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

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, Thidar Myint 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 (Grousselle et al. 2008). In addition, ghrelin stimulates insulin secretion in Holstein steers (Thidam-Myint 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 mono-gastric 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, Journel 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 (Journel 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-Lys^3]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 808 C until measure-

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, #AFPBS5), 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 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 poltron 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'-CAGCATGCTCTGAGTGGACTT-3', reverse 5'-CTCTGCACCTTCCTCCTGACT-3'; GOAT/MBOAT4: forward 5'-CGGGA-CAGGTTGTTTGGTTCC-3', reverse 5'-CCATGGGAAAGACACTGCTGTG-3'; GHSR: forward 5'-GGGCTCTCACCACATCATCTGTTGTAGCCAGCCAAGAGC-3', reverse 5'-TGGTGT-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-ΔΔCt 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 for 6 h (from pre- to postprandial response) was calculated by using a trapezoidal integration method and the AUC was measured 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 [α-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....
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 X 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 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 X day interaction (\( P < 0.05 \)). *\( P < 0.05 \) indicating significant differences between the HP and CNT diets.

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 X 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.
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).

**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

**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).
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 β 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 (Cai 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 (Schiotth et al. 1997, Depoortere et al. 2006) and is known to act as an agonist to the serotonin receptor 5-HT2b (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.

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 Kukawaya and Ms Than Shint (Department of Animal Science, Obihiro University of Agriculture and Veterinary Medicine) regarding the ghrelin RIA assay is also gratefully acknowledged.

References

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.


Received in final form 24 February 2014
Accepted 17 March 2014
Accepted Preprint published online 17 March 2014