Increased plasma ghrelin suppresses insulin release in wethers fed with a high-protein diet

T Takahashi¹, K Sato¹, S Kato¹, T Yonezawa¹, Y Kobayashi¹, Y Ohtani¹, S Ohwada², H Aso², T Yamaguchi², S G Roh¹ and K Katoh¹

¹Laboratory of Animal Physiology and ²Laboratory of Functional Morphology, Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amemiyamachi, Aoba-ku, Sendai 981-8555, Japan

Correspondence should be addressed to T Takahashi

Email
takahac@vmas.kitasato-u.ac.jp

Abstract

Ghrelin is a multifunctional peptide that promotes an increase of food intake and stimulates GH secretion. Ghrelin secretion is regulated by nutritional status and nutrients. Although a high-protein (HP) diet increases plasma ghrelin secretion in mammals, the mechanisms and the roles of the elevated ghrelin concentrations due to a HP diet have not been fully established. To clarify the roles of elevated acylated ghrelin upon intake of a HP diet, we investigated the regulation of ghrelin concentrations in plasma and tissues in wethers fed with either the HP diet or the control (CNT) diet for 14 days, and examined the action of the elevated plasma ghrelin by using a ghrelin-receptor antagonist. The HP diet gradually increased the plasma acylated-ghrelin concentrations, but the CNT diet did not. Although the GH concentrations did not vary significantly across the groups, an injection of ghrelin-receptor antagonist enhanced insulin levels in circulation in the HP diet group. In the fundus region of the stomach, the ghrelin levels did not differ between the HP and CNT diet groups, whereas ghrelin O-acyltransferase mRNA levels were higher in the group fed with HP diet than those of the CNT diet group were. These results indicate that the HP diet elevated the plasma ghrelin levels by increasing its synthesis; this elevation strongly suppresses the appearance of insulin in the circulation of wethers, but it is not involved in GH secretion. Overall, our findings indicate a role of endogenous ghrelin action in secretion of insulin, which acts as a regulator after the consumption of a HP diet.

Key Words

- ▶ ghrelin
- ▶ insulin
- ▶ growth hormone
- ▶ protein diet
- ▶ antagonist

Journal of Endocrinology
(2014) 221, 371–380

Introduction

Ghrelin, an endogenous ligand for growth hormone secretagogue receptor (GHSR), is produced primarily by the stomach (Kojima *et al.* 1999, Ariyasu *et al.* 2001) as well as the intestine, and then released into circulation. Ghrelin is a 28-amino acid peptide and is n-octanoylated on Ser 3 by ghrelin O-acyltransferase (GOAT/MBOAT4) (Kojima *et al.* 1999, Yang *et al.* 2008). This unique modification of n-octanoylation is essential for binding and activation of the GHSR 1a (Bednarek *et al.* 2000);

injection of the acylated ghrelin either peripherally or centrally stimulates GH secretion and increases food intake and body weight in rodents (Tschöp *et al.* 2000, Nakazato *et al.* 2001, Wren *et al.* 2001).

In ruminants such as sheep, it has been reported that ghrelin has the same effect in terms of a stimulation of GH secretion as seen in monogastric animals (Hashizume *et al.* 2005, ThidarMyint *et al.* 2006). However, i.c.v. injection of ghrelin did not show any orexigenic effect in

sheep (Iqbal *et al.* 2006). In contrast, a high dose (1 mg/sheep) of peripheral ghrelin, administered by injection, promotes feeding behavior in sheep (Grouselle *et al.* 2008). In addition, ghrelin stimulates insulin secretion in Holstein steers (ThidarMyint *et al.* 2006) and enhances glucose-induced insulin secretion in meal-fed sheep (Takahashi *et al.* 2006). In mouse B cells, ghrelin attenuates glucose-induced insulin release via activation of Kv channels and suppression of Ca^{2+} (Dezaki *et al.* 2004). Thus, the roles of ghrelin are slightly different in rodents and ruminants.

Ghrelin secretion is reflected by nutritional status and is thought to play an important role as a meal initiator in mammals. Negative energy balance, such as fasting, stimulates ghrelin secretion (Tschöp *et al.* 2000), whereas increased ghrelin levels can be suppressed by re-feeding (Tschöp *et al.* 2001). Moreover, ghrelin secretion is regulated by nutrients themselves (e.g. carbohydrate, fat, and protein in the diet) (Erdmann *et al.* 2003, Monteleone *et al.* 2003, Erdmann *et al.* 2004, Greenman *et al.* 2004) as well as the nutritional status of ruminants and monogastric animals. Interestingly, ingestion of a high-protein (HP) diet has been shown to increase plasma ghrelin levels in sheep, humans, and rats (Erdmann *et al.* 2003, Vallejo-Cremades *et al.* 2004, Takahashi *et al.* 2012) and ghrelin synthesis in rats (Vallejo-Cremades *et al.* 2004), even though a HP diet decreases GH secretion in wethers (Takahashi *et al.* 2012), suppresses food intake in humans and rodents (Paddon-Jones *et al.* 2008, Journal *et al.* 2012), enhances satiation more than a carbohydrate or fat diet does in humans (Westerterp-Plantenga *et al.* 1999), and induces complex signals including neuropeptides and metabolic hormones secreted from the gut in response to nutrients, blood amino acids, and metabolites (Journal *et al.* 2012). Thus, the effects of elevations of ghrelin levels following ingestion of a HP diet are highly controversial and the detailed mechanism by which the protein diet stimulates ghrelin secretion is unclear. In addition, the changes in plasma ghrelin concentration could transform the action of endogenous ghrelin on insulin secretion in association with the nutritional status, since it has been reported that ghrelin has both an inhibitory and stimulatory action on glucose-induced insulin secretion after feeding and during starvation (Takahashi *et al.* 2006, 2007). Furthermore, ghrelin is unlikely to be an important factor for rhythmic or nutrition-mediated GH secretion (Avram *et al.* 2005). We have also shown that a HP diet caused the dissociation between ghrelin and GH in wethers (Takahashi *et al.* 2012), whereas there was a negative correlation between ghrelin and insulin

concentrations (T Takahashi, Y Kobayashi, S Haga, Y Ohtani, K Sato, Y Obara, A Hagino, S G Roh and K Katoh, unpublished observations, 2012). However, there is no direct evidence regarding whether the differences of endogenous acylated-ghrelin levels by diets regulate insulin and GH secretion, because previous studies were focused on studying the role of paraphysiological ghrelin levels and the effect of ghrelin on glucose-induced insulin secretion. On the basis of these studies, we propose the hypothesis that a HP diet increases ghrelin and GOAT synthesis, and that the elevated acylated-ghrelin concentrations regulate insulin secretion rather than GH secretion in wethers. The results support a crucial role for ghrelin in the downstream mediation of feeding-induced insulin secretion. The results also indicate that the control of ghrelin secretion by protein in the diet makes a useful contribution to productivities, such as increases in feed intake, body growth, and meat quality in ruminants.

Materials and methods

Animals and diets

All procedures were approved by the Animal Care and use committee of Tohoku University (GSAS-20-8). We chose crossbred Suffolk×Corriedale wethers as model animals for the production and management of large animals. They were purchased from the Field Science Center, Tohoku University, and were housed in individual pens and had free access to water and mineral salts throughout the experiment. Animals were fed with a HP diet, containing 40% crude protein or a control (CNT) diet with 10% crude protein, based on the results described in our previous report (Takahashi *et al.* 2012). The wethers were fed either the HP or CNT diet daily at 1000 h for 14 days. Both HP and CNT diets provided the same energy levels (metabolizable energy (ME); 0.73 MJ/BW^{0.75}), and were sufficient for maintenance according to Japanese feeding standards for sheep.

Procedure for GHSR antagonist experiment

To examine if the difference observed in the plasma level of acylated ghrelin between the HP and CNT diet groups influences the action of ghrelin, the GHSR antagonist, [D-Lys³]GHRP-6 was administered by injection to the animals and the concentrations of insulin, GH, and glucose were measured in the plasma samples. We used 1-year-old-wethers ($n=3-4$, 28.4 ± 1.4 kg) for the antagonist tests. In a semi-randomized crossover design, the animals were assigned to four different groups according to the diet

and antagonist treatment. Saline injections were always given before the administration of the antagonist; the diet was randomized between wethers. This led to the consideration of the following four groups: saline with the HP diet; antagonist with the HP diet; saline with the CNT diet; and antagonist with the CNT diet. Before any treatment, each group was fed with either the HP or CNT diet for 13 days. After acclimatization to the diet for 13 days, a polyethylene catheter was placed into the jugular vein for blood sampling. On day 14, blood samples were taken every 10 min from 0900 to 1300 h over a 4 h period. Either 100 nmol/kg [D-Lys³]GHRP-6 (Sigma-Aldrich; [Dezaki et al. 2004](#), [Takahashi et al. 2007](#)) or saline were injected into the vein through the catheter 10 min before feeding and 20 min after feeding, because the effect of this dose disappeared in 60 min in our preliminary experiment (data not shown). The wethers were maintained on the same diet, there was a 3- to 5-day interval between saline and antagonist injections. Between diets, there was a 14-day washout interval, and after these 14 days, each group received the other diet, either the HP or CNT, for the next 13 days, which they had not received previously.

Preparations of blood samples and tissue collections

One-year-old wethers were used for tissue analysis ($n=18$, 39.4 ± 1.2 kg). Preprandial blood samples were collected from the jugular vein at 0950 h until the day of tissue collection (days 0, 1, 4, 8, 9, 10, 11, 12, and 13). The samples were collected in individual tubes containing aprotinin (50 KIU/ml) and heparin (10 U/ml). The blood samples were centrifuged at 5000 *g* for 10 min at 4 °C ([Kobayashi et al. 2006](#)). After centrifugation, acetic buffer, which contained 1 M CH₃COOH and 20 mM HCl, was added to the plasma to be used for assaying acylated ghrelin, and was frozen at –80 °C until extraction. The plasma samples were stored at –80 °C until measurements. The largest sources of ghrelin are in the gastrointestinal tract and ghrelin regulates insulin secretion via GHSR 1a in the pancreas. In addition, ruminants possess a multiple-chambered stomach and their digestion system is very distinct from those of monogastric animals, such as humans and rodents. To better understand the reason why plasma acylated ghrelin levels increased due to the HP diet, various tissues were collected to analyze the acylated-ghrelin concentration and mRNA expression levels. The wethers were killed by decapitation under anesthesia, and the pituitary glands, in which GHSR is expressed and GH secretion is regulated via GHSR, were removed immediately. Also, the rumen, reticulum, omasum, abomasum,

duodenum, jejunum, ileum, colon, and pancreas were collected from them. All tissue samples were rapidly frozen in liquid nitrogen after collections and stored at –80 °C until protein and RNA extraction was performed.

RIA for GH, ghrelin, and insulin, and biochemical analysis

Plasma GH concentrations were determined by RIA. Standards and samples were incubated with ¹²⁵I-labeled ovine GH (10 000 c.p.m./100 µl) and baboon anti-bovine GH antiserum (NIDDK, #AFB55), which were diluted to 1:500 000 with assay buffer, for 48 h at 27 °C. After 48 h, 100 µl of secondary antibody (2% anti-monkey IgG and 7% PEG; gifted by Prof. Matozaki of Gunma University) was added to these samples and incubated for 24 h at 27 °C. Following incubation, the bound and free antigens were separated by centrifugation at 800 *g* for 30 min at 4 °C and radioactivity found in the pellets was measured by using a gamma counter. The detection limit was 0.2 ng/ml. The intra-assay coefficient of variation for the GH was <10%. Human acylated ghrelin (Peptide Institute, Inc., Osaka, Japan #4372-s) was iodinated by the Chloramine-T method and purified using HPLC ([Tai et al. 1975](#), [ThidarMyint et al. 2006](#)) and was used as a standard. Acylated-ghrelin antiserum (Alpha Diagnostic International, San Antonio, TX, USA, #GHS11-A) was used at a dilution of 1:3000. The detection limit was 0.03 ng/ml. The intra-assay coefficient of variation for the ghrelin was less than 12%. Plasma concentrations of insulin were quantified by dextran-coated charcoal RIA as described previously ([Herbert et al. 1965](#), [Takahashi et al. 2012](#)). The detection limit was 0.13 µU/ml. The intra-assay coefficient of variation for the insulin was less than 10%. The plasma glucose concentrations were measured using commercially available kit (Glucose C-II Test-Wako; Wako Pure Chemical Co., Tokyo, Japan). All samples in each analysis were analyzed in the same assay to exclude the inter-assay variations.

Preparation of tissue for RIA

To analyze acylated-ghrelin contents in each tissue, after measuring the wet weights of the tissue, the samples were heated for 5 min in a microwave to inactivate intrinsic proteases. The heated samples were then immediately immersed in a tenfold volume of the acetic buffer. Then, these samples were homogenized with a polytron mixer and were extracted using the plasma acylated-ghrelin extraction method ([Sugino et al. 2002](#)). Extracted samples were suspended in RIA buffer and stored at –80 °C until

the acylated-ghrelin assay was performed. The extraction efficiencies for ghrelin were over 95%.

RNA isolation and quantitative RT-PCR

Total RNA was extracted from pituitary, pancreas, and abomasum tissues using the TRIzol reagent (Invitrogen). cDNA was synthesized from 500 ng of total RNA as described previously (Yonezawa *et al.* 2008). The primers used for PCR are as follows: *GHRL*: forward 5'-CAG-CCTGCTCTGGATGGACTT-3', reverse 5'-CTCTGCACCT-TCTCCTGACT-3'; *GOAT/MBOAT4*: forward 5'-CGGGA-CAGGTGTTTGGTTTC-3', reverse 5'-CCATGGGAAAGACACTGCTG-3'; *GHSR*: forward 5'-GGCTGCTCACCATCATGGTGT-3', reverse 5'-ACAGCCAGCATCTTCACGGTT-3'. The sequence of the primers used for *GAPDH* amplification was synthesized as described previously (Yonezawa *et al.* 2008). Quantitative PCR was carried out using SYBR Premix Ex Taq (Takara Bio, Inc., Tokyo, Japan) on a DNA engine Opticon 2 Continuous Fluorescence Detector (MJ Research, Inc., Waltham, MA, USA). The protocol for qPCR was as follows: 35 cycles of 10 s at 95 °C (denaturation), 20 s at 58 °C (annealing), and 20 s at 72 °C (extension). After PCR, melting curves were used to confirm the specificity of the single-target amplification. All tests were carried out in triplicate and each gene was normalized to *GAPDH*. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method.

Immunohistochemistry

To ascertain whether the HP diet increases the number of ghrelin-producing cells and the length of mucosa, we used immunohistochemistry to visualize ghrelin-positive cells in the fundus region of the abomasum. The tissue from the fundus region of the abomasum was treated with Zamboni's fixative overnight at 4 °C. They were dehydrated in a graded series of ethanol (80, 90, 95, and 100%) for a day each and treated with toluene for 6 h. Then, they were treated with a solution of toluene and paraffin overnight. Following this, the tissues were embedded in paraffin. Tissues were sectioned using a microtome (3-μm thickness), mounted on slides, deparaffinized in xylene, and dehydrated in a graded series of ethanol. The deparaffinized sections were washed with water and treated with H₂O₂ for 20 min to block the endogenous peroxidase activity. The slides were then washed with PBS and incubated in 3.0% normal goat serum for 30 min blocking. The sections were incubated overnight at 4 °C with acylated-ghrelin antibody (a generous gift from Dr Kojima) at a dilution of 1:2 000 000. Subsequently,

the sections were washed with PBS and incubated with Simplestain MAX-PO (R) (Nichirei Biosciences, Inc., Tokyo, Japan) for 30 min at room temperature as a secondary antibody. Next, they were rinsed with PBS and reacted with 3,3'-diaminobenzidine and H₂O₂ (Nichirei), and counterstained with Mayer's hematoxylin. We analyzed the number of ghrelin-positive cells per area (mm²) and measured the length of mucosa using Scion Image (Scion Corporation, Frederick, MD, USA).

Statistical analysis

All data are presented as mean ± S.E.M., and statistical significance is defined as $P < 0.05$. Differences in all hormone and metabolite concentrations between the two diets were analyzed by repeated-measure ANOVA to test the effects of diet, time, and diet × time. When there was a significant interaction for diet × time, we compared each group value with a Student's or paired *t*-test at the same sampling time. The area under the hormonal response curves (AUC) for the 4 h (from pre- to postprandial response) was calculated by using a trapezoidal integration method and the AUC was analyzed using the Tukey–Kramer *post hoc* test. The statistical analysis for quantitative PCR data was performed using Student's *t*-test between the treatments.

Results

Changes in plasma acylated-ghrelin concentrations in wethers on a HP diet intake

There were no differences in food intake, body weight gain, or the animals' feeding preference between the two diets (data not shown). We investigated if the preprandial acylated-ghrelin concentration in plasma increases in wethers on an HP diet. As shown in Fig. 1, the preprandial acylated-ghrelin concentrations gradually increased in the HP group, but not in the CNT group (Diet × Day; ANOVA: $P < 0.05$). In particular, the plasma ghrelin levels were significantly higher in the HP than the CNT group on days 10, 11, and 13 (Fig. 1; $P = 0.027$, $P = 0.031$, and $P = 0.048$ respectively).

Effect of GHSR antagonist [D-Lys³]GHRP-6 on the plasma levels of insulin, GH, and glucose

Administration of the GHSR antagonist to HP-diet-fed wethers significantly increased insulin concentrations after food intake compared with the HP group that received only a saline injection (Fig. 2A: Diet × Time; ANOVA: $P < 0.05$), even though the basal concentrations of insulin were

Figure 1

The changes in preprandial ghrelin concentrations in wethers fed with either a high-protein diet (HP, open circles) or a control diet (CNT, filled squares) for 14 days. All values are represented as mean $\pm$ s.e.m. ($n=5$ in each group) and were analyzed by repeated measures ANOVA and Student's *t*-test. There was a significant diet $\times$ day interaction ($P<0.05$). * $P<0.05$ indicating significant differences between the HP and CNT diets.

similar across the groups. In contrast, the insulin levels in the CNT group did not show any significant change between antagonist and saline treatment (Fig. 2B: Diet $\times$ Time; ANOVA: $P>0.10$). In addition, the AUC for insulin was considerably higher in antagonist-treated wethers on the HP diet than those for the other groups (Fig. 2C). GH concentrations in all four groups decreased with food intake, and further GH levels did not show any difference between the HP and CNT groups (data not shown). Glucose levels did not change in the saline- and antagonist-treated HP group (data not shown). In the CNT group, glucose levels of the wethers treated with GHSR antagonist also did not show any difference between the antagonist and the saline treatment (data not shown). We did not observe any change in acylated-ghrelin concentration in the antagonist and saline-treated HP or CNT groups (data not shown).

Protein and mRNA levels

In the abomasum fundus region, in which ghrelin is mainly produced, the acylated-ghrelin levels did not show any differences between the HP and the CNT groups (Fig. 3A; $P=0.17$). In contrast, the acylated-ghrelin levels in the pancreas and ileum of wethers fed with the HP diet were higher than those observed in wethers fed with the CNT diet (Fig. 3B; $P=0.045$ and $P=0.098$ respectively). On the basis of results for the acylated-ghrelin levels in abomasum fundus and the pancreas, *ghrelin* expression levels were measured using qPCR. There was a twofold increase in the *ghrelin* expression in the abomasum of the

HP group compared with the CNT group (Fig. 4A; $P=0.024$), whereas the mRNA levels of *ghrelin* in the pancreas were unaltered (data not shown). Ghrelin *O*-acyltransferase (GOAT) is an essential enzyme required for

Figure 2

Effect of saline (filled diamonds) or GHSR antagonist (open triangles) on plasma insulin concentration following (A) a high-protein diet (HP) or (B) a control diet (CNT). (C) Area under the curve (AUC) for insulin. GHSR antagonist or saline was injected into the vein through a catheter 10 min before feeding and 20 min after feeding of either the HP or CNT diet respectively. All values represent mean $\pm$ s.e.m. ($n=3-5$ in each group) and were analyzed by repeated measures ANOVA and Student's *t*-test. There was a significant diet $\times$ time interaction for the HP group only ($P<0.05$). For the AUC, the data were analyzed using the Tukey-Kramer test. * $P<0.05$; ** $P<0.01$ indicate significant differences between the saline and GHSR antagonist treatments. The letters above the bars (a and b) indicate the differences between the various treatment that were statistically significant ($P<0.05$) according to the Tukey-Kramer test.

Figure 3

Ghrelin concentrations in the abomasum fundus (A) and in the various tissue samples (B) from wethers that were fed with a high-protein diet (HP, white bars) or a control diet (CNT, black bars). All values are represented as mean $\pm$ S.E.M. ($n=5-6$ in each group); data were analyzed by Student's *t*-test. * $P < 0.05$ for the HP vs CNT.

binding octanoate to proghrelin, and is expressed in tissues that produce ghrelin. Therefore, *GOAT* expression levels are also elevated by the HP diet. As expected, the mRNA levels of *GOAT* in the abomasum of the HP diet group were dramatically higher than those in the CNT group (Fig. 4B; 5.1-fold increase; $P=0.017$). We next determined whether the elevation of plasma acylated ghrelin following the HP diet reflects changes in the *GHSR 1a* mRNA expression levels. The mRNA levels of *GHSR 1a* were to a certain extent reduced in the pituitary and pancreas of the HP group, this difference, however, was not statistically significant (data not shown).

Immunohistochemistry

Specific ghrelin immune-positive staining was observed in both HP and CNT diet groups, and strong immunostaining was mainly seen at the bottom of gastric mucosa. There was no significant difference in the number of

ghrelin-producing cells and the length of mucosa between the HP and the CNT groups (data not shown).

Discussion

We found that a HP diet elevates plasma acylated-ghrelin concentrations, which strongly suppresses the appearance of insulin in circulation via GHSR in wethers. Ghrelin, primarily produced and acylated by *GOAT*/*MBOAT4* in X/A-like cells of stomach, acts via GHSR 1a on the pituitary and the hypothalamus to stimulate the release of GH, inducing food intake and adiposity (Kojima *et al.* 1999, Tschöp *et al.* 2000, Nakazato *et al.* 2001, Wren *et al.* 2001, Yang *et al.* 2008). Our results indicated that the protein diet gradually raised the plasma acylated-ghrelin concentrations and that the levels were significantly higher in wethers on the HP diet than in those on the CNT diet from day 11. Earlier studies have shown that during starvation, the ghrelin levels in stomach decrease and the *ghrelin* mRNA expression levels in stomach increase (Sato *et al.* 2005), which is why it is assumed that an increase in active ghrelin in plasma is derived from cytoplasmic ghrelin released from the gastric ghrelin cells. Although we did not observe significant decreases in ghrelin contents of the stomach in the HP group, the elevated plasma ghrelin levels due to the HP diet might be caused through the release of ghrelin from stomach as indicated by the increase in *ghrelin* and *GOAT* mRNA expression. The increases in both mRNA expressions might be involved in the protein diet- and/or microbes-derived amino acids, because amino acids are required for synthesis of proteins, such as hormones and enzymes, and continuous i.v. infusion of mixed amino acids raises plasma ghrelin concentrations in sheep (Sugino *et al.* 2010). These

Figure 4

Ghrelin (A) and *ghrelin O-acyltransferase (GOAT)* (B) mRNA levels in the abomasum fundus of wethers that were fed with a high-protein diet (HP, white bars) or a control diet (CNT, black bars). All values are represented as mean $\pm$ S.E.M. ($n=5$ in each group); data were analyzed by Student's *t*-test. * $P < 0.05$ indicating significant differences between the HP and CNT groups.

observations provide evidence that a HP diet is able to act at the abomasum fundus level to control ghrelin synthesis and release, but it does not affect the signaling pathway involved in the regulation of the release of stored ghrelin. However, we cannot rule out the possibility that the HP diet stimulates ghrelin secretion from the duodenum. Previous reports indicate that because ghrelin is also produced in the duodenum – the second richest source of ghrelin (Date *et al.* 2000a, Ariyasu *et al.* 2001) – increased serum ghrelin concentrations are associated with the trophic effects on the intestine of an enriched protein diet (Vallejo-Cremades *et al.* 2005). Interestingly, ghrelin was detected in the rumen, reticulum, and omasum, even though it is mainly secreted from the abomasum. This finding is consistent with previous results that showed the distribution of *ghrelin* mRNA expression (Huang *et al.* 2006, Wang *et al.* 2014). However, in this study, they do not contribute to the elevation of ghrelin concentration by the HP diet, because their levels were very low compared with the abomasum and duodenum. Thus, further research is required to identify the roles and specific cell types in these tissues. As for the effects of other possible nutrients, high-carbohydrate and high-fat diets might regulate plasma ghrelin levels, since ingestion of concentrate diet suppresses postprandial ghrelin levels to a greater extent than a roughage diet intake in wethers (Takahashi *et al.* 2008), and the consumption of a high-fat diet led to a decrease in active ghrelin levels in mice (Briggs *et al.* 2010).

Feeding the wethers with an HP diet for 14 days increased the pancreatic ghrelin levels. Both ghrelin and GHSR are expressed and located in the pancreas (Ueberberg *et al.* 2009), and ghrelin is also released from pancreas (Dezaki *et al.* 2004). This indicates that the protein diet mainly elicits ghrelin secretion through these events in the stomach; however, ghrelin from duodenum and pancreatic tissues may act on its own in an autocrine/paracrine manner.

Our data showing the inhibitory action of ghrelin on insulin secretion are consistent with previous reports on similar studies in humans, rodents, and sheep. *In vitro*, ghrelin suppresses glucose-induced insulin secretion in a dose-dependent manner in isolated pancreatic islets (Egido *et al.* 2002, Colombo *et al.* 2003, Dezaki *et al.* 2004) and INS-1 cell lines (Wierup *et al.* 2004), indicating that in the pancreas, ghrelin may directly act on the B cells as a paracrine or autocrine factor and thereby regulate insulin secretion. Both *in vitro* and *in vivo* studies have shown that ghrelin inhibits glucose-induced insulin secretion in humans, rodents, and in starved sheep

(Reimer *et al.* 2003, Takahashi *et al.* 2007, Tong *et al.* 2010). For these reasons, it is believed that increased ghrelin levels in the pancreas and in blood directly suppress insulin secretion via GHSR 1a. However, it is also known that ghrelin enhanced glucose-induced insulin secretion in meal-fed sheep, and that an i.v. infusion of high-dose ghrelin after feeding stimulates insulin secretion in heifers (Takahashi *et al.* 2006, ThidarMyint *et al.* 2006). As these experiments have been carried out under postprandial conditions, the action of ghrelin on insulin secretion might be dependent on the changes in nutritional conditions.

In addition to the above reports, there is some evidence to indicate that ghrelin may act indirectly on insulin secretion. In rats, ghrelin inhibits glucose-induced insulin secretion when it was infused into the portal vein, but not into the femoral vein (Cui *et al.* 2008). Hepatic vagotomy or coinfusion with both atropine and ghrelin into the portal vein diminishes the inhibitory effect of ghrelin on glucose-induced insulin secretion. Thus, ghrelin indirectly inhibits glucose-induced insulin secretion via the hepatic portal system and the vagus nerve. Furthermore, when unacylated ghrelin (UAG) was injected into rats, the UAG enhanced the increase in insulin concentrations in the portal and peripheral circulation (Gauna *et al.* 2007). This effect was completely blocked by the coadministration of acylated ghrelin. GHSR 1a antagonist, [D-Lys³]GHRP-6, alone or in combination with acylated ghrelin and UAG, strongly enhanced the portal insulin response. Although we did not measure the UAG concentrations, the HP diet might elevate the UAG concentrations as well as acylated ghrelin. Therefore, the antagonist might remove the inhibitory effect of acylated ghrelin on insulin secretion and the elevated UAG might then stimulate insulin secretion by pancreatic islets.

The plasma GH concentrations did not show any differences, even though ghrelin strongly stimulates GH secretion *in vivo* and *in vitro* in humans, rodents, and ruminants and the HP diet increased the plasma ghrelin concentrations. There were no effects on GH secretion and body length of ghrelin-knockout mice (Wortley *et al.* 2004). Similarly, ghrelin is not involved in rhythmic or nutritionally mediated GH secretion (Avram *et al.* 2005). In addition, we have previously reported that the circadian changes in the plasma ghrelin concentrations were similar to the variations in the GH concentrations in a concentrated diet, but this was not the case for a timothy hay diet (Takahashi *et al.* 2008). Taken together, these reports and our result indicate that endogenous ghrelin is

unlikely to be important for GH secretion after feeding. However, chronic ghrelin administration decreases GH secretion (Date *et al.* 2000b), indicating that high concentrations in the circulation might cause a resistance similar to that for leptin secretion by high-fat diets. In fact, in the fasting state, the i.v. injection of ghrelin obviously does not stimulate GH secretion compared with satiety state in sheep, even though its injection dose is comparatively high (6.6 µg/kg BW; Takahashi *et al.* 2009). From these reports, we cannot rule out the possibility that GH did not display any differences between the diet groups because of the ghrelin resistance caused by the HP diet.

Furthermore, the ghrelin antagonist that was used in this study might be involved in the regulation of insulin and GH secretion. The reasons may be understood as follows: [D-Lys³]GHRP-6 is nonselective GHSR 1a antagonist in rodents (Schiöth *et al.* 1997, Depoortere *et al.* 2006) and is known to act as an agonist to the serotonin receptor 5-HT_{2b} (Depoortere *et al.* 2006) which regulates pancreatic B cell proliferation (Kim *et al.* 2010). [D-Lys³]GHRP-6 also binds to all the four melanocortin receptors which are involved in alterations of insulin levels (Fan *et al.* 2000), ghrelin-induced food intake, and GH release in mice (Shaw *et al.* 2005). Moreover, there has been no report looking at the degree of [D-Lys³]GHRP-6 binding affinity to GHSR 1a in sheep and other ruminants. Thus, further studies are needed to determine the effect of GHSR antagonist itself.

In conclusion, our study demonstrates that a high-protein diet elevated ghrelin concentrations in plasma through the release of newly synthesized ghrelin with no changes to stored ghrelin in abomasum fundus. Ghrelin regulates secretion of insulin on feeding, rather than GH secretion, in sheep. Suppressing insulin secretion by elevated ghrelin following HP diet intake is proposed to be beneficial for preventing damaging overproduction of insulin in B cells after feeding.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was partly supported by a grant-in-aid from the Japan Society for the Promotion of Science (JSPS).

Author contribution statement

The authors have made the following declarations about their contributions. The experiments were conceived and designed by: T Y, Y K, T T,

H A, and K K. The experiments were performed by T T, K S, S K, Y O, and S O. Essential reagents were provided by S O, H A, and T Y. The data were analyzed by T T. The paper was written by T T and S G R.

Acknowledgements

The authors are very grateful to Prof. T Matozaki (Molecular and Cellular Regulation, Gunma University) and Prof. M Kojima (Molecular Genetics, Institute of Life Science, Kurume University) for providing the antibody for the GH and ghrelin assay respectively. They also appreciate the technical support of H Kamiyama. The technical advice of Prof. H Kuwayama and Ms Than Than Sint (Department of Animal Science, Obihiro University of Agriculture and Veterinary Medicine) regarding the ghrelin RIA assay is also gratefully acknowledged.

References

- Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S *et al.* 2001 Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *Journal of Clinical Endocrinology and Metabolism* **86** 4753–4758. (doi:10.1210/jcem.86.10.7885)
- Avram AM, Jaffe CA, Symons KV & Barkan AL 2005 Endogenous circulating ghrelin does not mediate growth hormone rhythmicity or response to fasting. *Journal of Clinical Endocrinology and Metabolism* **90** 2982–2987. (doi:10.1210/jc.2004-1785)
- Bednarek MA, Feighner SD, Pong SS, McKee KK, Hreniuk DL, Silva MV, Warren VA, Howard AD, Van Der Ploeg LH & Heck JV 2000 Structure–function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. *Journal of Medicinal Chemistry* **43** 4370–4376. (doi:10.1021/jm0001727)
- Briggs DI, Enriori PJ, Lemus MB, Cowley MA & Andrews ZB 2010 Diet-induced obesity causes ghrelin resistance in arcuate NPY/AgRP neurons. *Endocrinology* **151** 4745–4755. (doi:10.1210/en.2010-0556)
- Colombo M, Gregersen S, Xiao J & Hermansen K 2003 Effects of ghrelin and other neuropeptides (CART, MCH, orexin A and B, and GLP-1) on the release of insulin from isolated rat islets. *Pancreas* **27** 161–166. (doi:10.1097/00006676-200308000-00009)
- Cui C, Ohnuma H, Daimon M, Susa S, Yamaguchi H, Kameda W, Jimbu Y, Oizumi T & Kato T 2008 Ghrelin infused into the portal vein inhibits glucose-stimulated insulin secretion in Wistar rats. *Peptides* **29** 1241–1246. (doi:10.1016/j.peptides.2008.02.022)
- Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K & Nakazato M 2000a 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* **141** 4255–4261. (doi:10.1210/endo.141.11.7757)
- Date Y, Murakami N, Kojima M, Kuroiwa T, Matsukura S, Kangawa K & Nakazato M 2000b Central effects of a novel acylated peptide, ghrelin, on growth hormone release in rats. *Biochemical and Biophysical Research Communications* **275** 477–480. (doi:10.1006/bbrc.2000.3342)
- Depoortere I, Thijs T & Peeters T 2006 The contractile effect of the ghrelin receptor antagonist, D-Lys3-GHRP-6, in rat fundic strips is mediated through 5-HT receptors. *European Journal of Pharmacology* **537** 160–165. (doi:10.1016/j.ejphar.2006.03.043)
- Dezaki K, Hosoda H, Kakei M, Hashiguchi S, Watanabe M, Kangawa K & Yada T 2004 Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating Ca²⁺ signaling in β-cells: implication in the glycemic control in rodents. *Diabetes* **53** 3142–3151. (doi:10.2337/diabetes.53.12.3142)
- Egido EM, Rodriguez-Gallardo J, Silvestre RA & Marco J 2002 Inhibitory effect of ghrelin on insulin and pancreatic somatostatin secretion.

- European Journal of Endocrinology* **146** 241–244. (doi:10.1530/eje.0.1460241)
- Erdmann J, Lippel F & Schusdziaira V 2003 Differential effect of protein and fat on plasma ghrelin levels in man. *Regulatory Peptides* **116** 101–107. (doi:10.1016/S0167-0115(03)00195-2)
- Erdmann J, Töpsch R, Lippel F, Gussmann P & Schusdziaira V 2004 Postprandial response of plasma ghrelin levels to various test meals in relation to food intake, plasma insulin, and glucose. *Journal of Clinical Endocrinology and Metabolism* **89** 3048–3054. (doi:10.1210/jc.2003-031610)
- Fan W, Dinulescu DM, Butler AA, Zhou J, Marks DL & Cone RD 2000 The central melanocortin system can directly regulate serum insulin levels. *Endocrinology* **141** 3072–3079. (doi:10.1210/endo.141.9.7665)
- Gauna C, Kiewiet RM, Janssen JA, van de Zande B, Delhanty PJ, Ghigo E, Hofland LJ, Themmen AP & van der Lely AJ 2007 Unacylated ghrelin acts as a potent insulin secretagogue in glucose-stimulated conditions. *American Journal of Physiology. Endocrinology and Metabolism* **293** E697–E704. (doi:10.1152/ajpendo.00219.2007)
- Greenman Y, Golani N, Gilad S, Yaron M, Limor R & Stern N 2004 Ghrelin secretion is modulated in a nutrient- and gender-specific manner. *Clinical Endocrinology* **60** 382–388. (doi:10.1111/j.1365-2265.2004.01993.x)
- Grouselle D, Chaillou E, Caraty A, Bluet-Pajot MT, Zizzari P, Tillet Y & Epelbaum J 2008 Pulsatile cerebrospinal fluid and plasma ghrelin in relation to growth hormone secretion and food intake in the sheep. *Journal of Neuroendocrinology* **20** 1138–1146. (doi:10.1111/j.1365-2826.2008.01770.x)
- Hashizume T, Horiuchi M, Nonaka S, Kasuya E, Kojima M, Hosoda H & Kangawa K 2005 Effects of ghrelin on growth hormone secretion *in vivo* in ruminants. *Regulatory Peptides* **126** 61–65. (doi:10.1016/j.regpep.2004.08.010)
- Herbert V, Lau KS, Gottlieb CW & Bleicher SJ 1965 Coated charcoal immunoassay of insulin. *Journal of Clinical Endocrinology and Metabolism* **25** 1375–1384. (doi:10.1210/jcem-25-10-1375)
- Huang ZG, Xiong L, Liu ZS, Qiao Y, Dai R, Xie Z, Liu SR, Shi GQ & Liu GQ 2006 The tissue distribution and developmental changes of ghrelin mRNA expression in sheep. *Yi Chuan Xue Bao* **33** 808–813. (doi:10.1016/S0379-4172(06)60114-5)
- Iqbal J, Kurose Y, Canny B & Clarke IJ 2006 Effects of central infusion of ghrelin on food intake and plasma levels of growth hormone, luteinizing hormone, prolactin, and cortisol secretion in sheep. *Endocrinology* **147** 510–519. (doi:10.1210/en.2005-1048)
- Journel M, Chaumontet C, Darcel N, Fromentin G & Tomé D 2012 Brain responses to high-protein diets. *Advances in Nutrition* **3** 322–329. (doi:10.3945/an.112.002071)
- Kim H, Toyofuku Y, Lynn FC, Chak E, Uchida T, Mizukami H, Fujitani Y, Kawamori R, Miyatsuka T, Kosaka Y *et al.* 2010 Serotonin regulates pancreatic β cell mass during pregnancy. *Nature Medicine* **16** 804–808. (doi:10.1038/nm.2173)
- Kobayashi Y, Onodera M, Yoshida M, Sugino T, Katoh K & Obara Y 2006 Postprandial changes in plasma concentrations of growth hormone and ghrelin around weaning in the goat. *General and Comparative Endocrinology* **148** 368–374. (doi:10.1016/j.ygcen.2006.04.009)
- Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H & Kangawa K 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402** 656–660. (doi:10.1038/45230)
- Monteleone P, Bencivenga R, Longobardi N, Serritella C & Maj M 2003 Differential responses of circulating ghrelin to high-fat or high-carbohydrate meal in healthy women. *Journal of Clinical Endocrinology and Metabolism* **88** 5510–5514. (doi:10.1210/jc.2003-030797)
- Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K & Matsukura S 2001 A role for ghrelin in the central regulation of feeding. *Nature* **409** 194–198. (doi:10.1038/35051587)
- Paddon-Jones D, Westman E, Mattes RD, Wolfe RR, Astrup A & Westerterp-Plantenga M 2008 Protein, weight management, and satiety. *American Journal of Clinical Nutrition* **87** 1558S–1561S.
- Reimer MK, Pacini G & Åhrén B 2003 Dose-dependent inhibition by ghrelin of insulin secretion in the mouse. *Endocrinology* **144** 916–921. (doi:10.1210/en.2002-220819)
- Sato T, Fukue Y, Teranishi H, Yoshida Y & Kojima M 2005 Molecular forms of hypothalamic ghrelin and its regulation by fasting and 2-deoxy-D-glucose administration. *Endocrinology* **146** 2510–2516. (doi:10.1210/en.2005-0174)
- Schiöth HB, Muceniec R & Wikberg JE 1997 Characterization of the binding of MSH-B, HB-228, GHRP-6 and 153N-6 to the human melanocortin receptor subtypes. *Neuropeptides* **31** 565–571. (doi:10.1016/S0143-4179(97)90002-0)
- Shaw AM, Irani BG, Moore MC, Haskell-Luevano C & Millard WJ 2005 Ghrelin-induced food intake and growth hormone secretion are altered in melanocortin 3 and 4 receptor knockout mice. *Peptides* **26** 1720–1727. (doi:10.1016/j.peptides.2004.12.026)
- Sugino T, Hasegawa Y, Kikkawa Y, Yamaura J, Yamagishi M, Kurose Y, Kojima M, Kangawa K & Terashima Y 2002 A transient ghrelin surge occurs just before feeding in a scheduled meal-fed sheep. *Biochemical and Biophysical Research Communications* **295** 255–260. (doi:10.1016/S0006-291X(02)00654-X)
- Sugino T, Kawakita Y, Fukumori R, Hasegawa Y, Kojima M, Kangawa K, Obitsu T & Taniguchi K 2010 Effects of glucose and amino acids on ghrelin secretion in sheep. *Animal Science Journal* **81** 199–204. (doi:10.1111/j.1740-0929.2009.00733.x)
- Tai HH, Korsch B & Chey WY 1975 Preparation of 125 I-labeled secretin of high specific radioactivity. *Analytical Biochemistry* **69** 34–42. (doi:10.1016/0003-2697(75)90562-X)
- Takahashi H, Kurose Y, Kobayashi S, Sugino T, Kojima M, Kangawa K, Hasegawa Y & Terashima Y 2006 Ghrelin enhances glucose-induced insulin secretion in scheduled meal-fed sheep. *Journal of Endocrinology* **189** 67–75. (doi:10.1677/joe.1.06310)
- Takahashi H, Kurose Y, Sakaida M, Suzuki Y, Kobayashi S, Sugino T, Kojima M, Kangawa K, Hasegawa Y & Terashima Y 2007 Ghrelin differentially modulates glucose-induced insulin secretion according to feeding status in sheep. *Journal of Endocrinology* **194** 621–625. (doi:10.1677/JOE-07-0206)
- Takahashi T, Kobayashi Y, Hasegawa S, Touno E, Otani Y, Haga S, Itoh F, Katoh K & Obara Y 2008 Different responses in postprandial plasma ghrelin and GH levels induced by concentrate or timothy hay feeding in wethers. *Domestic Animal Endocrinology* **34** 432–439. (doi:10.1016/j.domaniend.2007.12.003)
- Takahashi H, Kurose Y, Suzuki Y, Kojima M, Yamaguchi T, Yoshida Y, Ogino M, Hodate K, Azuma Y, Sugino T *et al.* 2009 Ghrelin differentially modulates the GH secretory response to GHRH between the fed and fasted states in sheep. *Domestic Animal Endocrinology* **37** 55–60. (doi:10.1016/j.domaniend.2009.02.003)
- Takahashi T, Kobayashi Y, Haga S, Ohtani Y, Sato K, Obara Y, Hagino A, Roh SG & Katoh K 2012 A high-protein diet induces dissociation between plasma concentrations of growth hormone and ghrelin in wethers. *Journal of Animal Science* **90** 4807–4813. (doi:10.2527/jas.2011-4596)
- ThidarMyint H, Yoshida H, Ito T & Kuwayama H 2006 Dose-dependent response of plasma ghrelin and growth hormone concentrations to bovine ghrelin in Holstein heifers. *Journal of Endocrinology* **189** 655–664. (doi:10.1677/joe.1.06746)
- Tong J, Prigeon RL, Davis HW, Bidlingmaier M, Kahn SE, Cummings DE, Tschöp MH & D'Alessio D 2010 Ghrelin suppresses glucose-stimulated insulin secretion and deteriorates glucose tolerance in healthy humans. *Diabetes* **59** 2145–2151. (doi:10.2337/db10-0504)
- Tschöp M, Smiley DL & Heiman ML 2000 Ghrelin induces adiposity in rodents. *Nature* **407** 908–913. (doi:10.1038/35038090)
- Tschöp M, Wawarta R, Riepl RL, Friedrich S, Bidlingmaier M, Landgraf R & Folwaczny C 2001 Post-prandial decrease of circulating human ghrelin levels. *Journal of Endocrinological Investigation* **24** RC19–RC21.

- Ueberberg B, Unger N, Saeger W, Mann K & Petersenn S 2009 Expression of ghrelin and its receptor in human tissues. *Hormone and Metabolic Research* **41** 814–821. (doi:10.1055/s-0029-1233462)
- Vallejo-Cremades MT, Gómez-García L, Chacatas-Cortesao M, Moreno C, Sánchez M, De Miguel E & Gómez De Segura IA 2004 Enriched protein diet-modified ghrelin expression and secretion in rats. *Regulatory Peptides* **121** 113–119. (doi:10.1016/j.regpep.2004.04.016)
- Vallejo-Cremades MT, Gómez de Segura IA, Gómez-García L, Pérez-Vicente J & De Miguel E 2005 A high-protein dietary treatment to intestinally hypotrophic rats induces ghrelin mRNA content and serum peptide level changes. *Clinical Nutrition* **24** 904–912. (doi:10.1016/j.clnu.2005.05.018)
- Wang W, Cheng L, Guo J, Ma Y & Li F 2014 Expression of Ghrelin in gastrointestinal tract and the effect of early weaning on Ghrelin expression in lambs. *Molecular Biology Reports* **41** 909–914. (doi:10.1007/s11033-013-2935-2)
- Westerterp-Plantenga MS, Rolland V, Wilson SA & Westerterp KR 1999 Satiety related to 24 h diet-induced thermogenesis during high protein/carbohydrate vs high fat diets measured in a respiration chamber. *European Journal of Clinical Nutrition* **53** 495–502. (doi:10.1038/sj.ejcn.1600782)
- Wierup N, Yang S, McEvilly RJ, Mulder H & Sundler F 2004 Ghrelin is expressed in a novel endocrine cell type in developing rat islets and inhibits insulin secretion from INS-1 (832/13) cells. *Journal of Histochemistry and Cytochemistry* **52** 301–310. (doi:10.1177/002215540405200301)
- Wortley KE, Anderson KD, Garcia K, Murray JD, Malinova L, Liu R, Moncrieffe M, Thabet K, Cox HJ, Yancopoulos GD *et al.* 2004 Genetic deletion of ghrelin does not decrease food intake but influences metabolic fuel preference. *PNAS* **101** 8227–8232. (doi:10.1073/pnas.0402763101)
- Wren AM, Small CJ, Abbott CR, Dhillo WS, Seal LJ, Cohen MA, Batterham RL, Taheri S, Stanley SA, Ghatei MA *et al.* 2001 Ghrelin causes hyperphagia and obesity in rats. *Diabetes* **50** 2540–2547. (doi:10.2337/diabetes.50.11.2540)
- Yang J, Brown MS, Liang G, Grishin NV & Goldstein JL 2008 Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* **132** 387–396. (doi:10.1016/j.cell.2008.01.017)
- Yonezawa T, Haga S, Kobayashi Y, Katoh K & Obara Y 2008 Unsaturated fatty acids promote proliferation via ERK1/2 and Akt pathway in bovine mammary epithelial cells. *Biochemical and Biophysical Research Communications* **367** 729–735. (doi:10.1016/j.bbrc.2007.12.190)

Received in final form 24 February 2014

Accepted 17 March 2014

Accepted Preprint published online 17 March 2014