DH, Thompson JC, Wolf SE. Effects of incremental starvation on gut mucosa. *Dig Dis Sci* 2003;**48**:765–9
6 Yamamoto T, Nakahigashi M, Saniabadi AR. Review article: diet and inflammatory bowel disease – epidemiology and treatment. *Aliment Pharmacol Therap* 2009;**30**:99–112
7 Takahashi H, Ando T, Watanabe O, Maeda O, Ishiguro K, Ohmiya N, Niwa Y, Goto H. Usefulness of an elemental diet in Crohn's disease. *Inflammopharmacology* 2007;**15**:15–7
8 Baksheev L, Fuller PJ. Humoral factors in intestinal adaptation. *Trends Endocrinol Metabol* 2000;**11**:401–5
9 Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999;**402**:656–60
10 Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 2000;**141**:4255–61
11 Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJS, Dean DC, Melillo DG, Patchett AA, Nargund R, Griffin PR, DeMartino JA, Gupta SK, Schaeffer JM, Smith RG, Van der Ploeg LH. A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* 1996;**273**:974–7
12 Kojima M, Kangawa K. Ghrelin: structure and function. *Physiol Rev* 2005;**85**:495–522
13 Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* 2008;**132**:387–96
14 Kojima M, Hosoda H, Matsuo H, Kangawa K. Ghrelin: discovery of the natural endogenous ligand for the growth hormone secretagogue receptor. *Trends Endocrinol Metab* 2001;**12**:118–22
15 Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. *Nature* 2000;**407**:908–13
16 Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S. A role for ghrelin in the central regulation of feeding. *Nature* 2001;**409**:194–8
17 Tschop M, Wawarta R, Riepl RL, Friedrich S, Bidlingmaier M, Landgraf R, Folwaczny C. Post-prandial decrease of circulating human ghrelin levels. *J Endocrinol Invest* 2001;**24**:RC19–21
18 Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML. Circulating ghrelin levels are decreased in human obesity. *Diabetes* 2001;**50**:707–9
19 Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S, Shirakami G, Usui T, Shimatsu A, Doi K, Hosoda H, Kojima M, Kangawa K, Nakao K. Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *J Clin Endocrinol Metab* 2001;**86**:4753–8
20 Andreis PG, Malendowicz LK, Trejter M, Neri G, Spinazzi R, Rossi GP, Nussdorfer GG. Ghrelin and growth hormone secretagogue receptor are expressed in the rat adrenal cortex: evidence that ghrelin stimulates the growth, but not the secretory activity of adrenal cells. *FEBS Lett* 2003;**536**:173–9
21 Jeffery PL, Herington AC, Chopin LK. Expression and action of the growth hormone releasing peptide ghrelin and its receptor in prostate cancer cell lines. *J Endocrinol* 2002;**172**:R7–11
22 Fadrique B, Lopez JM, Bermudez R, Gomez De Segura IA, Vazquez I, De ME. Growth hormone plus high protein diet promotes adaptation after massive bowel resection in aged rats. *Exp Gerontol* 2001;**36**:1727–37
23 Krsek M, Rosicka M, Haluzik M, Svobodova J, Kotrlikova E, Justova V, Lacinova Z, Jarkovska Z. Plasma ghrelin levels in patients with short bowel syndrome. *Endocr Res* 2002;**28**:27–33
24 Gonzalez-Rey E, Chorny A, Delgado M. Therapeutic action of ghrelin in a mouse model of colitis. *Gastroenterology* 2006;**130**:1707–20
25 Beck B, Musse N, Stricker-Krongrad A. Ghrelin, macronutrient intake and dietary preferences in long-evans rats. *Biochem Biophys Res Commun* 2002;**292**:1031–5
26 Erdmann J, Lippl F, Schusdziarra V. Differential effect of protein and fat on plasma ghrelin levels in man. *Regul Pept* 2003;**116**:101–7
27 Vallejo-Cremades MT, Gomez-Garcia L, Chacatas-Cortesao M, Moreno C, Sanchez M, De Miguel E, Gomez De Segura IA. Enriched protein diet-modified ghrelin expression and secretion in rats. *Regul Pept* 2004;**121**:113–9
28 O'morain C. Elemental diets in the treatment of Crohns-disease. *Proc Nutr Soc* 1979;**38**:403–8
29 Brzozowski T, Konturek PC, Konturek SJ, Kwiecien S, Drozdowicz D, Bielanski W, Pajdo R, Ptak A, Nikiforuk A, Pawlik WW, Hahn EG. Exogenous and endogenous ghrelin in gastroprotection against stress-induced gastric damage. *Regul Pept* 2004;**120**:39–51
30 Gronqvist B, Engstrom B, Grimelius L. Morphological studies of the rat small intestine after jejuno–ileal transposition. *Acta Chir Scand* 1975;**141**:208–17
31 Park JM, Kakimoto T, Kuroki T, Shiraishi R, Fujise T, Iwakiri R, Fujimoto K. Suppression of intestinal mucosal apoptosis by ghrelin in fasting rats. *Exp Biol Med (Maywood)* 2008;**233**:48–56
32 Kakimoto T, Fujise T, Shiraishi R, Kuroki T, Park JM, Ootani A, Sakata Y, Tsunada S, Iwakiri R, Fujimoto K. Indigestible material attenuated changes in apoptosis in the fasted rat jejunal mucosa. *Exp Biol Med* 2008;**233**:310–6
33 Drazen DL, Vahl TP, D'Alessio DA, Seeley RJ, Woods SC. Effects of a fixed meal pattern on ghrelin secretion: evidence for a learned response independent of nutrient status. *Endocrinology* 2006;**147**:23–30
34 Fukuda H, Mizuta Y, Isomoto H, Takeshima F, Ohnita K, Ohba K, Omagari K, Taniyama K, Kohno S. Ghrelin enhances gastric motility through direct stimulation of intrinsic neural pathways and capsaicin-sensitive afferent neurones in rats. *Scand J Gastroenterol* 2004;**39**:1209–14
35 Sibilia V, Torsello A, Pagani F, Rapetti D, Lattuada N, Locatelli V, Bulgarelli I, Guidobono F, Netti C. Effects of hexarelin against acid-independent and acid-dependent ulcerogens in the rat. *Peptides* 2004;**25**:2163–70
36 Dahly EM, Miller ME, Lund PK, Ney DM. Postreceptor resistance to exogenous growth hormone exists in the jejunal mucosa of parenterally fed rats. *J Nutr* 2004;**134**:530–7
37 Tung YCL, Hewson AK, Carter RN, Dickson SL. Central responsiveness to a ghrelin mimetic (GHRP-6) is rapidly altered by acute changes in nutritional status in rats. *J Neuroendocrinol* 2005;**17**:387–93
38 Dixit VD, Schaffer EM, Pyle RS, Collins GD, Sakthivel SK, Palaniappan R, Lillard JW Jr, Taub DD. Ghrelin inhibits leptin- and activation-induced proinflammatory cytokine expression by human monocytes and T cells. *J Clin Invest* 2004;**114**:57–66
39 Hataya Y, Akamizu T, Hosoda H, Kanamoto N, Moriyama K, Kangawa K, Takaya K, Nakao K. Alterations of plasma ghrelin levels in rats with lipopolysaccharide-induced wasting syndrome and effects of ghrelin treatment on the syndrome. *Endocrinology* 2003;**144**:5365–71
40 Isomoto H, Ueno H, Saenko VA, Mondal MS, Nishi Y, Kawano N, Ohnita K, Mizuta Y, Ohtsuru A, Yamashita S, Nakazato M, Kohno S. Impact of *Helicobacter pylori* infection on gastric and plasma ghrelin dynamics in humans. *Am J Gastroenterol* 2005;**100**:1711–20
41 Peracchi M, Conte D, Terrani C, Pizzinelli S, Gebbia C, Cappiello V, Spada A, Bardella MT. Circulating ghrelin levels in celiac patients. *Am J Gastroenterol* 2003;**98**:2474–8
42 Sharma V, McNeill JH. The emerging roles of leptin and ghrelin in cardiovascular physiology and pathophysiology. *Curr Vasc Pharmacol* 2005;**3**:169–80

(Received August 7, 2009, Accepted November 13, 2009)