Effects of glucose and volatile fatty acids on blood ghrelin concentrations in calves before and after weaning

R. Fukumori,* T. Mita,* T. Sugino,*† Y. Hasegawa,† M. Kojima,‡ K. Kangawa,§ T. Obitsu,* and K. Taniguchi*

*Graduate School of Biosphere Science, Hiroshima University, Higashi–Hiroshima 739-8528, Japan; †School of Veterinary Medicine and Animal Science, Kitasato University, Towada 034-8628, Japan; ‡Institute of Life Science, Kurume University, Kurume 839-0864, Japan; and §National Cardiovascular Center Research Institute, Osaka 565-8565, Japan

ABSTRACT: The effects of feeding and intravenous injections of glucose and VFA on blood ghrelin concentrations were investigated in calves before and after weaning. Eight Holstein bull calves were fed whole milk, allowed free access to solid feeds, and weaned at 7 wk. Measurements were carried out at 2, 4, 6, 9, 11, and 13 wk, at which time jugular blood samples were taken from 4 calves through a catheter from 10 min before to 120 min after their morning feed at 10 min intervals (Exp. 1). An additional 4 calves of the same age were intravenously injected with glucose (1.0 mmol·kg BW−1) and a solution of VFA (2.4 mmol·kg BW−1, acetate:propionate:butyrate in a 6:3:1 ratio) using a catheter, and jugular blood samples were taken temporally relative to the injection time (Exp. 2). In Exp. 1, blood ghrelin concentrations decreased \( P < 0.05 \) after feeding at all ages. However, preprandial ghrelin concentrations were less \( P = 0.025 \) and the degree of postprandial depression of ghrelin tended to be greater during the postweaning period \( P = 0.084 \) than during the preweaning period. Blood glucose concentrations increased after feeding during the preweaning period \( P < 0.05 \), whereas blood acetate concentrations increased during the postweaning period \( P < 0.05 \). In Exp. 2, injection of VFA induced a greater decrease in blood ghrelin concentrations than glucose injection throughout the entire period \( P < 0.05 \). These results indicate that weaning reduces the basal concentration of blood ghrelin because the circulating VFA derived from ruminal fermentation may more strongly depress blood ghrelin concentrations than glucose.

Key words: glucose, gut hormone, ruminant, short-chain fatty acid

INTRODUCTION

Ghrelin, mainly secreted by the abomasum in ruminants (Hayashida et al., 2001), can stimulate GH secretion in both nonruminants (Kojima et al., 1999, Takaya et al., 2000) and ruminants (Hosoda and Kangawa, 2008). Plasma ghrelin concentrations are related to feeding: a preprandial increase and a postprandial decline in circulating ghrelin concentrations have been observed in humans (Cummings et al., 2001) and sheep (Sugino et al., 2002). Some reports propose that circulating metabolites affect the depression of circulating ghrelin concentrations. In nonruminants, both oral and intravenous doses of glucose depressed plasma ghrelin concentrations (Nakagawa et al., 2002, Shiiya et al., 2002). In ruminants, however, the major energy substrate is VFA, and plasma glucose concentrations do not increase postprandially. In a previous paper, VFA (acetate, propionate, and butyrate) injections depressed plasma ghrelin concentrations in wethers (Fukumori et al., 2011), whereas glucose did not in sheep (Sugino et al., 2010). Thus, it is likely that the inhibiting factor for ghrelin secretion may differ between nonruminants and ruminants. In young calves, the digestive function substantially changes around the time of weaning. Preweaning, calves ingest milk lactose to absorb glucose through the intestines as their primary energy source, whereas postweaning, calves are fed solid feeds to absorb VFA produced by rumen microbes across the
rumen epithelium. Therefore, weaning appears to markedly affect postprandial regulatory metabolism, as well as the concentrations of circulating hormones. In addition, it is unclear whether ghrelin in young preruminant calves inherently possesses a physiological responsiveness towards VFA.

The objectives of the present study were to determine the effects of weaning on blood ghrelin concentrations, and to elucidate the effects of intravenous glucose and VFA injections on blood ghrelin concentrations around the weaning period.

MATERIALS AND METHODS

The procedures used in the present study were carried out in accordance with the principles and guidelines for animal use set by Hiroshima University, and formulated to comply with regulations of the Japanese Ministry of Education, Culture, Sports and Technology. All experiments were approved by the Animal Care and Use Committee of Hiroshima University.

Experimental Animals and Management

Eight Holstein bull calves (birth BW: 49.3 ± 1.7 kg) were kept in metabolism cages at 20°C under a 14 h light:10 h dark cycle and equally divided for the 2 experiments. The calves were fed colostrum starting from just after birth up to d 4. Thereafter, they were fed whole milk equivalent to 10% of birth BW, and allowed voluntary intake of calf starter [CP 20%, TDN 75% (DM)] and Klein grass hay (Panicum coloratum L.) during the preweaning period (0 to 6 wk). After weaning at 7 wk, they were fed calf starter and Klein grass hay equivalent to an energy supply of 0.7 kg of daily BW gain according to the Japanese Feeding Standard for Dairy Cattle (Agriculture, Forestry, and Fisheries Research Council Secretariat, 2006). The calves were allowed free access to water and were provided with feed at 0900 and 1700 h. Throughout the experiment, the feed intake of each calf was recorded daily, and the BW of each calf was recorded weekly. The DE intake was estimated by the Standard Tables of Feed Composition in Japan (Japan Livestock Industry Association, 2009). Each calf was fitted a jugular vein catheter (Argyle 16G CV catheter kit; Nippon Sherwood Medical Industries Ltd., Tokyo, Japan) filled with 200 U/mL heparinized saline at least 1 d before the experiment. The experiments were carried out at 2, 4, and 6 wk (preweaning) and at 9, 11, and 13 wk (postweaning). A diagram outlining the experimental design is shown in Figure 1.

Blood Collection

Exp. 1: Effect of Weaning on Blood Ghrelin Concentrations. Examination was conducted on the first day of each week (Figure 1). Blood samples (5 mL) were taken at 10-min intervals from –10 min to 120 min relative to the morning feeding time (0900 h), and feed was provided after sampling at 0 min. For the measurement of ghrelin, glucose, and acetate basal values, another blood sample was obtained at 0700 h.

Exp. 2: Effects of Intravenous Glucose and VFA Injections on Blood Ghrelin Concentrations. Examinations were conducted on the first and fourth days of each week (Figure 1). Glucose solution was prepared by dissolving D-glucose (Nacarai Tesque, Inc., Kyoto, Japan) in 50 mL of 0.9% saline and adjusting the pH to 7.4. A mixture of acetate, propionate, and butyrate (molar ratio, 6:3:1, respectively) was prepared by dissolving sodium acetate, sodium propionate, and sodium butyrate (Nacarai Tesque, Inc.) in 50 mL of 0.9% saline and adjusting to pH 7.4. This mixture ratio of VFA reflected the approximate ratio produced from the rumen. The amount of glucose injected, 1.0 mmol·kg BW⁻¹, mimicked postprandial hyperglycemia of milk-fed calves, whereas that of VFA injected, 2.4 mmol·kg BW⁻¹, adjusted the energy content equal to that of glucose. The injection order of glucose and VFA solution was changed by calf and week to avoid measurement values being affected by age (Figure 1). Injections were administered after feed deprivation for 16 h. Each solution was injected within 1 min into the jugular vein catheter, after which 10 mL of saline was injected to flush the catheter. Blood samples (5 mL) were obtained at –10, 0, 1.5, 3, 6, 9, 12, 15, 20, 25, 30, 40, 50, and 60 min relative to the injection time.

Laboratory Analyses

A portion of the blood samples was used for hematocrit determination by centrifuging at 13,000 × g for 5 min at 20°C, and the rest was immediately placed in tubes with heparin (10 U/mL of blood; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and aprotinin (500 KIE/mL of blood; Sigma-Aldrich, Inc., Tokyo, Japan). The extracted blood samples were centrifuged
at 2,330 × g for 15 min at 4°C. Harvested plasma was stored at −80°C until assayed.

Plasma ghrelin concentrations were measured with a time-resolved fluorimunoassay. Plasma bioactive ghrelin concentrations were measured with a competitive solid phase immunoassay using europium-labeled synthetic rat ghrelin, polyclonal antirat ghrelin, and polystyrene microtiter strips (Nalge Nunc Int., Tokyo, Japan) coated with antirabbit γ-globulin (Sugino et al., 2004). The polyclonal antirat ghrelin has a raised N-terminal fragment of ghrelin, which is almost identical among species (Hayashida et al., 2001). Intra- and interassay CV were 7 and 6%, respectively. The least measurable concentration and 50% inhibitory concentration in this assay system were 0.025 and 0.831 ng/mL, respectively.

Whole blood glucose concentrations were determined in Exp. 1 using a glucose analyzer (GA-1151; Arkray, Inc., Kyoto, Japan). Plasma acetate concentrations were determined every 20 min in Exp. 1 using a commercially available kit (F kit acetate; R-Biopharm AG, Darmstadt, Germany). Each glucose and acetate assay was performed once. The intraassay CV of glucose and acetate were 1 and 5%, respectively.

Calculations and Statistics

The values of blood ghrelin and acetate concentrations in whole blood were calculated based on this formula: blood concentration = plasma concentration·[(100 – hematocrit (%))·100⁻¹]⁻¹.

All data were analyzed with the JMP program package (SAS Inst. Inc., Cary, NC).

In Exp. 1, although comparisons of basal blood concentrations (at 0700 h) of ghrelin, glucose, and acetate were conducted with the raw data, postprandial time series values of blood ghrelin, glucose, and acetate were normalized to the respective baseline of each calf (mean value from –10 and 0 min). Values were expressed as the least squares mean of 4 calves with SE or SEM. Data were analyzed by ANOVA for repeated measures by mixed models. For the statistical analysis of time series data in each week, the model included time as a fixed effect and calf as a random effect. If the time effect was significant, then temporal changes after feeding were evaluated by multiple comparisons using Tukey’s test. For the statistical analysis of basal blood concentrations, decremental area of ghrelin, and incremental areas of glucose and acetate, the models included week as a fixed effect and calf as a random effect. If the week was significant, then differences among weeks were evaluated by Tukey’s test. The effect of weaning (2, 4, and 6 wk vs. 9, 11, and 13 wk) was tested by preplanned contrasts. For all statistical inferences a P < 0.05 was considered significant.

In Exp. 2, postinjection values of blood ghrelin were normalized to the respective baseline of each calf [mean value of preinjection (−10 and 0 min)]. Values were expressed as least squares mean of 4 calves with SE or SEM. Data were analyzed by ANOVA for repeated measures by mixed models. For the statistical analysis of time series data in each week, the model included time as a fixed effect and calf as a random effect. If the time effect was significant, then differences among weeks were evaluated by Tukey’s test. The comparison of the decremental area of ghrelin, the models included week as a fixed effect and calf as a random effect. If the week was significant, then differences among weeks were evaluated by Tukey’s test. The comparison of the decremental area between glucose and VFA injection in each week was evaluated by a paired t-test. Preplanned contrasts were used to test the effect of weaning (2, 4, and 6 wk vs. 9, 11, and 13 wk). For all statistical inferences a P < 0.05 was considered significant and P < 0.10 was considered a trend.

RESULTS

Feed Intake and BW

The data for DMI, DE intake per metabolic BW, and BW are presented in Figure 2.

Exp. 1: Effect of Weaning on Blood Ghrelin Concentrations. Basal concentrations of blood ghrelin, glucose, and acetate are shown in Table 1. Weaning decreased basal values of ghrelin and glucose (P = 0.025 and P < 0.001, respectively), but increased acetate (P =

| Table 1. Basal blood ghrelin, glucose, and acetate concentrations at 0700 h in calves before and after weaning at 7 wk |
|-----------------|--------|--------|--------|--------|--------|--------|--------|--------|
| Item            | Week   | SEM    | P-value |
|                 | 2      | 4      | 6      | 9      | 11     | 13     | Wk²    | Weaning³ |
| Ghrelin, ng/mL  | 0.556  | 0.427  | 0.683  | 0.354  | 0.380  | 0.321  | 0.156  | 0.150   | 0.025   |
| Glucose, mg/dL  | 96.9a  | 89.1a  | 82.0ab | 74.0bc | 72.4bc | 61.9c  | 4.41   | <0.001  | <0.001  |
| Acetate, mM     | 0.081b | 0.078b | 0.112b | 0.144b | 0.167a | 0.187a | 0.043  | 0.045   | 0.035   |

a–c Within a row, means without a common superscript differ (P < 0.05).

1 Data are shown as the least squares means and SEM (n = 4).

2 Effect of week.

3 Effect of weaning tested by preplanned contrasts.
There were effects of week on blood glucose and acetate ($P < 0.001$ and $P = 0.045$, respectively) because they gradually changed with age.

The responses of ghrelin, glucose, and acetate to feeding are shown in Figure 3 and Table 2. The time series data for ghrelin, glucose, and acetate (Figure 3) are presented only for wk 6 (preweaning) and 13 (postweaning) to allow clear distinctions. Blood ghrelin concentrations

Table 2. Decremental area of blood ghrelin and incremental areas of glucose and acetate (% of baseline) after feeding in calves before and after weaning at 7 wk$^1$

<table>
<thead>
<tr>
<th>Item</th>
<th>Week</th>
<th>SEM</th>
<th>$P$-value</th>
<th>Wk$^2$</th>
<th>Weaning$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decremental area (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ghrelin</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>44.5</td>
<td>58.5</td>
<td>53.6</td>
<td>60.6</td>
<td>64.6</td>
</tr>
<tr>
<td>Incremental area (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>31.5$^a$</td>
<td>22.5$^a$</td>
<td>23.7$^a$</td>
<td>3.22$^b$</td>
<td>2.26$^b$</td>
</tr>
<tr>
<td>Acetate</td>
<td>25.8</td>
<td>37.7</td>
<td>10.9</td>
<td>101</td>
<td>119</td>
</tr>
</tbody>
</table>

$^a,b$Within a row, means without a common superscript differ ($P < 0.05$).

$^1$Data are shown as the least squares mean and SEM ($n = 4$).

$^2$Effect of week.

$^3$Effect of weaning tested by preplanned contrasts.
after feeding compared with that of the baseline (−10 and 0 min) were decreased in both wk 6 and wk 13 (P < 0.05, Figure 3). The other week data not presented in Figure 3 (wk 2, 4, 9, and 11), also showed similar changes (P < 0.05). The incremental areas of ghrelin after feeding during the postweaning period were less than that during the preweaning period (P = 0.084, Table 2). The incremental areas of glucose during the postweaning period were greater than that during the preweaning period (P = 0.011).

Exp. 2: Effects of Intravenous Glucose and VFA Injections on Blood Ghrelin Concentrations. The data for Exp. 2 are presented in Figure 4 and Table 3. The time series data for ghrelin (Figure 4) are presented only at wk 6 and 13. Blood ghrelin concentrations after VFA injections were reduced compared with that of the baseline (−10 and 0 min) in both wk 6 and 13 (P < 0.05, Figure 4B), but blood ghrelin concentrations after glucose injection did not change significantly compared with that of the baseline at both wk 6 and 13. The other week data not presented in Figure 4 also did not show temporal changes after glucose injection (data not shown, P > 0.05). The decremental areas of ghrelin after VFA injections were greater (P < 0.05) compared with that after glucose injections in all weeks except for wk 4 (Table 3). There were no effects of week and weaning on the decremental areas of ghrelin in both VFA and glucose injections [Glucose injection: wk effect (P = 123), weaning effect (P = 0.942), VFA injection: wk effect (P = 0.802), weaning effect (P = 0.352)].

DISCUSSION

Nonruminants are observed to have postprandial increases in plasma glucose and insulin concentrations. In contrast, weaned ruminants are fed solid feed and absorb VFA as their primary energy source. An increment in VFA absorption induces a postprandial increase of plasma insulin concentrations and a decrease of GH in sheep (Matsunaga et al., 1999). Horino et al. (1968) demonstrated that such VFA-stimulatory action on insulin secretion was specific to ruminants. Thus, VFA may have an important regulatory role with regard to hormone secretion in ruminants. A previous study demonstrated that blood ghrelin concentrations were decreased by intravenous injection of VFA in adult wethers (Fukumori et al., 2011). In Exp. 1, a smaller increase in blood glucose concentrations and a greater increase in blood acetate concentrations after feeding were observed during the postweaning period. Although blood concentrations of propionate and butyrate were not measured in this study, a previous report showed that peripheral blood VFA concentrations, including acetate, propionate, and butyrate, increased after feeding (Quigley et al., 1992). Blood acetate, propionate, and butyrate concentrations were obviously greater in postweaning calves compared with that in preweaning calves (Klotz and Heitmann, 2007). These data suggest that the absorbed energy source changes from glucose to VFA at the time of weaning. In Exp. 2, isocaloric glucose and VFA were intravenously administered. As a result, VFA caused a more pronounced decrease of blood ghrelin concentrations than that of glucose throughout the experimental period. Therefore, the present study has clearly demonstrated that ghrelin secretion is more strongly suppressed by VFA than by glucose even in preweaning calves. The decreases in blood ghrelin concentrations after glucose injections were only slight, even in preruminant calves. In contrast, VFA injections decreased blood ghrelin concentrations even in preruminant calves that did not possess the rumen fermentation function. These results are consistent with previous studies in adult sheep, which
demonstrated that intravenous VFA injections decreased ghrelin concentrations (Fukumori et al., 2011), but intravenous glucose infusion did not (Sugino et al., 2010). These data suggest that ruminants may inherently possess ghrelin responses to VFA, but not to glucose. In Exp. 1, decreased basal ghrelin concentrations were observed during the postweaning period, which was consistent with a previous report (Kobayashi et al., 2006). In addition, a postprandial decrease of ghrelin during the postweaning period tended to be greater compared with that during the preweaning period. This phenomenon may be explained by the increased absorption of VFA after weaning. As for ghrelin responses to feeding, Miura et al. (2004) reported that postprandial plasma ghrelin concentrations did not decrease in 3-mo-old postweaning calves, unlike the current study. In their report, calves were fed 1 kg of concentrate and 3 kg of hay, whereas calves of the same age as those in the current study were fed about 2.4 kg of concentrate and 1 kg of hay. High concentrate diets more rapidly ferment and produce VFA compared with high roughage diets. Therefore, the dietary differences between the 2 experiments may affect the rumen fermentation and the ghrelin response to feeding. However, basal blood ghrelin concentrations did not clearly decrease with age after weaning, although basal blood acetate concentrations gradually increased after weaning. Thus, blood ghrelin concentrations may also be mediated by other factors.

During the preweaning period, postprandial decreases in ghrelin concentration were observed, although such decreases tended to be lesser than those during the postweaning period. The mechanisms underlying postprandial ghrelin depression in milk-fed calves have remained unclear because hyperglycemia did not induce ghrelin depression. In preweaning calves, other factors may mediate ghrelin depression because circulating ghrelin concentrations were found to be potentially affected by AA, fatty acids, insulin, and the nervous system (McCowen et al., 2002, Nishi et al., 2005, Foster-Schubert et al., 2008, Hosoda and Kangawa, 2008, Sugino et al., 2010, Fukumori et al., 2012). Therefore, further research is necessary to elucidate the effects of nutrients on blood ghrelin concentrations in preweaning calves.

In the previous study by Katoh et al. (2004), plasma GH concentrations in milk-fed calves increased postprandially, whereas those in postweaning calves did not. Although ghrelin has been well known to stimulate GH secretion (Hosoda and Kangawa, 2008), the present study showed that plasma ghrelin concentrations decreased even after milk feeding. The relationship between ghrelin and GH secretion in milk-fed calves cannot be explained in the present study and the postprandial GH increase in milk-fed calves would be regulated by other several factors.

Throughout this study, postweaning calves had a greater DE intake than preweaning calves, which may also affect the greater decrease in blood ghrelin concentrations (Wertz-Lutz et al., 2007). The changes in blood ghrelin concentrations by glucose and VFA in Exp. 2 were the result of single injections, which did not mimic realistic postprandial nutrient absorption. Therefore, further studies are required to examine the effect of weaning on blood ghrelin concentrations by equalized energy intake levels and by long-term infusion of nutrients at levels similar to those encountered postprandially.

In conclusion, this study demonstrated for the first time that preweaning calves inherently possessed a ghrelin response to VFA. Intravenous glucose injection did not decrease blood ghrelin concentrations in either pre- or postweaning calves. This finding suggests that the greater postprandial ghrelin depression observed during the postweaning period may be related to increased VFA absorption.

**LITERATURE CITED**


Cummings, D. E., J. Q. Purnell, R. S. Frayo, K. Schmidova, B. E. Wisse, and D. S. Weigle. 2001. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Dia-

---

### Table 3. Decremental area of blood ghrelin (% of baseline) after intravenous injection of glucose or VFA in calves before and after weaning at 7 wk

<table>
<thead>
<tr>
<th>Item</th>
<th>Week 2</th>
<th>Week 4</th>
<th>Week 6</th>
<th>Week 9</th>
<th>Week 11</th>
<th>Week 13</th>
<th>SEM</th>
<th>Wk²</th>
<th>Weaning³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose4</td>
<td>16.7</td>
<td>35.9</td>
<td>14.6</td>
<td>26.0</td>
<td>16.1</td>
<td>19.0</td>
<td>6.30</td>
<td>0.123</td>
<td>0.942</td>
</tr>
<tr>
<td>VFA5</td>
<td>54.2</td>
<td>54.6</td>
<td>57.4</td>
<td>61.1</td>
<td>62.7</td>
<td>55.0</td>
<td>6.74</td>
<td>0.802</td>
<td>0.352</td>
</tr>
</tbody>
</table>

*Mean is different with VFA injection except for 4 wk (P < 0.05).

1 Data are shown as the least squares means and SEM (n = 4).

2 Effect of week.

3 Effect of weaning tested by preplanned contrasts.

4 Decremental area of ghrelin after glucose injection.

5 Decremental area of ghrelin after VFA injection.
betes 50:1714–1719.