Prolonged, moderate nutrient restriction in beef cattle results in persistently elevated circulating ghrelin concentrations

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ABSTRACT: Four ruminally cannulated steers (BW 581 ± 12.8 kg) were used in a crossover design to determine the effects of prolonged, moderate nutrient restriction on plasma ghrelin concentrations and to establish the relationship of plasma ghrelin concentrations with hormones and metabolites indicative of nutritional status and end products of rumen fermentation. A high-grain diet was offered at 240% of the intake needed for BW maintenance (2.4×M) or 80% of the intake needed for BW maintenance (0.8×M). To standardize, all steers were acclimated to 2.4×M before initiation of the treatment periods. During period 1, 2 steers continued at 2.4×M, whereas intake for the remaining 2 steers was restricted to 0.8×M. On d 7, 14, and 21 after initiation of the restriction, serial blood samples were collected at 15-min intervals via indwelling jugular catheter and were assayed for ghrelin, GH, NEFA, insulin, and glucose concentrations. Rumen fluid was collected at hourly intervals for evaluation of pH and VFA concentrations. After period 1, steers were weighed, the treatments were switched between steer groups, and the intake amounts were recalculated. Intake of 2.4×M was established for previously restricted cattle, and period 2 was then conducted as described for period 1. Data were analyzed statistically as repeated measures in time, and stepwise regression was used to define the relationship of plasma ghrelin with hormones, metabolites, and end products of rumen fermentation. Throughout the 21-d treatment period, plasma ghrelin concentrations were elevated (P ≤ 0.001) for steers offered the 0.8×M diet. Plasma GH and NEFA concentrations were increased (P ≤ 0.001) and insulin concentrations were decreased (P ≤ 0.001) for steers offered 0.8×M, indicating a catabolic state throughout the treatment period. Stepwise regression indicated that the fluctuation in plasma ghrelin was correlated weakly with hormones and metabolites indicative of nutritional status as well as end products of carbohydrate fermentation in the rumen. These data are consistent with the hypothesis that plasma ghrelin concentrations are elevated for cattle experiencing prolonged, moderate nutrient restriction that results in a catabolic state. Fluctuations in plasma ghrelin concentrations, however, have a weak relationship with hormones, metabolites, and end products of rumen fermentation.

Key words: cattle, ghrelin, nutrient restriction

INTRODUCTION

Inadequate nutrient intake relative to demand for maintenance, production, or both can result in poor performance and metabolic disorders. Understanding the regulation of nutrient intake and expenditure is therefore important. Ghrelin is a peptide hormone synthesized by abomasal and ruminal tissues of cattle (Hayashida et al., 2001; Gentry et al., 2003). Ghrelin stimulates DMI through neuropeptides in the hypothalamus (Inui, 2001; Nakazato et al., 2001; Shintani et al., 2001) and influences energy metabolism and body composition (Tschöp et al., 2000). Plasma ghrelin concentrations increase with acute DMI deprivation in cattle (Wertz-Lutz et al., 2006) and sheep (Sugino et al., 2002a,b). Iqbal et al. (2006) found that exogenous ghrelin did not influence DMI in sheep; however, Wertz-Lutz et al. (2006) reported an increase in time spent feeding and a tendency for increased DMI with pulse doses of ghrelin in cattle. Plasma ghrelin concentrations as a result of prolonged DMI restriction that

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results in BW loss have not been established for cattle. Circulating ghrelin concentrations decreased in rodents with infusion of glucose (GLU; Tschöp et al., 2000). Intragastric GLU infusion did not decrease plasma ghrelin concentrations when gastric emptying was prevented, indicating that GLU absorption is necessary to influence plasma ghrelin concentrations (Williams et al., 2003). For ruminants, the majority of GLU is synthesized from propionate metabolism in the liver, not absorbed from the small intestine (Fahey and Berger, 1988; Forbes, 1995). Relative proportions of propionate can be manipulated (Fahey and Berger, 1988), and as much as 40% of dietary starch can escape rumen fermentation in specific dietary situations (Orskov, 1986). This experiment was designed 1) to evaluate the effects of prolonged, moderate DMI restriction on plasma ghrelin concentrations, and 2) to establish the relationship of plasma ghrelin concentrations with end products of carbohydrate digestion.

**MATERIALS AND METHODS**

**Dietary Treatments**

Dietary treatments for this experiment were 2 amounts of a high-energy diet (Table 1). The amount of DMI was 80% of that necessary to meet the NE\textsubscript{m} requirement of a given steer and was calculated as described below by using equations from the Nutrient Requirements of Beef Cattle (NRC, 2000). To determine the amount of DMI necessary to meet the NE\textsubscript{m} requirement (Mcal/d), the equation 0.077 × empty BW, kg\textsuperscript{0.75} was used (NRC, 2000). This NE\textsubscript{m} requirement was then divided by the energy density of the diet (Mcal/kg) to determine the amount of feed (kg/d) necessary to meet the NE\textsubscript{m} requirement of each particular steer based on its own BW. The amount of DMI needed to meet the NE\textsubscript{m} requirement was then multiplied by 2.4 to determine the target amount of DMI for the steers in the positive nutrient balance (2.4×M) or multiplied by 0.80 to determine the amount of DMI assigned to the negative nutrient balance (0.8×M) treatment. Once the given amount of feed for each steer was determined, the MP content of the feed was estimated on the basis of degradability of the dietary protein (43.4%), calculated by using the Beef NRC (2000) equation. The amount of dietary MP consumed was then compared with the MP required for maintenance of the BW, which was calculated by using the equation 3.8 g of MP × BW, kg\textsuperscript{0.75} (NRC, 2000).

**Animals and Procedures**

This experiment was conducted in a climate-controlled metabolism facility at South Dakota State University, and all animal procedures were approved by the Institutional Animal Care and Use Committee.

Four ruminally cannulated (3-yr-old) Angus cross-bred steers (BW, 581.4 ± 12.8 kg), each fitted with an indwelling jugular catheter, were used in a crossover design to evaluate the effects of prolonged, moderate nutrient restriction on plasma hormone and metabolite concentrations and their relationship with ruminal fermentation.

During a 23-d preexperimental adaptation period, steers were acclimated to the climate-controlled facility. Equal aliquots of feed were offered twice daily at 0800 and 2000 h, and this 12-h feeding interval was maintained throughout the experiment. As a means of standardizing the steers before initiation of the first period, DMI of the common high-grain finishing diet (Table 1) was increased gradually during the acclimation period until DMI was 240% of the amount required to meet the NE\textsubscript{m} requirement of each steer. Because the sole source of fiber in this diet was beet pulp, an inadequate amount of functional fiber began to result in acidosis and bloat. For this reason, 0.23 kg of wheat straw and 50 g of sodium bicarbonate were given daily to each steer beginning 2 d before initiation of period 1 and continuing for the remainder of the experiment. Wheat straw was weighed into individual paper bags and delivered through the rumen cannula to ensure that an equal amount of wheat straw was consumed by each steer. Sodium bicarbonate was mixed into the diet for the morning feeding.

When all steers had reached 240% of the DMI required to meet NE\textsubscript{m}, treatment period 1 was initiated. The experiment was conducted as a crossover design with two 21-d treatment periods. During treatment period 1, 2 steers were continued at 2.4×M and the remaining 2 steers were limited to 80% of the DMI needed to meet the NE\textsubscript{m} requirement. Serial blood and rumen fluid samples were collected on d 7, 14, and 21 after initiation of the treatment period. After period

**Table 1. Composition of the experimental diet**

<table>
<thead>
<tr>
<th>Ingredient, % DM basis</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beet pulp</td>
<td>20.00</td>
</tr>
<tr>
<td>Corn</td>
<td>65.00</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>5.67</td>
</tr>
<tr>
<td>DDGS\textsuperscript{1}</td>
<td>5.00</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.00</td>
</tr>
<tr>
<td>Trace mineral salt\textsuperscript{2}</td>
<td>0.30</td>
</tr>
<tr>
<td>Vitamin A, D, E\textsuperscript{3}</td>
<td>0.0055</td>
</tr>
<tr>
<td>Zinc sulfate\textsuperscript{4}</td>
<td>0.0056</td>
</tr>
<tr>
<td>Rumensin\textsuperscript{5}</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Calculated nutrient composition, DM basis

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP, %</td>
<td>12.5</td>
</tr>
<tr>
<td>Degradable intake protein, %</td>
<td>43.4</td>
</tr>
<tr>
<td>NE\textsubscript{m}, Mcal/kg</td>
<td>2.1</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Dried distiller’s grains with solubles.

\textsuperscript{2}Contained: NaCl, 94.0 to 98.5%; Zn, 0.35%; Fe, 0.20%; Co, 0.005%; Mn, 0.20%; Cu, 0.30%; and I 0.007%.

\textsuperscript{3}Contained 30,000 IU/g of vitamin A, 500 IU/g of vitamin E, and 500,000 IU/g of vitamin D3.

\textsuperscript{4}35.54% Zn.

\textsuperscript{5}Formulated to contain 32 g/t.
1, dietary treatments were switched between steer groups, steers were weighed, and DMI was recalculated on the basis of the BW recorded at the end of period 1 and the new treatment assignment. A DMI of 2.4×M again was established during a 14-d crossover period between treatment periods, and then a second 21-d treatment and sampling period was conducted as described for period 1.

**Blood and Rumen Fluid Collection**

During each 21-d treatment period, blood and rumen fluid samples were collected at 7, 14, and 21 d after the DMI restriction was invoked. An indwelling jugular catheter was inserted on d 6 of the treatment period, as described previously by Wertz-Lutz et al. (2006). Indwelling catheter patency was maintained by using 2.9% sodium citrate, and catheters were replaced only when patency failed. Catheters that failed between collection days were removed, and new catheters were inserted 1 d before the next collection period. On each sampling day, blood samples were collected at 15-min intervals from 0700 to 1145, 1300 to 1345, 1600 to 1645, and 1800 to 1845. A 10-mL aliquot of blood was collected into a glass tube containing K$_3$EDTA (12.15 mg supplied by a 15% solution) for plasma separation. Blood collected from each steer throughout the collection period was less than 1% of the total estimated blood volume of a steer. Tubes were placed on ice and then centrifuged at 4°C for 20 min at 1,100 × g within 1 h of collection. A 1.0-mL aliquot of plasma was treated with 50 μL of 1 N HCl and 100 μg of phenylmethanesulfonyl fluoride (Sigma-Aldrich, St. Louis, MO) to preserve the integrity of the octanoyl moiety of ghrelin. Plasma samples were stored at −20°C for subsequent measurement of ghrelin. The remaining plasma was separated into 1.0-mL aliquots and stored at −20°C for subsequent analyses of GH, NEFA, insulin (INS), and GLU.

Steers were surgically modified by using a 2-stage (laparotomy and rumenotomy) rumen fistulation technique and fitted with a 10-cm flexible rumen cannula (Bar Diamond Inc., Parma, ID). Steers were prepared for laparotomy by clipping excess hair and scrubbing the surgical site with disinfecting soap. Steers were then anesthetized by using 15 mL of lidocaine in 1-mL aliquots in a continuous reverse-7 pattern dorsal and cranial to the incision site to block peripheral sensation from reaching the central nervous system. The incision site was traced in the paralumbar fossa, using the cannula as a pattern. The hide was removed from this section, and the exposed fat, muscle, and peritoneum were separated by using blunt dissection, thereby exposing the rumen epithelium. A portion of the rumen epithelium from the dorsal blind sac was externalized, and the hide, peritoneum, and rumen epithelia were sutured together. The surgical site was cleaned with a topical disinfectant, and steers were given an injection of long-acting penicillin (Agri Labs, St. Joseph, MO) according to the label directions.

After the laparotomy, the steers were allowed a 14-d recovery for adhesion of the tissue layers to occur before the rumenotomy was performed. During this time, steer body temperature was monitored and the externalized rumen epithelium was observed for infection. Steers were given penicillin G on alternate days for the first 7 d of recovery. After the 14-d recovery, the exposed, keratinized rumen epithelium was removed and the cannula was inserted into the fistula. Before being used experimentally, steers were allowed at least 14 d of recovery after cannula insertion.

Rumen fluid (50 mL), collected at the beginning of each hour in which blood samples were collected, was strained through 4 layers of cheesecloth and 1 layer of nylon mesh. Rumen fluid pH was recorded immediately after collection, and a 5-mL aliquot of rumen fluid was acidified with 1 mL of 25% metaphosphoric acid. Acidified rumen fluid was allowed to stand for 30 min and then centrifuged at 20,000 × g for 30 min. The resulting acidified supernatant was aliquoted into microcentrifuge tubes and frozen at −20°C for subsequent quantification of molar proportion of VFA via gas chromatography (Erwin et al., 1961). An HP 5890A chromatograph with a flame ionization detector was used (Hewlett-Packard, Palo Alto, CA). Volatile fatty acids were resolved by using a 30-m DB-FFAP, fused-silica capillary column with an i.d. of 0.25 mm (Alltech, Deerfield, IL), and an isothermal run with an oven temperature of 140°C, an injector temperature of 250°C, and a detector temperature of 230°C. Helium was used as the carrier gas, with a flow rate of 1.5 mL/min.

**Analyses of Hormones and Metabolites**

Because of the pulsatile nature of ghrelin and GH, these analyses were performed on plasma samples collected at 15-min intervals. Subsequent to quantification, plasma ghrelin or GH concentration data were pooled by hour and the average was used for statistical analyses. Plasma ghrelin concentrations were measured by using duplicate 100-μL plasma samples, with an active rat ghrelin RIA, according to the procedures provided by the manufacturer (Linco Research, St. Charles, MO). This assay is specific for the first 11 AA of the N-terminus of ghrelin, including the octanoyl moiety, and has been validated for bovine plasma (Wertz et al., 2003). An intact octanoyl moiety is believed to be essential for binding to the GH secretagogue receptor and biological activity (Inui, 2001). The precipitate was collected by centrifugation at 1,500 × g, and the supernatant was discarded. Assay tubes containing pellets were counted for 1 min on a gamma counter (Wizard 1470 Automatic Gamma Counter, PerkinElmer, Wellesley, MA). Average intraassay CV for the ghrelin assay was 3.9%, whereas the interassay assay CV was 10.9%. The detectable range for the
Nutrient restriction increases ghrelin

The first objective of this experiment was to determine whether plasma ghrelin concentrations were elevated persistently throughout a prolonged (21-d), moderate nutrient restriction that resulted in loss of BW, ghrelin assay was from 5 to 160 pg/tube, and the average recovery was 93.1%.

Growth hormone concentration was quantified in the second aliquot of plasma by using an ovine GH RIA. Plasma GH concentrations were determined by using triplicate 200-μL samples of plasma with materials and procedures provided by A. F. Parlow, National Hormone and Peptide Program (Torrance, CA), and methodology outlined by Wertz-Lutz et al. (2006). The minimal detectable concentration was 0.15 ng/tube. Intraassay CV was 5.3%, interassay CV was 11.0%, and recovery was 89%.

Insulin and NEFA concentrations were measured for plasma samples collected at 1-h intervals. Plasma INS concentrations were determined by using duplicate 25-μL aliquots of plasma with a Linco Ultra-Sensitive Human Insulin RIA (Linco Research), and bovine INS (Sigma-Aldrich) was used as the standard, according to the instructions of the assay manufacturer. The bovine INS standard was validated compared with the human INS standard before initiation of the analyses. The precipitate was collected by centrifugation at 1,500 × g, and the supernatant was discarded. Assay tubes containing pellets were counted for 1 min on a gamma counter (PerkinElmer). The minimal detectable amount of INS was 0.006 ng/tube and the maximal detectable amount of INS was 0.10 ng/tube. Insulin recovery was 96.5% and interassay and intraassay CV for the INS assay were 8.8 and 3.5%, respectively. Plasma NEFA concentrations were determined with triplicate plasma aliquots with a colorimetric assay according to the manufacturer’s procedures (Wako Chemicals US Inc., Richmond, VA). The detectable range of the NEFA assay was 62.5 to 1,000 μEq/L with an average intraassay CV of 1.7% and average interassay CV of 2.8%. Plasma GLU concentrations were assayed in triplicate by using a GLU oxidase kit (Sigma-Aldrich). The detectable range for the GLU assay was from 25 to 100 mg/dL, and the average intraassay CV was 1.4% and average interassay CV was 15.3%.

Statistical Analyses

To verify that differences in NEm and MP intake resulted from the invoked differences in DMI, these variables and BW change were analyzed statistically as a crossover design with a model that accounted for variation attributable to sampling period, steer, and amount of DMI using the MIXED procedure (SAS Inst. Inc., Cary, NC). Differences in the characterization variables that resulted from amount of DMI were separated by using Fisher’s t-test. Plasma ghrelin, GH, INS, and NEFA concentrations and rumen VFA concentrations and pH were analyzed statistically as repeated measures in time by using the MIXED procedure of SAS, with independent errors that accounted for error correlation during the sampling times. The model included length of treatment (7, 14, or 21 d), sampling time relative to feeding, amount of DMI (0.8×M vs. 2.4×M), steer, period, and the interactions of length of treatment, sampling time relative to feeding, and amount of DMI as independent variables. The interaction of length of treatment, amount of DMI, and sampling time was not significant. Because the 3-way interaction was not significant for any of the measured dependent variables, data were reported for the interaction of amount of DMI × length of treatment period and amount of DMI × sampling time. Differences in least squares means for plasma ghrelin, GH, INS, NEFA, and rumen VFA concentrations and pH were separated by using Fisher’s t-test. The data set was then divided by dietary treatment, and Pearson correlation and stepwise regression were performed to characterize the relationship between plasma hormones and metabolites and end products of rumen fermentation for steers in the different nutritional states.

RESULTS

Nutritional State of Steers

Imposed dietary treatments resulted in less (P ≤ 0.002) DMI of the common compositional diet for steers assigned to the 0.8×M treatment compared with those assigned to the 2.4×M treatment (3.9 and 10.4 ± 0.18 kg/d, respectively). This DMI restriction resulted in a net energy intake that was below the NEm requirement for 0.8×M (−1.0 Mcal/d) and a net energy intake that was different (P ≤ 0.001) from that of the steers assigned to the 2.4×M (9.0 Mcal/d) treatment. The DMI restriction also resulted in MP intake below that required to meet the maintenance requirement for steers in the 0.8×M treatment (−107 g/d below the maintenance requirement). Metabolizable protein intake also was less (P ≤ 0.001) for steers on the 0.8×M diet compared with those on the 2.4×M diet (500 g/d in excess of the maintenance requirement). Energy and protein intake below that required for BW maintenance resulted in decreased (P ≤ 0.001) BW for steers offered the 0.8×M diet (−49.4 ± 6.6 kg average BW change) compared with those offered the 2.4×M diet (58.0 ± 6.6 kg average BW change). There was a significant period effect for BW change (P ≤ 0.01) whereby steers assigned to the 2.4×M treatment gained more and those assigned to the 0.8×M treatment lost less during period 2. This period effect can be explained by the bias of gastrointestinal tract fill at the interim weigh period. A period effect was not observed for DMI, NEm intake, or MP intake.

Length of Restriction on Hormone and Metabolite Profiles

The first objective of this experiment was to determine whether plasma ghrelin concentrations were elevated persistently throughout a prolonged (21-d), moderate nutrient restriction that resulted in loss of BW,
and to determine the relationship of ghrelin with hormones and metabolites indicative of nutritional status. Plasma GH and NEFA concentrations were elevated \((P \leq 0.001)\) for steers on the 0.8×M diet throughout the 21-d treatment period (Figures 1A and 1B, respectively). In contrast, plasma INS concentrations were lower \((P \leq 0.001)\) for steers on the 0.8×M diet throughout the 21-d treatment period (Figure 1C). A dietary treatment \(\times\) length of treatment interaction resulted for plasma INS concentrations \((P \leq 0.01)\). Plasma INS concentrations were similar across the 21-d sampling period for steers on the 0.8×M diet, whereas plasma INS concentrations were greater at d 7 and 14 compared with d 21 for steers on the 2.4×M diet. Plasma GH, NEFA, and INS concentrations along with BW change indicated that steers in the 0.8×M treatment were in a catabolic state throughout the 21-d treatment period (Figure 1D). There was an interaction of dietary treatment \(\times\) length of treatment \((P \leq 0.01)\) for plasma ghrelin concentrations. Plasma ghrelin concentrations for steers in the 2.4×M treat-
ment were similar regardless of length of treatment, whereas plasma ghrelin concentrations for those in the 0.8×M diet treatment were greater at d 14 compared with those on d 7 and 21 of DMI restriction. An interaction of dietary treatment × length of treatment also resulted ($P \leq 0.03$) for plasma GLU concentrations (Figure 1E). For steers on the 0.8×M diet, plasma GLU concentrations were less ($P \leq 0.05$) on d 14 compared with d 7 and 21, whereas plasma GLU concentrations in steers on the 2.4×M diet decreased linearly ($P \leq 0.05$) as the length of treatment progressed. Plasma GLU concentrations on d 21 were similar between treatment groups regardless of DMI of the high-grain diet. These data are consistent with the hypothesis that plasma ghrelin concentrations are elevated persistently for cattle experiencing a prolonged, moderate energy and protein restriction sufficient to result in a catabolic state and the loss of BW.

A period effect ($P \leq 0.001$) resulted for plasma ghrelin, GLU, and INS concentrations. The difference in plasma ghrelin, GLU, and INS concentrations between steers in the 0.8×M and 2.4×M treatments numerically was greater for period 2 compared with period 1, which suggests that carryover effects may exist. The experimental design, however, did not permit testing the interaction of period × dietary treatment, and this potential carryover effect warrants further investigation.

**Fluctuation of Plasma Hormones and Metabolites Relative to Feeding**

The second objective of this experiment was to evaluate the relationship of plasma ghrelin concentrations with plasma hormone and metabolite concentrations as well as characteristics of carbohydrate fermentation in the rumen during a 12-h feeding interval for cattle in a positive nutritional state and those subjected to a prolonged, moderate DMI restriction. There was no 3-way interaction of dietary treatment × length of treatment × sampling time relative to feeding for any of the measured variables. For this reason, data were pooled for d 7, 14, and 21 and are reported as the interaction of dietary treatment × sampling time relative to feeding (Figures 2 and 3). Even though differences in plasma ghrelin and GH concentrations (Figures 2A and 2B, respectively) resulted from the main effects of dietary treatment and sampling time relative to feeding, the interaction of dietary treatment × sampling time relative to feeding was not a significant source of variation for these variables. Plasma ghrelin concentrations were elevated ($P < 0.001$) for steers on the 0.8×M diet compared with those on the 2.4×M diet (averages across sampling times were 181.8 and 86.0 ± 4.8 pg/mL, respectively) throughout the 12-h sampling period. Plasma GH concentrations also were elevated ($P < 0.001$) for steers offered the 0.8×M diet compared with the 2.4×M diet (averages across sampling times were 15.0 and 9.4 ± 0.45 ng/mL, respectively) throughout the 12-h sampling period. However, regardless of dietary treatment, plasma ghrelin and GH concentrations fluctuated as a result of sampling time relative to feeding ($P < 0.003$). Plasma ghrelin concentrations were elevated ($P \leq 0.05$) before feeding at 0800 and 2000, and reached a nadir between the 2 feeding times (Figure 2A). Average plasma GH concentration the hour before the 0800 feeding was greater ($P \leq 0.05$) than plasma GH concentrations throughout the remainder of the sampling period (Figure 2B). Although GH was elevated at the 0800 feeding along with ghrelin, plasma GH was not elevated before the 2000 feeding despite increasing ghrelin concentrations.

An interaction of dietary treatment × sampling time relative to feeding ($P \leq 0.05$) was also observed for plasma INS concentrations (Figure 2C). Plasma INS concentrations increased subsequent to feeding and then returned to baseline for steers on the 2.4×M diet; however, this pattern was not observed for those on the 0.8×M diet. An interaction of dietary treatment × sampling time relative to feeding also resulted for plasma NEFA concentrations ($P < 0.001$). For steers offered the 2.4×M diet, plasma NEFA concentrations were similar regardless of sampling time relative to feeding (Figure 2D). However, for those offered the 0.8×M diet, plasma NEFA concentrations were elevated before the 0800 and 2000 feeding times and reached a nadir between feeding.

**Fluctuation of Rumen Fermentation Characteristics Relative to Feeding**

The interaction of dietary treatment × sampling time relative to feeding was not a significant source of variation for ruminal pH or VFA concentrations (Figure 3A to 3H). Ruminal pH was lower ($P < 0.001$) for steers on the 2.4×M treatment compared with those on the 0.8×M treatment (Figure 3A). Ruminal acetate (61.7 and 50.8 ± 0.39%) and butyrate (14.3 and 7.7 ± 0.28%) concentrations were greater ($P < 0.001$) for steers fed the 0.8×M diet compared with the 2.4×M diet (Figure 3B and 3C, respectively). However, both acetate and butyrate concentrations were similar regardless of sampling time relative to feeding. Ruminal propionate (19.4 and 37.9 ± 0.42%) and valerate (1.2 and 2.1 ± 0.07%) concentrations were less ($P < 0.001$) for steers in the 0.8×M treatment compared with those in the 2.4×M treatment (Figures 3D and 3E, respectively). Ruminal valerate concentrations were similar regardless of sampling time relative to feeding; however, ruminal propionate concentrations were greater ($P \leq 0.05$) 3 and 5 h postprandial compared with pre-feeding propionate concentrations at the 0800 feeding. Ruminal isovalerate (2.3 and 0.9 ± 0.07%) and isobutyrate (1.2 and 0.6 ± 0.02%) concentrations were greater ($P < 0.001$) for steers in the 0.8×M treatment compared with those in the 2.4×M treatment (Figures 3F and 3G, respectively). For both isovalerate and iso-
butyrate, ruminal concentrations were elevated ($P \leq 0.05$) at sampling times before both the 0800 and 2000 feedings and reached a nadir between feedings. Acetate:propionate (Figure 3H) was greater ($P < 0.001$) for steers offered the 0.8×M diet compared with those offered the 2.4×M diet (3.3 and 1.4 ± 0.05, respectively). Acetate:propionate was greater ($P \leq 0.05$) before the 0800 feeding compared with the remainder of the sampling times relative to feeding.

A period effect resulted ($P \leq 0.003$) for ruminal pH, acetate, butyrate, and valerate concentrations and the ratio of acetate to propionate. Ruminal pH was lower for period 2 compared with period 1. This shift in ruminal pH explains the shift in ruminal VFA profile and
Figure 3. Characteristics of ruminal carbohydrate fermentation in beef cattle exposed to a prolonged energy and protein restriction sufficient to result in BW loss. Volatile fatty acid concentrations are expressed as moles of an individual VFA per 100 mol of total VFA in the rumen fluid. TRT = 0.8×M = 80% of the DMI needed to meet the energy requirement for maintenance of BW, or 2.4×M = 240% of the DMI needed to meet the requirement for maintenance of BW; TIME = sampling time relative to the feeding times of 0800 and 2000; TRT×TIME = interaction of the main effects; NS = not significant.
the decreased acetate-to-propionate ratio. The authors do not have an explanation for the period effect on ruminal fermentation characteristics. However, unlike the plasma hormone data, the magnitude of difference between the 2 treatment groups for period 1 and period 2 appeared similar.

**Relationship of Plasma Hormones, Metabolites, and Characteristics of Rumen Fermentation**

Correlations between plasma ghrelin concentrations and other hormones and metabolites indicative of nutritional status or end products of carbohydrate fermentation in the rumen were weak. Pearson correlations indicated that for steers in the 2.4×M treatment, plasma GH concentrations (Pearson coefficient = 0.40), plasma GLU concentrations (Pearson coefficient = 0.29), and ruminal acetate concentrations (Pearson coefficient = 0.27) were correlated positively (P ≤ 0.01) with plasma ghrelin concentration, whereas plasma NEFA (Pearson coefficient = −0.23) and ruminal propionate (Pearson coefficient = −0.24) concentrations were correlated negatively (P ≤ 0.05) with plasma ghrelin concentrations. Stepwise regression indicated that the fluctuation in ghrelin for steers in the 2.4×M treatment was explained (P ≤ 0.01) by plasma GH concentrations (partial R² = 0.15) and, to a lesser extent, by plasma GLU (partial R² = 0.07) and ruminal acetate (partial R² = 0.07) concentrations.

In contrast, the fluctuation in plasma ghrelin concentrations for steers in the 0.8×M treatment was correlated positively (P ≤ 0.05) with plasma NEFA concentrations (Pearson coefficient = 0.20) and with ruminal isovalerate (Pearson coefficient = 0.25) and valerate (Pearson coefficient = 0.21) concentrations, but negatively correlated (P ≤ 0.05) with plasma INS (Pearson coefficient = −0.53) and GLU concentrations (Pearson coefficient = −0.31), ruminal acetate concentrations (Pearson coefficient = −0.23), and the acetate-to-propionate ratio (Pearson coefficient = −0.23). Stepwise regression indicated that the fluctuation in plasma ghrelin concentration was explained (P ≤ 0.01) by plasma INS concentrations (partial R² = 0.28) and, to a lesser extent, by ruminal acetate concentrations (partial R² = 0.03).

**DISCUSSION**

**Relationship of Ghrelin with Nutritional Status During Prolonged DMI Restriction**

The nutritional status of animals is influenced by nutrient intake relative to nutrient expenditure. As a result of the importance of nutrient intake to the efficiency and body composition of production livestock, a substantial amount of research has been conducted regarding the regulation of DMI in livestock (Baile and Della-Fera, 1981; Baile and McLaughlin, 1987). Regulation of DMI can be described as short term, as it pertains to the feeding pattern within a daily feeding interval, or long term, as it pertains to changes in BW over a longer period of time. Characterizing hormones that are involved in the regulation of DMI, and therefore nutritional status, in both the short term and long term is important to understanding the regulation of feed efficiency and composition of gain, both of which have an economic impact on the beef industry.

Recently, the peptide hormone ghrelin has been investigated because of its potent orexigenic influence in animals. Ghrelin is synthesized by abomasal and ruminal tissues of cattle (Hayashida et al., 2001; Gentry et al., 2003), and ghrelin has been reported to cross the blood-brain barrier (Lee et al., 2002). It has been suggested that ghrelin may convey peripheral nutritional status to the central nervous system and communicate a need for greater DMI or decreased energy expenditure. In rodents, ghrelin stimulates DMI through neuropeptides in the hypothalamus (Inui, 2001; Nakazato et al., 2001; Shintani et al., 2001). Additionally, ghrelin is reported to influence energy metabolism, including decreased fat utilization to meet energy requirements, which results in altered body composition that favors increased body fat in rodents (Tschöp et al., 2000). Researchers also have demonstrated a direct effect of ghrelin on GLU uptake by adipose tissue (Patel et al., 2006).

Most research that has been conducted with livestock to evaluate the relationship of plasma ghrelin with nutritional status has been done with short-term periods of complete feed deprivation without sufficient length to result in differences in body composition. Plasma ghrelin concentrations for sheep, offered feed but not allowed to consume it, remained elevated for the 3-h sampling period compared with those of sheep allowed to consume the offered feed (Sugino et al., 2002a). Wertz-Lutz et al. (2006) demonstrated elevated plasma ghrelin concentrations that persisted for 48 h in mature beef cattle completely deprived of feed. However, plasma ghrelin concentrations have not been measured beyond 48 h of feed deprivation in cattle. Salen et al. (2003) demonstrated elevated plasma ghrelin concentrations that persisted through 48 h of feed deprivation but that declined as feed deprivation continued through 72 h in young pigs. Because plasma ghrelin concentrations have not been measured during prolonged feed deprivation in cattle and there is evidence in young pigs that ghrelin in response to feed deprivation may be transient, the present experiment was conducted.

More often than complete feed deprivation, ruminant livestock encounter periods of inadequate nutrient intake relative to the nutrient demand for maintenance or production. Inadequate nutrient intake can limit growth and milk production or alter the composition of end-products with economic value (meat or milk). For these reasons, the first objective of this experiment was to determine whether long-term (21 d), moderate energy and protein restriction sufficient to result in decreased BW would result in persistently elevated
plasma ghrelin concentrations in mature beef cattle. In the current experiment, plasma ghrelin concentrations were elevated throughout the 21-d nutrient restriction. Body weight loss along with decreased plasma INS concentrations and elevated plasma NEFA and GH concentrations indicated that steers on the 0.8×M diet were mobilizing body tissue stores to meet nutrient requirements not met by dietary intake. Data from the current experiment are consistent with the hypothesis that plasma ghrelin concentrations remain elevated with prolonged, moderate DMI restriction sufficient to result in energy and protein deficiency and mobilization of body tissue. On the basis of these data, we speculate that ghrelin may have a role in conveying long-term nutrient status and in altering body composition.

Further investigation into the relevance of persistently elevated plasma ghrelin concentrations in nutrient-restricted cattle is warranted because research with other species has demonstrated that ghrelin influences body composition. Tschöp et al. (2000) demonstrated that the administration of ghrelin altered the use of carbohydrates and fat as metabolic fuels, which contributed to increased adiposity in rodents. In addition to the indirect effect of ghrelin on the adiposity via its influence on energy expenditure, researchers have demonstrated a direct effect of ghrelin on adipocyte proliferation, differentiation, and GLU uptake. Roh et al. (2002) reported that ghrelin decreased proliferation of preadipocytes from ovine subcutaneous tissue, but stimulated the differentiation of these preadipocytes. Choi et al. (2003) also reported that ghrelin stimulated preadipocyte differentiation in rat adipose tissue. Additionally, Patel et al. (2006) demonstrated that ghrelin stimulated deoxyglucose uptake by rodent epididymal adipocytes in the presence, but not the absence, of INS.

The relevance of persistently elevated plasma ghrelin concentrations also is dependent on expression of the GH secretagogue receptor (GHS-R) to which ghrelin binds. The GHS-R has been identified in a variety of animal tissues, including the hypothalamus, pituitary, adipose tissue, liver tissue, and skeletal muscle (Wang et al., 2002). Kurose et al. (2005) demonstrated that although plasma ghrelin concentrations were greater, expression of the GHS-R in the arcuate nucleus of the hypothalamus was less in sheep grown to achieve a high, compared with a low, body fat composition. These data are consistent with the hypothesis that GHS-R expression is dependent on the nutrient status of the animal. Expression of the GHS-R also has been reported in some, but not all, adipose depots in rodents (Patel et al., 2006). Expression of the GHS-R in adipose, liver, and muscle tissues for meat-producing animals has not been demonstrated. However, expression of the GHS-R in these tissues relative to nutritional status warrants further investigation to explain the relevance of persistently elevated plasma ghrelin concentrations in nutrient-restricted cattle.

In the current experiment, an interaction of nutrient intake × length of treatment occurred for plasma ghrelin concentrations. Plasma ghrelin concentrations were greater for steers on the 0.8×M diet on d 14 compared with those on d 7 and 21. This may reflect the experimental design, because DMI needed to meet 80% of the NEm requirement was calculated at the beginning of each period and that amount of DMI was maintained throughout the 21-d period. As steers lost weight throughout the period, DMI would have supplied greater than 80% of the NEm requirement that was based on initial BW. Decreased ghrelin concentrations at d 21 may be explained by the nutrient intake becoming nearer to that required for BW maintenance. Nutrient intake nearing that required for maintenance of BW, however, does not explain the lower plasma ghrelin concentration at d 7 of nutrient restriction.

Rumen distention also must be considered as a plausible explanation for the interaction of dietary treatment × length of treatment. Sugino et al. (2003) reported that cholinergic activity of the vagus nerves suppressed ghrelin secretion and suggested that distension of the rumen may regulate ghrelin secretion. However, Arnold et al. (2006) acknowledged that phasic increases in ghrelin influenced the activity of some load-sensing vagal afferents but concluded that the acute eating stimulatory effect of ghrelin did not require vagal afferent signaling. As a means of standardizing before beginning the initial treatment-sampling period, all steers in the current experiment were adapted to an intake amount that would supply 240% of the NEm requirement before the restriction was invoked. Intake amount did differ for the 2 treatment groups throughout the treatment period such that the amount of feed consumed was greater for steers on the 2.4×M diet compared with those on the 0.8×M diet, and therefore distention and suppression of ghrelin should be greater. In the current experiment, no objective measure of rumen distention was made; however, it may explain the nutrient intake × length of treatment interaction. Additionally, a decrease in plasma GLU concentration also resulted for steers in the 0.8×M treatment on d 14, and because GLU has been reported to influence plasma ghrelin concentrations in monogastric animals, it cannot be ruled out as a mediator of ghrelin in ruminants despite differences in GLU metabolism between monogastric and ruminant animals.

**Relationship of Ghrelin with Plasma Hormones, Metabolites, and Characteristics of Rumen Fermentation Within a 12-h Feeding Interval**

Postprandial plasma ghrelin concentrations decreased with the consumption of a high-carbohydrate, high-fat, or high-protein diet in humans, but the greatest suppression occurred with the ingestion of a high-carbohydrate diet (Tannousdit et al., 2006). For rodents, infusion and absorption of GLU have been reported to decrease plasma ghrelin concentrations (Tschöp et al., 2000; Williams et al., 2003). In contrast to monogastric animals, most dietary carbohydrate is
fermented to VFA in the rumen; thus, little carbohydrate reaches the small intestine, where it would be absorbed as GLU. Ruminants generate the majority GLU from the metabolism of propionate in the liver; therefore, plasma GLU concentrations fluctuate less postprandially (Fahey and Berger, 1988; Forbes, 1995). Additionally, ruminal VFA have been implicated as regulators of DMI (Bhattacharya and Alulu, 1975; Sheperd and Combs, 1998; Bradford and Allen, 2007). We hypothesized that propionate in ruminants is analogous to GLU in monogastric animals and should be correlated negatively to plasma ghrelin concentrations over a 12-h feeding interval.

Although acetate, propionate, and butyrate have been implicated as potential regulators of DMI, their effect has been varied. Bhattacharya and Alulu (1975) demonstrated that intraruminal infusion of acetate, propionate, or butyrate decreased DMI of both a high-grain and a high-roughage diet in sheep, whereas Quigley and Heitmann (1991) demonstrated no effects of propionate infusion into the portal vein on DMI, regardless of whether lambs were in positive or negative energy balance. Likewise, Deetz and Wangsness (1981) demonstrated no effect of intravenous propionate infusion on DMI in sheep. Sheperd and Combs (1998) reported that intraruminal propionate infusion decreased DMI to a greater extent than did acetate in dairy cows, whereas Bhattacharya and Alulu (1975) demonstrated that acetate was more efficacious than propionate in decreasing DMI. Oba and Allen (2003a,b) reported a conflicting response to propionate infusion on DMI in lactating dairy cows. In one experiment, propionate decreased DMI, whereas DMI was not affected by propionate infusion in the other experiment. Oba and Allen (2003a,b) speculated that differences in the oxidative capacity of the liver may explain the differing responses to propionate infusion. The ineffectiveness of intravenous or portal infusion of propionate in decreasing DMI in ruminants, and the inconsistent efficacy of intraruminal propionate infusion in suppressing DMI may indicate an indirect role of propionate in the regulation of DMI that is perhaps mediated by the physiological state of the animal. It is important to note that infusion of VFA does not completely suppress DMI. Reported suppression of DMI as a result of VFA infusion ranges from 3 to 58% (Bhattacharya and Alulu, 1975; Sheperd and Combs, 1998; Bradford and Allen, 2007). If indeed ghrelin is an orexigenic peptide in ruminant animals, altering body composition, or regulating energy expenditure. Because ruminant livestock frequently encounter periods in which nutrient intake is not sufficient to meet the nutrient requirement for maintenance, production, or both, further defining the role of ghrelin is important to understanding the regulation of nutrient intake and expenditure in ruminant animals. Because nutrient intake and expenditure influence feed efficiency and composition of gain, a greater understanding of the role of ghrelin may allow for improvements in feed efficiency and composition of gain.

In the current experiment, in which DMI was controlled, plasma ghrelin concentrations differed as a result of the quantity of a common diet consumed or the nutrients it supplied, but the interaction of sampling time relative to feeding and quantity of feed consumed was not significant because plasma ghrelin concentrations were elevated before both feeding times, with a nadir in between feedings regardless of the quantity of DMI. Rumen distention has been addressed earlier in the discussion and also is relevant to the discussion of acute regulation of DMI because distention and ghrelin suppression would be expected to be greatest after a meal and to lessen as feed is digested and the next feeding time approaches.

In summary, plasma ghrelin concentrations are persistently elevated with prolonged nutrient restriction sufficient to result in a loss of BW. Fluctuation in plasma ghrelin concentrations during a feeding interval was not completely explained by the fluctuation of other hormones or metabolites indicative of nutritional status or by fluctuations in ruminal VFA concentrations or ruminal pH. These data are consistent with the hypothesis that, although plasma ghrelin concentrations are elevated with prolonged nutrient restriction, factors in addition to GH, INS, NEFA, GLU, or end products of ruminal carbohydrate fermentation account for the fluctuation in plasma ghrelin concentration during a 12-h feed interval.

Elevated plasma ghrelin concentrations with prolonged nutrient restriction imply a potential role for ghrelin in conveying the long-term nutritional status in ruminants, altering body composition, or regulating energy expenditure. Because ruminant livestock frequently encounter periods in which nutrient intake is not sufficient to meet the nutrient requirement for maintenance, production, or both, further defining the role of ghrelin is important to understanding the regulation of nutrient intake and expenditure in ruminant animals. Because nutrient intake and expenditure influence feed efficiency and composition of gain, a greater understanding of the role of ghrelin may allow for improvements in feed efficiency and composition of gain.

LITERATURE CITED


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