A high-protein diet induces dissociation between plasma concentrations of growth hormone and ghrelin in wethers


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ABSTRACT: High-carbohydrate or high-fat diets have been demonstrated to change ghrelin concentrations in plasma; however, there remains a need to clarify the effects of dietary protein on the interaction between circulating GH and ghrelin concentrations in the ruminant. In this study, we investigated the postprandial changes in plasma concentrations of GH and ghrelin and their interactions when wethers were fed either a high-protein (HP; 40% CP) or a low-protein (LP; 10% CP) diet for 2 wk. The wethers were divided into 2 groups and fed once a day for 2 wk in a randomized crossover design. Each diet contained the same level of ME. Blood was collected from the animals at specific times over 24 h to measure hormones and metabolites. Feeding once a day caused a prompt reduction in the GH and ghrelin concentrations regardless of the type of diet that the wethers consumed. The preprandial concentrations (P = 0.04), area under the curve (AUC; P = 0.04), and incremental AUC (iAUC; P = 0.06) for ghrelin in HP-fed wethers were or tended to be greater than those in LP-fed wethers although concentrations for GH were the same for both diets (P = 0.23). In addition, the time it took for the postprandial ghrelin concentrations to recover to the preprandial concentrations was greater in HP-fed wethers than in LP-fed wethers although this was not true for GH concentrations. Similarly, as for ghrelin, postprandial increase (P < 0.001) and AUC (P = 0.03) for insulin concentration was greater in the HP-fed wethers than in the LP-fed wethers. From these findings, we concluded that dietary proteins (or some other derived metabolites) may dissociate the interaction between plasma concentrations of GH and ghrelin in wethers.

Key words: diurnal change, ghrelin, growth hormone, high-protein diet, low-protein diet, wether

INTRODUCTION

The gastric hormone ghrelin, an endogenous ligand for the GH secretagogue receptor (GHS-R), stimulates GH secretion (Kojima et al., 1999). Ghrelin also stimulates appetite and BW gain in both humans and rodents (Nakazato et al., 2001) and regulates gastric acid secretion and motility (Masuda et al., 2000; Date et al., 2001). These results support the hypothesis that ghrelin plays an important role in regulating metabolism, body composition, and energy expenditure. In addition, plasma concentrations of ghrelin are affected by nutrition and feeding behavior in humans, rodents, and sheep (Cummings et al., 2001; Tschöp et al., 2001; Sugino et al., 2002a), suggesting its physiological role as a hunger signal that initiates food intake, which may partly show seasonality (Iqbal et al., 2006; Grouselle et al., 2008); therefore, plasma concentrations of ghrelin are increased by food deprivation or restriction (Wertz-Lutz et al., 2006; Bradford and Allen, 2008). As the frequency of feedings per day increases, the plasma concentrations of ghrelin decrease in sheep (Sugino et al., 2002b). In addition, plasma concentrations of ghrelin were greater in overweight than in normal or lean sheep (Kurose et al., 2005) although they were increased in lean humans (Shiiya et al., 2002).

It was recently found that the postprandial decrease in plasma concentrations of ghrelin in wethers fed a concentrate diet was greater than that in wethers fed a rough-
age diet whereas the total GH secretion in the roughage-fed group was greater than that in the concentrate-fed group (Takahashi et al., 2008), indicating that the interaction between ghrelin and GH concentrations are diet dependent. Consistent with these findings, plasma concentrations of ghrelin were increased by a high-protein (HP) diet and consumption of essential AA in humans, pigs, and rats (Knerr et al., 2003; Vallejo-Cremades et al., 2004; Zhang et al., 2007; Nakahara et al., 2010). In contrast, 1 study reported that a HP diet had no effect on postprandial plasma concentrations of ghrelin (Greenman et al., 2004); therefore, there remains a need to clarify the detailed effects of dietary protein on the interaction between circulating GH and ghrelin concentrations (the somatotropic status) in the ruminant. In this study, we investigated the postprandial changes in the plasma concentrations of GH and ghrelin for 24 h when wethers were fed either a HP (40%) or low-protein (LP; 10%) diet for 2 wk, with each diet having the same level of ME.

**MATERIALS AND METHODS**

All procedures involving animals were approved by the Animal Care and Use Committee of Tohoku University (GSAS-20-8).

**Animals**

Five 1-yr-old wethers (Suffolk × Corriedale) weighing 38.7 ± 2.2 kg were used. The animals (n = 5) were housed in individual pens (136 by 60 cm) under a 12-h light–dark cycle with free access to water and mineral salts throughout the experiment.

**Experimental Procedures**

The 2 diets, one of which was HP and starch free and the other LP and high starch, were offered to the animals using a randomized crossover design (Table 1). The ratio of CP to the total volume of the HP and LP diets was 40 and 10%, respectively. Both diets were fed at the same ME level (11 MJ). Animals were offered fixed amounts (575 g) of each diet once daily between 1000 and 1200 h for 14 d. The BW were measured on d 0 and 13. On d 14, blood samples were taken at intervals over 24 h. We set a 2-wk washout interval between the HP and LP experiments. During the interval period, the animals were fed alfalfa-hay cubes. The diets were sufficient for maintenance, as shown in Table 1, and were based on the Japanese feeding standards for sheep (AFFRC, 1996).

**Collection and Preparation of Blood Samples**

On d 13 of each dietary period (1 d before blood was sampled), a polyethylene catheter was placed in the left jugular vein of each animal. On d 14, blood samples were collected every 15 min from 0900 to 1300 h, every 1 h from 1300 to 2300 h, and every 2 h from 2300 to 0900 h for 24 h. The blood samples (8 mL) were divided into 2 tubes for ghrelin assay and for assays of other hormones and metabolites. For the ghrelin assay, the blood samples (2.0 mL) were put into tubes containing heparin (10 units/mL) and aprotinin [50 Killikrein inhibitor units (KIU)/mL]. For the other assays, the remaining blood samples (6 mL) were put into tubes containing only heparin (10 units/mL). All blood samples were centrifuged at 5,000 × g for 10 min at 4ºC (Kobayashi et al., 2006) to separate the plasma. The separated plasma samples were stored at –80ºC before measurement of the plasma concentrations of glucose, NEFA, urea N, GH, IGF-I, and insulin. Plasma used for ghrelin analysis was treated with an acetic acid buffer (1 M acetate and 20 mM HCl) at a ratio of 1:1 and stored at –80ºC.

**Biochemical Analyses**

Nutrient values of individual ingredients were analyzed by Meiji Feed Co., Ltd. (Tokyo, Japan). Plasma concentrations of hormones and metabolites were quantified in duplicate. The plasma concentrations of glucose, NEFA, and urea N were measured using an enzymatic assay kit according to the manufacturer’s instructions (Glucose C-II Test-Wako, NEFA C-Test-Wako, and Urea Nitrogen B-Test-Wako, respectively; Wako Pure Chemical Industries, Osaka, Japan). The reaction of the assay mixture was performed in a 96-well microtiter plate, and the absorbance was measured using a plate reader. The intra- and interassay CV for the glucose, NEFA, and urea N assays were <5.0%. The detec-

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<th>Table 1. Ingredients and chemical compositions of high-protein (HP) and low-protein (LP) diets fed daily to wethers</th>
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<td><strong>Item</strong></td>
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1Nutrient % of fed DM.
tion limits for the glucose, NEFA, and urea N were 0.76 μg/well, 0.66 nEq/well, and 0.19 μg/well, respectively.

To eliminate interassay variations in each hormone assay, all samples were analyzed in the same assay. Plasma concentrations of GH were measured by double-antibody RIA. Ovine GH (AFP-12855B; National Hormone and Peptide Program, Harbor-University of California, Los Angeles Medical Center, Torrance, CA) was used as the radiiodinated antigen and standard. The GH antiserum (AFP-C0123080; National Hormone and Peptide Program) was used at a dilution of 1:160,000 (Kobayashi et al., 2006; Takahashi et al., 2008). The detection limit was 0.02 ng/tube. The intra-assay CV for the GH was <10%.

Plasma samples for IGF-I assay were extracted using acid-ethanol methods according to the method of Daughaday et al. (1980) with slight modification. The plasma samples were mixed with an acid solution (87.5% ethanol and 12.5% 2 M HCl, v/v) at a ratio of 1:4, and incubated at room temperature for 30 min. The samples were then centrifuged at 1,860 × g for 30 min at 4°C. The supernatant was neutralized with 1.0 M Tris base, pH 7.2, at a ratio of 25:7. The neutralized samples were diluted with an assay buffer (0.04 M sodium phosphate containing 0.01 M EDTA, 0.25% BSA, 0.02% proteamine SO4, and 0.02% NaN3, pH 7.5) at a ratio of 1:10. After the extraction, the concentrations of IGF-I were measured by RIA. Human IGF-I (PT10011; Toyobo, Osaka, Japan) was used as the standard and 125I-labeled human IGF-I (NEX241; PerkinElmer Life Sciences, Boston, MA) was used as the radiiodinated antigen. The IGF-I antiserum (UB2-495; National Hormone and Peptide Program) was used at a dilution of 1:42,000 (Kobayashi et al., 2006; Takahashi et al., 2008). The detection limit was 0.003 ng/tube. The intra-assay CV for the IGF-I was <10%.

Plasma concentrations of insulin were quantified by dextran-coated charcoal RIA as described previously (Herbert et al., 1965, Kobayashi et al., 2006). Bovine pancreas insulin (I-5500; Sigma-Aldrich Co., St. Louis, MO) was used as the standard and 125I-labeled porcine insulin (NEX104; PerkinElmer Life Sciences) was used as the radiiodinated antigen. Insulin antiserum (I-6136; Sigma-Aldrich) was used at a dilution of 1:42,000 (Kobayashi et al., 2006; Takahashi et al., 2008). The detection limit was 0.026 μunit/tube. The intra-assay CV for the insulin was <10%.

Plasma concentrations of ghrelin were determined by double-antibody RIA. Bovine ghrelin (gift from Dr. F. Itoh) was used as the standard and 125I-labeled human ghrelin (IM347; GE Healthcare Life Sciences, Piscataway, NJ) was used as the radiiodinated antigen. Ghrelin antiserum (GHS11-A; Alpha Diagnosti International Inc., San Antonio, TX) was used at a dilution of 1:3,000. The detection limit was 0.003 ng/tube. The intra-assay CV for the insulin was <12%.

**Statistical Analyses**

All data are presented as means ± SE of individual means and the statistical significance was defined as P < 0.05. Data were analyzed using a repeated measures ANOVA to test the effects of diet, time, and diet × time. When there was an interaction in diet × time, we compared the means with a paired t-test at the same sampling time. The area under the curve (AUC) for 24 h was calculated by using a trapezoidal integration method. The incremental AUC (iAUC) for each hormone was calculated for 0 to 23 h after feeding. For calculating the iAUC, the mean concentration of the 5 preprandial values from –1 to 0 h was used as the basal value. All data were analyzed using SAS (SAS Inst. Inc., Cary, NC).

**RESULTS**

All animals completely consumed the diets offered regardless of diet types. In addition, neither the HP nor LP diet given for 2 wk affected the BW of the animals (Table 2).

There was no interaction in diet × time for plasma concentrations of GH. Although the concentrations in both groups promptly decreased after feeding, there were no differences between the 2 diets (Figure 1A; P = 0.23). The GH AUC and iAUC for the HP vs. LP diets were 3,274 ± 224 vs. 4,168 ± 674 ng·min/mL (P = 0.16) and –608 ± 260 vs. –218 ± 493 ng·min/mL (P = 0.54), respectively (data not shown).

The plasma concentration of IGF-I in HP-fed wethers was greater than that in LP-fed wethers only at 5 h after feeding (Figure 1B) and there were no differences between the 2 diets (data not shown).

For plasma concentrations of ghrelin, there was an interaction (P < 0.001) between diet × time. The preprandial concentrations of ghrelin in LP-fed wethers were greater than those in HP-fed wethers (P = 0.04; Figure 2A). The plasma concentrations of ghrelin in both groups drastically decreased after feeding, as observed for GH (Figure 1A). Thereafter, the plasma concentrations of ghrelin in both groups gradually recovered to their basal concentrations (Figure 2B). From 0 to 6.0 h after feeding, the ghrelin concentrations in HP-fed wethers were greater than those in LP-fed wethers (P <

| Table 2. Changes in the BW of wethers fed with high-protein (HP) and low-protein (LP) diets for 2 wk1 |
|---------------------------------|------------------|------------------|
| Diet                           | d 02             | d 132            |
| HP, kg                         | 39.1 ± 2.3       | 38.9 ± 2.4       |
| LP, kg                         | 38.2 ± 2.0       | 39.1 ± 2.0       |

1Means ± SE (n = 5).

2Day 0 and d 13 refer to the day before the experiment was performed and samples collected.
The AUC for the HP-fed wethers was greater than that for the LP-fed wethers (1,948 ± 228 vs. 1,217 ± 75 ng·min/mL; *P* = 0.04; data not shown). In addition, the iAUC for the HP-fed wethers tended to be greater than that for the LP-fed wethers (–851 ± 297 vs. –42 ± 157 ng·min/mL; *P* = 0.06; data not shown). Furthermore, both baselines were normalized to 0% and the recovery of postprandial ghrelin concentrations were examined between both diet groups (Figure 2B). Figure 2B shows that the time it took for postprandial concentrations of ghrelin to recover to preprandial concentrations was longer in HP-fed wethers than in LP-fed wethers.

For plasma concentrations of insulin, there was an interaction between diet × time (*P* < 0.001; Figure 3). The plasma concentrations of insulin in HP-fed wethers reached its peak at 120 min after feeding whereas concentrations in LP-fed wethers reached its peak at 135 min after feeding. Although the preprandial plasma concentrations of insulin did not differ between the diets (*P* = 0.26), there were differences in the postprandial concentrations at the 10 observation times (Figure 3). From 1.5 to 2.0 h after feeding, the plasma concentrations of insulin in the HP-fed wethers were greater than those in the LP-fed wethers (*P* < 0.05). In addition, AUC for the HP-fed wethers was greater than that for the LP-fed wethers (1,981 ± 223 vs. 1,282 ± 49 μ units·min/mL; *P* = 0.03; data not shown) although the iAUC did not differ between the diets (389 ± 204 vs. 2 ± 75 μ units·min/mL; *P* = 0.15; data not shown).

The plasma concentrations of glucose in HP-fed wethers were greater (*P* < 0.05) at 2.0 and 3.0 h after feeding than those in LP-fed wethers (Figure 4A). Although there was no difference (*P* > 0.10) in the plasma concentrations of NEFA between the treatments (Figure 4B), the plasma concentrations of urea N in HP-fed wethers were greater (*P* < 0.05) than those in LP-fed wethers (Figure 4C).

**DISCUSSION**

A new finding in this study was that the protein levels in diets could modify plasma concentrations of ghrelin without changing GH concentrations. Giving wethers a HP diet for 14 d increased basal ghrelin concentrations but delayed the recovery time to basal concentrations after a rapid postprandial decrease in ghrelin concentrations. In
sheep, Sugino et al. (2002b) suggested that the preprandial rise in ghrelin concentrations indicates the role of ghrelin as a hunger signal to induce food intake. In fact, a peripheral high-dosage ghrelin injection stimulated feeding behavior in sheep (Grouselle et al., 2008). The acylated form of ghrelin is directly involved in feed intake and BW gain and has been shown to be the signal for increased adiposity and feeding (Soares and Leite-Moreira, 2008). It has been reported that the ingestion of a HP diet, tryptophan, and AA increased the plasma concentration and expression of ghrelin mRNA in the fundus (Erdmann et al., 2003; Knerr et al., 2003; Vallejo-Cremades et al., 2004, 2005; Zhang et al., 2007). Increased plasma concentrations of ghrelin after prolonged nutrient restriction in beef cattle imply that there is a potential role for ghrelin in conveying long-term nutritional status, altering body composition, or regulating energy expenditure (Wertz-Lutz et al., 2008). In addition, the concentration of acylated ghrelin at 6.0 h postprandial was 57% greater in pigs fed raw rice than in pigs fed raw corn (Menoyo et al., 2011). The alteration of the plasma concentrations of ghrelin may change the signal transduction system for feeding and nutritional status (Tschöp et al., 2000; Nakazato et al., 2001; Dezaki et al., 2006; Grouselle et al., 2008).

Despite the rise in plasma concentrations of ghrelin in HP-fed wethers, there were no differences in the basal concentrations for plasma GH. In addition, the postprandial suppression of ghrelin concentrations lasted longer than that of GH in HP-fed wethers. This finding is consistent with the reports that follow. The genetic deletion of ghrelin caused no effects on serum concentrations of GH and body lengths in mice (Wortley et al., 2004). Moreover, ghrelin is unlikely to be important for rhythmic or nutritionally mediated GH secretion (Avram et al., 2005). There was no correlation between the circulating concentrations of ghrelin and GH in sheep (Tolle et al., 2002). The circadian alterations in plasma concentrations of ghrelin were similar to those in GH concentrations in sheep only when a concentrate diet was given but never when a roughage diet was given (Sugino et al., 2002a; Takahashi et al., 2008). These findings could indicate that there is not necessarily an interaction between circulating ghrelin and GH concentrations in sheep; therefore, the precise mechanisms for the dissociation between plasma concentrations of ghrelin and GH in sheep remain to be clarified. It has been reported, however, that IGF-I regulates ghrelin secretion (Vallejo-Cremades et al., 2004). In fact, in the present study, the plasma concentrations of IGF-I in the HP-fed group were slightly greater than those in LP-fed group although the increased IGF-I in the HP-fed group might not be enough to affect plasma concentrations of ghrelin.

In rats, subcutaneous injection of insulin or 20% glucose solution decreased ghrelin concentrations but increased leptin concentrations (Nakahara et al., 2010); however, in the present study, it is unlikely that the increased glucose concentration caused the decreased ghrelin concent-
trations. Moreover, other studies in ruminants revealed that glucose infusion during hyperglycemic-hyperinsulinemic clamp did not alter ghrelin concentrations in sheep (Sugino et al., 2010). Also, short-chain fatty acids (SCFA) as the main substrates for energy production in ruminants have been demonstrated to inhibit ghrelin secretion (Fukumori et al., 2011). In fact, the LP diet used in the present study has greater carbohydrate contents and therefore suggests the possibility for a greater SCFA production compared with HP diet. Therefore, the differences of ghrelin concentrations between the diet groups might be attributed to the suppressive and stimulatory effects of HP and LP diets, respectively, on ghrelin secretions due to potential changes in SCFA production. Moreover, in the LP diet group, the effect of glucose derived from this diet was not marked on concentrations. Despite the previous findings demonstrating that an AA mixture increased GH release from primary cultured anterior pituitary cells from ruminant species by increasing cellular Ca ion concentrations (Ohata et al., 1997; Katoh and Obara, 2001). Another explanation could be that an increase in ruminal ammonia production raised the plasma concentrations of urea N, which might affect the control mechanisms of the somatotropic axis.

The plasma concentrations of insulin in HP-fed wethers were greater than those in LP-fed wethers. Kuhara et al. (1991) demonstrated that an intravenous infusion of AA elevated plasma concentrations of insulin and GH in sheep. In addition, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinoctropic polypeptide (GIP) are known as gut-derived incretin hormones and potentially stimulate glucose-dependent insulin secretion (Baggio and Drucker, 2007). The plasma concentrations of GLP-1 and GIP were also increased by HP diets or AA (Fieseler et al., 1995; Raben et al., 2003; Nilsson et al., 2007); therefore, the increased insulin concentrations in HP-fed wethers could be a result of the increased protein-derived AA or the induced secretion of unknown hormones or both.

In the present study, the postprandial changes in plasma concentrations of GH and ghrelin were measured for 24 h during which wethers were fed either a HP (40%) or LP (10%) diet containing the same level of ME. Our findings revealed that plasma concentrations of ghrelin and insulin might be increased by a HP diet although this is not the case for GH; therefore, the dissociation in ghrelin and GH caused by a HP diet remains to be clarified with reference to the control of the somatotropic axis by nutrients in sheep. It is likely that many nutrient factors control the ruminant somatotropic axis.

LITERATURE CITED


