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論文内容要旨

Study on the Disposition of Vitamin E and Expression of α-Tocopherol-Related Genes during Weaning and Peripartum in Cattle

（離乳および分娩移行期牛におけるビタミンE体内動態とα-トコフェロール関連遺伝子発現特性に関する研究）

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Chapter 1.

Introduction

1. Metabolism in Calves during Weaning

The weaning period in ruminants has important factors that show dynamic and dramatic changes. The metabolic and endocrine changes during weaning are related to the quantity and quality of production and health in dairy and beef cattle. Pre-ruminant animals require glucose from milk as the main energy source during the neonatal period. After weaning, with rumen development, the metabolism of carbohydrates in ruminants dramatically changes, and it differs in several aspects from that in pre- and non-ruminants. In particular, ruminants are only able to absorb a very small amount of glucose from the intestinal tract, whereas considerable amounts of volatile fatty acids (**VFAs**), mainly acetate, propionate, and butyrate, are absorbed from the forestomach (Herdt, 1988). Nonetheless, the metabolic changes and physiological adaptation in weaning production systems are not well understood in calves.

2. Metabolism and Stress in Dairy Cows during Peripartum

Calving is an unavoidable event for the production of milk and young cattle in livestock systems. Dramatic changes in metabolism, endocrine status, physiological stress, and immune function occur during peripartum (from late pregnancy to early lactation) in dairy cows. The initiation of lactogenesis and acceleration of milk yield by the activation of mammary gland functions demand high amounts of nutrients and energy, whereas a reduction in feed intake at this time induces a negative energy balance and the mobilization of adipose tissue. Furthermore, parturition and increased metabolic activity result in induced oxidative stress and depletion of antioxidant defenses around the time of calving (Castillo *et al*., 2005; Konvicna *et al*., 2015). The majority of production diseases (mastitis, ketosis, digestive disorders, and laminitis) occur during early lactation (Ingvartsen *et al*., 2003). Therefore, the nutritional and metabolic control and prevention of production diseases in peripartum are important challenges for dairy cow welfare, health, and performance.

3. Effect of α-Tocopherol on the Health and Performance of Cattle

Alpha-tocopherol (**aT**), the most biologically active form of Vitamin E (**VE**) in animals, is a lipid-soluble antioxidant (Brigelius-Flohe and Traber, 1999). It is useful in animal production, but it cannot be synthesized in the mammalian body, and must therefore be provided through the diet or by supplementation. Oral administration or injection of aT has positive effects on the immune system and growth in calves (Higuchi *et al*., 2013; Krueger *et al*., 2014; Otomaru *et al*., 2013). Furthermore, in a previous study (H. Ishizaki, S. Haga, and M. Nakano. NARO Institute of Livestock and Grassland Science, Nasushiobara, Japan, personal communication), aT oral administration for 2 weeks prevented the plasma cortisol surge in steer calves stressed by truck transportation. In dairy cows, blood VE levels decline gradually throughout prepartum, reaching the lowest levels (hypovitaminosis E) around calving (Weiss, 1998). Several studies have documented that lower blood aT concentrations are associated with the incidence of peripartum diseases such as mastitis, retained fetal membranes, and left displaced abomasum (Politis *et al*., 2012; Qu *et al*., 2013). Supplementation of aT enhances immune function and reduces the risk of mastitis during the periparturient period in dairy cows (Politis *et al*., 2012).

4. α-Tocopherol-Related Genes

Alpha-tocopherol shows tissue-specific distribution (Combs, 2008; Hidiroglou *et al*., 1988), and this distribution property might affect the aT potencies for each tissue. However, the molecular mechanisms underlying this tissue distribution and the effect of aT are unknown in cattle. Pioneering studies conducted with humans and rodents have identified and characterized some aT-related molecules, such as aT transfer protein (**αTTP**), afamin (**AFM**), tocopherol-associated protein (**TAP**), scavenger receptor class B, Type 1 (**SR-B1**, a high-density lipoprotein (**HDL**) receptor), ATP-binding cassette transporter A1 (**ABCA1,** a HDL transporter) and cytochrome P450 family 4, subfamily F, polypeptide 2 (**CYP4F2**) (Yamauchi *et al*., 2001; Mardones *et al*., 2002; Voegele *et al*., 2002; Traber, 2007). As described above, the expression of six aT-related genes (αTTP, AFM, TAP, SR-BI, ABCA1, and CYP4F2 mRNA) in various tissues could regulate aT metabolism, transportation, and tissue distribution in cattle.

5. Aim of the Present Study

Therefore, the aims of this study were as follows:

1. To clarify the tissue distribution of six aT-related gene expressions and to elucidate the changes in the gene expression levels and aT disposition in relation to aT administration in cattle.
2. To investigate the hepatic aT-related gene expressions and the changes in metabolism of the rumen and liver in pre-ruminant and ruminant calves in weaning production systems.
3. To investigate the changes in metabolism, stress, and the expression of aT-related genes in the mammary gland and liver from late pregnancy to early lactation in dairy cows.

Chapter 2.

Tissue Distribution of α-Tocopherol-Related Gene Expressions and the Changes in Disposition of Vitamin E and these Gene Expression Levels of Weaned Calves in Response to α-Tocopherol Administration

1. Tissue Distribution of the aT-Related Gene Expressions

This study was to investigate mRNA expressions of six aT-related genes (αTTP, AFM, TAP, SR-BI, ABCA1, and CYP4F2) in 20 major tissues, including the liver (metabolic), testis (reproductive), adrenal gland (endocrine), thymus, spleen, lymph node (immune), and gastrointestinal tract (digestive and absorptive) tissues, in Japanese Black (**JB**) beef calves ($n = 5$). The levels of αTTP, AFM, TAP, and ABCA1 mRNA were significantly greater in the liver than in the other 19 tissues ($P < 0.05$). CYP4F2 mRNA was most highly expressed in the liver and testis. The results show that the liver may be an important tissue in the regulation of aT transportation and metabolism in cattle.

2. Changes in Disposition of VE and aT-Related Gene Expression Levels in Response to aT Administration

This study was conducted to elucidate changes in disposition of VE and gene expression patterns after oral administration of aT in JB beef calves. Fourteen male JB calves were divided into three groups. Alpha-tocopherol was orally administered to 4 (15 IU/kg BW/d) and 5 (30 IU/kg BW/d) calves for 2 wk. The control group consisted of 5 calves that did not receive an aT supplement (0 IU/kg BW/d). The hepatic aT content significantly increased in a dose-dependent manner ($P < 0.05$). However, no significant differences in biliary and serum aT concentrations were found between the 15 and 30 IU/kg BW/d groups. The serum aT concentration plateaued within 1 wk in both the 15 and 30 IU/kg BW/d groups. In addition, the hepatic expression levels of AFM and SR-B1 mRNA in the 30 IU/kg BW/d group were significantly greater than those in the 0 IU/kg BW/d group ($P < 0.05$). These results suggested that the liver regulates the amount of aT secreted into circulation in response to high aT administration.

Chapter 3.

Expression of Hepatic α-Tocopherol-Related Genes in Relation to the Change in Metabolism between Pre-Ruminant and Ruminant Calves in Weaning Production Systems

1. Basal Plasma Concentrations of Metabolites and Hormones around the Time of Weaning

First, we investigated the changes in basal plasma concentrations of metabolites and hormones in Holstein calves around the time of weaning in early weaning production systems. Twenty male Holstein calves (at 1, 3, 8, 13, and 19 wk, $n = 4$ each) were monitored. Calves were weaned at 6 wk. Plasma acetate and β-hydroxy-butyrate (**BHBA**) concentrations were very low until weaning, increased after weaning, and remained high until 13 wk. Plasma glucose concentrations decreased from 1 to 8 wk, but increased at 19 wk. The plasma cortisol concentration, which was at a maximum at 1 wk, rapidly decreased thereafter ($P < 0.05$). These results suggested that calves began to absorb and utilize considerable amounts of VFAs from the forestomach to meet energy demands after weaning, and carbohydrate metabolism could shift from pre-ruminant to ruminant without variations of basal insulin and glucagon status in early weaning production systems.

2. Expression of Genes Related to Rumen Functions between Pre- and Post-Weaning Calves

To investigate the development of rumen functions for metabolic adaptation from pre- to ruminant, the levels of monocarboxylic acid transporter 1 (**MCT1**) and mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase 2 (**HMGCS2**) mRNA, and plasma BHBA were measured. Pre- (4 wk, $n = 4$) and post-weaning (13 wk, $n = 4$, weaned in 7–8 wk) Holstein calves were used. In addition, pre- (5 wk, $n = 5$) and post-weaning (18 wk, $n = 5$, weaned in 13 wk) JB calves were used. The expression levels of MCT1 and HMGCS2 mRNA, and plasma BHBA concentrations in post-weaning calves were significantly greater ($P < 0.05$) than those in pre-weaning calves of both breeds. These results suggested that the variations of rumen gene expressions between pre- and post-weaning calves might represent changes in metabolism and development of ruminant digestive functions.

3. Alpha-Tocopherol Status and Hepatic aT-Related Gene Expression between Pre-and Post-Weaning Calves

This study was conducted to elucidate the hepatic aT-related gene expression in pre- and post-weaning calves in weaning production systems. The same calves were used as above. The expression levels of AFM and CYP4F2 mRNA in post-weaning JB calves were significantly downregulated ($P < 0.05$) than in pre-weaning, although no significant differences were found the serum and liver aT levels between pre- and post-weaning. Pre-weaning Holstein calves consumed high amounts of aT from commercial milk replacer and starter diets, and the serum and hepatic aT levels in pre-weaning calves were significantly higher ($P < 0.05$) than those in post-weaning. However, the expression level of aT-related genes between pre- and post-weaning calves were not significantly different. These results suggested that VE disposition and aT-related gene expression pattern are different between Holstein and JB calves in weaning production systems.

4. Glucose and Lipid Metabolism and Hepatic Related Genes Expression between Pre-and Post-Weaning Calves

This study was conducted to determine the changes in hepatic glucose and lipid metabolism in calves in weaning production systems. The same calves were used as above. The expression levels of glucose transporter 2 (**GLUT2**) and pyruvate carboxylase (**PC**) mRNA in post-weaning JB calves were significantly lower ($P < 0.05$) than those in pre-weaning JB calves. However, the expression levels of phosphoenolpyruvate carboxykinase in cytosol and mitochondria (**PEPCK-C** and **-M**) in post-weaning were significantly greater ($P < 0.05$) than those in pre-weaning JB calves. In lipid metabolism, plasma lipid parameter levels in post-weaning calves were significantly lower ($P < 0.05$) than pre-weaning JB calves, and the hepatic apoA1 and B mRNA levels were significantly downregulated ($P < 0.05$) in post-weaning than in pre-weaning JB calves. These results suggested that the hepatic glucose and lipid metabolism in especially JB calves were influenced by weaning and aging in weaning production systems.

Chapter 4.

The Disposition of Vitamin E and Expression Levels of α-Tocopherol-Related Genes during Peripartum in Dairy Cows

1. Blood Concentrations of VE, Metabolites, Hormones, and Stress Markers from Late Pregnancy to Early Lactation in Multiparous Holstein Dairy Cows

First, we confirmed the changes in VE, metabolism, and physiological status in dairy cows from late pregnancy to early lactation. Twenty-eight multiparous Holstein cows were monitored during peripartum. The dry matter intake and aT intake temporarily decreased around calving. The milk yield markedly increased after parturition. Serum aT levels declined gradually throughout prepartum, reaching the lowest levels after calving, and the levels gradually recovered in propartum. Alpha-tocopherol in colostrum was markedly greater than that in transition and mature milk. The plasma energy and lipid metabolite levels, and hormone levels showed that metabolism and endocrine status dramatically changed during the peripartum. Around calving, oxidative and physical stress drastically occurred, and liver functions were impaired from calving to lactation.

2. Lactogenesis and aT Transportation in the Mammary Gland from Late Pregnancy to Early Lactation in Multiparous Holstein Dairy Cows

This study was conducted to investigate our hypothesis that hypovitaminosis E around calving time occurred from the temporarily active aT transportation from the blood into colostrum across the mammary gland. Ten multiparous Holstein dairy cows were used. Mammary tissue was obtained by conscious biopsies at –8, –1, 0, and 6 wk of after parturition. Milk protein gene expressions were confirmed. In addition, a blood sample from the coccygeal artery and milk vein of five out of ten cows were collected at –8, –4, –1, 0, 3, and 6 wk of after parturition to calculate the mammary extraction ratio. After calving, the extraction ratio of the milk lipid substrate, BHBA, in the mammary gland was significantly elevated 1 wk before calving and the specific transporter, MCT1, mRNA levels were also upregulated from –1 wk of parturition. The mRNA expression levels of αTTP, TAP, SR-BI, and CYP4F2 significantly changed during peripartum and milk aT level after parturition (colostrum) was significantly greater than those at other times ($P < 0.05$). However, this arterial-venous differences method could not confirm the temporal extraction of aT in the mammary gland for aT-rich colostrum production. This study firstly reported the expression of aT-related genes in bovine mammary glands, and these results suggested that aT-related genes play an important role in the transfer of aT from blood to colostrum.

3. Hepatic Metabolism, Stress, and aT-Related Gene Expression from Late Pregnancy to Early Lactation in Multiparous Holstein Dairy Cows

This study was conducted to confirm our hypothesis that hypovitaminosis E around calving time occurs as a result of the dysfunction of VE transportation in the liver to circulation. Seven multiparous Holstein dairy cows were used. Liver biopsies were performed in –4, –1, 0, 1, and 4 wk after parturition. In the liver around calving time, the expression levels of endoplasmic reticulum (**ER**) stress marker genes and the acute-phase response molecule, haptoglobin, mRNA were drastically increased ($P < 0.05$). However, the expression levels of key antioxidant enzymes were significantly downregulated ($P < 0.05$). Furthermore, the hepatic αTTP, TAP, and AFM mRNA levels were also significantly downregulated around calving time ($P < 0.05$). Serum aT levels were the lowest around calving time, but hepatic aT levels did not change during peripartum. These results suggested that ER and oxidative stress, and acute-phase responses are induced during peripartum, which leads to an inhibition of the functional expression of aT-related genes in the liver and the dysfunction of VE transportation from the liver to circulation.

<u>Chapter 5.</u>

Summary

In summary, these studies highlight the disposition of VE and the expression of aT-related genes during weaning and peripartum in cattle as follows:

1. The liver may play an important role in the regulation of aT, as inferred from the high expression levels of the aT-related genes in cattle.

2. The hepatic expression of aT-related genes and aT metabolism in calves might be associated with the adaptation of hepatic energy and lipid metabolism, and rumen development during weaning.

3. Changes in the aT-related gene expressions in the liver and mammary gland during peripartum suggest that several factors have interactive effects on hypovitaminosis E around calving time in dairy cows:

   A) Decreasing dry matter intake and aT intake

   B) Increasing aT transfer from the blood to colostrum across the mammary gland

   C) Decreasing blood HDL level as aT carrier

   D) Increasing systemic oxidative stress

   E) Decreasing hepatic aT secretion into circulation

Appendix

This doctoral thesis includes the contents derived from two of our original papers as follows:

1, **Satoshi Haga（CA）**, Sumiko Fujimoto, Tomo Yonezawa, Kazuya Yoshioka, Hiroyuki Shingu, Yosuke Kobayashi, Tatsuyuki Takahashi, Yoshihisa Otani, Kazuo Katoh, Yoshiaki Obara. Changes in hepatic key enzymes of dairy calves in early weaning production systems. *Journal of Dairy Science* 91:3156-3164. 2008.

2, **Satoshi Haga (CA)**, Miwa Nakano, Hiroshi Ishizaki, Sanggun Roh, Kazuo Katoh. Expression of α-tocopherol-associated genes and α-tocopherol accumulation in Japanese Black (*Wagyu*) calves with and without α-tocopherol supplementation. *Journal of Animal Science* 93:4048-4057. 2015.

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論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

ビタミンEには子牛の肺炎や下痢、乳牛の周産期疾病の発症・損耗リスクを低減する効果がある。自然界にはビタミンE同族体が8種類存在するが、ヒトや実験動物同様、ウシにとってもα-トコフェロール（aT）が最も生物活性が高いとされており、ウシの健全性に強く関与する重要な微量栄養素である。子牛のaT摂取不足や乳牛の低aT血症化は生産のマイナス要因になるが、補給されたaTの組織分布や低aT血症化に関与するaT体内動態メカニズムは分かっていない。一方、ヒトやげっ歯類において、aT体内動態を特異的に制御する「aT関連遺伝子」の存在が明らかになりつつあるが、ウシにおける知見は乏しい。ウシaT関連遺伝子発現特性の基礎的解明は、aTを活用した更なる健全かつ効率的な乳肉用牛生産技術の開発に資すると考えられる。そこで著者は、ウシaT関連遺伝子の組織発現特性がaT体内動態に関与するという仮説を立て、離乳や分娩移行期牛に焦点を当て、aT体内動態とaT関連遺伝子の組織発現特性の関連性を明らかにする研究を行った。

著者はまず黒毛和種離乳仔ウシを供試し、aT関連遺伝子発現とaT含有量のウシ組織分布を調べ、肝臓をはじめ、副腎、精巣、小腸領域等における特性を明らかにした。また、aT補給時の血中aT濃度増加の上限を示し、肝臓がaT血液循環を制御していること、またaT補給により、aT関連遺伝子の組織発現が調節されることを新規に見出し、ウシにおけるaT関連遺伝子発現特性が、aT体内動態を制御するメカニズムの一端を担っていることを示唆した。

次に著者は、人工哺乳による早期離乳方式で飼養されたホルスタイン雄仔ウシおよび自然哺乳による母子分離離乳方式で飼養された黒毛和種雄子ウシを供試し、離乳移行期における離乳や成長という変化が仔ウシの肝臓におけるaT関連遺伝子発現およびaT体内動態に及ぼす影響について、種差や哺乳離乳方式を加味しつつ、離乳前後で比較し検討した。その結果、特に黒毛和種仔ウシでは離乳前後問わず、血中aT濃度が低く、aT摂取不足となりやすい黒毛和種子牛の飼養環境が示唆された。また、肝臓におけるaT関連遺伝子発現が離乳前後で変化することを明らかにし、この時に肝臓における脂質代謝、糖代謝関連遺伝子の発現も変化することから、離乳前後におけるpre-ruminantからruminantへの移行時期に変化する肝臓の代謝特性がaT関連遺伝子発現にも影響し得ることを示唆した。

最後に、著者は周産期の乳牛に発生する低aT血症化とaT関連遺伝子の組織発現変化の関連性を調べる実験を行った。周産期乳牛に起きる低aT血症化の要因として、aT摂取量の減少や酸化ストレス増大によるaT消費の増加、血中aT担体であるHDL濃度の減少だけでなく、乳腺の初乳合成期におけるaT関連遺伝子発現の変化および肝機能低下に伴う肝aT関連遺伝子発現抑制が低aT血症化の要因になっている可能性を、肝臓および乳腺バイオプシー法など国内において類稀な研究手法を駆使して明らかにし、aT関連遺伝子発現が乳牛の健全性に強く関与するものであることを示した。

以上のように、著者が本研究で得た、離乳および分娩移行期牛におけるビタミンE体内動態とaT関連遺伝子発現特性に関する知見は、著者の仮説を支持するものであり、極めて新規的かつ生産現場も考慮した応用性への示唆に富むものである。よって、審査員一同は、本論文の著者が博士（農学）の学位を授与されるのに値するものと判定した。