Study on the Physiological Functions of Two Endocrine Factors Derived from the Liver, ANGPTL8 and Chemerin, in Ruminants

論文内容要旨

Study on the Physiological Functions of Two Endocrine Factors Derived from the Liver, ANGPTL8 and Chemerin,in Ruminants.

（反芻動物における肝臓由来内分泌因子 ANGPTL8 および Chemerin の生理的機能に関する研究）

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Introduction

Nutritional metabolism in ruminants is tightly associated with productive traits, for example, milk production in dairy cows and meat production in beef cattle. Thus, the synthesis of milk is based on the influx of nutrients such as glucose, lipids, and amino acids. Likewise, the development of marbling in muscle largely is dependent on both lipid and protein metabolism. The liver is essential to these production processes, and also plays a role in the control of metabolism in peripheral tissues, including muscle and adipose tissues, in an endocrine manner (Iroz et al., 2015).

Recent endocrinological studies have identified various hormone-like endocrine factors from peripheral tissues and organs other than endocrine glands. These hormone-like endocrine factors are referred to as peptide hormones and cytokines. The discovery of these endocrine factors has undermined the long-standing model in which metabolism in peripheral tissues, such as the liver, adipose tissue, and skeletal muscle, is controlled by the secretion of hormones from the endocrine glands, and led to the development of a new model in which various tissues secrete a range of hormone-like factors depending on their metabolic state, which results in the construction of a regulatory axis to maintain homeostasis in the whole body (Ouchi et al., 2011; Pedersen and Febbraio, 2012).

Endocrine factors secreted from the liver are called hepatokines and mainly function to control glucose and lipid metabolism (Stefan and Häring, 2013). To date, a range of hepatokines have been identified: IGF-1, a well-known hormone derived from the liver with growth factor-like effects, is now considered a hepatokine (Musso et al., 2015); fetuin-A; fibroblast growth factor 21 (FGF21); selenoprotein-P (SeP); and angiopoietin-like proteins (ANGPTLs). These hepatokines have regulatory

effects on insulin sensitivity, glucose metabolism, and lipid metabolism (Stefan and Häring, 2013). Thus, increased understanding of hepatic endocrine function will provide new insights into the regulatory pathways of nutritional metabolism. This is particularly the case as the liver plays a pivotal role by sending out signals that reflect its metabolic state, such as elevated insulin resistance or accumulation of excess lipid under high dietary energy intake.

The present study focuses on two novel hepatokines namely, angiopoietin-like protein 8 (ANGPTL8) and chemerin. ANGPTL8 is predominantly expressed in and secreted from the liver and adipose tissues in humans and mice (Quagliarini et al., 2012). The most important physiological consequence of ANGPTL8 expression and secretion is the regulation of lipid metabolism, especially the control of blood fatty acid and triglyceride levels (Wang et al., 2013). The behaviors of chemerin and ANGPTL8 in humans and rodents suggest that these hepatokines also regulate glucose and lipid metabolism in ruminants and consequently have significant roles in productive traits. Chemerin is a secreted chemoattractant protein, which is highly expressed in the liver and adipose tissues of humans and rodents (Wittamer et al., 2003; Roh et al., 2007; Takahashi et al., 2011). This chemokine is also reported to have physiological effects especially on glucose metabolism, such as insulin secretion, peripheral insulin sensitivity, and glucose uptake (Sell et al., 2009; Takahashi et al., 2011; Wargent et al., 2015). These physiological effects are considered to be mainly mediated by the chemerin receptor, chemokine-like receptor 1 (CMKLR1), although chemerin has other receptors, namely C-C chemokine receptor-like 2 (CCRL2) and G protein-coupled receptor 1 (GPR1) (Rourke et al., 2013). Currently, however, there is little precise information on the expression and

physiological function of these hepatokines in ruminants. Here, growing calves and dairy cows were used as animal models as their nutritional metabolism is dramatically but accurately controlled during weaning, growth, pregnancy and lactation. The aims of this study were:

1. To investigate expression of ANGPTL8 in weaning calves and growing cattle;
2. To investigate expression of ANGPTL8 in lactating/ dry-off dairy cows and periparturient cows.
3. To investigate expression of chemerin and its receptors in weaning calves and growing cattle;
4. To investigate expression of chemerin and its receptors in lactating/ dry-off dairy cows and periparturient cows;

Results and discussions

Chapter 1. Expression of ANGPTL8 in weaning calves and growing cattle

We examined the expressional levels of *ANGPTL8* in (1) pre-weaning and post-weaning calves, and (2) growing and fattening cattle, to elucidate its role in the regulation of lipid metabolism in young calves. *ANGPTL8* mRNA expression was observed in the liver and adipose tissues of Holstein calves. ANGPTL8 protein was detected in the liver and plasma, and found to localize at the cytosol of hepatocytes of Japanese Black calves, which indicate ANGPTL8 is a hepatokine secreted from liver in cattle. The expressional level of *ANGPTL8* in the liver increased in post-weaning Japanese Black calves, whereas plasma ANGPTL8 protein level had tendency to increase in the same calves. Plasma level of phospholipid had tendency to decrease, and plasma acetoacetate level decreased in post-weaning calves. Meanwhile, the expressional levels of lipoprotein lipase (LPL) decreased in perirenal adipose tissue of post-weaning calves, compared to pre-weaning calves. Wang *et al.* reported a potential inhibitory function of ANGPTL8 on LPL (Wang et al., 2013). The data indicates that the upregulation of ANGPTL8 expression was a physiological adaptation to weaning in which plasma phospholipid declined because of the deprivation of dietary lipid intake, resulting in inhibition of peripheral LPL activity to decrease the uptake of lipid into tissues in such a low energy state caused by weaning.

Next, we examined the expressional alteration of ANGPTL8 in weaned growing cattle. In growing Japanese Black cattle, the expressional level of ANGPTL8 in the liver peaked at 17 months of age, whereas there were no significant differences in its expressional level between the groups fed hay or concentrates throughout the

experimental period. Hepatic GPAT1 expressional level also peaked at 17 months of age, although expressional levels of FAS and LDLR were not controlled by feeding and age. Thus, the elevated expression of *ANGPTL8* observed in calves at 17 months of age might contribute to the inhibition of lipid uptake in skeletal muscle and result in facilitating skeletal muscle growth. The results from weaning calves and growing cattle indicate that hepatic *ANGPTL8* expression is controlled dynamically by physiological transition such as weaning and growth.

Chapter 2. Expression of ANGPTL8 in lactating/ dry-off dairy cows and periparturient cows.

The mobilization of fatty acids from adipose tissues and triglycerides from liver to the mammary gland plays an essential pathway providing lipids for lactogenesis. Lipid metabolism in dairy cows is controlled by metabolic hormones such as insulin and growth hormone. Due to its function in controlling blood lipid levels, ANGPTL8 is suggested to regulate lactogenic metabolism in dairy cows. Here, we examined the pattern of expression of *ANGPTL8* in these cattle.

The level of hepatic *ANGPTL8* mRNA was dramatically reduced on the day of parturition and at 1 week after parturition but returned to the levels seen at preparturition after 4 weeks. Plasma level of ANGPTL8 slightly decreased at the day of parturition and returned to original level in 1 week postpartum. The level of plasma triglycerides was lower on the day of parturition and at 1 week after parturition, whereas plasma NEFA levels were elevated at the same periods. Plasma insulin level decreased in postpartum period. Treatment of cultured bovine hepatocytes with insulin revealed this hormone downregulates ANGPTL8 mRNA,

indicating insulin controls peripartum ANGPTL8 expression in liver of cows. Furthermore, changes in Hepatic ANGPTL8 mRNA and plasma triglycerides showed similar patterns of change during the periparturient period, suggesting that downregulation of ANGPTL8 contributed to the elevation of lipid uptake into the mammary gland, which facilitated the synthesis of milk lipids.

Chapter 3. Expression of chemerin and its receptors in weaning calves and growing cattle

First, we investigated gene expression of chemerin and chemerin receptors in various bovine tissues. The highest level of chemerin mRNA was observed in the liver of the Holstein/Friesian cattle, whereas lower expression was detected in other tissues including perirenal adipose tissue, adrenal gland, lung, spleen, and duodenum. Chemerin protein was detected in liver and plasma, indicating chemerin is a hepatokine secreted from liver in cattle. *CMKLR1* was highly expressed in the lung, the liver, the adrenal gland, and the spleen; lower levels of expression were found in perirenal adipose tissue, longissimus muscle, rumen, and duodenum. *CCRL2* was highly expressed in the lung, ileum, cecum, and rectal colon; lower levels of expression were also identified in the liver, perirenal adipose tissue, longissimus muscle, and duodenum. In contrast, *GPR1* was predominantly expressed in the liver and forestomach (rumen, reticulum, and omasum).

Next, the physiological effect of chemerin was examined by administration of a peptide analogue to sheep. The plasma insulin levels were sharply elevated after administration of 500 μg/head of the peptide analogue (Figure 1A). Consistently, plasma glucose and non-esterified fatty acid (NEFA) levels slightly declined after

administration (Figure 1B and 1C). Plasma triglyceride levels also increased rapidly after dministration but returned to the basal level within 30 min (Figure 1D). These data suggested that chemerin, as a hepatokine, regulated insulin secretion and consequently controlled plasma glucose and NEFA levels.

To investigate the expressional changes in chemerin and its receptors mRNA levels during weaning period, they were measured in liver skeletal muscle and four depots of adipose tissue of pre-weaning (1.5 months of age) and post-weaning (3.5 months of age) Japanese Black calves. The level of chemerin mRNA in mesenteric adipose tissue was higher in post-weaning calves. Interestingly, hepatic chemerin protein content was less abundant in post-weaning calves. However, there was no difference in plasma levels of chemerin between pre-weaning and post-weaning calves. Hepatic *CCRL2* and *GPR1* mRNA levels were higher in post-weaning calves Simultaneously, hepatic pyruvate carboxylase (*PC*) mRNA levels were lower in post-weaning calves, whereas hepatic phosphoenolpyruvate carboxykinase 2 (*PCK2*) increased. Meanwhile, the mRNA levels of the autophagy-related genes, atrogin 1 and muscle-RING finger protein-1 (*MuRF1*), were not altered in post-weaning calves. Plasma insulin concentrations did not differ between the two groups of calves; however, plasma levels of some factors involved in hepatic metabolism were altered. Plasma levels of aspartate aminotransferase (AST), alanine transaminase (ALT), and lactate dehydrogenase (LDH), the major enzymes indicating hepatic condition, were significantly elevated in post-weaning calves. The plasma levels of phospholipids, which are secreted mainly from the liver as very low-density lipoproteins (VLDL) tended to decrease in post-weaning calves ($P = 0.09$). Acetoacetate, beta-hydroxybutyrate, and the levels of total ketone bodies significantly increased in

post-weaning calves. Takahashi et al suggested chemerin has inhibitory effect on expression of genes related to hepatic gluconeogenesis such as PCK and subsequently attenuates gluconeogenesis in liver (Takahashi et al., 2011). These results suggested that stress to the liver caused by abrupt weaning performed in this study might have affected the levels of hepatic chemerin protein. Decreased hepatic chemerin might upregulated PCK expression in liver of weaned calves.

We further investigated the expressional changes in chemerin and chemerin receptors in growing Japanese Black cattle fed different diet (hay or concentrate). Chemerin mRNA levels in liver was higher in calves fed a concentrate diet than in those fed hay. The amount of chemerin protein in the liver was also higher in the concentrate-fed group. The levels of hepatic *CMKLR1* mRNA gradually decreased as the calves grew up. Hepatic *PC* mRNA levels were lower in concentrate-fed calves at 10 months of age. In contrast to expressional change in *CMKLR1*, hepatic P*CK2* expression elevated in 31 months of age in both group. These results indicated higher dietary energy intake is a factor for upregulation of hepatic chemerin expression in young weaned cattle, and chemerin signaling could be involved in hepatic gluconeogenesis in growing cattle.

Chapter 4. Expression of chemerin and its receptors in lactating/ dry-off dairy cows and periparturient cows.

Chemerin has been reported to exacerbate peripheral insulin sensitivity and to stimulate lipolysis in adipose tissue (Roh et al., 2007; Ernst et al., 2010). Previously, we reported that chemerin has a positive effect on lactogenesis in immortalized bovine mammary epithelial cells in vitro (Suzuki et al., 2015). Here, we investigated

the physiological role of chemerin in the metabolism of dairy cows. The level of hepatic chemerin mRNA was elevated in the dry period compared to the early/middle/late-lactation period, whereas *CCRL2* remained at a high level at this period. Hepatic *PC* mRNA level decreased at middle and late-lactation period, although the expressional levels of *PCK1* and *PCK2* did not change during experiment period. Thus, chemerin signaling was not supposed to have a significant role in hepatic glucose metabolism through lactation period in cows.

Focusing on peripartum period (-4 weeks to 4 weeks relative to parturition, in this study), hepatic chemerin mRNA expression slightly decreased at the day of parturition, and recovered thereafter. On the other hand, plasma chemerin level slightly decreased at partum day, but elevated in postpartum period. There were no differences in the expressional levels of CMKLR, CCRL2 and GPR1 in peripartum period. Plasma insulin level dramatically declined at the day of parturition and kept low levels in postpartum period. Because the pattern of plasma chemerin level in peripartum period was not consistent with the change inplasma insulin, it seemed that chemerin has minimal effect on the regulation of insulin secretion in this period. But, another physiological role of chemerin was suggested in postpartum period, such as lipolytic effect to provide NEFA from adipose tissue or inhibitory effect on insulin signaling during lactation.

We also investigated the local function of chemerin within mammary gland because chemerin has chemoattractant ability which is essential for mammary gland immunity and involution. Expressions of the chemerin, *CMKLR1*, *CCRL2* and *GPR1* mRNA were detected in the mammary grand as well as in the liver and adipose tissues of Holstein-Friesian dairy cows. Protein of chemerin, CMKLR1, and CCRL2

were localized in mammary epithelial cells and stroma cells, detected by IHC. We found that the mRNA expression of chemerin was elevated at the late-lactation period to the dry-off period in the mammary gland, compared to the early/middle-lactation period. *CMKLR1* and *CCRL2* expressions were elevated at the dry-off period. *CD68* (Cluster of Differentiation 68) expression appeared to increase at the late-lactation and dry-off periods although this change was not significant ($P = 0.12$). In cultured bovine mammary epithelial cells (MAC-T cells), the treatment with TNF-α, a potent proinflammatory cytokine, dramatically induced chemerin and CCRL2 expression. Considering the chemotactic property of chemerin to antigen presenting cells (Wittamer et al., 2003; Zabel et al., 2005), these results suggest that gene expression of chemerin began to increase from the late-lactation period and reached a peak at the dry-off period in order to facilitate the infiltration of immune cells into the mammary gland. Otherwise, the release of chemerin is supposed to be induced during inflammation in which proinflammatory cytokines are robustly released within mammary gland. These results together support the active role of chemerin in the maintenance of mammary gland function in mammary gland involution and immune response.

Conclusion

In summary, the present study provided following novel information about expressional regulation and physiological roles of the ANGPTL8 and chemerin as hepatokines in weaning calves, growing cattle and dairy cows;

1. *ANGPTL8* was highly expressed in the liver and secreted into blood in cattle. Hepatic *ANGPTL8* expression was upregulated after weaning, whereas LPL expression in adipose tissue declined, indicating the role of ANGPTL8 to suppress lipid uptake into adipose tissue in postweaning period. In growing cattle, there was no difference in hepatic ANGPTL8 expression between cattle fed hay and those fed concentrate diet. However, its expression was elevated at 17 months of age when skeletal muscle develops most robustly in growing cattle. This result suggests elevated hepatic *ANGPTL8* contributes to the development of skeletal muscle by inhibiting lipid accumulation in muscle.
2. *ANGPTL8* mRNA level in the liver was lower in early postpartum period in dairy cows. Simultaneously, plasma *TG* level was decreased, and plasma NEFA level was elevated. Plasma insulin level also decreased in postpartum period, which was suggested to be a regulator of hepatic *ANGPTL8* in peripartum period. These results indicate that decreased *ANGPTL8* expression might contribute to the decline in plasma triglycerides levels by facilitating triglyceride uptake by peripheral tissues.
3. Chemerin was highly expressed in the liver and secreted into blood. CMKLR1 and CCRL2 were expressed in a variety of tissues, whereas GPR1 was expressed mainly in liver and forestomach. The administration of a chemerin analogue peptide sharply stimulated insulin secretion in sheep.

4. Hepatic chemerin protein level was downregulated after weaning without transcriptional regulation. Plasma levels of AST, ALT, and LDH were elevated in postweaning calves. Hepatic *PCK2* expression was upregulated in the same calves. In growing cattle, chemerin expression in liver elevated in concentrate-fed cattle, compared to hay-fed cattle. Hepatic *CMKLR1* expression gradually decreased and *PCK2* expression elevated as cattle grew up. These results suggest that hepatic chemerin signaling was regulated by weaning, growth and dietary energy level to maintain glucose homeostasis in weaning calves and growing cattle.
5. In dairy cows, hepatic chemerin expression was elevated in the dry-off period. During peripartum period, hepatic chemerin expression decreased at parturition. On the other hand, expression of the chemerin gene in the mammary gland was elevated from the late-lactation period to the dry-off period. *CD68* expression was also increased in the dry off-period. These results shown chemerin has minimal effect on insulin signaling or glucose metabolism in peripartum dairy cows. However, chemerin was supposed to have regulatory role involution and immune response in mammary gland.

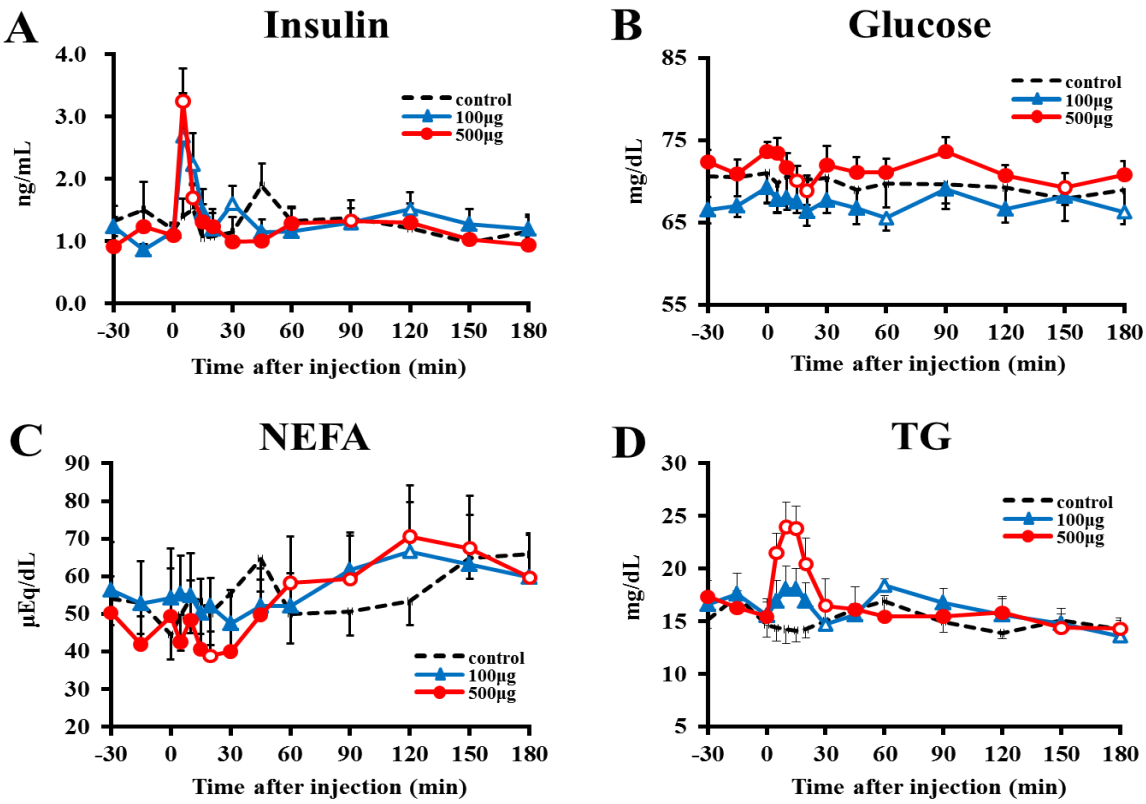

Figure 1. Plasma levels of insulin (A), glucose (B), NEFA (C) and TG (D) in cross-bred young sheep (n=6) weighing 23.4 ± 0.6 kg after administration of human chemerin analogue peptide (NH_2-yFLPsQFa(Tic)S-COOH; 100 μg or 500 μg/head, saline as control). Insulin levels were measured by radio-immuno assay and metabolites levels were measured by enzymatic colorimetric assay. Levels are represented as mean ± SE and open symbols indicate significant differences from the average of pre-administration levels ($P < 0.05$, paired *t*-test). (Adapted from Suzuki Y et al., 2012)

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