

Postprandial changes in plasma GH and insulin concentrations, and responses to stimulation with GH-releasing hormone (GHRH) and GHRP-6 in calves around weaning

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Abstract

Changes in plasma concentrations of GH and insulin in response to feeding and stimulation with GH-releasing hormone (GHRH) or GH-releasing peptide (GHRP-6, a ligand for endogenous GH secretagogue receptors) were compared between 3-week-old (milk-fed) and 12-week-old (concentrate and hay-fed) calves. Feeding of a milk-replacer diet in 3-week-old animals significantly increased the basal (prefeeding) concentrations of GH, insulin and glucose in plasma, whereas feeding of concentrate and hay in 12-week-old animals did not cause a significant change in these traits. However, in the animals maintained on a milk-replacer diet until 12 weeks of age, postprandial plasma GH concentrations and AUC (area under the curve) were not different from those in the age-matched weaned

group. The venous injection of either GHRH (0.25 µg/kg) or GHRP-6 (2.5 µg/kg) significantly increased plasma GH concentrations in both 3- and 12-week-old animals, but GH AUC was significantly greater in 3-week-old than in 12-week-old animals. Insulin concentration was transiently but significantly increased by the injection of GHRP-6 only in 12-week-old animals, the AUC being greater in 12-week-old than 3-week-old animals. From these results, we conclude that postprandial levels of plasma GH and insulin concentrations are altered after weaning and by aging, and that the quality of diets or development of the neuroendocrine functions in the digestive–pituitary system may be involved in this alteration.

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Introduction

Weaning, after birth, is the most drastic event that neonates have to experience, because they are obliged to change feedstuffs from liquid milk to solid particles, even if it occurs gradually. This means that weaning has to change the digestive and metabolic functions to meet the change in the quality of the diet. We recently found that weaning abolished leptin expression in the rumen and abomasum in calves. Similarly, the expression of CCKA and CCKB receptors in the foregut of calves was also suppressed after weaning (Yonekura *et al.* 2002). In addition, when calves were fed a milk-replacer diet until 13 weeks of age, leptin expression in the epithelium of the rumen and abomasum was still detected throughout (Yonekura *et al.* 2002), and carbonic anhydrase activity and ion secretion in the parotid gland also remained at preweaning levels (Kitade *et al.* 2002). With regard to glucose metabolism, when veal calves were fed milk without solid feed, such as concentrates or roughage, plasma glucose and insulin concentrations were greater than those of weanlings (Blum & Hammon 1999).

For the ruminant, growth hormone (GH), as well as insulin, as in other domestic animals, is indispensable for

the control of the metabolism and function of bone, liver, skeletal and cardiac muscles, adipose tissue, reproductive organs and various other tissues or organs (Simmen *et al.* 1998, Clark & Rogol 1999). The somatotrophic axis principally consists of hypothalamic (growth hormone-releasing hormone (GHRH) and somatostatin (SRIF)) and peripheral hormones, such as ghrelin and leptin, and insulin-like growth factor-I (IGF-I). Pituitary GH secretion, which alters in a pulsatile manner in all of the animal species studied so far, is mainly stimulated by two hormones (GHRH and ghrelin (or GH secretagogues: GHS)), whereas it is suppressed by SRIF (Lamberts 1999, Tannenbaum & Epelbaum 1999) and IGF-I (Katoh *et al.* 2004). The responsiveness to GHRH in the bovine fetus pituitary cells seems greater than that in adult cells, since the basal GH release was age-dependently reduced in ovine pituitary cells isolated at different ages and the maximum release was obtained in a 70-day-old fetus (Blanchard *et al.* 1988). Therefore, it is likely that GH secretion in response to GHRH stimulation declines with the aging process, although it is not known how the response to GHRP changes and why basal GH levels decline in an age-dependent manner.

Weaning is a critical point for neonates because not only do they have to change diets but also the endocrine system has to adapt to this change. In the present study, therefore, we observed changes in the secretion of GH and insulin in response to feeding and stimulation with GHRH and GHS (GH-releasing peptide: GHRP-6) in 3-week-old (milk-fed) and 12-week-old (concentrate and hay-fed) calves. We also compared the endocrine responses to feeding between the animals maintained on a milk-replacer diet until 12 weeks of age and those of weaned, age-matched control animals.

Materials and Methods

Animals

Holstein calves were treated according to 'Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences' (Physiological Society of Japan), and the present experiment was approved by the Animal Care Committee of Tohoku University.

Four to six male calves for each experiment were fed with their dams for 1 week after birth to ensure sufficient intake of colostrum before starting the experimental feeding. In experiments 1 and 3, the animals were fed a milk-replacer diet until 4 weeks of age, milk-replacer and calf-starter diets after 4 weeks of age, and then calf starter and hay from 7 to 13 weeks of age, as described in our previous reports (Kitade *et al.* 2002, Yonekura *et al.* 2002). In experiment 2, the animals were fed only a milk-replacer diet until 12 weeks of age. All calves were fed twice daily at 1000 and 1600 h.

Experimental design

Experiment 1: effects of feeding At 3 and 12 weeks of age, at 1 day before blood sampling, calves were fitted with a catheter (type V-1; Top, Tokyo, Japan) in the left jugular vein under local anesthesia. The catheter was filled with sterile iso-osmotic sodium citrate (3.8% w/v) solution before and throughout the sampling. The blood sampling (4 ml each) was done every 15 min from 30 min before to 90 min after feeding (0930–1130 h). Animals were fed a milk-replacer diet (2000 ml) at the age of 3 weeks and, at 12 weeks of age, had free access to a calf-starter diet and hay for 30 min twice daily at 1000 and 1600 h. Blood samples were centrifuged at 8000 g for 15 min, and blood plasma was divided into three portions and stored at -30°C until analysis.

Experiment 2: effects of feeding in animals maintained on a milk-replacer diet Five calves were maintained on a milk-replacer diet until 12 weeks of age, as previously described (Kitade *et al.* 2002, Yonekura *et al.* 2002). Another five animals were weaned at 7 weeks old and fed until 12 weeks of age, as age-matched controls.

They were subjected to blood sampling at 12 weeks of age, as described in experiment 1.

Experiment 3: effects of venous injection of GHRH or GHRP-6 At the ages of 3 and 12 weeks, on the same day as for experiment 1, blood sampling (4 ml each) was conducted every 15 min for 90 min after the venous injection of hGHRH (0.25 $\mu\text{g}/\text{kg}$ body mass) or GHRP-6 (2.5 $\mu\text{g}/\text{kg}$ body mass) (1130–1400 h). The dose of GHRH (Peptide Research Institute, Osaka, Japan) was determined from our previous report (Matsunaga *et al.* 1997), whereas the dose of GHRP-6 (Peptide Research Institute) employed was 10 times greater than that of GHRH, because the potency of GHRP for GH-releasing activity has been found to be 10-fold less than that of GHRH (Ohata *et al.* 1997). The morning feed was given at 1000 h, but the afternoon feed was given after the final sample had been taken. The sampling and storage method of blood plasma was the same as described for experiment 1.

Analyses

The plasma concentrations of GH, insulin and IGF-I were measured by RIA, as previously described (Kuhara *et al.* 1991). The glucose concentration was determined by the glucose oxidase method, using a commercial kit (Glucose CII-Test; Wako Pure Chemicals, Osaka, Japan).

Statistics

The data at each sampling time were expressed as the mean \pm S.E.M. For the GH and insulin secretion curve data, AUC (area under the curve) was calculated and expressed as ng per min/ml and μU per min/ml, respectively.

The significance of the differences was determined by paired *t*-test to analyze the data within each hormone concentration curve, and by Student's *t*-test between two groups (Zar 1984).

Results

Experiment 1: effects of feeding

Feeding of a milk-replacer diet in the 3-week-old group significantly increased the basal GH concentration, the peak value being 45.3 ± 7.5 ng/ml ($n=6$) at 60 min after feeding. In contrast, feeding of concentrate and hay in the 12-week-old group did not cause a significant change in GH concentrations ($n=4$). Consequently, there were significant differences between the two groups for 15–90 min after feeding. The AUC of GH for the 3-week-old group was significantly greater than that for the 12-week-old group (5547.9 ± 563.3 vs 1547.5 ± 404.1 ng per min/ml respectively; $P=0.0009$), as shown in Fig. 1B.

Plasma insulin concentration levels before feeding were significantly greater for the 12-week-old group than for

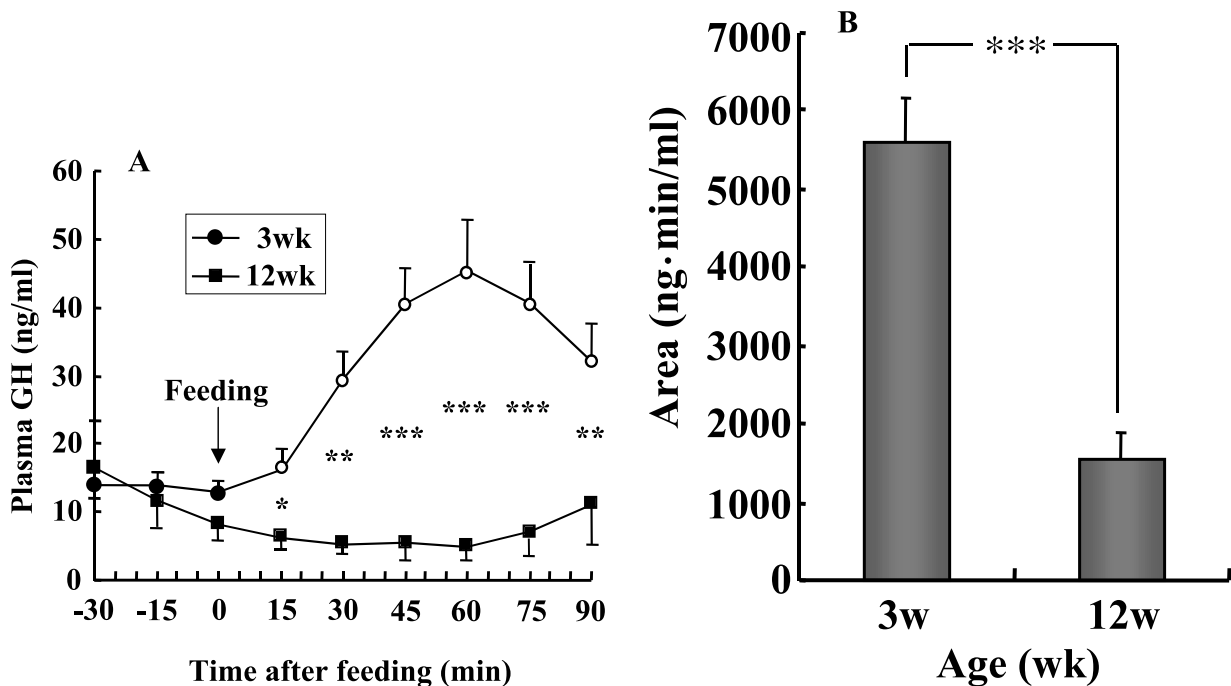


Figure 1 Postprandial changes in plasma GH concentrations (A) and GH AUC (B) in 3-week-old ($n=6$) and 12-week-old ($n=4$) calves. Animals were given a milk-replacer diet at 3 weeks of age, or concentrate and hay at 12 weeks of age. The values are represented as the mean \pm S.E.M. Open symbols indicate a significant difference from the prefeeding level. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

the 3-week-old group, as shown in Fig. 2A (4.1 ± 1.1 vs 16.1 ± 2.3 $\mu\text{U/ml}$ at 0 min respectively; $P=0.0008$). Feeding caused a significant increase in insulin concentrations in the 3-week-old group, the peak value being 34.1 ± 4.7 $\mu\text{U/ml}$ at 75 min after feeding. For the 12-week-old group, a nonsignificant increase in plasma insulin concentrations was noted after feeding. However, the AUC of insulin was still greater for the 12-week-old group than for the 3-week-old group (3033.5 ± 424.5 vs 1637.7 ± 105.0 $\mu\text{U per min/ml}$ respectively; $P=0.0088$), as shown in Fig. 2B.

As depicted in Fig. 3, the prefeeding glucose level was significantly greater for the 12-week-old group than for the 3-week-old group (78.6 ± 3.6 vs 56.1 ± 5.5 mg/dl at 0 min respectively; $P=0.0166$). Feeding caused a significant increase in the 3-week-old group (from 55.1 ± 6.2 to 89.3 ± 3.5 mg/dl) only at 90 min after feeding.

IGF-I concentrations in plasma sampled just before feeding were slightly greater for the 12-week-old group than for the 3-week group (29.7 ± 4.2 vs 20.4 ± 3.9 ng/ml respectively; $P=0.1390$; not shown).

Experiment 2: effects of feeding in the animals maintained on a milk-replacer diet

The GH levels in the animals maintained on a milk-replacer diet until 12 weeks of age were not significantly

different from that of the age-matched, weaned animals (Fig. 4A). Therefore, there was no significant difference in the GH AUC between the age-matched control group ($n=5$) and the group maintained on milk replacer ($n=5$) (1147.5 ± 531.1 vs 1557.7 ± 326.8 ng per min/ml respectively; $P=0.1797$; not shown).

In contrast, the postprandial insulin level of the group maintained on a milk-replacer diet drastically and significantly increased after feeding (Fig. 4B). The insulin AUC was, therefore, significantly greater for the group maintained on a milk-replacer diet than for the control group (11250.4 ± 1750.3 vs 3125.2 ± 375.6 $\mu\text{U per min/ml}$ respectively; $P=0.0001$; not shown).

As also shown in Fig. 4C, there were no significant changes in postprandial glucose levels in the weaned control group. However, the glucose level of the group maintained on a milk-replacer diet significantly increased, and was significantly greater ($P=0.0004$) than that of the control group at 30–90 min after feeding (Fig. 4C).

Plasma concentrations of IGF-I before feeding were significantly greater in the group maintained on a milk-replacer diet than those of the control group (57.0 ± 7.8 vs 31.4 ± 4.3 ng/dl respectively; $P=0.0017$).

Experiment 3: effects of injection of GHRH or GHRP-6

The GH levels during the preinjection period were significantly greater in the 3-week-old group than those in

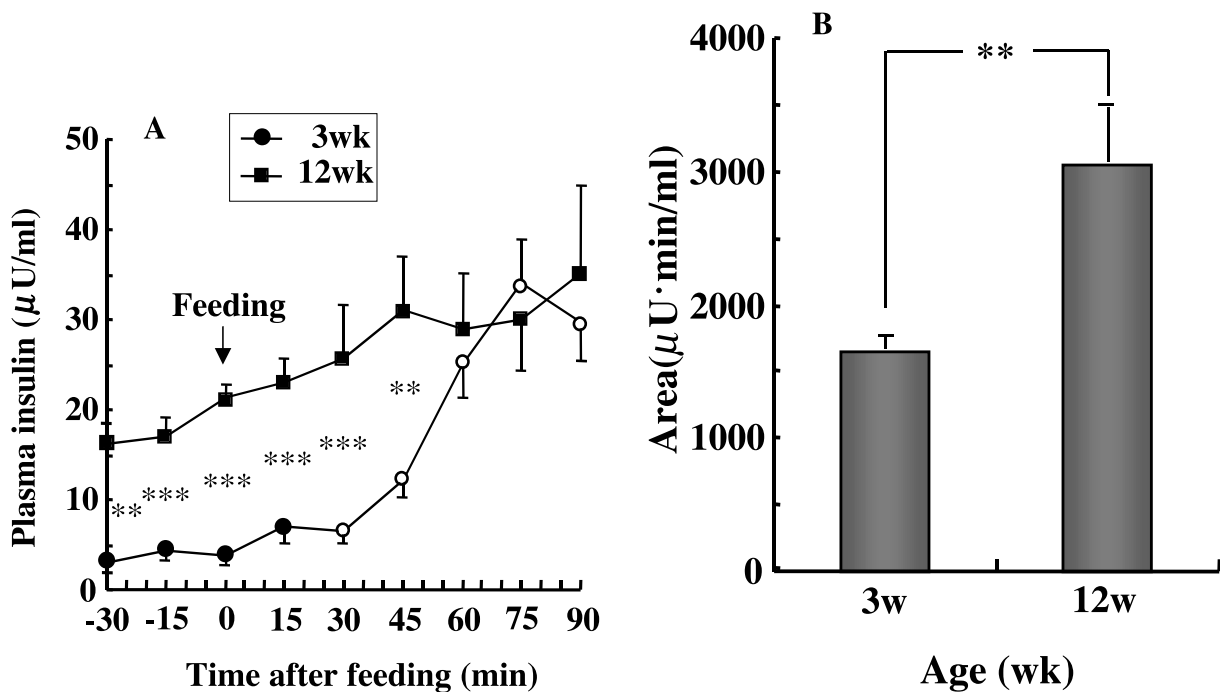


Figure 2 Postprandial changes in plasma insulin concentrations (A) and insulin AUC (B) in 3-week-old ($n=6$) and 12-week-old ($n=4$) calves. Animals were fed as in Fig. 1. The values are represented as the mean \pm S.E.M. Open symbols indicate a significant difference from the prefeeding level. ** $P<0.01$; *** $P<0.001$.

the 12-week-old group, as shown in Fig. 5. The GHRH injection ($0.25 \mu\text{g}/\text{kg}$) significantly increased the GH level by 151 and 139 ng/ml respectively, for the 3-week and

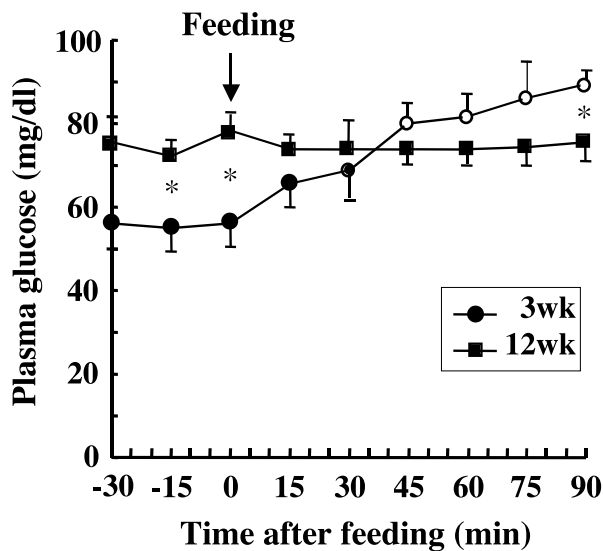


Figure 3 Postprandial changes in plasma glucose concentrations in 3-week-old ($n=6$) and 12-week-old ($n=4$) calves. Animals were fed as in Fig. 1. The values are represented as the mean \pm S.E.M. Open symbols indicate a significant difference from the prefeeding level. * $P<0.05$.

12-week groups. Similarly, the GHRP-6 injection ($2.5 \mu\text{g}/\text{kg}$) also significantly increased the GH level, by 85 and 82 ng/ml respectively, for the 3-week and 12-week groups. The AUC for 3-week-old animals was greater than that for 12-week-old animals in both cases.

The injection of GHRH slightly increased (Fig. 6A), but GHRP-6 injection significantly increased (Fig. 6B) the insulin level of the 12-week-old group. The insulin AUC for GHRH and GHRP-6 injection for the 12-week-old group was significantly greater than that for 3-week-old group ($P<0.01$).

Plasma glucose concentration was not significantly changed by the injection of GHRH or GHRP-6 (not shown).

Discussion

The present study clearly demonstrated that postprandial changes in plasma GH concentrations in 3-week-old (preweaning) calves are different from those in 12-week-old (postweaning) calves. Feeding commonly reduces plasma GH levels, as shown in sheep (Bassett 1974a,b, Driver & Forbes 1981, Trenkle 1989, Thomas *et al.* 1991, Matsunaga *et al.* 1998, 1999), calves (Moseley *et al.* 1988) and humans (Merimee & Fineberg 1974, Ishizuka *et al.* 1983, Jaffe *et al.* 1998), although it seemed to increase with feeding in lambs (Driver & Forbes 1981), steers

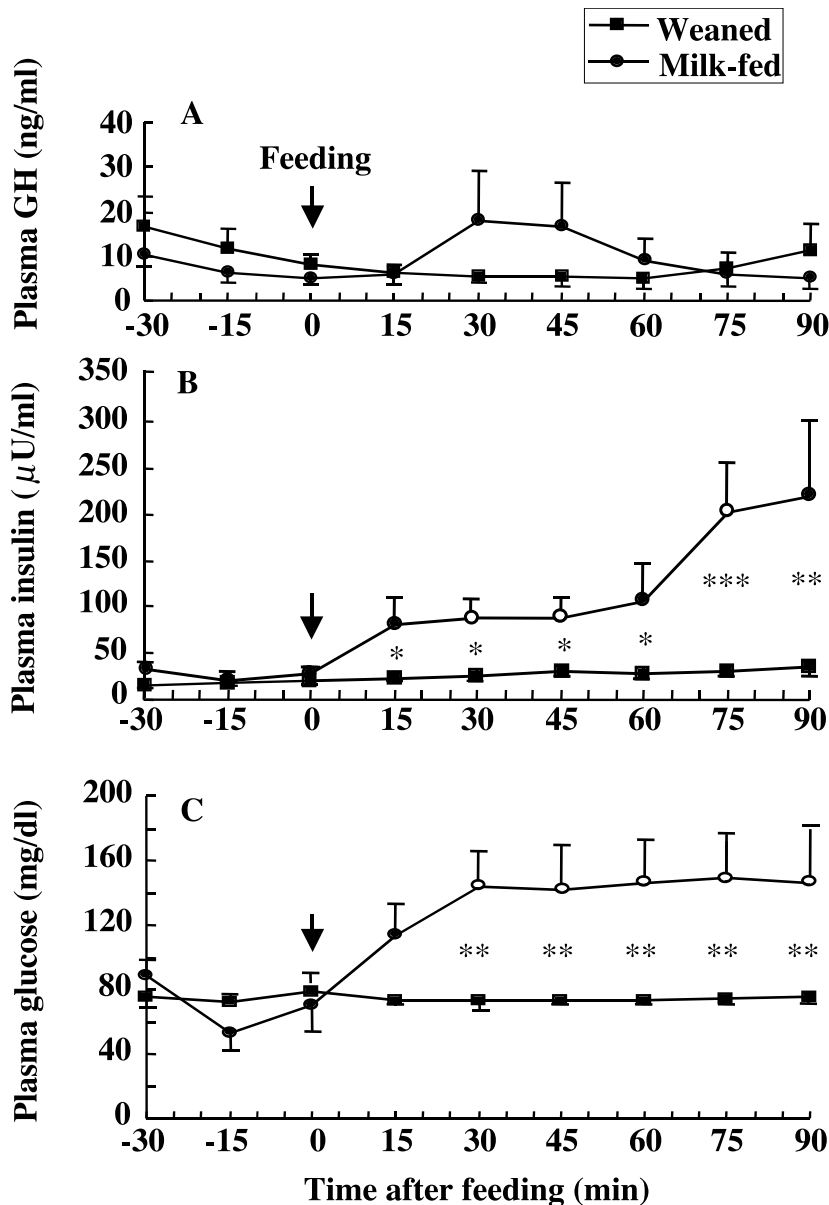


Figure 4 Postprandial changes in plasma GH (A), insulin (B) and glucose (C) concentrations in weaned ($n=5$) or milk-fed ($n=5$) animals at 12 weeks of age. Unweaned, milk-fed animals were fed a milk-replacer diet until 12 weeks of age, whereas weaned (control) animals were fed concentrate and hay. The values are represented as the mean \pm S.E.M. Open symbols indicate a significant difference from the prefeeding level. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

(Moseley *et al.* 1988) and rats (Tannenbaum *et al.* 1976). Feeding-induced reductions in plasma GH levels become more apparent in adult animals under restricted feeding or at low feeding frequency during the day, because these conditions are known to raise basal GH levels and pulse amplitude (Thomas *et al.* 1990, 1991), and to reduce SRIF synthesis in the hypothalamus of sheep (Thomas

et al. 1991, Henry *et al.* 2001). Feeding also reduces GHRH-induced GH release, and the response is mimicked by the anticipation of being fed, by distension of the rumen with a water-filled balloon, and sham-feeding (Trenkle 1989).

In the present study, the postprandial increase in GH levels shown in the milk-fed preweaning animals was

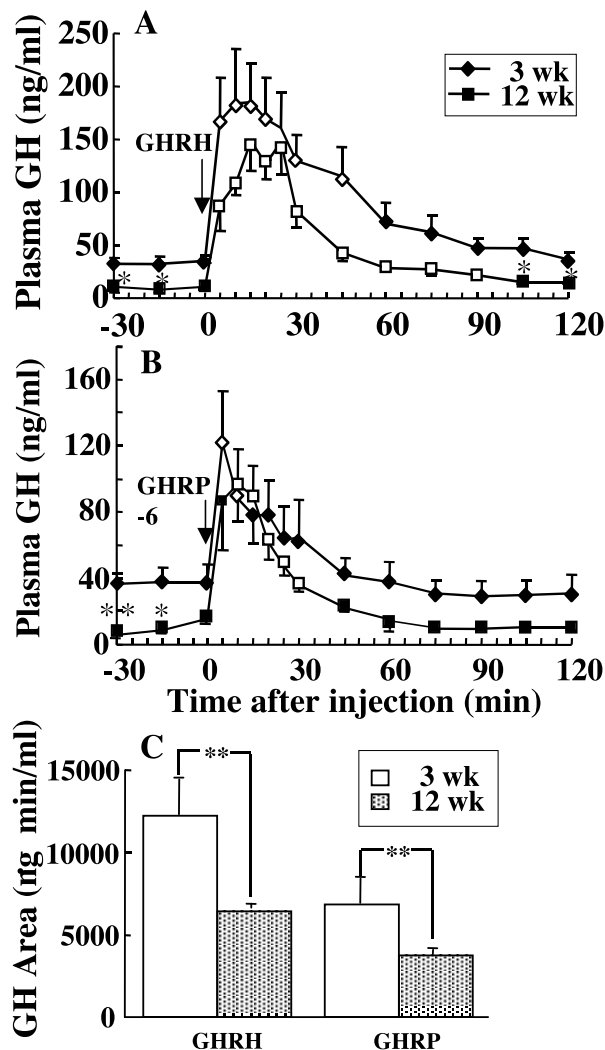


Figure 5 Changes in plasma GH concentrations (A and B) and AUC (C) in response to the intravenous administration of GHRH (0.25 µg/kg) (A) or GHRP-6 (2.5 µg/kg) (B) in 3-week-old ($n=6$) and 12-week-old ($n=4$) calves. The animals were fed as in Fig. 1, and given either GH stimulant 2 h after feeding. The values are represented as the mean \pm S.E.M. Open symbols indicate a significant difference from the preweaning level. * $P<0.05$; ** $P<0.01$.

abolished at 12 weeks of age, even though they were maintained on a milk-replacer diet. Therefore, the postprandial GH increase shown in preweaning animals seems to be dependent on age-associated processes. A significantly greater IGF-I concentration for the 12-week-old animals maintained on the milk-replacer diet may have caused a greater inhibitory feedback action on GH release from the pituitary gland than in age-matched, weaned animals, as suggested by *in vitro* results with goat anterior pituitary cells (Katoh *et al.* 2004). In a previous study (Hammon & Blum 1997), however, postprandial GH response was not dependent on plasma IGF-I levels.

In the present study, basal and GHRH- or GHRP-stimulated GH secretion was greater for 3-week-old than for 12-week-old animals. We used GHRH as a central GH stimulant released from the hypothalamus and GHRP as a peripheral stimulant. In previous reports (Blanchard *et al.* 1988, Silverman *et al.* 1989), the basal GH release was age-dependently reduced in ovine pituitary cells isolated at different ages, and the maximum GH release was obtained in a 70-day-old fetus (term=147 days), in response to administration of GHRH and somatostatin. This and our preliminary study (Katoh & Obara 2001) indicate that bovine somatotrophs differentiate and possess GHRH receptors and the relevant intracellular signaling system at an early state of gestation, and that GH secretion in response to GHRH stimulation declines with the aging process in this species.

The mechanism underlying the difference in postprandial GH levels between pre- and postweaning animals has yet to be clarified. It is known that ingested milk directly comes down into the omasum by passing through the reticular (esophageal) groove in preweaning, milk-fed animals (Ruckebusch 1988). The efferent limb of the groove-opening reflex contains cholinergic parasympathetic fibers, the transmitter of which is vasoactive intestinal peptide (VIP) (Reid *et al.* 1991). Therefore, the digestive tract is apparently able to detect and respond to the chemical and physical quality of diets, and to change a neural reflex to control GH secretion. Alternatively, Plouzek *et al.* (1988) reported that hypophysial stalk transection reduced the GH increase induced by GHRH stimulation, but feeding further reduced the GHRH-induced response in calves. These findings imply that the postprandial reduction in GH level is partly mediated by peripheral factors, as well as by the central nervous system. We postulated a physiologic role of short-chain fatty acids produced by microbes in the forestomachs (the reticulo-rumen), and have shown their involvement in the inhibitory mechanism for the postprandially reduced GH levels (Matsunaga *et al.* 1998, 1999, Katoh *et al.* 1999, Ishiwata *et al.* 2000, Katoh & Obara 2001). In addition, we recently demonstrated that leptin mRNA expression was increased by addition of acetate to the medium of primary cultured bovine anterior pituitary cells (Yonekura *et al.* 2003). Leptin is known as an inhibitor of GH secretion induced by GHRH in sheep pituitary cells (Roh *et al.* 1998).

It has been shown that feeding is accompanied by a reciprocal increase in peripheral plasma insulin, glucagon and SRIF concentrations (Bassett 1974b, Matsunaga *et al.* 1999). In the present study, the postprandial insulin response was greater in preweaning than that in postweaning animals, although basal levels were significantly greater in postweaning than in preweaning animals. It is likely that the postprandial increase in insulin concentration was due to milk ingestion in preweaning calves because the milk replacer contained a high concentration of proteins and fats as ingredients (24% proteins and 21% fats). Milk feeding

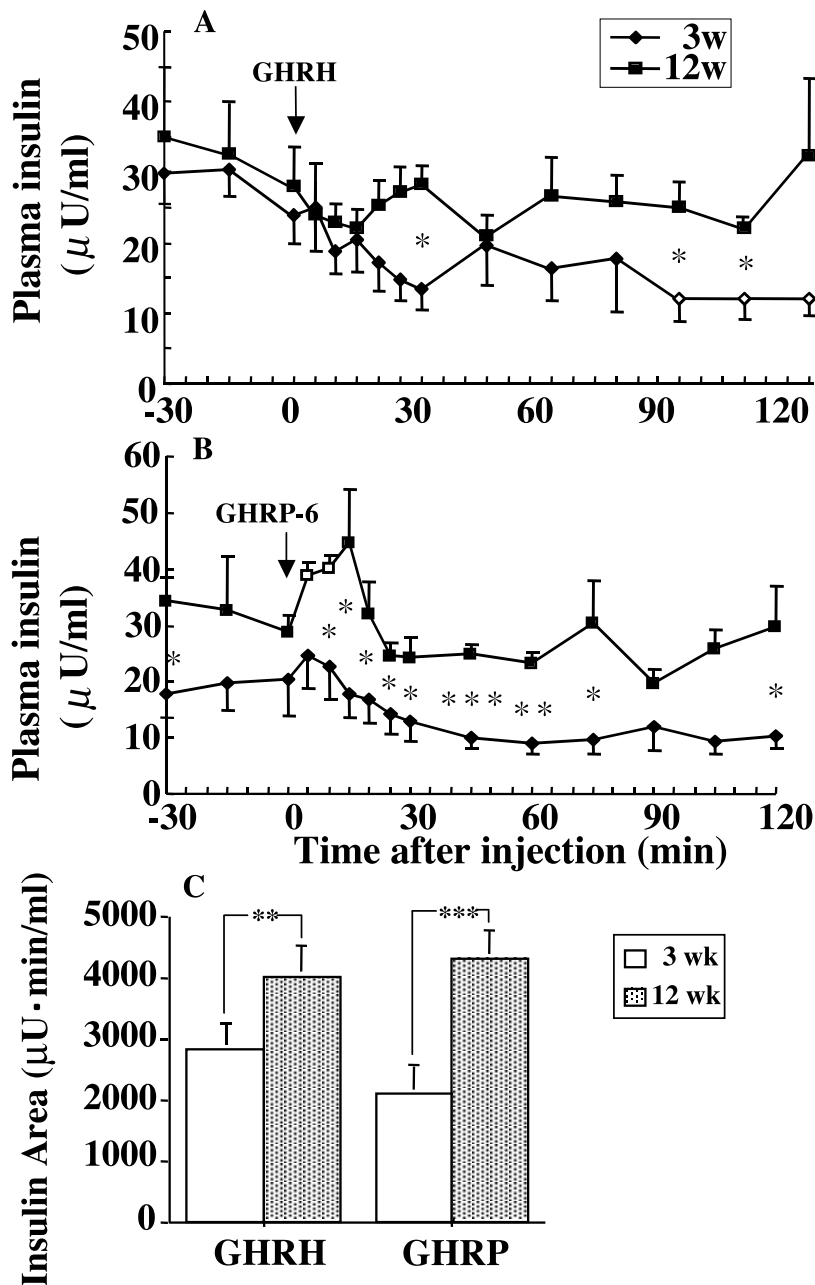


Figure 6 Changes in plasma insulin concentrations (A and B) and AUC (C) in response to the intravenous administration of GHRH (0.25 µg/kg) (A) or GHRP-6 (2.5 µg/kg) (B) in 3-week-old ($n=6$) or 12-week-old ($n=4$) calves. The animals were fed as in Fig. 1, and given either GH stimulant 2 h after feeding. The values are represented as the mean \pm S.E.M. Open symbols indicate a significant difference from the preweaning level. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

caused hyperinsulinemia and hyperglycemia in the group maintained on a milk-replacer diet, as shown in the present experiment (Fig. 4) and a previous study (Blum & Hammon 1999). Higher basal glucose levels may be, at least in part, due to higher SGLT-1 expression in the small

intestine and also to higher hepatic gluconeogenesis in milk-fed animals. This is likely because lambs maintained on a milk-replacer diet showed no reduction in SGLT-1 expression relative to age-matched animals (Lescal-Matys *et al.* 1993).

In 12-week-old calves, the GHRP-6 injection transiently but significantly increased plasma insulin concentrations. This may be caused by the direct action of GHRP on β cells, because rat pancreatic islets have been shown to synthesize ghrelin and GHS receptors (Guan *et al.* 1997), and ghrelin increases insulin release (Adeghate & Ponery 2002). This is not always the case in the human, however, because ghrelin has been shown to cause hyperglycemia and insulin suppression (Svensson *et al.* 1998, Broglio *et al.* 2001).

In conclusion, the feeding of milk in 3-week-old calves significantly increased plasma concentrations of GH, insulin and glucose, whereas feeding of concentrate and hay in 12-week-old animals did not cause a significant change in these parameters. However, feeding-induced GH secretion in the animals maintained on a milk-replacer diet until 12 weeks of age was not different from that in the age-matched, weaned group. The difference in postprandial responses may be explained by aging, by the difference in processes accompanied by quality of diets, or by altered neuroendocrine functions in the digestive–pituitary axis.

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