ANGIOPOIETIN-LIKE PROTEIN 4 IN BOVINE PHYSIOLOGY

by

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Abstract

Angiopoietin-like protein 4 (ANGPTL4) is a 55-kDa secreted glycoprotein which is an important factor for regulation of energy and lipid metabolism. Plasma ANGPTL4 has the ability to inhibit lipoprotein lipase (LPL) function by preventing it from catalyzing hydrolysis of lipoprotein triglyceride, which contributes to ANGPTL4's ability to decrease fat storage. Furthermore, research in mice suggests that gut microbes suppress gastrointestinal ANGPTL4 production, and that decreased plasma ANGPTL4 concentrations promote fat storage. In our previous work, we found that bovine ruminal epithelial cells expressed ANGPTL4 to a greater extent than liver hepatocytes, which are usually considered the predominant source of circulating ANGPTL4. Therefore, 3 studies were conducted to evaluate the hypothesis that ruminal expression and plasma concentrations of ANGPTL4 could be influenced by alterations in ruminal fermentation. The first and second studies utilized dietary treatments intended to alter ruminal fermentability. Diets with relatively low or high forage content were fed to 12 non-lactating dairy cows (study 1) and 8 beef cattle (study 2) prior to collection of ruminal fluid and ruminal tissue samples. The results suggested that increasing the dietary concentrate decreased ruminal expression of ANGPTL4 but did not significantly alter plasma ANGPTL4 concentrations. The third study was designed to assess whether effects of diet fermentability on ruminal ANGPTL4 synthesis are mediated by changes in volatile fatty acid concentrations. In this study, 6 lactating cows were infused with acetate, propionate, or butyrate in a Latin square design. Results showed that ANGPTL4 expression was not significantly altered by volatile fatty acid infusions, but that expression was correlated with ruminal pH and total volatile fatty acid concentration. The mechanism by which ANGPTL4 regulates intracellular lipid metabolism also remains unclear. Although ANGPTL4 is known to associate with β1 and β5 integrins, it is unknown if these extracellular matrix proteins mediate the effects of ANGPTL4 in adipose tissue or muscle. The objective of the last experiment was to detect the ANGPTL4 receptor or mediator in muscle satellite cells and adipose tissue. We successfully expressed recombinant bovine ANGPTL4 with a cell free glycoprotein synthesis system. However, we did not detect the ANGPTL4–receptor complex following exposure to bovine adipose tissue explants or cultured bovine muscle satellite cells. Overall, these research projects determined that the ruminal

ANGPTL4 production is influenced by fermentation, but it remains unclear whether fermentation products or direct host/microbe interactions are responsible. Finally, it will be important to identify the ANGPTL4 receptor or mediator to better understand the downstream regulatory mechanisms involved in mediating the metabolic effects of ANGPTL4.

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Chapter 1 - Literature Review

Background

Angiopoietin-like protein 4 (ANGPTL4) research has been focusing on lipid metabolism and cancer research in recent years. It is a plasma protein, also known as the fasting induced adipose factor (FIAF), which is expressed under the regulation of peroxisome proliferator-activated receptors (PPARs). Angiopoietin-like protein 4 is predominately expressed in adipose tissue and liver, but can be detected in various other tissues (Kersten et al., 2000). It belongs to the Angptl family, which includes 7 proteins. Angiopoietin-like protein 4 expression and secretion can be driven by the nutritional status of human and mouse subjects. Plasma ANGPTL4 concentration can be elevated during fasting or by high fat diets (Kersten et al., 2000). Furthermore, ANGPTL4 acts as a signal to prevent fat storage and stimulate fat mobilization (Mandard et al., 2005).

Angiopoietin-like proteins share similar molecular characteristics, including an NH_2 terminal coiled-coil domain (CCD) and COOH-terminal fibrinogen-like domain (FLD; Hato et al., 2008). The CCD of ANGPTL4 can act to bind to lipoprotein lipase (LPL) to inhibit its function. The binding structure transforms LPL from an active dimer form to an inactive monomer form (Sukonina et al., 2006; Yau et al., 2009). Furthermore, in the mouse model, central administration of the ANGPTL4 FLD caused a significant reduction in food intake and loss in body weight, indicating that Angptl4-induced anorexia and weight loss may be mediated by the FLD (Kim et al., 2010).

Bovine ANGPTL4 is a glycoprotein of about 55 kDa, and its deglycoselylated form is 42kDa. It is secreted by multiple tissues; it is most abundantly expressed in the liver, followed by adipose tissue, and the GI tract in bovine tissues. Interestingly, expression in the bovine GI tract is approximately 10% of the abundance in liver and adipose tissue (Mamedova et al., 2010).

Angptl4 Regulation through Peroxisome Proliferator Activated Receptors

Expression of ANGPTL4 is stimulated by the peroxisome proliferator activated receptors (PPARs) which are ligand activated transcription factors. Three subtypes of PPARs have been named PPARα, PPARβ/δ, and PPARγ. PPARs can be activated by lipids and respond through

peroxisome proliferator elements (PPRE). PPARs and PPRE modulate genes related with lipid metabolism (Gurr et al., 2008). PAPRα is abundantly expressed in liver, adipose tissue, muscle, heart and various immune cells, PPARβ/δ in heart, muscle, keratinocytes, liver and skin, and PPARγ primarily in adipose tissue. PPARs play an important role in regulation of energy and glucose metabolism, inflammation, and wound healing. PPARs heterodimerize with retinoid X receptors and bind specific transcriptional elements to regulate DNA transcription, especially that of genes involved in to fatty acid transport and oxidation, triglyceride synthesis and transport, and ketogenesis (Kersten et al., 2000; Gurr et al., 2008; Staiger et al., 2009; Goh et al 2010; Sanderson et al., 2010). Recently, the PPARs have been targeted for therapeutic purposes, such as dyslipidemia and insulin resistant. Activation of ANGPTL4 expression through PPARs is mediated by different PPAR isoforms in different tissues (Sanderson et al., 2010).

Effects of ANGPTL4 Overexpression

Angiopoietin-like protein 4 overexpression mice had increased ANGPTL4 mRNA expression in white adipose tissue (WAT) and brown adipose tissue (BAT). Angiopoietin-like protein 4 overexpressing mice also had greater skeletal muscle and heart Angptl4 mRNA expression, but not in the liver (Yu et al., 2004; Mandard et al., 2006). The results showed that ANGPTL4 overexpression reduced body weight and fat mass in gonadal fat (WAT), perirenal fat (WAT), and BAT. The WAT mass was decreased by about 50% in ANGPTL4 overexpression mice and was not because of diminishing food intake compared to wild type mice. In a fasted state, ANGPTL4 overexpression mice have higher plasma TG, FFA, glycerol, total cholesterol, HDL-cholesterol, VLDL, and HDL concentrations. The adipose triglyceride lipase, which works with hormone sensitive lipase to stimulate lipolysis, was increased by 50% at the mRNA level in ANGPTL4 overexpression mice (Mandard et al., 2006). Furthermore, the study revealed that PGC-1α and PPARα, nuclear factors involved in fatty acid oxidative metabolism, were up-regulated in WAT. Angiopoietin-like protein 4 is also stimulated by high fat diet (HFD). In ANGPTL4 overexpression mice fed HFD, increased lipolysis, elevated plasma lipid levels, and TG accumulation in the liver were observed (Ge et al., 2004; Mandard et al., 2006). Angiopoietin-like protein 4 acts as a signal to prevent fat storage and stimulate fat mobilization (Mandard et al., 2006).

Functions of ANGPTL4

Angiopoietin-like protein 4 is related to several different research areas, including metabolism, cancer, and diabetes research. Angiopoietin-like protein 4 expression is altered by nutritional status. Plasma ANGPTL4 concentrations can be elevated for a short time between meals (Kersten et al., 2009). The functions of ANGPTL4 may differ depending on whether it is acting in local tissue or circulating in the bloodstream. Cancer researchers are interested in the potential use of targeted Angptl4 therapy to suppress angiogenesis and minimize blood supply locally around tumor cells (Pudua et al., 2008). From a nutritional standpoint, gut microbes may influence gastrointestinal expression of ANGPTL4 and potentially influence host metabolism (Aronsson et al., 2010).

ANGPTL4 Alters Lipoprotein Lipase Function

Lipoprotein lipase is a key enzyme responsible for fat storage. It functions to hydrolyze triglyceride from triglyceride-rich lipoprotein, including circulating very low-density lipoproteins and chylomicrons. Lipoprotein lipase releases free fatty acids and glycerol to be utilized in local tissue or systemically. Overexpression of human LPL in skeletal muscle and liver of transgenic mice caused insulin resistance (Ferreira et al., 2001; and Kim et al., 2001). Conversely, LPL deficiency results in hypertriglyceridemia and low levels of high-density lipoprotein (Havel et al., 1960; Rodebell et al., 1964; Miles et al., 2001). Angiopoietin-like protein 4 inhibits LPL activity in vitro and injecting mice with recombinant ANGPTL4 potently increased plasma triglyceride concentrations by inhibiting triglyceride clearance (Yoshida et al., 2002). Tissue-specific regulation of LPL in relation to nutritional state directs the flow of triglyceride-fatty acids to different tissues according to needs. In the fed state, LPL is activated by insulin for clearance of chylomicrons, and increasing free fatty acid release allows for tissue uptake and subsequent storage in adipose tissue and incorporation into milk fat in the mammary gland. In contrast, LPL activity in skeletal and cardiac muscle is down-regulated by insulin. During fasting, the liver elevates VLDL secretion and LPL activity is down-regulated in adipose tissue and up-regulated in skeletal and cardiac muscle, allowing these tissues to use free fatty acids for oxidation (Gurr et al., 2008). Angiopoietin-like protein 4 can inhibit LPL function, which suppresses clearance of triglyceride-rich lipoproteins and increases serum TG (Yoshida et al., 2002). However, overexpression of ANGPTL4 in mice also stimulates adipose tissue

lipolysis, further increasing free fatty acids (FFA) in circulation (Mandard et al., 2005). Furthermore, mice intravenously injected with ANGPTL4 also have increased plasma FFA (Yoshida et al., 2002).

Nutritional Status Affects ANGPTL4 Expression

As a plasma protein, ANGPTL4 is secreted into the bloodstream and acts as an endocrine factor. The plasma concentration of ANGPTL4 is increased by fasting (Kersten et al., 2000). The animal's fasting condition increases lipolysis and FFA release; subsequently, the FFA activate PPAR and increase ANGPTL4 production (Kersten et al., 1999; Staiger et al., 2009). Mice fed a high fat diet have altered ANGPTL4 expression (Kersten et al., 2000; Aronsson et al., 2010; Georgiadi et al., 2010). Additionally, results of one study suggested that fatty acid profile might also influence ANGPTL4 expression in the GI tract (Fleissner et al., 2010). Overexpression of ANGPTL4 in vivo has shown effects on plasma TG concentrations independent of VLDL-TG secretion (Mandard et al., 2006). The ANGPTL4 overexpression in the fed state increased plasma levels of FFA, glycerol, and TG. After 24 h of fasting, the Angptl4 overexpression mice had a 24-fold increase in plasma ANGPTL4 and dramatically increased plasma VLDL. In addition to decreasing LPL activity, ANGPTL4 also reduces hepatic lipase activity by as much as 50% (Lichtenstein et al., 2007). Angiopoietin-like protein 4 decreased cholesterol ester clearance and uptake of fatty acid and cholesterol into tissue. Subsequently, it increases cholesterol synthesis in liver due to inhibition of LPL- and HL-dependent hepatic cholesterol uptake, increasing plasma cholesterol concentration and significantly decreasing hepatic cholesterol content (Lichtenstein et al., 2007).

Plasma ANGPTL4 levels are increased by fasting, caloric restriction, and endurance exercise (Kersten et al., 2009). Skeletal muscle secreted ANGPTL4 was induced via PPAR-δ. PPARδ is stimulated by long chain fatty acids (LCFAs) and enhances ANGPTL4 expression. Plasma LCFAs elevated ANGPTL4 concentration during fasting due to WAT lipolysis; it released LCFAs mediating ANGPTL4 production in skeletal muscle. This results in a positive feedback loop between adipose tissue and skeletal muscle; more ANGPTL4 inhibition of LPL prevents the release and subsequent storage of FFA from circulating TG and continues to increase WAT lipolysis (Staigeret al., 2009). Consequently, FFA concentrations in plasma are elevated.

Central administration of ANGPTL4 into the hypothalamus of mice revealed that it is involved in mechanisms controlling food intake and body weight (Kim et al., 2010). Hypothalamic ANGPTL4 is down-regulated by fasting and oral administration of lipid, glucose, and protein of mice. Central administration of ANGPTL4 caused a modest suppression of food intake and body weight gain in the 24 h post-injection. Furthermore, the central administration increased energy expenditure and suppressed energy intake, both promoting negative energy balance. Hypothalamic ANGPTL4 depresses AMPK activity and subsequently increases energy expenditure and suppresses food intake. AMPK acts as recovery factor from an energy depleted condition. Hypothalamic ANGPTL4 is responsive to insulin, food intake, and leptin (Kim et al., 2010).

Gut Microbe Influence on ANGPTL4 Production

The gastrointestinal microbial flora exists in a dynamic eco-system. Recently some studies have shown that the gut microbiota is associated with obesity, regulation of energy harvest and storage, and inflammation (Velagapudi et al., 2010). Gut microbiota may promote metabolic disease and stimulate intestinal angiogenesis (Stappenbeck et al., 2002). Composition of gut microbe populations also can be altered by different diets, including high fat diets or diets with a higher percentage of carbohydrate (Crawford et al., 2009; Velagapudi et al., 2010). One important function of gastrointestinal microbes is to break down structural polysaccharides to provide short chain fatty acids (SCFA) for the host to absorb to optimize energy harvest (Flint et al., 2008). Therefore, gut microbes stand on two sides of the energy balance equation, by affecting energy harvest and by influencing expression of genes that regulate energy storage and expenditure (Bäckhed et al., 2007).

One mouse study showed that obesity alters microbial ecology, and the obese microbiome has enhanced capacity for energy utilization (Turnbaugh et al., 2006). Studies of germ-free (GF) mice and colonized mice, GF mice infused with microbes, also showed that the gut microbe community promotes adiposity in previously GF mice (Velagapudi et al., 2010). Germ free mice have decreased TG storage in adipose tissue and hepatic TG levels compared with conventionally-raised (CV) mice (Velagapudi et al., 2010). The presence of microbes increases hepatic triglyceride production. GF mice have higher fatty acid oxidation and decreased lipogenesis. Moreover, microbes potentially suppress ANGPTL4 expression and

promote triglyceride deposition in adipocytes, because of the absence of LPL inhibition by ANGPTL4 (Bäckhed et al., 2004).

A study of GF and CV mice showed microbe modulation of the liver gene transcriptome and serum lipidome, also associated with adipose tissue fat storage. The serum metabolite profile reflected intestinal microbial metabolism (Velagapudi et al., 2010). The presence of microbes in CV mice resulted in higher energy harvesting and elevated production of phosphatidylcholine, one activator of PPARα, which also influences lipid metabolism and clearance (Chakravarthy et al, 2009; Velagapudi et al., 2010). In CV mice, ANGPTL4 functions as an LPL inhibitor, which decreases lipid clearance, phosphatidylcholine, triglyceride, and VLDL production in the liver. It is associated with lower serum TG concentration and increased TG storage in the adipose tissue (Velagapudi et al., 2010).

Germ-free and CV mice were fed with a low fat diet (LFD) or a high fat diet (HFD) in energy expenditure research. It was discovered that GF mice had lower energy expenditure (Fleissner et al., 2010). There was no difference in body weight gain between GF and CV mice when fed the LFD. Under HFD feeding, GF mice had greater increases in body fat and body weight compare to CV mice. GF mice had increased Angptl4 mRNA expression in the intestine with a western diet and HFD. However, there was no difference in circulating ANGPTL4 concentration compared with CV mice (Fleissner et al., 2010). These results are in contrast to the Bäckhed (2007) study; in this case, the GF mice were not protected from obesity despite enhanced intestinal ANGPTL4 expression. One factor that may have contributed to the different results in these studies is that they used different mouse strains. Also, the basal diets bed in the 2 studies had different lipid and carbohydrate composition in the high fat diets. It is also important to recognize that in the study by Fleissner et al. (2010), the circulating ANGPTL4 concentration was not altered by GF status, potentially explaining the lack of protection from obesity. In conclusion, the gut microbiota contributes to host metabolism and energy harvest from the diet. The presence of gut microbes appears to suppress ANGPTL4 production in the gut.

ANGPTL4 modulates angiogenesis

Angiopoietin-like protein 4 can function as either an anti- or pro-angiogenic factor. It can coordinate with the extracellular matrix to modulate blood vessel and lymphatic vessel leakiness (Hato et al., 2008). Angiopoietin-like protein 4 plays a role in prenatal partitioning of intestinal

lymphatic and blood vessels through the downstream signal *Prox1*. *Prox1* is a required regulator for lymphatic angiogenesis, and ANGPTL4 deficiency caused depression of *Prox1* expression (Bäckhed et al., 2007). Angiopoietin-like protein 4 is also associated with wound healing, involving extracellular communication to promote the re-epithelialization step of this process. In wound healing, proliferation and keratinocyte migration is followed by the inflammatory stage (Goh et al., 2010a). Keratinocytes produce ANGPTL4, stimulated by activation of PPARβ/δ after wounding, and ANGPTL4 subsequently coordinates cell-matrix communication. Furthermore, ANGPTL4 deficient mice had a delayed re-epithelialization process during wound healing. Angiopoietin-like protein 4 interacts with matrix proteins vitronectin and fibronectin to coordinate delayed proteolytic degradation in the wound bed and modulate the wound healing processes (Goh et al., 2010a).

Angiopoietin-like protein 4 also interacts with integrins β1 and β5, which modulate cell migration, intracellular signal transduction, and the wound healing process. Binding of ANGPTL4 with integrin β1 activated the FAK-Src-PAK1 signaling pathway that controls cell adhesion and enhances migration in the extracellular matrix (ECM). It also potentially increases endothelial permeability (Goh et al., 2010ab). Angiopoietin-like protein 4 is elevated in many human tumor cells and is secreted by proliferating tumorous epithelial cells. By binding to ECM integrins, ANGPTL4 modulates O_2^- production through activation of the FAK and Rac1 pathway. Nox1 (NADPH oxidase) is the major mediator for autocrine modulation of O_2^- production in tumor cells by ANGPTL4. Angiopoietin-like protein 4 increases reactive oxygen species (ROS) levels, which in turn stimulates tumor growth, invasiveness, and metastasis. Suppression of ANGPTL4 expression impairs tumor growth and enhances tumor apoptosis (Zhu et al., 2011).

Angiopoietin-like protein 4 plays a role in tumor metastasis via control of vascular permeability and cell junction leakiness. Angiopoietin-like protein 4 acts as a critical target gene for TGFβ, via the Smad pathway, increasing the chance of breast tumor metastasis to the lung. Tumor cell-derived ANGPTL4 increases gaps between cell to cell junctions located in the vascular endothelium, which increases the permeability of lung capillaries (Padua et al., 2008). Furthermore, ANGPTL4 transcription in human cell is activated by PPAR ligands and TGFβ. TGFβ signaling is influenced by PPARβ/δ pathway via TGF enhancer and PPAR enhancer

ligand. Thus, the TGFβ-inducible upstream enhancer stimulated the activation of ANGPTL4 gene (Kaddatz et al., 2010).

Other research provided evidence that ANGPTL4 acts as an antiangiogenic factor under some controlled conditions. In an in vitro experiment, ANGPTL4 and vascular endothelial growth factor (VEGF) interacted to reduce normal vascular proliferation and tube formation of vascular endothelial cells (Ito et al., 2003). In contrast, adipose tissue growth requires a capillary network to increase circulation around adipose tissue. For enhanced vascular circulation around adipose tissue, VEGF and ANGPTL4 have co-activator functions on PPARγ. An in vitro study showed ANGPTL4 inhibition of VEGF expression, but ANGPTL4 and VEGF both increase PPARγ expression. Furthermore, upregulated ANGPTL4 expression stimulates endothelial cell growth and differentiation (Gealekman et al., 2008). Cardiovascular disorders are associated with hypoxia, caused by deficient blood supply, which also stimulates ANGPTL4 expression (Cazes et al., 2006). Hypoxic conditions stimulate endogenous ANGPTL4 accumulation in the ECM, and ECM association with ANGPTL4 causes decreases in cell adhesion, cell migration and tube sprouting (Cazes et al., 2006).

Angiopoietin-like protein 4 regulates vascular integrity, capillary proliferation, and interacts with the ECM to influence cell migration and adhesion. Researchers have shown ANGPTL4 to have pro- and anti-angiogenesis effects under different tissues and conditions, and also provided insight into the role of ANGPTL4 as part of complex cell-to-cell communication.

ANGPTL4 May Act as an Inflammatory Factor

Adipocytes secrete numerous adipokines and adipose tissue plays a key role in the endocrine system. It mediates lipid and glucose metabolism and some adipokines act as inflammatory mediators, such as TNFα, IL-1β, and IL-6. Angiopoietin-like protein 4 is also one of the adipokines regulating part of lipid metabolism (Trayhurn et al., 2010). Its expression can be stimulated by hypoxia, similar to leptin, IL-6, and VEGF (Wang et al., 2007). Increased body fat is associated with low-grade inflammation, and obese subjects have lower oxygen pressure in adipocyte. Hypoxia induces inflammatory adipokine production and induces an inflammatory response in macrophages. Research investigating interactions between preadipocytes, adipocytes, and adipose tissue macrophages revealed that inflammatory responses in macrophage under hypoxic conditions inhibit adipocyte differentiation. Hypoxia also activated endoplasmic

reticulum stress (ER stress) and oxidative stress to stimulate inflammation in adipocytes (Wood et al., 2009; Trayhurn et al., 2010).

Angiopoietin-like protein 4 expression is stimulated in response to dietary saturated fat, especially with high fat diets (Kersten et al., 2000). Interestingly, recent research has showed that ANGPTL4 limits the inflammatory response induced by saturated fat, inhibiting macrophage uptake of fatty acids in the mesenteric lymph node. Angiopoietin-like protein 4 dramatically reduced foam cell formation, reducing chyle-induced ER stress and limiting expression of inflammation genes in this scenario (Lichtenstein et al., 2010). Dietary saturated fatty acid (SFA) can be transported to cells as circulating FFAs or hydrolyzed to fatty acids from chylomicron or VLDL by LPL. Angiopoietin-like protein 4 acts as an anti-inflammatory agent by inhibiting LPL in mesenteric lymph nodes, which results in decreased hydrolysis of triglycerides carried by chylomicrons, and subsequently, decreased uptake of free SFA into the macrophages. Inhibition of SFA uptake by mesenteric lymph node macrophages decreases proinflammatory effects caused by SFA and prevents their conversion to foam cells. The authors further demonstrated that chyle-mediated activation of ER stress triggers inflammation in macrophages, which increased signaling by recombinant ANGPTL4 (Lichtenstein et al., 2010).

Hypoxia stimulates ANGPTL4 expression (Wang et al., 2007). It binds to ECM integrins and further modulates oxygen production and ROS levels (Zhu et al., 2011). However, the receptor or mediator located in other tissues is still remains unknown. In particular, ANGPTL4 influences downstream kinase activity and signaling pathways in the muscle and adipose tissues. It is important to identify the receptor or mediator in the muscle or adipose tissue to better understand how ANGPTL4 influences lipid metabolism.

CONCLUSION

Bovine ANGPTL4 is expressed in different types of tissue. Research on human and mouse subjects has shown that Angptl4 plays important roles in lipid metabolism, modulation of energy harvest, regulation of angiogenesis, and potentially antagonism of proinflammatory factors. Research on ANGPTL4 in humans and mice has showed that it is important in lipid metabolism and its expression can be altered in as little as 24 hours to shift lipid metabolism. Angiopoietin-like protein 4 stimulates adipose tissue lipolysis and limited clearance of TG in plasma. Several studies have shown that gastrointestinal ANGPTL4 expression is mediated by microbes, and showed different responses in high fat and low fat diets when microbes were present. Ruminants have a different digestive system that depends on microbes to digest most nutrients, and it will be interesting to further investigate ANGPTL4 expression associated with the ruminal microbiota and feed composition in cattle. Furthermore, dairy cattle during the transition to lactation experience negative energy balance and tissue mobilization to provide nutrients to support milk synthesis. Therefore, because of the large number of commensal microbes in the rumen, and the importance of lipolysis in the physiology of lactating cows, ANGPTL4 may play a central role in bovine metabolism.

REFERENCES

Aronsson, L., Y. Huang, P. Parini, M. Korach-André, J. Håkansson, J.-Å. Gustafsson, S. Pettersson, V. Arulampalam, and J. Rafter. 2010. Decreased fat storage by *lactobacillus paracasei* is associated with increased levels of angiopoietin-like 4 protein (ANGPTL4). PLoS ONE 5(9):E13087.

Bäckhed, F., H. Ding, T. Wang, L. V. Hooper, G. Y. Koh, A. Nagy, C. F. Semenkovich, and J. I. Gordon. 2004. The gut microbiota as an environmental factor that regulates fat storage. Proc. Natl. Acad. Sci. 101(44):15718-15723.

Bäckhed, F., P. A. Crawford, D. O'Donnell, and J. I. Gordon. 2007. Postnatal lymphatic partitioning from the blood vasculature in the small intestine requires fasting-induced adipose factor. Proc. Natl. Acad. Sci. 104(2):606-611.

Bäckhed, F., J. K. Manchester, C. F. Semenkovich, and J. I. Gordon. 2007. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc. Natl. Acad. Sci. 104(3):979-984.

Cazes, A., A. Galaup, C. Chomel, M. Bignon, N. Brechot, S. Le Jan, H. Weber, P. Corvol, L. Muller, S. Germain, and C. Monnot. 2006. Extracellular matrix-bound angiopoietin-like 4 inhibits endothelial cell adhesion, migration, and sprouting and alters actin cytoskeleton. Circ. Res. 99(11):1207-1215.

Chakravarthy, M. V., I. J. Lodhi, L. Yin, R. R. V. Malapaka, H. E. Xu, J. Turk, and C. F. Semenkovich. 2009. Identification of a physiologically relevant endogenous ligand for PPARα in liver. Cell 138(3):476-488.

Crawford, P. A., J. R. Crowley, N. Sambandam, B. D. Muegge, E. K. Costello, M. Hamady, R. Knight, and J. I. Gordon. 2009. Regulation of myocardial ketone body metabolism by the gut microbiota during nutrient deprivation. Proc. Natl. Acad. Sci. 106(27):11276-11281.

Ferreira, L. D. M. C.-B., L. K. Pulawa, D. R. Jensen, and R. H. Eckel. 2001. Overexpressing human lipoprotein lipase in mouse skeletal muscle is associated with insulin resistance. Diabetes 50(5):1064-1068.

Fleissner, C. K., N. Huebel, M. M. El-Bary, G. Loh, S. Klaus,and M. Blaut. 2010. Absence of intestinal microbiota does not protect mice from diet-induced obesity. Br. J. Nutr. 104(6):919-930.

Flint, H. J., E. A. Bayer, M. T. Rincon, R. Lamed, and B. A. White. 2008. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat. Rev. Micro. 6(2):121-131.

Ge, H., G. Yang, L. Huang, D. Motola, T. Pourbahrami, and C. Li. 2004. Oligomerization and regulated proteolytic processing of angiopoietin-like protein 4. J. Biol. Chem. 279(3):2038-2045.

Gealekman, O. 2008. Enhanced angiogenesis in obesity and in response to PPARγ activators through adipocyte VEGF and ANGPTL4 production. Am. J. Physiol. Endocrinol. Metab. 295(5):E1056.

Goh, Y. Y., M. Pal, H. C. Chong, P. Zhu, M. J. Tan, L. Punugu, C. R. I. Lam, Y. H. Yau, C. K. Tan, R.-L. Huang, S. M. Tan, M. B. Y. Tang, J. L. Ding, S. Kersten, and N. S. Tan. 2010. Angiopoietin-like 4 interacts with integrins β1 and β5 to modulate keratinocyte migration. Am. J. Pathol. 177(6):2791-803.

Goh, Y. Y., M. Pal, H. C. Chong, P. Zhu, M. J. Tan, L. Punugu, C. K. Tan, R.-L. Huang, S. K. Sze, M. B. Y. Tang, J. L. Ding, S. Kersten, and N. S. Tan. 2010. Angiopoietin-like 4 interacts with matrix proteins to modulate wound healing. J. Biol. Chem. 285(43):2999-3009.

Gurr, M. I., J. L. Harwood, and K. N. Frayn. 2008. Lipid transport. Lipid Biochemistry. Blackwell Science Ltd. P.170-214.

Hato, T., M. Tabata, and Y. Oike. 2008. The role of angiopoietin-like proteins in angiogenesis and metabolism. Trends Cardiovas. Med. 18(1):6-14.

Kaddatz, K., T. Adhikary, F. Finkernagel, W. Meissner, S. Müller-Brüsselbach, and R. Müller. 2010. Transcriptional profiling identifies functional interactions of TGFβ and PPARβ/δ signaling. J. Biol. Chem. 285(38):29469-29479.

Kersten, S., S. Mandard, N. S. Tan, P. Escher, D. Metzger, P. Chambon, F. J. Gonzalezi, B. Desvergne, and W. Wahli. 2000. Characterization of the fasting-induced adipose factor FIAF, a novel peroxisome proliferator-activated receptor target gene. J. Biol. Chem. 275(37):28488-28493.

Kersten, S. 2005. Regulation of lipid metabolism via angiopoietin-like proteins. Biochem. Soc. Transactions 33(5):1059-1062.

Kersten, S., L. Lichtenstein, E. Steenbergen, K. Mudde, H. F. J. Hendriks, M. K. Hesselink, P. Schrauwen, and M. Muller. 2009. Caloric restriction and exercise increase plasma ANGPTL4 levels in humans via elevated free fatty acids. Arterioscler. Thromb. Vasc. Biol. 29(6):969-974.

Kersten, S., J. Seydoux, J. M. Peters, F. J. Gonzalez, B. Desvergne, and W. Wahli. 1999. Peroxisome proliferator–activated receptor α mediates the adaptive response to fasting. J. Clin. Invest. 103(11):1489-1498.

Kim, H.-K., B.-S. Youn, M.-S. Shin, C. Namkoong, K. H. Park, J. H. Baik, J. B. Kim, J.-Y. Park, K.-U. Lee, Y.-B. Kim, and M.-S. Kim. 2010. Hypothalamic Angptl4/Fiaf is a novel regulator of food intake and body weight. Diabetes 59(11):2772-2780.

Kim, I., H.-G. Kim, H. Kim, H.-H. Kim, S. K. Park, C.-S. Uhm, Z. H. Lee, and G. Y. Koh. 2000. Hepatic expression, synthesis and secretion of a novel fibrinogen/angiopoietin-related protein that prevents endothelial-cell apoptosis. Biochem. J. 346(3):603-610.

Kim, J. K., J. J. Fillmore, Y. Chen, C. Yu, I. K. Moore, M. Pypaert, E. P. Lutz, Y. Kako, W. Velez-Carrasco, I. J. Goldberg, J. L. Breslow, and G. I. Shulman. 2001. Tissue-specific overexpression of lipoprotein lipase causes tissue-specific insulin resistance. Proc. Natl. Acad. Sci. 98(13):7522-7527.

Lichtenstein, L., F. Mattijssen, N. J. de Wit, A. Georgiadi, G. J. Hooiveld, R. van der Meer, Y. He, L. Qi, A. Köster, J. T. Tamsma, N. S. Tan, M. Müller, and S. Kersten. 2010. Angptl4 protects against severe proinflammatory effects of saturated fat by inhibiting fatty acid uptake into mesenteric lymph node macrophages. Cell Metab. 12(6):580-592.

Mamedova, L. K., K. Robbins, B. J. Johnson, and B. J. Bradford. 2010. Tissue expression of angiopoietin-like protein 4 in cattle. J. Anim. Sci. 88(1):124-130.

Mandard, S., F. Zandbergen, E. Straten, W. Wahli, F. Kuipers, M. Mùller, and S. Kersten. 2005. The fasting-induced adipose factor/angiopoietin-like protein 4 is physically associated with lipoproteins and governs plasma lipid levels and adiposity. J. Biol. Chem. 281(2):934-944.

Mandard, S., F. Zandbergen, N. S. Tan, P. Escher, D. Patsouris, W. Koenig, R. Kleemann, A. Bakker, F. Veenman, W. Wahli, M. Müller, and S. Kersten. 2004. The direct peroxisome proliferator-activated receptor target fasting-induced adipose factor (FIAF/PGAR/ANGPTL4) is present in blood plasma as a truncated protein that is increased by fenofibrate treatment. J. Biol. Chem. 279(33):34411-34420.

Miles, J. M., Y. Park, and W. S. Harris. 2001. Invited review: lipoprotein lipase and triglyceride-rich lipoprotein metabolism. Nutr. Clin. Pract. 16(5):273-279.

Osborn, O., D. D. Sears, and J. M. Olefsky. 2010. Fat-induced inflammation unchecked. Cell Metab. 12(6):553-554.

Padua, D., X. Zhang, Q. Wang, C. Nadal, W. L. Gerald, R. R. Gomis, and J. Massague. 2008. TGFβ primes breast tumors for lung metastasis seeding through angiopoietin-like 4. Cell 133(1):66-77.

Rodbell, M. 1966. Metabolism of isolated fat cells. J. Biol. Chem. 241(1):130-139.

Samuel, B. S., A. Shaito, T. Motoike, F. E. Rey, F. Backhed, J. K. Manchester, R. E. Hammer, S. C. Williams, J. Crowley, M. Yanagisawa, and J. I. Gordon. 2008. Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. Proc. Natl. Acad. Sci. 105(43):16767-16772.

Sanderson, L. M. and S. Kersten. 2010. PPARs: important regulators in metabolism and inflammation. Nuclear Receptors. Vol. 8. Springer Netherlands. P.259-285.

Sonnenburg, W. K., D. Yu, E.-C. Lee, W. Xiong, G. Gololobov, B. Key, J. Gay, N. Wilganowski, Y. Hu, S. Zhao, M. Schneider, Z.-M. Ding, B. P. Zambrowicz, G. Landes, D. R. Powell, and U. Desai. 2009. GPIHBP1 stabilizes lipoprotein lipase and prevents its inhibition by angiopoietin-like 3 and angiopoietin-like 4. J. Lipid Res. 50(12):2421-2429.

Staiger, H., C. Haas, J. Machann, R. Werner, M. Weisser, F. Schick, F. Machicao, N. Stefan, A. Fritsche, and H.-U. Häring. 2009. Muscle-derived angiopoietin-like protein 4 is induced by fatty acids via peroxisome proliferator–activated receptor (PPAR)-δ and is of metabolic relevance in humans. Diabetes 58(3):579-589.

Stappenbeck, T. S., L. V. Hooper, and J. I. Gordon. 2002. Developmental regulation of intestinal angiogenesis by indigenous microbes via paneth cells. Proc. Natl. Acad. Sci. 99(24):15451-15455.

Sukonina, V., A. Lookene, T. Olivecrona, and G. Olivecrona. 2006. Angiopoietin-like protein 4 converts lipoprotein lipase to inactive monomers and modulates lipase activity in adipose tissue. Proc. Natl. Acad. Sci. 103(46):17450-17455.

Trayhurn, P., F. P. Heredia, B. Wang, and I. S. Wood. 2010. The role of hypoxia in adipocyte function and dysfunction. Novel insights into adipose cell functions. Springer Berlin Heidelberg. P.45-60.

Turnbaugh, P. J., R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis, and J. I. Gordon. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444(7122):1027-1131.

Velagapudi, V. R., R. Hezaveh, C. S. Reigstad, P. Gopalacharyulu, L. Yetukuri, S. Islam, J. Felin, R. Perkins, J. Boren, M. Oresic, and F. Backhed. 2010. The gut microbiota modulates host energy and lipid metabolism in mice. J. Lipid Res. 51(5):1101-1112.

Wang, B., I. Wood, and P. Trayhurn. 2007. Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. Eur. J. Physiol. 455(3):479-492.

Wood, I. S., F. P. de Heredia, B. Wang, and P. Trayhurn. 2009. Cellular hypoxia and adipose tissue dysfunction in obesity. Proc. Nutr. Soc. 68(04):370-377.

Yau, M.-h., Y. Wang, K. S. L. Lam, J. Zhang, D. Wu, and A. Xu. 2009. A highly conserved motif within the NH2-terminal coiled-coil domain of angiopoietin-like protein 4 confers its inhibitory effects on lipoprotein lipase by disrupting the enzyme dimerization. J. Biol. Chem. 284(18):11942-11952.

Yoshida, K., T. Shimizugawa, M. Ono, and H. Furukawa. 2002. Angiopoietin-like protein 4 is a potent hyperlipidemia-inducing factor in mice and inhibitor of lipoprotein lipase. J. Lipid Res. 43(11):1770-1772.

Zhu, P., M. Tan, R.-L. Huang, C. Tan, H. Chong, M. Pal, C. R. I. Lam, P. Boukamp, J. Pan, S. Tan, S. Kersten, H. Li, J. Ding, and N. Tan. 2011. Angiopoietin-like protein 4 elevates the prosurvival lntracellular O_2^-:H_2O_2 ratio and confers anoikis resistance to tumors. Cancer Cell 19(3):401-415.

Chapter 2 - Effects of Dietary Fermentability and Volatile Fatty Acids on Ruminal Angiopoietin-Like Protein 4 Expression

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ABSTRACT

Angiopoietin-like protein 4 (ANGPTL4) is a 55-kDa glycoprotein. It is produced by a number of different tissues, including adipose tissue, liver, muscle, and gastrointestinal tissues. Ruminal epithelium expresses ANGPTL4 at similar levels as liver, which has the highest expression level compared to other tissues. Ruminal microbes could influence on ANGPTL4 production via changes in volatile fatty acid (VFA) production, or through direct microbe/epithelium interactions. The objective of this study was to evaluate the effects of dietary concentrate inclusion and VFA infusions on plasma ANGPTL4 abundance and papillae ANGPTL4 expression. Experiment 1 involved 12 non-lactating Holstein cows which were randomly assigned to either low concentrate (LC: 8% concentrate) or high concentrate (HC: 64% concentrate) diets. Diets were fed for at least 28 days prior to euthanasia and ruminal tissue collection. In experiment 2, 8 beef heifers were randomly assigned to 2 groups; one group was fed a diet containing 90% concentrate, and the other a diet which contained 60% concentrate. These diets were fed for 75 d prior to euthanasia and ruminal tissue collection (700 kg final bodyweight). In experiment 3, 6 ruminally cannulated lactating Holstein cows were randomly assigned to treatment sequence in replicated 3×3 Latin squares and fed a standard lactation diet. Initially, cows were infused with 10 mol/d sodium acetate, sodium propionate, or sodium butyrate for 2 d. However, during period (P) 1, both dry matter intake and calculated energy intake were decreased by infusions ($P < 0.01$) relative to pre-treatment. Therefore, infusion rates were decreased to 5 mol/d for P2 and P3. The VFA treatments did not directly affect ANGPTL4 expression, but ruminal butyrate concentration was positively related to plasma ANGPLT4 concentration. Results of 3 experiments indicated that 1) production of ANGPTL4 in ruminal tissue decreased in response to increased dietary fermentability; 2) ruminal ANGPTL4 was associated with ruminal pH and total ruminal VFA concentration; and 3) ruminal ANGPTL4 was positively associated with plasma ANGPTL4.

Key Words: ANGPTL4, cattle, volatile fatty acids

INTRODUCTION

Ruminant lipid and energy metabolism is regulated by the complex interaction of nutrition, physiology, and genomic factors, including both the animal and its commensal microbiota. In the last decade, angiopoietin-like-protein 4 (ANGPTL4) research has largely focused on its roles in the regulation of lipid and energy metabolism in mice and humans. One key effect of plasma ANGPTL4 is its inhibition of lipoprotein lipase (LPL) function (Yau et al., 2009). Furthermore, ANGPTL4 also stimulates adipose tissue lipolysis, increases oxidation of fatty acids, and decreases white adipose tissue mass (Hato et al., 2008; Kersten et al., 2005). Angiopoietin-like protein 4 is a widely-expressed protein in different tissues, and is especially abundant in liver and adipose tissue. Human and mouse studies showed that fasting, exercise, or the consumption of a high fat diet can elevate plasma ANGPTL4 concentrations (Kersten et al., 2005; Kersten et al., 2009; and Mandard et al., 2006).

Recent studies have highlighted the dramatic effects of gut microbes on host metabolism and inflammatory pathways (Greiner et al., 2010). Microbe colonization of germ-free mice was discovered to suppress ANGPTL4 expression in the ileum and decrease plasma ANGPTL4 concentrations (Bäckhed et al., 2004). Furthermore, suppression of ANGPTL4 after colonization was associated with increased LPL function and fat storage in the adipose tissue (Bäckhed et al., 2004). These findings suggest that host-microbe interactions in the gut play an important role in metabolic physiology, and that ANGPTL4 may be a key player in these interactions. If such interactions are important in nonruminant animals, it is likely that are even more critical in the ruminant, because of the massive and essential population of commensal bacteria, protozoa, and fungi that inhabit the rumen. The fermentability of diets fed to ruminants can dramatically alter the ruminal environment and microbe ecology (Russell et al., 2001), and in turn, the behavior, productivity, and health of the animal (Bradford et al., 2004; Nagaraja et al., 2007). Therefore, it is of great interest to understand whether ANGPTL4 mediates any of the effects of ruminal microbes on the host animal.

Previous work demonstrated that ANGPTL4 is expressed in many bovine tissues, but that protein abundance of ANGPTL4 was quite high in the epithelial layer of the rumen wall (Mamedova et al., 2010). This finding raised the possibility that interactions between microbes and ruminal epithelial cells alter expression of ANGPTL4 in the rumen wall. Because changes in diet fermentability have such dramatic effects on microbial ecology and the profile of

metabolites produced in the rumen, such diet changes provide a simple tool to assess whether these shifts influence ANGPTL4 expression and concentrations. However, the many changes induced by shifts in diet fermentability make it difficult to pinpoint mechanisms driving alterations in epithelial function. Intraruminal VFA infusions offer the ability to independently test the effects of a change in metabolite concentrations with corresponding differences in microbial populations or ruminal pH. Therefore, 3 experiments were carried out with the objective of understanding how dietary fermentability and specific VFA influence ruminal production and plasma concentrations of ANGPLT4. We hypothesized that ruminal environmental changes would influence both ruminal production and systemic concentrations of ANGPTL4.

MATERIALS AND METHODS

Experimental Design and Treatment

Experiment 1

Experiment 1 evaluated the effects of dietary fermentability on ANGPTL4. The study design and sample collection have been described previously (Penner et al., 2009; Taniguchi et al., 2010). Briefly, 12 non-lactating, non-gestating and ruminally cannulated Holstein cows were housed in tie-stalls for the study. The animal experiments were performed at the Lethbridge Research Center of Agriculture and Agri-Food Canada and study protocols were approved by the Canadian Council on Animal Care (Ottawa, ON, Canada). Cows were randomly assigned to either a low concentrate diet (LC: 8% concentrate, 92% forage) or a high concentrate diet (HC: 64% concentrate, 36% forage). Cows were fed treatment diets (Table 2.1) for at least 23 d before slaughter, and then ruminal pH monitoring devices (Penner et al., 2006) were used to collect pH data every 30 s for 3 days. Ruminal fluid was collected at the end of dietary treatment, and frozen at -20°C for GC analysis. The method of ruminal fluid collection and gas chromatography analysis for VFA concentration has been described previously (Penner et al., 2009). Cattle were then euthanized and samples of ruminal epithelium were collected and frozen at -80°C for subsequent analysis.

Experiment 2

This experiment was designed to assess whether ANGPTL4 is influenced by diet composition in beef cattle. Beef heifers were housed at the Lethbridge Research Center of Agriculture and Agri-Food Canada and study protocols were approved by the Canadian Council on Animal Care (Ottawa, ON, Canada). Eight beef heifers were either fed a diet with 90% concentrates (DM basis) or one including 60% concentrates for 75 d (Table 2.2). At the end of this feeding period, ruminal pH was monitored continuously (Penner et al., 2006) for 6 d before animals were euthanized (700 kg final weight). Ruminal tissue samples were collected and frozen at -80°C for subsequent analysis.

Experiment 3

Six ruminally cannulated multiparous lactating Holstein cows from the Kansas State University Dairy Teaching and Research Facility were randomly assigned to treatment sequence

in replicated 3×3 Latin squares. Experimental procedures were approved by the Kansas State University Animal Care and Use Committee. Beginning 7 d before treatments started, cows were housed in tie-stalls and fed a standard lactation diet formulated to meet all dietary requirements (NRC, 2001), as shown in Table 2.3. Cows were fed for ad libitum intake once daily (1600 h) and milked 3 times daily (200 h, 1000 h, and 1800 h). Dry matter intake was recorded daily throughout the experiment. The treatments were intraruminal infusions of sodium acetate, sodium propionate, and sodium butyrate (Sigma-Aldrich, St. Louis, MO). Treatment periods were 2 d in length, with 5 d between subsequent periods.

At the initiation of the experiment, treatments were infused by intraruminal bolus (2 L, 0.83 M, pH 6.0) once every 4 h during the 2 d treatment periods, for a total infusion rate of 10 mol/d. This infusion rate was expected to decrease feed intake by no more than 10% (Bradford et al., 2007). However, during the first period of treatment, dry matter intake (DMI) and mean calculated metabolizable energy (ME) intakes were decreased by 24% and 19%, respectively, compared to pre-treatment data (Table 2.4). Therefore, in periods 2 and 3, the infusion rate was decreased to 5 mol/d by decreasing the infusion volume to 1 L per bolus.

Ruminal pH was measured with indwelling pH meters (Kahne Limited, Auckland, New Zealand), which recorded pH data every 5 min during treatment periods. Ruminal fluid was collected immediately before and after treatment periods by sampling ruminal contents from 6 sites throughout the rumen and squeezing it though a nylon screen (1 mm pore size) to collect ruminal fluid. Ruminal fluid samples were frozen at -20°C until analysis as previously described (Mullins et al., 2010).

Blood samples were collected into K_3 EDTA-containing tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) immediately before and after infusion periods (900 h). Plasma samples were harvested by centrifuging at 2,000 × *g* for 15 min immediately after sample collection and were frozen at -20°C until analysis. Ruminal papillae were collected immediately after each infusion period. Approximately 1/3 of the rumen contents were removed via the cannula to allow access to the ventral rumen wall. The ventral sac of the rumen was then pulled toward the cannula, and approximately 20 papillae were excised using surgical scissors. Papillae samples were immediately frozen in liquid nitrogen and subsequently stored at −80°C until analysis.

Papillae and Plasma Analyses

Western Blot Analysis

The Western blot method was described previously (Mamedova et al., 2010). Approximately 40 mg papillae samples were homogenized with radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) and X1 protease inhibitor cocktail (Thermo Scientific, Rockford, IL). Samples were centrifuged at 15,000 × *g* for 10 min at 4°C, and the supernatant was collected for total protein assay and Western blot. Total protein concentration was determined with a Coomassie protein assay kit (Pierce, Rockford, IL). Forty micrograms of supernatant protein or 1 μL of plasma samples were denatured in Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA) including 5% 2-mercaptoethanol (Sigma-Aldrich Inc.). The mixture was incubated at 82°C for 7 min according to the manufacturer's directions for tissue isolates, but plasma samples were incubated at 95 °C for decreased background signal. Then, proteins were separated by SDS-PAGE on a 4% to 12% Tris-glycine gel. The separated proteins were dry-transferred to a nitrocellulose membrane (iBlot, Invitrogen, Carlsbad, CA). Membranes were blocked for 2 h in a solution of phosphate buffered saline (PBS) containing 0.05% Tween-20 and 5% non-fat dry milk. Membranes were then incubated with a goat anti-ANGPTL4 antibody (1:1,000 dilution; Santa Cruz Biotechnology) followed by an anti-goat IgG secondary antibody (1:10,000 dilution; Santa Cruz Biotechnology). Both incubations were performed in Tris-HCl buffer, pH 7.5, with 0.05% Tween-20, and after each antibody incubation, membranes were washed with Tris-HCl buffer 5 times for a total of 15 min. Angiopoietin-like protein 4 was detected by using chemiluminescence (West-Dura, Thermo Scientific) and relative protein abundance was determined by densitometry (Image J, NIH software, http://rsbweb.nih.gov/nih-image/).

Enzyme-linked Immunosorbent Assay (ELISA)

In experiment 3, plasma ANGPTL4 concentrations were measured using a human ANGPTL4 ELISA kit (RayBiotech Inc., Norcross, GA) which was previously validated for use in bovine plasma (Khan et al., 2011). Standards included in the kit (0–20,000 pg/mL) were used and the procedure was carried out according to manufacturer's instructions.

Quantitative Real Time PCR

The method of determining ANGPTL4 mRNA abundance by quantitative real-time PCR was described by Mamadova and others (2010). Briefly, approximately 20 mg papillae samples were homogenized in 1 ml of QIAzol lysis reagent, and total RNA was extracted by using affinity columns (RNeasy Lipid Tissue Mini Kit, Qiagen, Valencia, CA). Two micrograms of total RNA were reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystem, Foster City, CA). Quantitative RT-PCR was performed in 96-well plates using Fast SYBR Green Master Mix (Applied Biosystems) with an ABI Prism 7500 instrument (Applied Biosystems). Angiopoietin-like protein 4 mRNA abundance was determined relative to the internal control gene ribosomal protein subunit 9 (RPS9; Mamedova et al., 2010). Forward and reverse primers were included at 200 nM final concentrations, and primer sequences were as follows: bovine ANGPTL4 forward, GATGGCTCCGTGGACTTTAACC; reverse, GGATGTGATGCACCTTCTCCAG; bovine RPS9 forward, GAACAAACGTGAGGTCTGGAGG; reverse, ATTACCTTCGAACAGACGCCG. Samples were analyzed in triplicate with a standard shuttle PCR protocol: 30 s at 95°C and 30 s at 60°C for 40 cycles.

STATISTICAL ANALYSIS

Data were analyzed using the REML procedure of JMP (version 8.0, SAS Institute, Cary, NC). The model used for experiment 1 included the fixed effect of treatment and the random effect of block. For experiment 2, the model included the fixed effect of treatment. Ruminal ANGPTL4 mRNA abundance data in experiment 2 were log-transformed prior to analysis to achieve a normal distribution of residuals, and reported means are back-transformed. Data from experiment 3, period 1 were analyzed separately from periods 2 and 3 because of the different doses used. In experiment 3, period 1 data were modeled with the fixed effect of treatment and period 2 and 3 data were fit to a model including the fixed effect of treatment and the random effects of cow and period. For each regression analysis, the distribution of Cook's D statistic was visually checked and outliers were removed from the analysis. No more than 2 data points were removed from any regression. Statistical tests were declared significant at $P < 0.05$ and tendencies were declared at $0.05 \leq P < 0.10$.

RESULTS

Experiment 1 and 2

Experiments 1 and 2 evaluated the effects of dietary fermentability on ANGPTL4 expression. The compositions of diets are shown in Tables 2.1 and 2.2. The high concentrate, more fermentable diet in experiment 1 tended to decrease ANGPTL4 mRNA by 55% ($P = 0.08$, Figure 2.1). However, there were no dietary effects on protein abundance in ruminal epithelium ($P = 0.46$, Figure 2.2) or plasma ($P = 0.34$, Figure 2.3). Across treatments, ANGPTL4 mRNA tended to be positively correlated with ruminal pH ($P = 0.07$, Figure 2.4), and was inversely correlated with total ruminal VFA concentration ($P = 0.03$, Figure 2.5).

In experiment 2, ruminal epithelium ANGPTL4 protein abundance was decreased by the high concentrate (highly fermentable) diet ($P < 0.01$, Figure 2.6), but there was no effect on plasma ANGPTL4 abundance ($P = 0.39$). The relative ANGPTL4 protein abundance in the ruminal epithelium was also positively correlated with ruminal pH ($P < 0.01$, Figure 2.7). The results from experiment 1 and 2 indicated that increased ruminal fermentation caused decreases in ANGPTL4 expression in the ruminal epithelium.

Experiment 3

Dry Matter Intake and Metabolizable Energy Intake

In period 1 of this study, the VFA infusion rate was 10 mol/d, which significantly decreased DMI and ME intake compared to the days prior to treatment (both $P < 0.01$, Table 2.4). In periods 2 and 3, the VFA infusion rate was decreased to 5 mol/d in an attempt to avoid altering ME intake during infusions. As expected, the lower infusion rate did not significantly alter DMI ($P = 0.17$, Table 2.5) or ME intake ($P = 0.46$, Table 2.5).

Ruminal pH and VFA

During experiment 3, mean ruminal pH was above 6.0 for all treatments. Treatments did not alter the mean or SD of ruminal pH at either infusion rate (Tables 2.4 and 2.5). In addition to ruminal pH, VFA concentrations in ruminal fluid were measured (Tables 2.6 and 2.7). In general, few effects of treatment on ruminal VFA concentrations were found. Propionate concentrations tended to be lower during treatment periods at both infusion rates ($P = 0.09$ and P

= 0.05). Also, ruminal valerate concentrations were lower during treatment periods 2 and 3 compared to pre-treatment concentrations (*P* = 0.02, Table 2.7), but valerate did not differ between individual treatments (*P* = 0.43). At the lower infusion rate, butyrate infusion tended to increase ruminal butyrate concentrations (*P* = 0.10, Table 2.7); however, other treatments did not result in significant elevations in ruminal concentrations of the infused VFA. No treatment effects were observed for total VFA concentrations, and there was no correlation found between mean ruminal pH and total VFA concentration (*P* = 0.56, Figure 2.8).

Papillae Parameters

Papillae ANGPTL4 protein abundance was not affected by treatments across the two infusion rates (*P* = 0.84). Furthermore, ANGPTL4 mRNA abundance was not significantly different for different VFA treatments (*P* > 0.27, Tables 2.8 and 2.9). Although no correlation between tissue ANGPTL4 and ME intake was observed (*P* = 0.30, Figure 2.9), tissue ANGPTL4 mRNA was negatively correlated with both ruminal pH (*P* = 0.03, Figure 2.10) and total ruminal VFA (*P* = 0.01, Figure 2.11). Interestingly, plasma ANGPTL4 concentrations tended to correlate with tissue ANGPTL4 abundance (*P* = 0.09, Figure 2.12).

Plasma Parameters

Plasma ANGPTL4 concentrations are displayed in Tables 2.8 and 2.9. There were no treatment effects at either infusion rate (*P* = 0.91 and *P* = 0.30). Furthermore, despite the significant impact of infusions on ME intake in period 1, there were no differences in treatment vs. pre-treatment plasma ANGPLT4 concentrations (*P* > 0.34). Plasma ANGPTL4 had an inverse relationship with ruminal pH (*P* < 0.01, Figure 2.13), with regression estimates ranging from 5.6 to 1.9 ng/mL as ruminal pH increases from 6.0 to 6.2. Plasma ANGPTL4 tended to correlate with ME intake (*P* = 0.06, Figure 2.14) and total ruminal VFA concentration (*P* = 0.06, Figure 2.15). We also analyzed the correlation between each individual ruminal VFA concentration and plasma ANGPTL4 concentration. There was a correlation between ruminal butyrate concentrations and plasma ANGPTL4 concentrations (*P* = 0.04, Figure 2.16), but no correlations were found between ruminal acetate or propionate and plasma or ruminal ANGPTL4 concentrations (*P* > 0.10).

DISCUSSION

Contribution of gastrointestinal ANGPTL4 to Systemic Concentrations

Angiopoietin-like protein 4 concentrations are influenced by nutritional status, and ANGPTL4, in turn, has effects on feed intake, plasma lipid parameters, triglyceride storage, and lipid clearance (Hato et al., 2008; Kersten et al., 2005& 2009; Mandard et al., 2005). Researchers used ANGPTL4 overexpression mice to demonstrate that ANGPTL4 can increase plasma triglyceride and cholesterol concentrations, inhibit LPL function, and prevent free fatty acid uptake by tissue (Lichtenstein et al., 2007). Expression of ANGPTL4 in the mouse hypothalamus was influenced by glucose, insulin, and leptin, and this synthesis of ANGPTL4 plays a role in the control of food intake and body weight (Kim et al., 2010).

Diet composition can influence ANGPTL4, as demonstrated by a study with mice supplemented with high fat diets. The study altered fat percentage (low fat diet: 17.2% fat; high fat diet: 43.0% fat; Western diet: 40.6% fat) of diets and fat percentage influenced intestinal ANGPTL4 mRNA expression when microbes were present in the gut (Fleissner et al., 2010). Also, ANGPTL4 expression was altered by differing fatty acid composition of the high fat diet (Fleissner et al., 2010). Research on mice raised germ-free and then colonized with commensal microbes suggested that the introduction of gut microbes suppresses ANGPTL4 expression in intestine (Bäckhed et al., 2004). It was suggested that gastrointestinal-derived ANGPTL4 influences circulating concentrations of this plasma factor (Bäckhed et al., 2004). In our findings, ruminal papillae ANGPTL4 abundance was positively, albeit weakly, correlated with plasma ANGPTL4 concentrations. Gastrointestinal-derived ANGPTL4 may make a meaningful contribution to the circulating ANGPTL4 pool, although this remains to be proven conclusively.

Diet Influences ANGPTL4 Expression in Ruminal Epithelium

Immunofluorescent detection demonstrated that the ruminal epithelial layer contained similar or greater quantities of ANGPTL4 compared to the liver (Mamedova et al., 2010), which has been seen as a key tissue source of ANGPTL4. In addition, analysis of gastrointestinal ANGPTL4 mRNA and protein showed that ANGPTL4 is widely expressed in the ruminant gastrointestinal tract (Mamedova et al., 2010). In the current study, we evaluated the influence of diet fermentability on ANGPTL4 in ruminal tissue and plasma. In cattle fed the highly fermentable diets, we observed a tendency for decreased ANGPTL4 mRNA abundance (Exp. 1)

and a significant decrease in ANGPTL4 protein abundance (Exp. 2) in ruminal tissue compared to cattle fed less fermentable, higher-forage diets.

Changing diet composition can alter the ruminal environment and microbial ecology. Alterations in ruminal fermentation can influence pH, VFA concentrations, and ammonia concentrations (Russell et al., 2001). The design of experiments 1 and 2 did not allow us to directly evaluate whether these factors were involved in the observed changes in ANGPTL4 synthesis. However, regression analysis showed positive correlation between ruminal pH and ANGPTL4 mRNA and protein abundance. In addition, ruminal ANGPTL4 mRNA was negatively related with total ruminal VFA. Volatile fatty acids are the predominant acids in the rumen, and as such, ruminal pH and VFA concentrations are often inversely related. Mechanistically, it seems likely that relationships between ruminal pH and ANGPTL4 expression are driven by changes in VFA concentrations. However, microbial ecology can also shift dramatically as the rumen becomes more acidic, because some species are more sensitive to inhibition by low pH than other species (Nagaraja et al., 2007). Therefore, the observed effects of diet on ruminal ANGPTL4 expression may have been driven by specific VFA, direct microbe/epithelium interactions, or by metabolites produced by particular species of ruminal microbes.

To test one possible mechanism mediating the effect of diet on ruminal ANGPTL4, we tested the effects of VFA infusion in experiment 3. However, we did not observe effects of individual VFA on either ruminal ANGPTL4 expression or plasma concentrations. Interestingly, we found that ruminal butyrate concentrations were negatively correlated with plasma ANGPTL4 concentrations; this correlation was not observed for ruminal acetate or propionate concentrations. Butyrate, which is a key product of ruminal fermentation, is also a primary energy source for ruminal epithelial tissue (Aschenbach et al, 2011; Kristensen et al., 2005). Recent work suggests that butyrate may alter cell function and signaling simply by serving as a nutrient source. Butyrate prevented autophagy in colonocytes, cells that also use butyrate as a primary energy source (Donohoe et al., 2011). Butyrate could also alter cellular function through its role as a histone deacetylase inhibitor (Davie et al., 2003) or through the membrane free fatty acid receptors 2 and 3, although these receptors are activated by multiple VFA (Stoddart et al., 2008).Surprisingly, however, the observed relationship between ruminal butyrate concentrations and plasma ANGPTL4 concentrations were not mirrored at the tissue level, making the proposed

direct effects of butyrate on ANGPTL4 synthesis less convincing. In general, VFA infusions failed to clearly prove or disprove the hypothesis that they mediate dietary effects on ruminal ANGPTL4 expression, largely because of the difficulty of finding a VFA infusion rate capable of significantly increasing ruminal VFA concentrations without altering energy intake.

Recent research demonstrated that probiotic microbes can stimulate ANGPTL4 expression in the gut (Aronsson et al., 2010). The mechanism underlying this effect appeared to be the release of a soluble factor by the microbes that can activate PPARα and PPARγ in epithelial cells. Results of the experiment also suggested that changing gastrointestinal secretion of ANGPTL4 can elevate serum ANGPTL4 concentrations (Aronsson et al., 2011). Because dietary changes can dramatically alter populations of microbes in the rumen, it is possible that unique signaling factors produced by a subset of ruminal microorganisms may have similar effects on ruminal epithelial cells.

Diet Influences Plasma ANGPTL4 in Ruminants

Bovine ANGPTL4 is not induced by fasting, in contrast to mouse and human responses (Bradford et al., 2009). Consistent with previous data from feed-restricted cattle, we found out that plasma ANGPTL4 concentrations were positively correlated with total ME intake in experiment 3. In contrast, there was no relationship observed between ruminal papillae ANGPTL4 and ME intake. Restricting feed intake of cattle decreased adipose tissue ANGPTL4 mRNA abundance, in contrast to the increased plasma concentrations observed in those animals (Bradford et al., 2009). This inconsistency suggests that adipose tissue may not be the most important tissue source of circulating ANGPTL4 in cattle. Circulating ANGPTL4 is potentially up-regulated by increased total energy intake in cattle, but whether this response is driven by increased secretion from adipose tissue, liver, muscle, the GI tract, or another tissue source remains to be determined. Interestingly, we observed a tendency for a positive correlation between ruminal ANGPTL4 and plasma ANGPTL4 concentrations, indicating that gastrointestinal sources of ANGPTL4 are worthy of continued investigation.

CONCLUSIONS

In 2 experiments, we determined that more highly fermentable diets suppress ruminal expression of ANGPTL4 in cattle. Although changes in ruminal VFA profiles provide one possible mechanism underlying this response, we failed to detect any effects of individual VFA infusions in experiment 3. Finally, regression analyses from these experiments demonstrated that ruminal butyrate concentration is negatively related and total energy intake is positively related to plasma ANGPLT4 concentration. These findings add to the evidence that microbial fermentation, whether through products of fermentation or direct host/microbe interactions, can alter the signals generated by gastrointestinal epithelial cells.

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REFERENCES

Aronsson, L., Y. Huang, P. Parini, M. Korach-André, J. Håkansson, J.-Å. Gustafsson, S. Pettersson, V. Arulampalam, and J. Rafter. 2010. Decreased fat storage by *lactobacillus paracaseiis* associated with increased levels of angiopoietin-like 4 protein (ANGPTL4). PLoS One 5(9):e13087.

Aschenbach, J. R. 2011. Ruminant nutrition symposium: role of fermentation acid absorption in the regulation of ruminal pH. J. Anim. Sci. 89(4):1092-1107.

Bäckhed, F., H. Ding, T. Wang, L. V. Hooper, G. Y. Koh, A. Nagy, C. F. Semenkovich, and J. I. Gordon. 2004. The gut microbiota as an environmental factor that regulates fat storage. Proc. Natl. Acad. Sci. 101(44):15718-15723.

Bäckhed, F., J. K. Manchester, C. F. Semenkovich, and J. I. Gordon. 2007. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc. Natl. Acad. Sci. 104(3):979-984.

Bradford, B. J. 2007. Short communication: rate of propionate infusion within meals does not influence feeding behavior. J. Dairy Sci. 90(5):2305-2308.

Bradford, B. J. and M. S. Allen. 2004. Milk fat responses to a change in diet fermentability vary by production level in dairy cattle. J. Dairy Sci. 87(11):3800-3807.

Bradford, B. J., L. K. Mamedova, K. J. Harvatine, and Y. R. Boisclair. 2009. Plasma angiopoietin-like protein 4 concentration is decreased by energy restriction in lactating dairy cattle. Proc. XI th international symposium on ruminant physiology, Clermont-Ferrand, France. 426-427 (Abstr.).

Davie, J. R. 2003. Inhibition of histone deacetylase activity by butyrate. J. Nutr. 133(7):2485S-2493S.

Donohoe, Dallas R., N. Garge, X. Zhang, W. Sun, Thomas M. O'Connell, Maureen K. Bunger, and Scott J. Bultman. 2011. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab. 13(5):517-526.

Dumas, M.-E. 2011. The microbial-mammalian metabolic axis: beyond simple metabolism. Cell Metab. 13(5):489-490.

Fleissner, C. K., N. Huebel, M. M. El-Bary, G. Loh, S. Klaus,and M. Blaut. 2010. Absence of intestinal microbiota does not protect mice from diet-induced obesity. Br. J. Nutr. 104(6):919-930.

Greiner, T. and F. Bäckhed. 2011. Effects of the gut microbiota on obesity and glucose homeostasis. Trends Endocrinol. Metab. 22(4):117-123.

Hato, T., M. Tabata, and Y. Oike. 2008. The role of angiopoietin-like proteins in angiogenesis and metabolism. Trends Cardiovas. Med. 18(1):6-14.

Khan, J., D. Graugnard, D. H. Keisler, B. J. Bradford, L. K. Mamedova, J. K. Drackley, and J. J. Loor. 2011. Hepatokine, growth hormone, and PPARα-regulated gene network expression in liver of peripartal cows fed two levels of dietary energy prepartum. J. Dairy Sci. 94 (E-Suppl. 1): 732 (Abstr.).

Kersten, S. 2005. Regulation of lipid metabolism via angiopoietin-like proteins. Biochem. Soc. T. 33(5):1059-1062.

Kersten, S., L. Lichtenstein, E. Steenbergen, K. Mudde, H. F. J. Hendriks, M. K. Hesselink, P. Schrauwen, and M. Muller. 2009. Caloric restriction and exercise increase plasma ANGPTL4 levels in humans via elevated free fatty acids. Arterioscl. Throm. Vas. 29(6):969-974.

Kim, H.-K., B.-S. Youn, M.-S. Shin, C. Namkoong, K. H. Park, J. H. Baik, J. B. Kim, J.-Y. Park, K.-U. Lee, Y.-B. Kim, and M.-S. Kim. 2010. Hypothalamic Angptl4/Fiaf is a novel regulator of food intake and body weight. Diabetes 59(11): 2772-2780.

Kristensen, N. B. 2005. Splanchnic metabolism of volatile fatty acids in the dairy cow. J. Anim. Sci. 80(1):3-10.

Mamedova, L. K. 2009. Production of angiopoietin-like protein 4 in ruminal tissue is decreased with increasing dietary fermentability. ADSA-CSAS-ASAS Joint Annual Meeting. J. Anim. Sci., Montreal, QC, Canada (Abstr.).

Mamedova, L. K., K. Robbins, B. J. Johnson, and B. J. Bradford. 2010. Tissue expression of angiopoietin-like protein 4 in cattle. J. Anim. Sci. 88(1):124-130.

Mandard, S., F. Zandbergen, E. Straten, W. Wahli, F. Kuipers, M. Mùller, and S. Kersten. 2005. The fasting-induced adipose factor/angiopoietin-like protein 4 is physically associated with lipoproteins and governs plasma lipid levels and adiposity. J. Biol. Chem. 281(2):934-944.

Mullins, C. R., K. N. Grigsby, D. E. Anderson, E. C. Titgemeyer, and B. J. Bradford. 2010. Effects of feeding increasing levels of wet corn gluten feed on production and ruminal fermentation in lactating dairy cows. J. Dairy Sci. 93(11):5329-5337.

Nagaraja, T. G. and E. C. Titgemeyer. 2007. Ruminal acidosis in beef cattle: the current microbiological and nutritional outlook and. J. Dairy Sci. 90(Supplement 1):E17-E38.

NRC. 2001. Nutrient requirements of dairy cattle. 7th rev. ed. National Research Council. Natl. Acad. Sci., Washington, DC.

Penner, G. B., K. A. Beauchemin, and T. Mutsvangwa. 2006. An evaluation of the accuracy and precision of a stand-alone submersible continuous ruminal pH measurement system. J. Dairy Sci. 89(6):2132-2140.

Penner, G. B., M. Taniguchi, L. L. Guan, K. A. Beauchemin, and M. Oba. 2009. Effect of dietary forage to concentrate ratio on volatile fatty acid absorption and the expression of genes related to volatile fatty acid absorption and metabolism in ruminal tissue. J. Dairy Sci. 92(6):2767-2781.

Rhoads, R. P. 2007. Effect of nutrition on the GH responsiveness of liver and adipose tissue in dairy cows. J. Endocrinol. 195(1):49-58.

Russell, J. B. and J. L. Rychlik. 2001. Factors that alter rumen microbial ecology. Science 292(5519):1119-1122.

Sonnenburg, W. K., D. Yu, E.-C. Lee, W. Xiong, G. Gololobov, B. Key, J. Gay, N. Wilganowski, Y. Hu, S. Zhao, M. Schneider, Z.-M. Ding, B. P. Zambrowicz, G. Landes, D. R. Powell, and U. Desai. 2009. GPIHBP1 stabilizes lipoprotein lipase and prevents its inhibition by angiopoietin-like 3 and angiopoietin-like 4. J. Lipid Res. 50(12):2421-2429.

Stoddart, L. A., N. J. Smith, and G. Milligan. 2008. International union of pharmacology. LXXI. free fatty acid receptors FFA1, -2, and -3: pharmacology and pathophysiological functions. Pharm. Rev. 60(4):405-417.

Sumner-Thomson, J. M., J. L. Vierck, and J. P. McNamara. 2011. Differential expression of genes in adipose tissue of first-lactation dairy cattle1. J. Dairy Sci. 94(1):361-369.

Taniguchi, M., G. B. Penner, K. A. Beauchemin, M. Oba, and L. L. Guan. 2010. Comparative analysis of gene expression profiles in ruminal tissue from Holstein dairy cows fed high or low concentrate diets. Comp. Biochem. Physiol. D 5(4):274-279.

Yau, M.-h., Y. Wang, K. S. L. Lam, J. Zhang, D. Wu, and A. Xu. 2009. A highly conserved motif within the NH2-terminal coiled-coil domain of angiopoietin-like protein 4 confers its inhibitory effects on lipoprotein lipase by disrupting the enzyme dimerization. J. Biol. Chem. 284(18):11942-11952.

Table 2.1 Experiment 1: Ingredient composition and nutrient analysis of the TMR[1].

	Treatment	
Item	LC	HC
Ingredient, %of DM		
Alfalfa hay	14.5	5.7
Rolled barley grain	4.1	44.3
Barley silage	77.2	30.1
Ground barley grain	2.0	16.9
Canola meal	1.2	1.3
Mineral and vitamin mix[2]	1.0	1.7
Canola oil	0.02	0.09
Nutrient composition		
DM	48.5	67.8
Ash, % of DM	9.8	6.7
CP, % of DM	12.5	12.9
NDF, % of DM	44.2	28.3
Forage NDF, % of NDF	97.0	59.0
Forage NDF, % of DM	42.9	16.7
Forage, %	91.7	35.8

[1]Table adopted from Penner et al., 2009
[2]Contained 36.3% Na, 55.7% Cl, 9,151 mg/kg of Zn, 2,294 mg/kg of Cu, 3,545 mg/kg of Mn, 33.1 mg/kg of Co, 82.4 mg/kg of I, 761.1 kIU vitamin A, 190.3 kIU vitamin D, and 4,507.0 kIU vitamin E.

Table 2.2 Experiment 2: Ingredient composition and nutrient analysis of the TMR.

Item	Diets	
	Backgrounding	Fishing
Ingredient composition, % diet DM		
Tempered barley-grain	40.0	85.0
Corn silage	55.0	10.0
Supplement	5.0	5.0
Nutrient composition		
DM, %	50.4	77.1
CP, % DM	10.0	14.1
NDF, % DM	27.9	20.8
ADF, % DM	15.6	7.5

Table 2.3 Experiment 3: Ingredient composition and nutrient analysis of the TMR.

	Item	
% DM	Alfalfa hay	18.74
	Corn silage	19.98
	Sweet Bran	33.16
	Cotton seed	4.67
	Finely rolled corn	10.65
	Finely rolled milo	4.59
	Expeller soybean meal[1]	4.30
	Menhaden fish meal	0.31
	Limestone	1.37
	Trace mineral salt	0.07
	Sodium bicarbonate	1.07
	Magnesium oxide	0.09
	Calcium soaps of palm fatty acids	0.82
	Vitamin/mineral pre-mix[2]	0.19
Nutrients[3]		
	DM, % as-fed	59.6
	CP	17.9
	NDF	31.0
	Starch	23.9
	NFC	47.5
	Ether extract	4.8

[1]SoyBest, Grain states Soya, West Point, NE.
[2]Composed of Vitamins A, D, E, Selenium, 4-Plex (Zinpro Crop.), Rumensin 80 (Elanco Corp.), and XP yeast (Diamond V, Inc.).
[3]Nutrients other than DM expressed as a percentage of diet DM.

Table 2.4 Experiment 3. Effects of VFA on intake and ruminal measures, study 1.

10 mol/d	Pre-treatment vs. Treatment[1]				Treatment[1]				
Item	Con	Trt	SEM	*P*-value	A	P	B	SEM	*P*-value
DMI, kg	26.0	19.8	1.6	**< 0.01**	16.9	22.4	20.1	2.7	0.46
Energy intake[2], MJ/d	281.9	229.5	17.8	**< 0.01**	191.5	258.0	238.9	29.7	0.39
Mean pH					6.10	6.14	6.18	0.06	0.67
SD pH					0.40	0.36	0.34	0.02	0.19

[1]VFA infusion 10 mol/d; A= acetate; P= propionate; B= butyrate.
[2] Metabolizable energy intake= Dietary intake + infusions.

Table 2.5 Experiment 3. Effects of VFA on intake and ruminal measures, study 2.

5 mol/d	Pre-treatment vs. Treatment[1]				Treatment[1]				
Item	Con	Trt	SEM	*P*-value	A	P	B	SEM	*P*-value
DMI, kg	24.3	22.8	2.0	0.17	26.3	19.0	23.1	1.9	0.12
Energy intake[2], MJ/d	262.6	254.4	21.7	0.46	289.2	213.2	260.6	20.9	0.14
Mean pH					6.09	6.16	6.08	0.04	0.48
SD pH					0.36	0.37	0.35	0.03	0.44

[1]VFA infusion 5 mol/d; A= acetate; P= propionate; B= butyrate.
[2] Metabolizable energy intake = Dietary intake + infusions.

Table 2.6 Experiment 3. Volatile fatty acids concentrations, study 1.

10mol/d	Pre-treatment vs. Treatment				Treatment[1]				
Item	Con	Trt	SEM	*P*-value	A	P	B	SEM	*P*-value
Acetate	62.2	61.9	1.9	0.91	65.8	60.1	59.9	3.5	0.49
Propionate	31.5	26.3	1.7	**0.09**	26.7	27.0	25.2	2.6	0.87
Butyrate	15.7	16.9	0.8	0.16	16.4	15.7	18.7	1.3	0.34
Isobutyrate	1.1	1.2	0.04	0.39	1.1	1.2	1.2	0.1	0.26
Isovalerate	1.4	1.5	0.1	0.17	1.5	1.6	1.4	0.2	0.67
Valerate	3.7	3.0	0.3	0.17	3.0	3.1	2.9	0.4	0.90
Total VFA	115.5	110.8	3.2	0.27	114.4	109.2	108.7	5.3	0.73

[1]VFA infusion 10 mol/d; A= acetate; P= propionate; B= butyrate.

Table 2.7 Experiment 3. Volatile fatty acids concentrations, study 2.

5 mol/d	Pre-treatment vs. Treatment				Treatment[1]				
Item	Con	Trt	SEM	*P*-value	A	P	B	SEM	*P*-value
Acetate	66.1	63.4	3.1	0.30	68.9	59.3	62.0	2.8	0.11
Propionate	30.2	27.3	1.8	**0.05**	26.9	28.4	26.5	1.3	0.52
Butyrate	15.7	16.2	1.1	0.59	14.6	15.4	18.7	1.2	**0.10**
Isobutyrate	1.1	1.2	0.1	0.30	1.2	1.2	1.2	0.1	0.83
Isovalerate	1.5	1.5	0.1	0.91	1.5	1.4	1.6	0.1	0.51
Valerate	3.2	2.8	0.2	**0.02**	2.6	2.9	2.9	0.2	0.43
Total VFA	117.8	112.3	5.6	0.20	115.4	108.5	113.0	4.6	0.51

[1]VFA infusion 5 mol/d; A= acetate; P= propionate; B= butyrate.

Table 2.8 Experiment 3. Effects of VFA on Angptl4 concentration and abundance in plasma and papillae, study 1.

10mol/d	Pre-treatment vs. Treatment				Treatments[1]				
Relative ANGPTL4 abundance	Con	Trt	SEM	*P*-value	A	P	B	SEM	*P*-value
Plasma ANGPTL4 concentration, ng/ml	3.22	3.58	0.81	0.34	3.35	4.19	2.84	2.10	0.91
Tissue ANGPTL4 abundance[2], AU					679.5	802	712.3	147.2	0.84
ANGPTL4 mRNA abundance[3], 10^{-3}AU					4.59	3.43	4.57	1.30	0.79

[1]VFA infusion 10 mol/d; A= acetate; P= propionate; B= butyrate.
[2]Papillae ANGPTL4 abundance. The Western blot densitometry results were combined image of glycosylated (approximately 55 kDa) and deglycosylated (approximately 42 kDa) forms of the protein.
[3]ANGPTL4 mRNA results were relative to bovine RPS9.

Table 2.9 Experiment 3. Effects of VFA on Angptl4 concentration and abundance in plasma and papillae, study 2.

5 mol/d	Pre-treatment vs. Treatment				Treatments[1]				
Relative ANGPTL4 abundance	Con	Trt	SEM	*P*-value	A	P	B	SEM	*P*-value
Plasma ANGPTL4 concentration, ng/ml	4.00	3.70	0.89	0.43	4.55	3.39	3.30	1.02	0.30
Tissue ANGPTL4 abundance[2], AU					1005.6	1085.6	1011.3	140.6	0.84
ANGPTL4 mRNA abundance[3], 10^{-3}AU					3.59	3.93	5.02	0.59	0.27

[1]VFA infusion 5 mol/d; A= acetate; P= propionate; B= butyrate.
[2]Papillae ANGPTL4 abundance. The Western blot densitometry results were combined image of glycosylated (approximately 55 kDa) and deglycosylated (approximately 42 kDa) forms of the protein.
[3]ANGPTL4 mRNA results were relative to bovine RPS9.

Figure 2.1.1 Experiment 1. Effect of diet on mRNA abundance of ANGPTL4 in ruminal epithelium. Dietary fermentability influences ruminal ANGPTL4 mRNA expression (*P* = 0.08). Cows were randomly assigned to high concentrate (64% concentrate, n=5) or low concentrate (8% concentrate, n=6) diets. Papillae samples for mRNA analysis were collected at the end of the 28 d dietary treatment. Values are means ± SEM; data were log-transformed for analysis and means displayed were back-transformed.

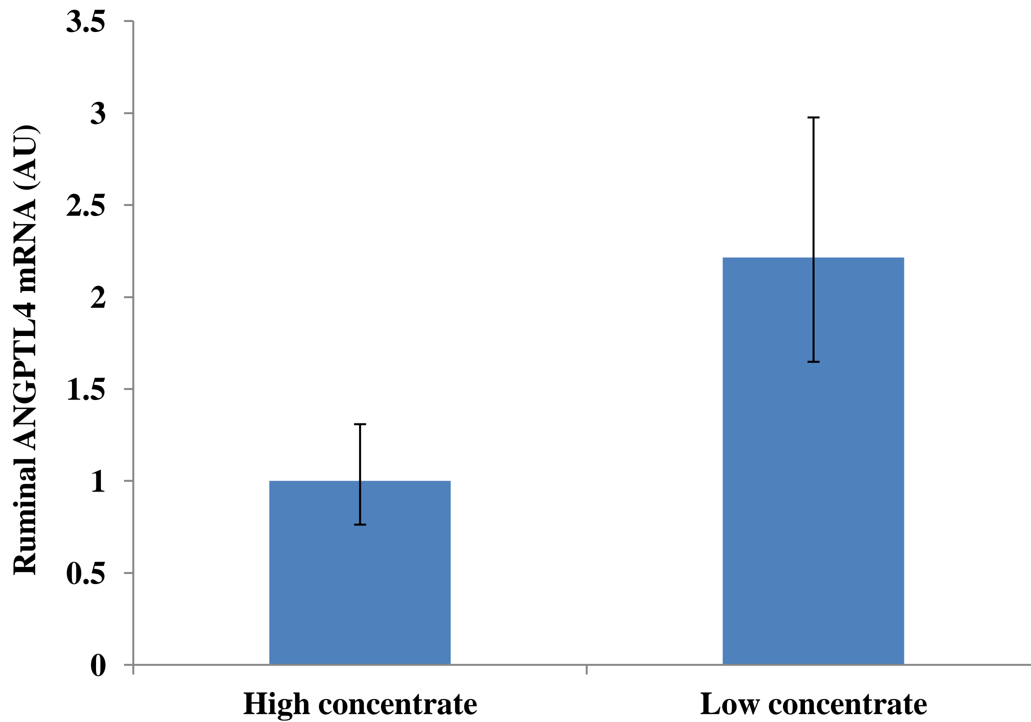


Figure 2.2.2 Experiment 1. Comparison between high and low dietary concentrate effects on ANGPTL4 abundance in ruminal epithelium. Cows were randomly assigned to high concentrate (64% concentrate, n=5) or low concentrate (8% concentrate, n=6) diets. Ruminal epithelium samples for Western blot analysis were collected at the end of the 28d dietary treatment. Values are means ± SEM. There was no dietary effect on ANGPTL4 abundance in ruminal epithelium, P = 0.46.

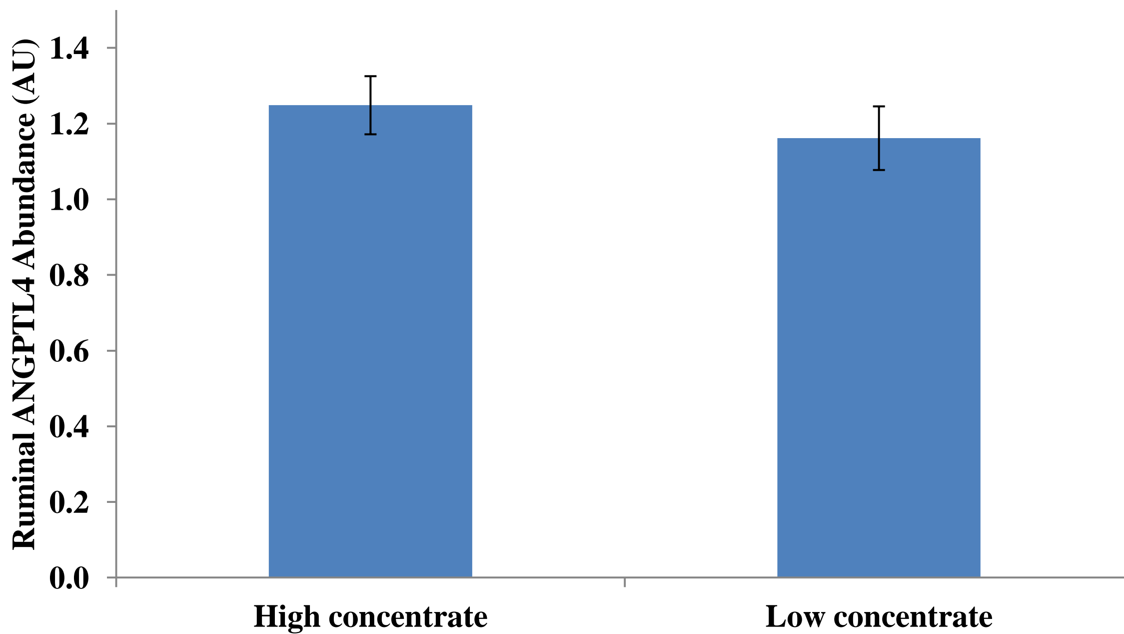


Figure 2.3 Experiment 1. Comparison between high and low dietary concentrate effects on ANGPTL4 abundance in plasma. Cows were randomly assigned to high concentrate (64% concentrate, n=5) or low concentrate (8% concentrate, n=6) diets. Plasma samples for Western blot analysis were collected at the end of the 28d dietary treatment before slaughter. Values are means ± SEM. There was no dietary effect on plasma ANGPTL4 concentration, P = 0.34.

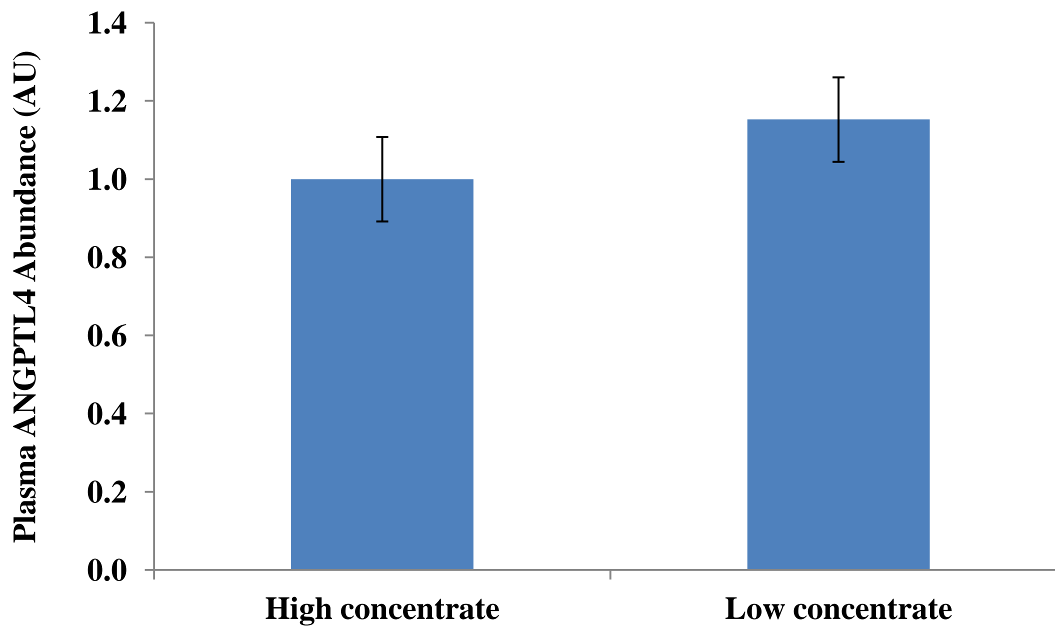


Figure 2.4 Experiment 1. Correlation between ruminal pH and ANGPTL4 mRNA. Cows (n = 11) were randomly assigned to high concentrate (64% concentrate) or low concentrate (8% concentrate) diets. The ruminal pH data were collected during the final 3 d of treatment, and papillae samples for mRNA analysis were collected at the end of the 28d dietary treatment. A tendency is shown for increased mRNA abundance (log scale) with higher ruminal pH, $P = 0.07$.

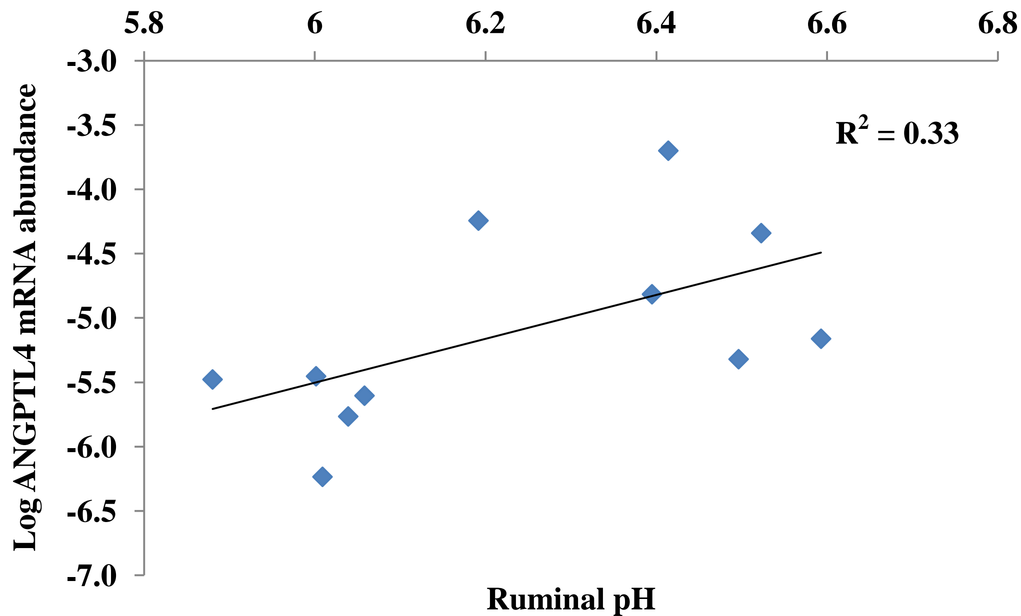


Figure 2.5 Experiment 1. Correlation between total ruminal VFA and ANGPTL4 mRNA. Cows (n = 10) were randomly assigned to high concentrate (64% concentrate) or low concentrate (8% concentrate) diets. The ruminal VFA data and papillae samples for mRNA analysis were collected at the end of the 28d dietary treatment. A significant relationship was detected between mRNA abundance (log scale) and ruminal VFA, $P = 0.03$.

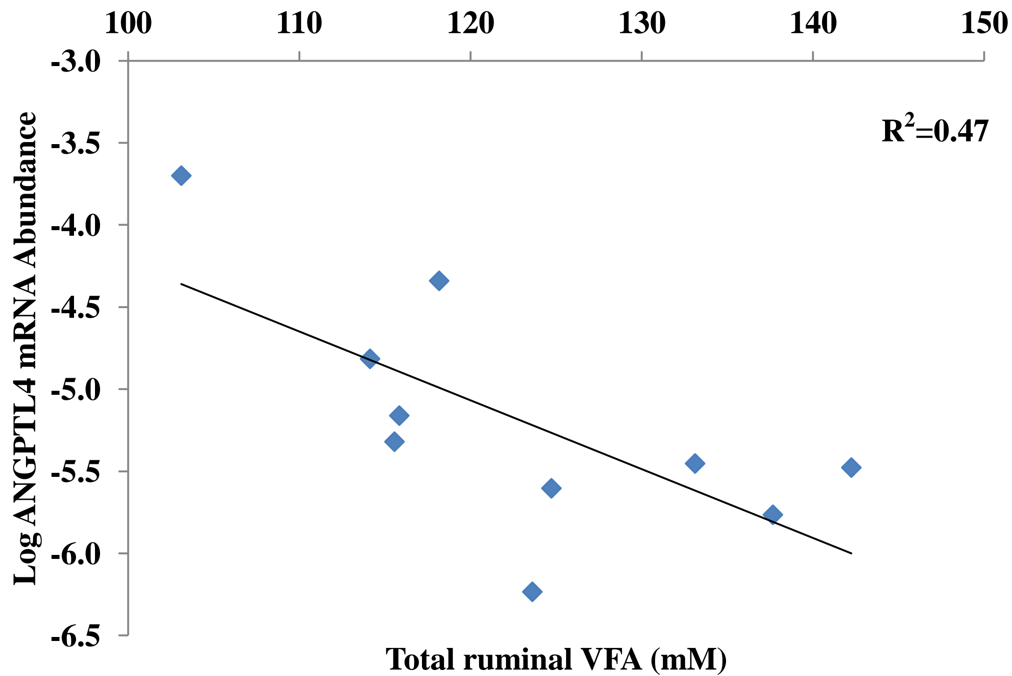


Figure 2.6 Experiment 2. Effects of dietary concentrate on ruminal and plasma ANGPTL4 abundance. Beef heifers were assigned to either a finishing diet (90% concentrate, n = 4) or a backgrounding diet (60% concentrate, n = 4) for 75 d. Ruminal epithelium and plasma samples for Western blot analysis were collected at the end of the dietary treatment. Values are means ± SEM. A significant effect was observed for ruminal ANGPTL4 abundance ($P < 0.01$), but no significant effect on plasma ANGPTL4 concentration was detected ($P = 0.39$).

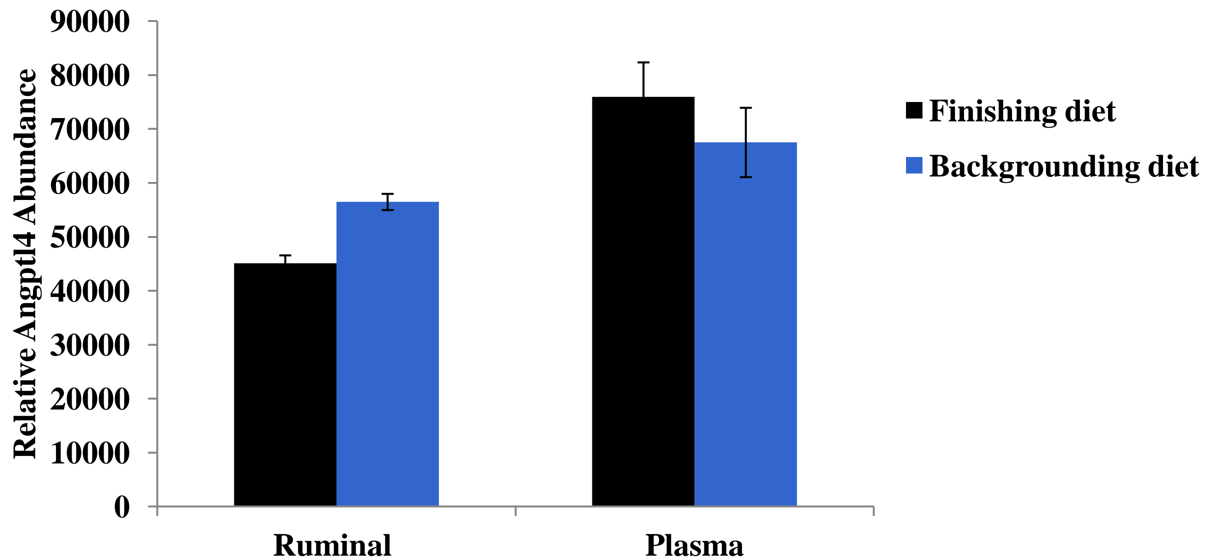


Figure 2.7 Experiment 2. Correlation between ruminal pH and ruminal ANGPTL4.
Beef heifers were assigned to either a finishing diet (90% concentrate, n = 4) or a backgrounding diet (60% concentrate, n = 4) for 75 d. The ruminal pH data were collected continuously over the final 6 d of treatment. Ruminal epithelium for Western blot analysis was collected at the end of the dietary treatment. A significant relationship between ruminal ANGPTL4 abundance and ruminal pH was detected ($P < 0.01$).

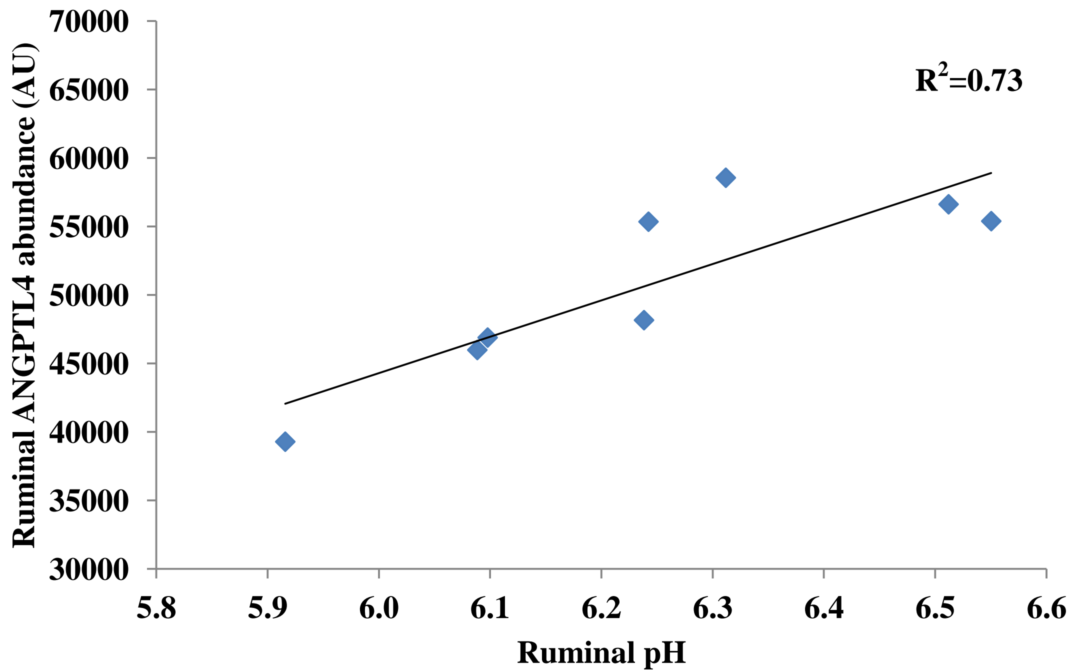


Figure 2.8 Experiment 3. Correlation between total ruminal VFA concentration and ruminal pH. Cows were infused with 10mol/d of acetate, butyrate, or propionate for 2 d in period 1 and 5 mol/d of acetate, butyrate, or propionate for 2 d in periods 2 and 3. The total VFA concentration data were collected after treatment, and the ruminal pH data were collected continuously for 2 d of treatment. There was no significant relationship (P = 0.56) between ruminal VFA concentration and pH.

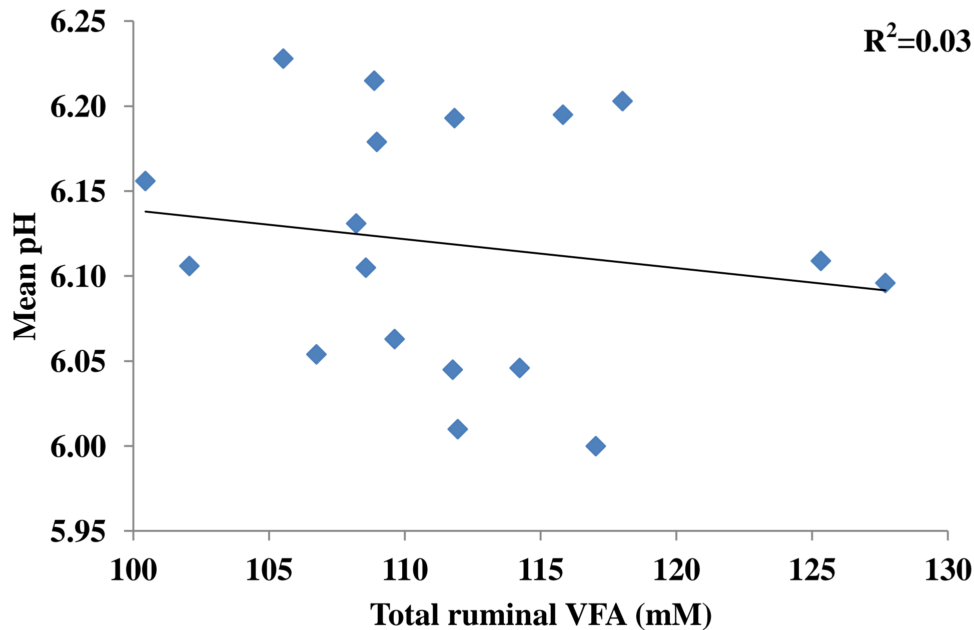


Figure 2.9 Experiment 3. Correlation between total ME intake and tissue ANGPTL4 abundance. Cows were infused with 10mol/d of acetate, butyrate, or propionate for 2 d in period 1 and 5 mol/d of acetate, butyrate, or propionate for 2 d in periods 2 and 3. Ruminal papillae for Western blot analysis were collected at the end of the infusion treatment. There was no significant relationship between ME intake and ruminal ANGPTL4 abundance (P = 0.30), n=16 (2 outliers removed).

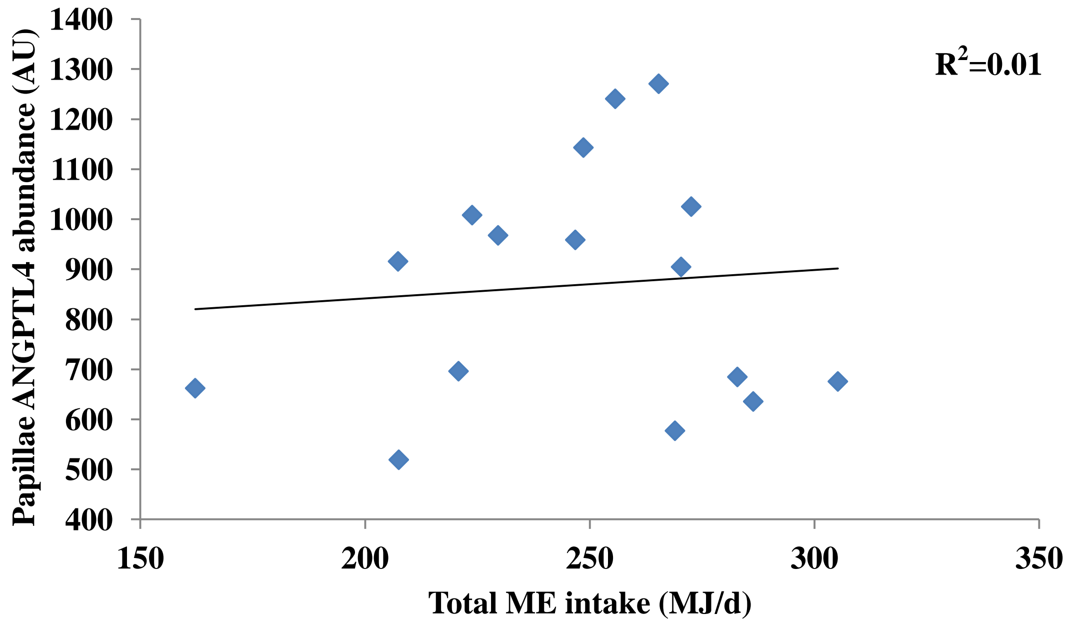


Figure 2.10 Experiment 3. Correlation between ruminal pH and papillae ANGPTL4 abundance. Cows were infused with 10mol/d of acetate, butyrate, or propionate for 2 d in period 1 and 5 mol/d of acetate, butyrate, or propionate for 2 d in periods 2 and 3. Ruminal papillae for Western blot analysis were collected at the end of the infusion treatment. A significant relationship was observed between papillae ANGPTL4 abundance and ruminal pH (*P* = 0.03), and n=17 (1 outlier removed).

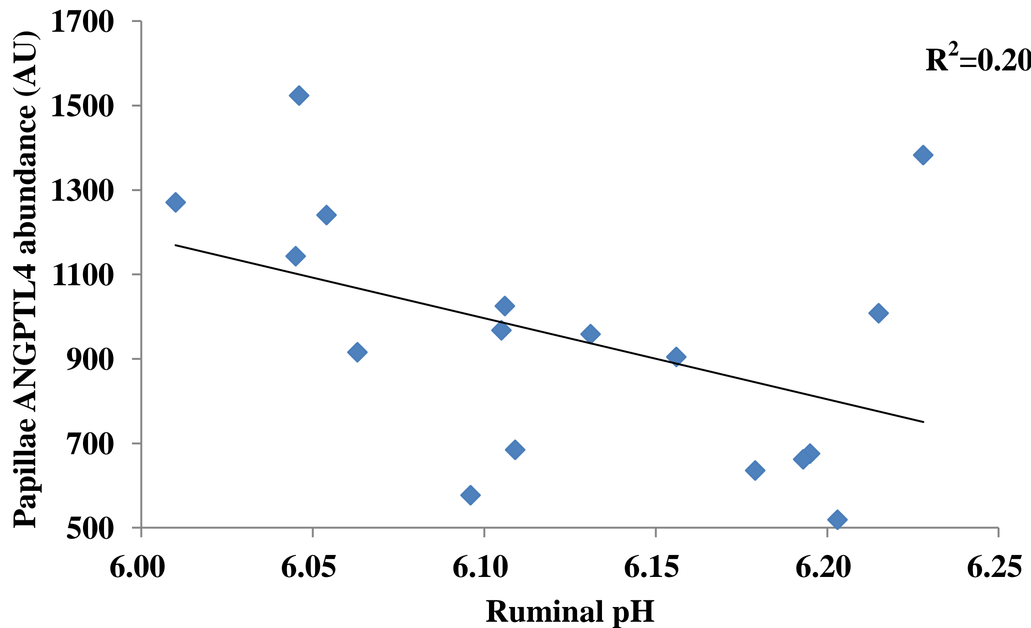


Figure 2.11 Experiment 3. Correlation between total ruminal VFA and papillae ANGPTL4 abundance. Cows were infused with 10mol/d of acetate, butyrate, or propionate for 2 d in period 1 and 5 mol/d of acetate, butyrate, or propionate for 2 d in periods 2 and 3. Ruminal VFA concentration data and ruminal papillae for Western blot analysis were collected at the end of the infusion treatment. A significant relationship is shown between papillae ANGPTL4 abundance and ruminal VFA (P = 0.01), n=17 (1 outlier removed).

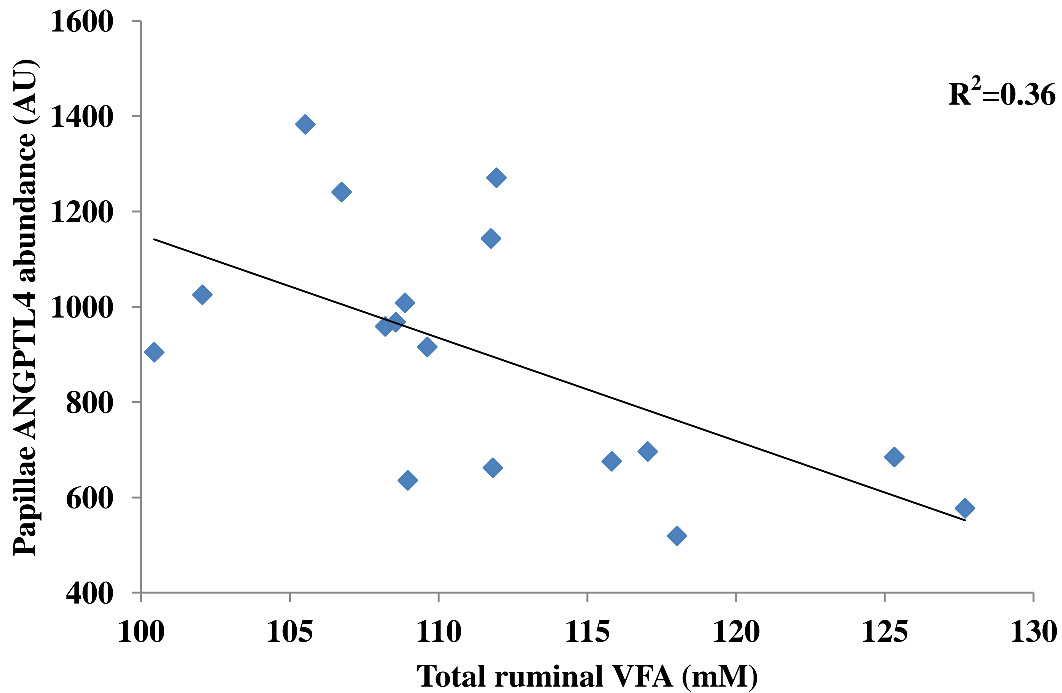


Figure 2.12 Experiment 3. Correlation between papillae ANGPTL4 abundance and plasma ANGPTL4 concentrations. Cows were infused with 10mol/d of acetate, butyrate, or propionate for 2 d in period 1 and 5 mol/d of acetate, butyrate, or propionate for 2 d in periods 2 and 3. Plasma samples and ruminal papillae for Western blot analysis were collected at the end of the infusion treatment. Plasma ANGPTL4 concentrations tended to be correlated with papillae ANGPTL4 abundance (*P* = 0.09), n=15.

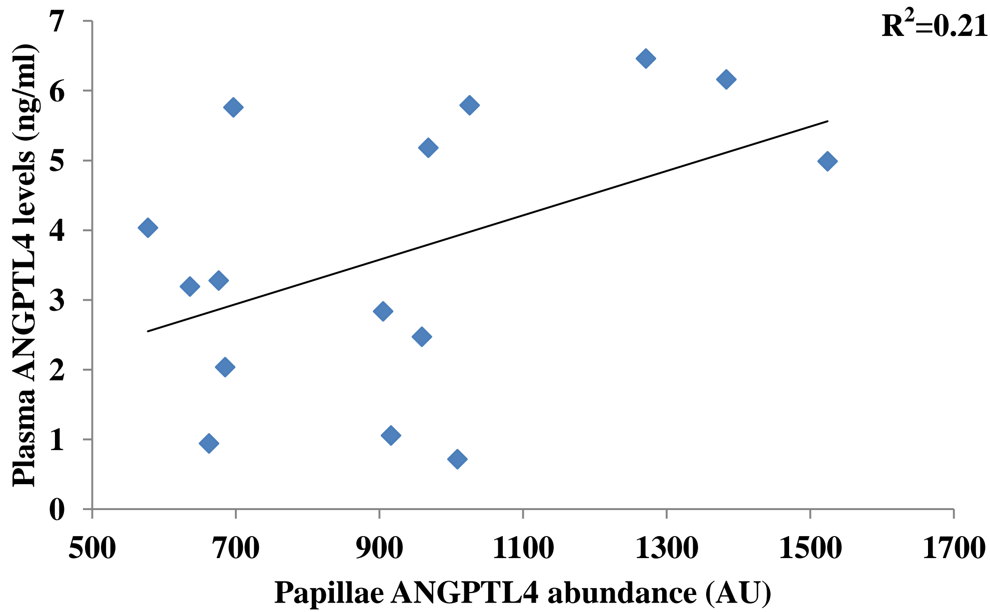


Figure 2.13 Experiment 3. Correlation between ruminal pH and plasma ANGPTL4 concentrations. Cows were infused with 10mol/d of acetate, butyrate, or propionate for 2 d in period 1 and 5 mol/d of acetate, butyrate, or propionate for 2 d in periods 2 and 3. The ruminal pH data were collected continuously for 2 d of treatment, and plasma samples were collected after treatment. Plasma ANGPTL4 concentrations were correlated with ruminal pH ($P < 0.01$), n=14 (1outlier removed).

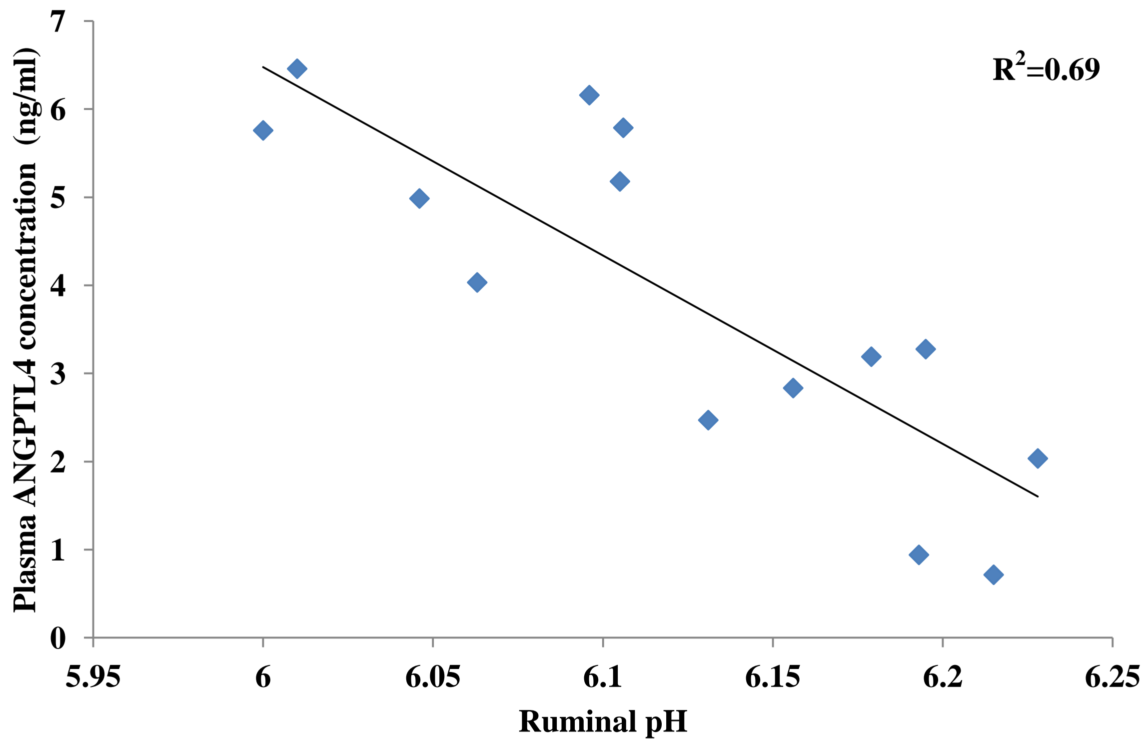


Figure 2.14 Experiment 3. Correlation between ME intake and plasma ANGPTL4. Cows were infused with 10mol/d of acetate, butyrate, or propionate for 2 d in period 1 and 5 mol/d of acetate, butyrate, or propionate for 2 d in periods 2 and 3. The plasma samples were collected after treatment. Plasma ANGPTL4 concentrations tended to be correlated with ME intake (P = 0.06), n=28 (2 outliers removed).

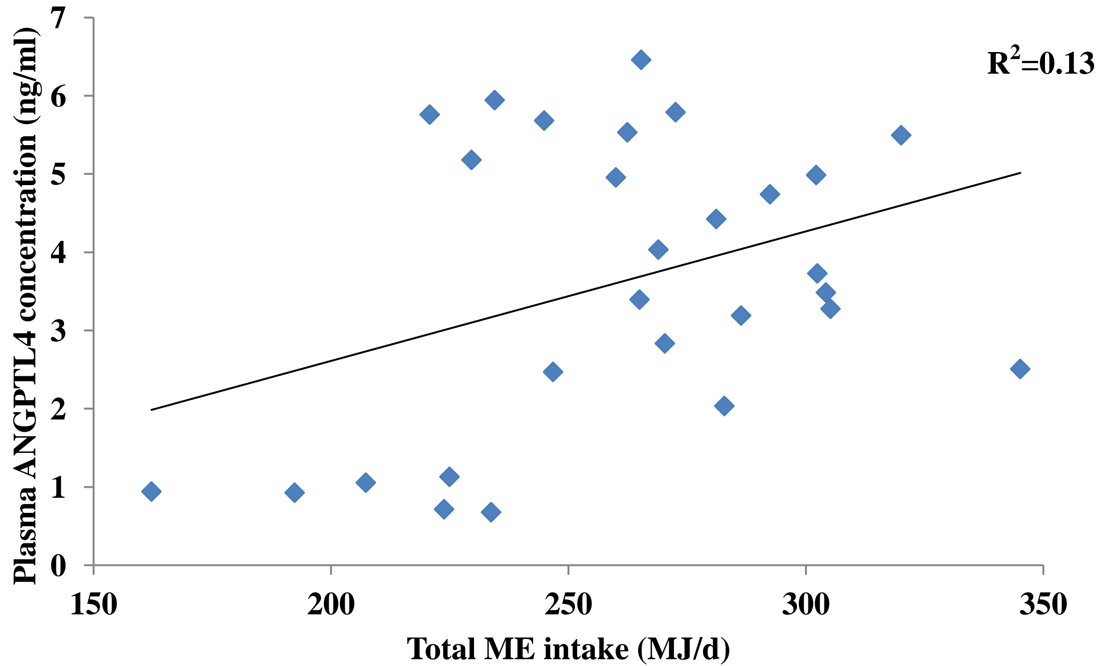


Figure 2.15 Experiment 3. Correlation between total ruminal VFA and plasma ANGPTL4. Cows were infused with 10mol/d of acetate, butyrate, or propionate for 2 d in period 1 and 5 mol/d of acetate, butyrate, or propionate for 2 d in periods 2 and 3. The ruminal VFA and plasma samples were collected after treatment. Plasma ANGPTL4 concentrations tended to be correlated with ruminal VFA concentrations (*P* = 0.06), n=29 (2 outliers removed).

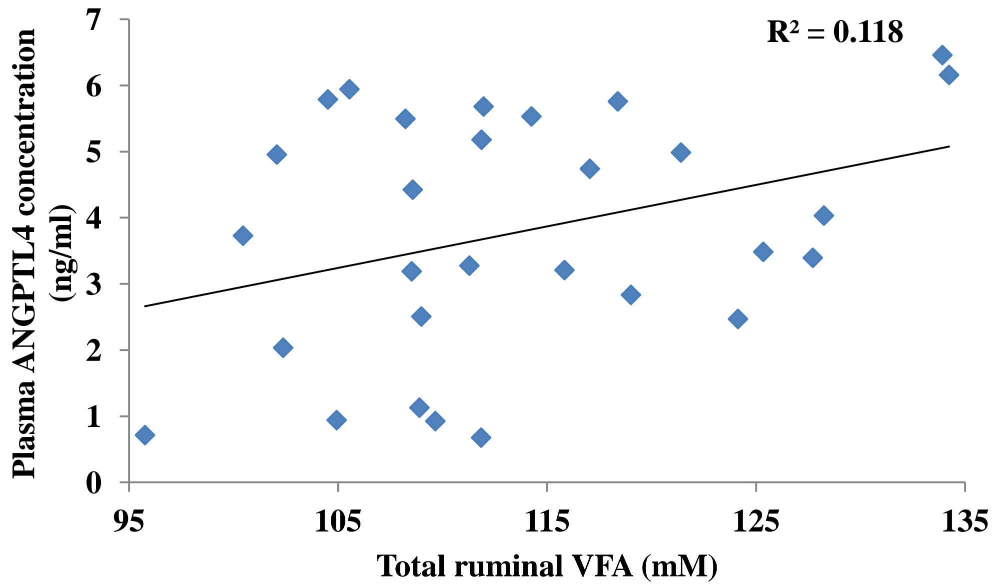
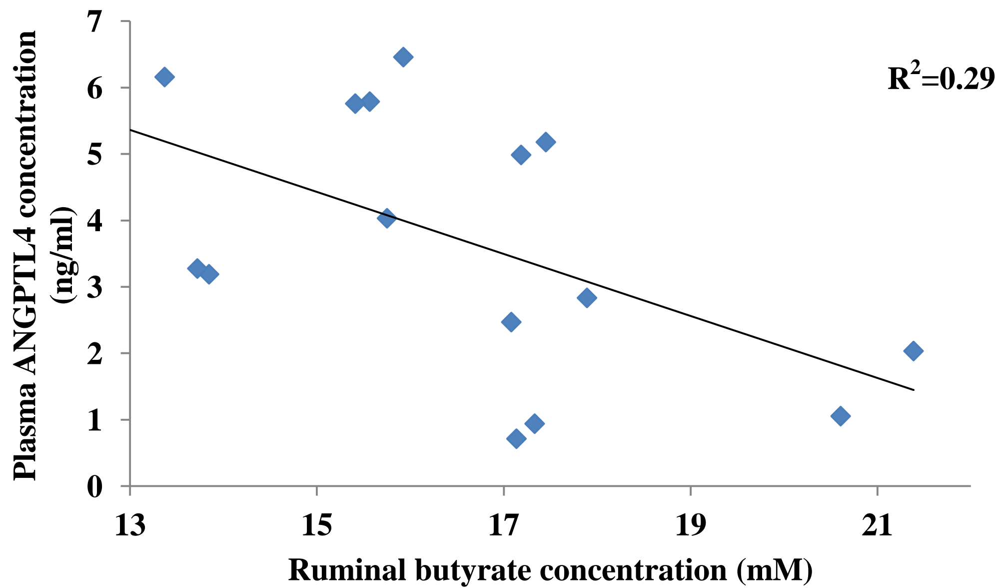


Figure 2.16 Experiment 3. Correlation between ruminal butyrate concentrations and plasma ANGPTL4 concentrations Cows were infused with 10mol/d of acetate, butyrate, or propionate for 2 d in period 1 and 5 mol/d of acetate, butyrate, or propionate for 2 d in periods 2 and 3. The ruminal butyrate and plasma data were collected after treatments. A significant relationship was observed between plasma ANGPTL4 and ruminal butyrate concentrations (P = 0.04), n=15.



Chapter 3 - Strategies to Identify a Receptor for Angiopoietin-like Protein 4

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ABSTRACT

Angiopoietin-like protein 4 (ANGPTL4) is an energy homeostasis mediator. It can suppress lipoprotein lipase function, stimulate adipose tissue lipolysis, and modulate food intake. In human muscle tissue, long chain fatty acids can stimulate ANGPTL4 production. Increasing ANGPTL4 can further stimulate adipose tissue lipolysis, which causes a positive feedback loop to form. Thus, ANGPTL4 acts as an endocrine factor in adipose tissue, muscle, and the hypothalamus. The regulation of cell communication and downstream protein production that is initiated by ANGPTL4 strongly suggests that a receptor is involved in these responses. Angiopoietin-like protein 4 had been found associated with integrins β1 and β5 in the extracellular matrix, and this association influences cell migration and the wound healing process. However, it is unclear if these matrix proteins mediate the metabolic responses to ANGPTL4. To be able to identify the ANGPTL4 receptor or mediator in muscle and adipose tissue, we synthesized bovine ANGPTL4 with L-photo-leucine to attempt to detect the protein-receptor/mediator interaction by UV cross-linking. We succeeded in synthesizing bovine ANGPTL4 with an in vitro cell-free glycoprotein expression system. However, we did not detect any ANGPTL4-receptor/mediator complexes by Western blot. Immunoprecipitation could be used in further studies to purify ANGPTL4-receptor/mediator complexes prior to detection. It is important to identify the ANGPTL4 receptor or mediator to further our understanding about the downstream pathways influenced by ANGPTL4 signaling.

Key words: ANGPTL4, receptor, mediator, protein synthesis

INTRODUCTION

The angiopoietin-like protein (ANGPTL) family has similar molecular characteristics with the angiopoietin family; both contain NH_2 terminal coiled-coil domains (CCD) and COOH-terminal fibrinogen-like domains (FLD; Hato et al., 2008). Angiopoietins can bind to Tie 1 and Tie 2 receptors by the FLD (Hato et al., 2008). However, the ANGPTLs only contain 4 to 6 cysteines in FLD, and these proteins therefore are unable to bind to Tie 1 and Tie 2 receptors (Oike et al., 2004). Angiopoietin-like protein 4 (ANGPTL4), a glycoprotein, was identified as a protein under transcriptional control of the nuclear receptors peroxisome proliferator-activated receptor (PPAR) α, γ, or β/δ (Kersten et al., 2009). It acts as an energy metabolism modulator. ANGPTL4 can stimulate lipolysis of adipose tissue and inhibits the function of lipoprotein lipase (Yoshida et al., 2002). These effects, in turn, increase plasma free fatty acid and glycerol concentrations (Mandard et al., 2006).

Muscle ANGPTL4 mRNA has been detected about 40% abundance compared to white adipose tissue. Long-chain fatty acids (LCFA) can stimulate PPARδ to produce ANGPTL4 by human myotubes (Dutton et al., 2008; Staiger et al., 2009). Myotube ANGPTL4 (mANGPTL4) also stimulates adipose tissue lipolysis. Therefore, LCFAs potentially mediate ANGPTL4 production in plasma though mANGPTL4 as a positive feedback loop under fasting status (Staiger et al., 2009).

Hypothalamic ANGPTL4 (hANGPTL4) can control food intake and body weight gain; also it can increase efficiency of energy use (Kim et al., 2010). The full length ANGPTL4 or its FLD alone both have the ability to regulate energy expenditure; they also suppress hypothalamic AMP-activated protein kinase (AMPK) function, which suppresses food intake and alters glucose production (Minokoshi et al., 2002; Yang et al., 2010; Kim et al., 2010). Interestingly, muscle derived ANGPTL4 has the opposite effect on AMPK (Kim et al., 2010; Minokoshi et al., 2002). Evidence suggests that fasting increases the muscle expression of ANGPTL4, which further increases lipolysis and fatty acid oxidation (Dutton et al., 2008). Angiopoietin-like protein 4 changes kinase activity and acts as a protein endocrine factor; these functions require a receptor or mediator.

Angiopoietin-like protein 4 deficient mice have been shown to have a delayed wound healing process and impaired prenatal intestinal lymphatic and blood vessel partitioning (Bäckhed et al., 2007; Goh et al., 2010). It also potentially has influences on Prox1, a required

gene for normal lymphagiogenesis, to maintain postnatal vessel partitioning. Deficiency of ANGPTL4 suppressed Prox1 expression (Bäckhed et al., 2007). Angiopoietin-like protein 4 is capable of binding integrins β1 and β5 in the extracellular matrix. It can activate the FAK-Src-PAK1 pathway, which influences cell migration and the wound re-epithelialization process (Goh et al., 2010). The mechanisms of regulation related to ANGPTL4 still remain unknown, especially in tissues critical to metabolism, such as muscle and adipose tissue.

Kawaguchi et al. (2007) published a method they used to identify the retinol binding protein (RBP) receptor. Retinol binding protein is a protein that transports vitamin A in the blood, but which also has signaling functions. Researchers had synthesized amine- and photo-reactive His-RBP, and then used UV crosslinking to bind RBP to interacting protein(s) in retinal pigment epithelium. After UV exposure, the covalent RBP-receptor complex was purified by the His tag of RBP. The RBP-receptor complex was identified as STRA6 by immunobloting and high-performance liquid chromatography, and this protein was later confirmed to be a functional RBP receptor (Kawaguchi et al., 2007). Likewise, ANGPTL4 signaling has been shown to modulate kinase activity and metabolite production, and is likely driven by changes in intracellular communication. However, the ANGPTL4 receptor is still not identified, and as a result, the mechanism of this regulation remains unclear. Our objective was to use methods similar to those employed by Kawaguchi et al. (2007) to identify the ANGPTL4 receptor or mediator (Figure 3.1).

MATERIALS AND METHODS

All experiment procedures with animals were approved by the Kansas State University Institutional Animal Care and Use Committee.

Bovine ANGPTL4 DNA Preparation

The bovine ANGPTL4 gene was sequenced and inserted into the pET-15b vector, resistant to ampicillin, by a commercial service provider (GenScript USA inc., Piscataway, NJ). This vector was transformed into E. coli strain DH5α (Invitrogen Corp., Carlsbad, CA), and grown on LB agar (Thermo Fisher Scientific Inc., Waltham, MA) containing 50 µg/mL ampicillin. After overnight culture at 37°C, a single colony was transferred into 10 mL LB broth (Thermo Fisher Scientific) containing 50 µg/mL ampicillin and cultured overnight at 37°C. Approximately 1.2 mL of bacterial culture was used for plasmid DNA extraction (Zyppy Plasmid Miniprep Kit, Zymo Research Crop., Irvine, CA) according to the manufacturer's directions.

Recombinant DNA

Restriction enzyme digestion of the pET-15b-ANGPTL4 and pT7CFE-CHis (Thermo Fisher Scientific) vectors was carried out with Nde I and BamH I (New England BioLabs Inc., Ipswish, CA) at 37°C overnight. Then gel electrophoresis was performed in 0.8% agarose gel (Agarose I, Amresco Inc., Solon, OH) with 70 V, 28 mA for 1.5 h. In addition to digested vectors, a 1 kb DNA ladder (GeneRuler., Thermo Fisher Scientific) was included on the gel. The expected 1245-bp ANGPTL4 and 3627-bp pT7CFE-CHis vector fragments were purified with a gel extraction kit (MinElute, Qiagen Inc.,Valencia, CA). The ANGPTL4 sequence and the pT7CFE-CHis vector fragment were then incubated with T4 DNA Ligase (Invitrogen) at 26°C for 1 h. The recombinant DNA, to be named to pT7CFE-CHis-ANGPTL4, was transformed into DH5α for amplification. This was followed by plasmid DNA extraction for subsequent use.

Synthesis of ANGPTL4

pT7CFE-CHis-ANGPTL4 was linearized by enzyme Spe I (New England BioLabs) overnight at 37°C. Linearized DNA was treated with proteinase K (100 µg/mL,MP Biomedicals LLC., Solon, OH) and 10% SDS at 50°C for 30 min. Then, a PCR clean-up kit (MinElute PCR purification, Qiagen) was used to recover pure linearized DNA. Linearized pT7CFE-CHis-

ANGPTL4 was used to synthesis RNA (MEGAscript, Invitrogen) at 37°C for 4 h, followed by the addition of 1 µL of TURBO DNase (Invitrogen) and incubation at 37°C for 15 min to remove template DNA. We recovered RNA by Ambion MEGAclear kit (Invitrogen). Quality and purity of RNA were measured by UV absorbance (NANODROP 1000 spectrophotometer, Thermo Fisher Scientific). Angiopoietin-like protein 4 RNA was then applied to the Human in Vitro Glycoprotein Expression system with 70 µM L-photo-leucine (Thermo Fisher Scientific) to synthesis ANGPTL4 carrying L-photo-leucine. After incubation at 28°C for 1.5 h, the solution containing ANGPTL4 was stored at -80°C until used. Protein concentration was determined with a Coomassie protein assay kit (Pierce, Rockford, IL).

Bovine Muscle Satellite Cell Isolation

The procedures of muscle satellite cell isolation have been described previously (Johnson et al., 1998). Briefly, we used sterilized equipment to obtain 500 g of semimembranosus muscle and transported the tissue to the cell culture laboratory immediately after cattle were slaughtered. All procedures were carried out under sterilized condition in a tissue culture hood. Muscle was ground in a sterile meat grinder, after removal of connective tissue. Then, muscle was incubated in Earl's Balanced Salt Solution (EBSS) with 0.1% pronase for 1 h at 37°C with frequent mixing. The mixture was centrifuged at 1,500 × *g* for 4 min. The pellet was suspended in phosphate buffered saline (PBS, Thermo Fisher Scientific), and the suspension was centrifuged at 500 × *g* for 10 min. The supernatant was collected to centrifuge at 1500 × *g* for 10 min to isolate the mononucleated cells. Then PBS was used to re-suspend and centrifuge the mixture twice. Finally, the mononucleated cells were suspended with Dulbecco's Modified Eagle Medium (DMEM, GIBCO, Invitrogen) containing 10% FBS, 1% penicillin streptomycin mixture, and 10% (v/v) dimethylsulfoxide (DMSO). Cells were frozen by gradient freezing: 4°C for 20 min, -20°C for 20 min, and -80°C overnight. Then, cells were stored in liquid nitrogen until used in subsequent procedures.

Adipose Tissue Preparation

Bovine adipose tissue was dissected from abdominal fat 10 min. after euthanasia. Adipose tissue was cut into about 40 mg pieces. Tissue was transferred to the tissue culture lab within 15 min using DMEM with 100 units penicillin and 0.1 mg streptomycin/mL. Before

adipose tissue was incubated with synthesized ANGPTL4, it was washed briefly with Hank's buffered salt solution (HBSS, Invitrogen).

UV Cross-Link

All UV exposure was carried out using a 365-nM UV bench lamp (UVP LLC., Upland, CA) with 10 cm distance between the bulb and the media.

Bovine muscle satellite cells were plated on 6 cm^2 cell culture plates which were pre-coated with growth factor reduced Matrigel (BD Biosciences). The culture medium was a 1:1 mixture of DMEM and low glucose DMEM with 5% FBS, 1% Normocin (InvivoGen, San Diego, CA), and 0.2% penicillin streptomycin (GIBCO, Invitrogen). Cells were incubated at 37°C, 5% CO_2 for 5 d. At 5d, cells were rinsed with cold Ca^{++} and Mg^{++} free HBSS. Then 1 mL of serum free OPTI-MEM (GIBCO, Invitrogen) and 30 µg synthesized ANGPTL4 were added to each plate and gently mixed. Cells were exposed to UV light repeatedly, with 10 min of UV exposure in a laminar flow hood followed by 2 min without UV in a cell culture incubator at 37°C, 5% CO_2. This cycle was continued for 1 h. At the same time, one control sample was also exposed to UV light, but without ANGPTL4 treatment. Another control was placed into the incubator without UV exposure or ANGPTL4. After 1 h of UV exposure, we removed the cell medium and washed cells with ice cold PBS. Then, cells were placed on ice and protein and RNA were isolated (PARIS, Ambion, Life Technologies, Carlsbad, CA). Protein was stored for use in Western blot analysis.

Six adipose tissue pieces were placed into 3.8 cm^2 culture plates (BD Biosciences, Franklin Lakes, NJ) with serum free OPTI-MEM (GIBCO, Invitrogen) and 100 µg synthesized ANGPTL4 was gently mixed into the media. Adipose tissues samples were exposed to UV light according to the same protocol described for bovine muscle satellite cells. After UV exposure, adipose tissue was rinsed briefly with Ca^{++} and Mg^{++} free HBSS. Approximately 40 mg of adipose tissue was homogenized with 0.3 ml radioimmunoprecipitation assay lysis buffer (RIPA, Santa Cruz Biotechnology, Santa Cruz, CA) with the addition of 1x protease inhibitor cocktail (PMSF, Thermo Scientific, Rockford, IL).

Western Blot Analysis

Samples were centrifuged at 15,000 rpm for 10 min at 4°C, and the supernatants were used for total protein assays and Western blots. Total protein concentration was determined with

a Coomassie protein assay kit (Pierce). A maximum of 60 μg of supernatant protein was denatured in Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA) including 5% 2-mercaptoethanol (Sigma-Aldrich Inc., Miamisburg, OH) for 7 min at 82°C. Sixty micrograms of total protein were separated by SDS-PAGE using a 4% to 12% Tris-glyscine gel. The separated proteins were transferred to nitrocellulose membranes (iBlot, Invitrogen) and membranes were blocked for 2 h in a solution of 1X PBS, 0.05% Tween-20, and 5% non-fat dry milk. Membranes were then incubated with a goat anti-ANGPTL4 antibody (1:1,000 dilution; Santa Cruz Biotechnology) followed by anti-goat IgG secondary antibody (1:10,000 dilution; Santa Cruz Biotechnology). All incubations were performed in Tris-HCl buffer, pH7.5, with 0.05% Tween-20. After each antibody incubation, membranes were washed with Tris-HCl buffer 5 times for a total of 15 min. Angiopoietin-like protein 4 was detected by using chemiluminescence (West-Dura, Thermo Scientific) and digital image capture (ChemiDoc-It Imaging System, UVP Inc., Upland, CA).

RESULTS AND DISCUSSION

Angiopoietin-like protein 4 acts as an endocrine factor to change and regulate cell communication, kinase activity, and gene expression (Goh et al., 2010; Staiger et al., 2009; Kim et al., 2010). As a protein, ANGPTL4 requires a receptor or some type of membrane-spanning mediator to alter intracellular signaling in target cells. Angiopoietin-like protein 4 can interact with the integrin β subunits of α5β1, αvβ5, and α3β1 (Goh et al., 2010). Integrin signaling can increase cell migration, proliferation, and survival (Avraamides et al., 2008). Also, integrins influence muscle and adipose tissue functions. Integrin β1 can influence adipocyte differentiation, embryogenesis, myoblast migration and proliferation, lymphangiogenesis, and tumor metastasis (Kawaguchi et al., 2003; Brakebusch et al., 2005; Avraamides et al., 2008).

In our study, we tried to find a mediator or a receptor which binds to the ANGPTL4 based on the methods of Kawaguchi et al. (2007). Bovine ANGPTL4 DNA was successfully recombined with the pT7CFE-CHis vector (Figure 3.2 and 3.4.1). We designed a sticky end in order for the ANGPTL4 fragment to combine with the pT7CFE-CHis vector. It should be noted that the pT7CFE-CHis also encodes a C-terminal His-tag, which may influence recombinant ANGPTL4 binding to mediators or receptors. Transcription from DNA to RNA was performed in order to generate RNA template for cell-free synthesis of recombinant bovine ANGPTL4. The cell-free protein synthesis system uses HeLa cell (human hybridoma) extracts to synthesize and modify glycoproteins. Enzymes in this system are supplemented with essential translation factors and inhibitors of certain phosphorylation reactions (Mikami et al., 2010). The sequence encoding ANGPTL4 also encoded an N-terminal 6-His tag and photo-reactive leucine was incorporated during the translation process. Figure 3.3 shows the synthesized protein sequence and amino acid composition. Angiopoietin-like protein 4 contained 10.9% leucine, making it the most abundant amino acid in this protein. Therefore, during protein synthesis, we chose to use L-photo-leucine (L-2-amino-4,4-azi-pentanoic acid) to replace leucine in the amino acid mixture.

After protein translation, the ANGPTL4 carried L-photo-leucine. This chemically modified photo-reactive amino acid is stable until exposed to UV light, at which time the reactive group covalently binds to nearby amino acids. Protein photo cross-linking by UV activation can be used to discover protein-protein interactions within mammalian cells (Hino et al., 2005; Suchanek et al., 2005). The synthesized ANGPTL4 was detected by Western blot before UV reaction (Figure 3.4.2). During synthesis of ANGPTL4, varying degrees of

glycosylation likely occurred. The functional ANGPTL4 is likely located between the 38 to 49 kDa standards that showed on the immunoblotting result. The higher molecular weight protein at 80 kDa and lower molecular weight proteins around 25 kDa are not the targeted protein. The ANGPTL4 antibody has good specificity for detection of ANGPTL4 in bovine tissues (Mamedova et al., 2010), but this does not necessarily mean that the antibody could not have bound to some of the human proteins included in the cell-free translation kit. Additionally, we do not know if ANGPTL4 was folded into a correct form in the cell-free system. Following the attempt to cross-link ANGPTL4 to a receptor or mediator on muscle satellite cell (Figure 3.5.1) and adipose tissue (Figure 3.5.2), we were not able to detect higher molecular weight bands on the Western blot, which would have been indicative of a cross-linked compound.

The study was not able to detect an ANGPTL4 receptor or mediator. It is possible that we were not able to synthesis the ANGPTL4 structure in its native form. Another possible problem is that neither the synthesized protein mixture nor the putative ANGPTL4-receptor complex (after UV exposure) was purified. The mixture provided a relatively low ANGPTL4 concentration that may have prevented us from having enough ANGPTL4-receptor complexes to be detected. The study could have been improved by increasing the protein concentration before the UV activation step and purifying the ANGPTL4-receptor complex after UV exposure by immunoprecipitation.

An alternative approach could be to use RNA interference (RNAi) to suppress β1 and β5 integrin gene expression in muscle satellite cells or adipocytes (i.e. differentiated 3T3-L1 mouse embryonic fibroblast-adipose like cells). Knockdown of integrin expression might change the cellular responses to ANGPTL4. If the integrins are the key mediators for ANGPTL4 in these cell types, their knockdown should eliminate effects of ANGPTL4 on muscle and adipose metabolism.

CONCLUSIONS

In the study, we used recombinant protein production techniques in combination with a photo reactive amino acid method to attempt to identify a receptor for ANGPTL4. Synthesizing ANGPTL4 with photo-reactive leucine using an in vitro transcription kit was successful. However, the results did not show any evidence of an ANGPTL4-receptor or -mediator complex by Western blot analysis. The ANGPTL4 had been found to regulate cell function and communication; it is also known that ANGPTL4 can associate with integrin β1 and β5 in the extracellular matrix (Goh et al., 2010). These responses indicated that a receptor or mediator might exist. Further experiments could use RNAi to knock down integrin β1 or β5 gene expression; if this treatment eliminated the cellular responses to ANGPTL4, then these proteins are likely responsible for the cellular effects of ANGPTL4. Another strategy is to purify higher concentrations of ANGPTL4-receptor or -mediator complex by immunoprecipitation, which would make ANGPTL4 more concentrated for immunoblot detection. It is important to identify the ANGPTL4 receptor or mediator(s) to understand the down-stream pathway and regulation mechanism for ANGPTL4.

REFERENCES

Avraamides, C. J., B. Garmy-Susini, and J. A. Varner. 2008. Integrins in angiogenesis and lymphangiogenesis. Nature Rev. Cancer 8(8):604.

Bäckhed, F., P. A. Crawford, D. O'Donnell, and J. I. Gordon. 2007. Postnatal lymphatic partitioning from the blood vasculature in the small intestine requires fasting-induced adipose factor. Proc. Natl. Acad. Sci. 104(2):606.

Brakebusch, C. 2005. β1 integrin function in vivo: adhesion, migration and more. Cancer Metastasis Rev. 24(3):403.

Dutton, S. and P. Trayhurn. 2008. Regulation of angiopoietin-like protein 4/fasting-induced adipose factor (Angptl4/FIAF) expression in mouse white adipose tissue and 3T3-L1 adipocytes. Br. J. Nutr. 100(01):18-26.

Goh, Y. Y., M. Pal, H. C. Chong, P. Zhu, M. J. Tan, L. Punugu, C. R. I. Lam, Y. H. Yau, C. K. Tan, R.-L. Huang, S. M. Tan, M. B. Y. Tang, J. L. Ding, S. Kersten, and N. S. Tan. 2010. Angiopoietin-like 4 interacts with integrins β1 and β5 to modulate keratinocyte migration. Am. J. Pathol. 177**:** 2791-2803.

Hannon, G. J. 2002. RNA interference. Nature 418(6894):244.

Hato, T., M. Tabata, and Y. Oike. 2008. The role of angiopoietin-like proteins in angiogenesis and metabolism. Trends Cardiovascul. Med. 18(1):6-14.

Hino, N., Y. Okazaki, T. Kobayashi, A. Hayashi, K. Sakamoto, S. Yokoyama1. 2005. Protein photo-cross-linking in mammalian cells by site-specific incorporation of a photoreactive amino acid. Nature Methods 2(3):201.

Johnson, B. J., N. Halstead, M. E. White, M. R. Hathaway, A. DiCostanzo, and W. R. Dayton. 1998. Activation state of muscle satellite cells isolated from steers implanted with a combined trenbolone acetate and estradiol implant. J. Anim. Sci. 76(11):2779-2786.

Kawaguchi, N., C. Sundberg, M. Kveiborg, B. Moghadaszadeh, M. Asmar, N. Dietrich, C. K. Thodeti, F. C. Nielsen, P. Möller, A. M. Mercurio, R. Albrechtsen, and U. M. Wewer. 2003. ADAM12 induces actin cytoskeleton and extracellular matrix reorganization during early adipocyte differentiation by regulating β1 integrin function. J. Cell Sci. 116(19):3893-3904.

Kawaguchi, R., J. Yu, J. Honda, J. Hu, J. Whitelegge, P. Ping, P. Wiita, D. Bok, and H. Sun. 2007. A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A. Science 315(5813):820-825.

Kersten, S. 2009. Angiopoietin-like proteins and lipid metabolism cellular lipid metabolism. P. 237-249. Springer Berlin Heidelberg.

Kim, H.-K., B.-S. Youn, M.-S. Shin, C. Namkoong, K. H. Park, J. H. Baik, J. B. Kim, J.-Y. Park, K.-U. Lee, Y.-B. Kim, and M.-S. Kim. 2010. Hypothalamic Angptl4/Fiaf is a novel regulator of food intake and body weight. Diabetes 59(11): 2772-2780.

Mamedova, L. K., K. Robbins, B. J. Johnson, and B. J. Bradford. 2010. Tissue expression of angiopoietin-like protein 4 in cattle. J. Anim. Sci. 88(1):124-130.

Mikami, S., T. Kobayashi, and H. Imataka. 2010. Cell-free protein synthesis systems with extracts from cultured human cells. Cell-free protein production. Humana Press 607:43-52.

Mikami, S., T. Kobayashi, M. Masutani, S. Yokoyama, and H. Imataka. 2008. A human cell-derived in vitro coupled transcription/translation system optimized for production of recombinant proteins. Protein Expres. Purif. 62(2):190-199.

Mikami, S., M. Masutani, N. Sonenberg, S. Yokoyama, and H. Imataka. 2006. An efficient mammalian cell-free translation system supplemented with translation factors. Protein Expres. Purif. 46(2):348-357.

Minokoshi, Y., Y.-B. Kim, O. D. Peroni, L. G. D. Fryer, C. Muller, D. Carling, and B. B. Kahn. 2002. Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. Nature 415(6869):339-343.

Oike, Y. 2004. Angiopoietin-related/angiopoietin-like proteins regulate angiogenesis. Int. J. Hematol. 80(1):21.

Premsrirut, Prem K., Lukas E. Dow, Sang Y. Kim, M. Camiolo, Colin D. Malone, C. Miething, C. Scuoppo, J. Zuber, Ross A. Dickins, Scott C. Kogan, Kenneth R. Shroyer, R. Sordella, Gregory J. Hannon, and Scott W. Lowe. 2011. A rapid and scalable system for studying gene function in mice using conditional RNA interference. Cell 145(1):145-158.

Staiger, H., C. Haas, J. Machann, R. Werner, M. Weisser, F. Schick, F. Machicao, N. Stefan, A. Fritsche, and H.-U. Häring. 2009. Muscle-derived angiopoietin-like protein 4 is induced by fatty acids via peroxisome proliferator–activated receptor (PPAR)-δ and is of metabolic relevance in humans. Diabetes 58(3):579-589.

Suchanek, M., Y. Okazaki, T. Kobayashi, A. Hayashi, K. Sakamoto, and S. Yokoyama. 2005. Photo-leucine and photo-methionine allow identification of protein-protein interactions in living cells. Nature Methods 2(4):261-267.

Yang, C. S., C. K. L. Lam, M. Chari, G. W. C. Cheung, A. Kokorovic, S. Gao, I. Leclerc, G. A. Rutter, and T. K. T. Lam. 2010. Hypothalamic AMPK regulates glucose production. Diabetes 59(10):2435-2443.

Figure 3.1 Description of ANGPTL4 photo cross-link with receptor. The model was adapted from Kawaguchi et al., 2007. The bovine ANGPTL4 RNA was used as a template for an in vitro cell-free glycoprotein expression system. It was supplemented with L-photo-leucine to synthesize His-tagged ANGPTL4 with several photo-reactive leucines incorporated into the protein. The synthesized ANGPTL4 was then cultured with bovine muscle satellite cells and adipose tissue. The mixtures were incubated for 1 h during exposure to 365 nm UV light to form a stable, cross-linked ANGPTL4/receptor complex. Then, we attempted to detect an ANGPTL4-receptor complex by Western blot.

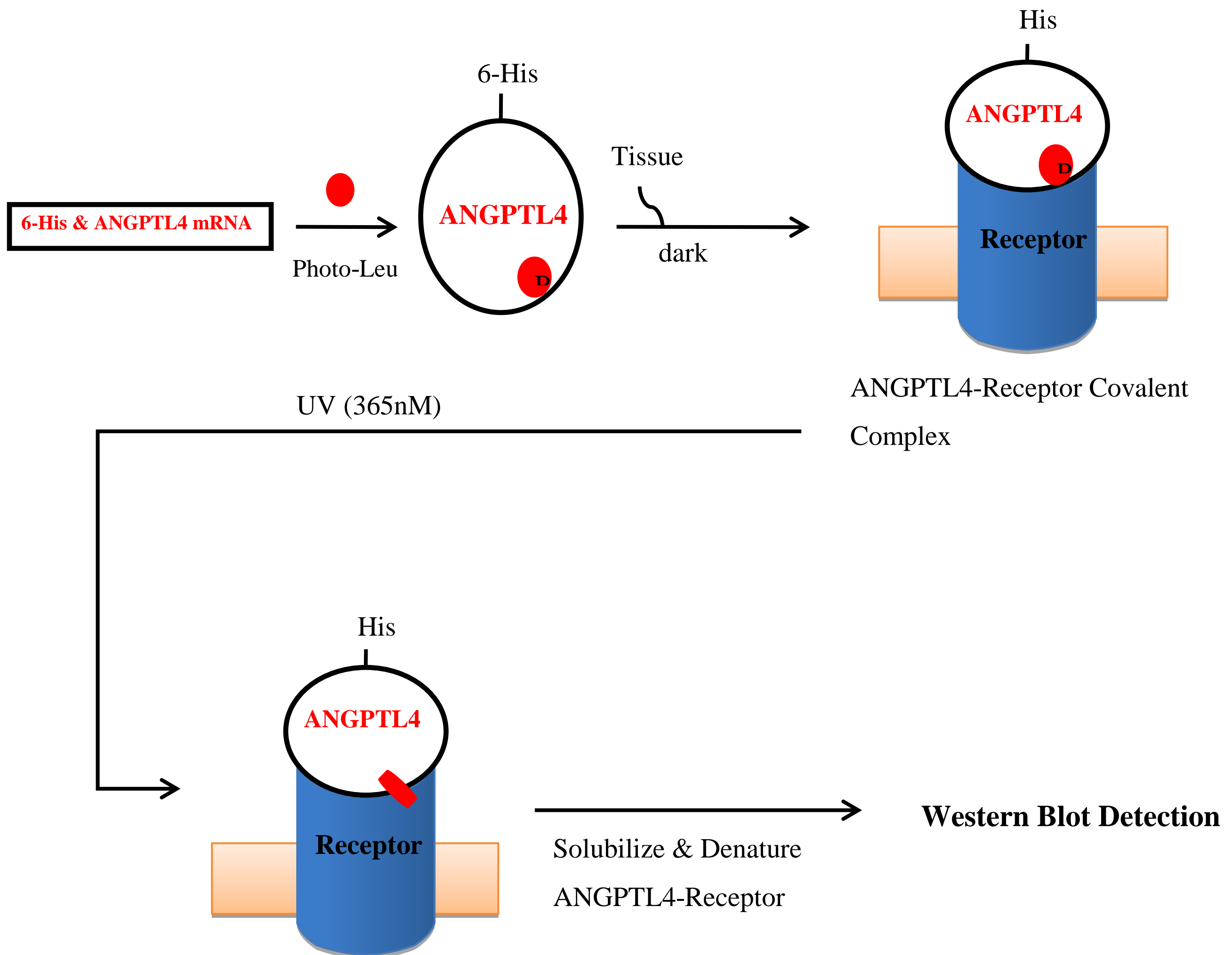


Figure 3.2 pT7CFE1-CHiS-ANGPTL4. The pT7CFE1-Chis vector and bovine ANGPTL4 were both cleaved using NdeI and BamH1, then separated by gel electrophoresis and purified by gel extraction. The two fragments, ANGPTL4 (1245bp) and PT7CFE1-Chis vector (3627bp), were connected by T4 ligase.

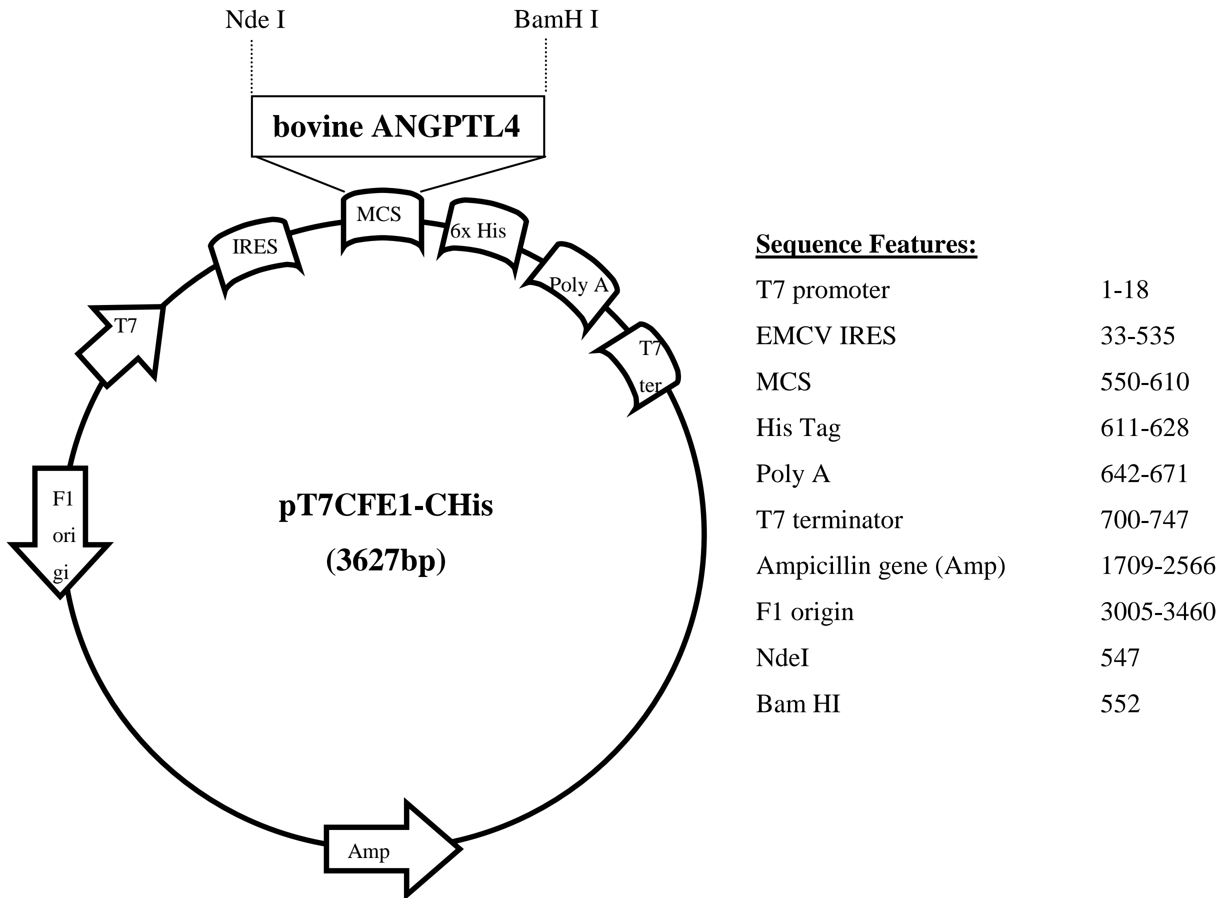
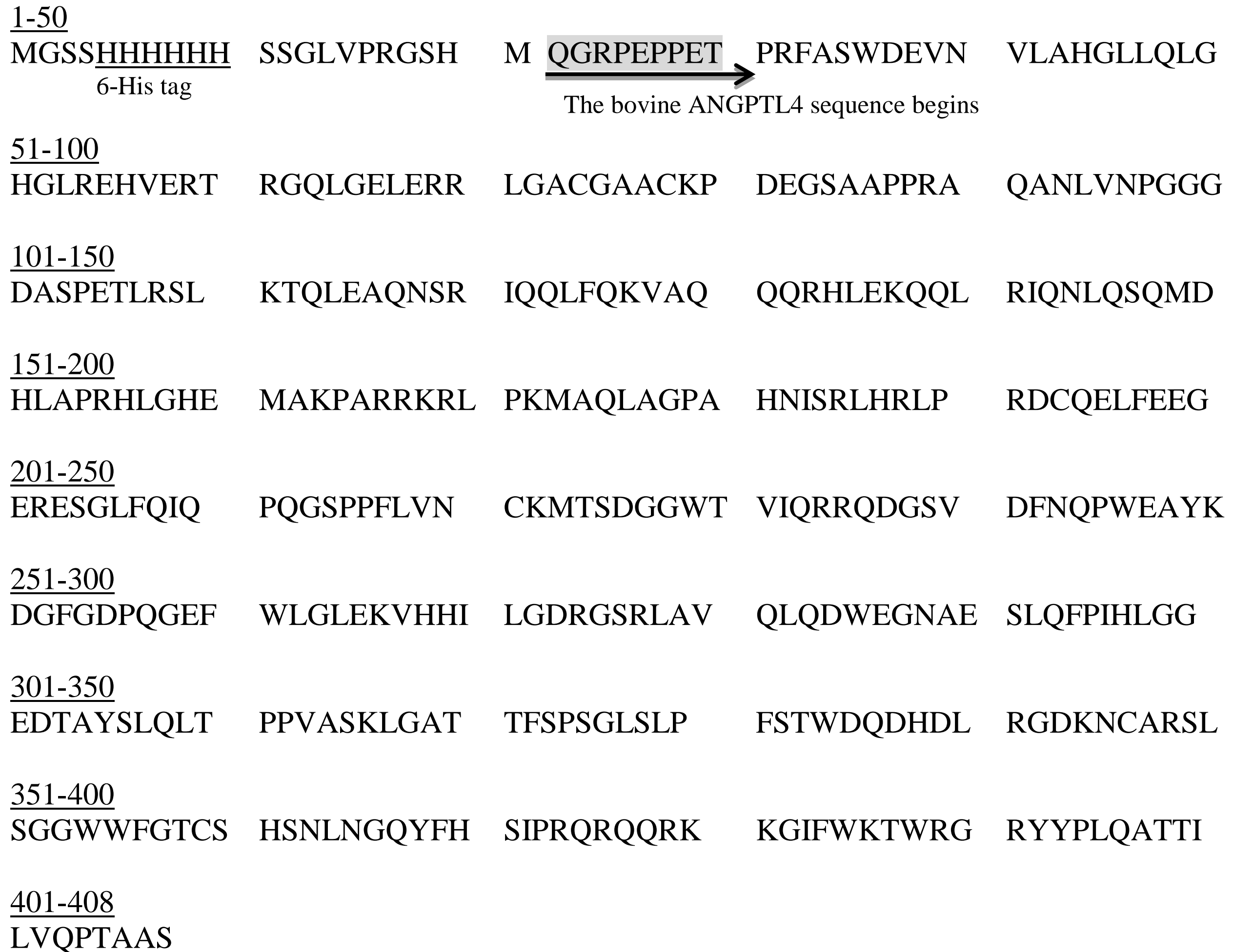


Figure 3.3 Synthesized ANGPTL4 sequence and amino acid composition. The synthesized ANGPTL4 carried with 6-His tag. The bovine ANGPTL4 sequence is begins at site 22. Synthesized ANGPTL4 contained 10.7% leucine (of total amino acids) which was substituted with L-photo leucine.



1-50
MGSSHHHHHH SSGLVPRGSH M QGRPEPPET PRFASWDEVN VLAHGLLQLG
6-His tag
The bovine ANGPTL4 sequence begins

51-100
HGLREHVERT RGQLGELERR LGACGAACKP DEGSAAPPRA QANLVNPGGG

101-150
DASPETLRSL KTQLEAQNSR IQQLFQKVAQ QQRHLEKQQL RIQNLQSQMD

151-200
HLAPRHLGHE MAKPARRKRL PKMAQLAGPA HNISRLHRLP RDCQELFEEG

201-250
ERESGLFQIQ PQGSPPFLVN CKMTSDGGWT VIQRRQDGSV DFNQPWEAYK

251-300
DGFGDPQGEF WLGLEKVHHI LGDRGSRLAV QLQDWEGNAE SLQFPIHLGG

301-350
EDTAYSLQLT PPVASKLGAT TFSPSGLSLP FSTWDQDHDL RGDKNCARSL

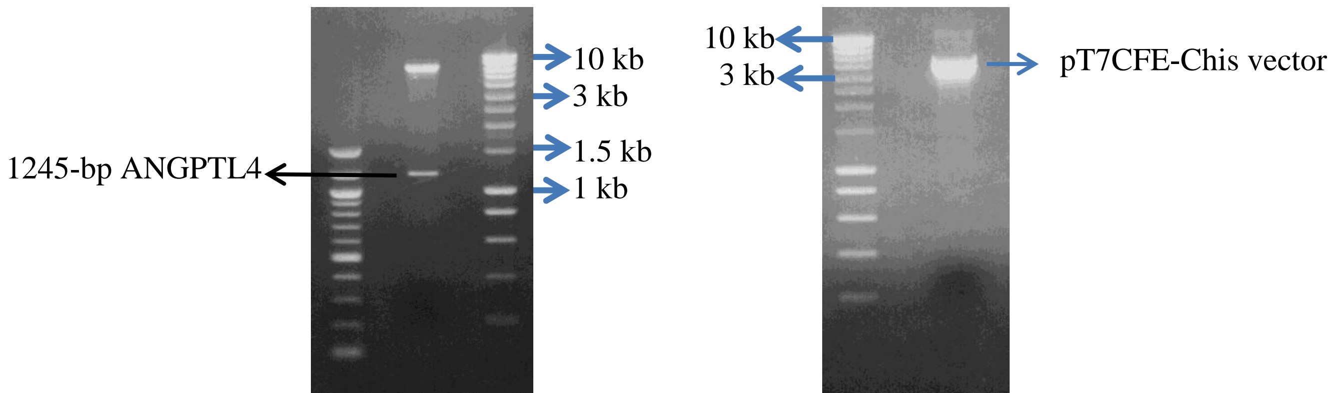
351-400
SGGWWFGTCS HSNLNGQYFH SIPRQRQQRK KGIFWKTWRG RYYPLQATTI

401-408
LVQPTAAS

Amino acid composition:

Ala	(A)	5.5%	Lys	(K)	3.7%
Arg	(R)	8.0%	Met	(M)	1.5%
Asn	(N)	3.0%	Phe	(F)	3.5%
Asp	(D)	4.2%	Pro	(P)	7.0%
Cys	(C)	1.5%	Ser	(S)	7.5%
Gln	(Q)	9.2%	Thr	(T)	4.0%
Glu	(E)	5.7%	Trp	(W)	2.5%
Gly	(G)	10.2%	Tyr	(Y)	1.2%
His	(H)	5.5%	Val	(V)	3.2%
Ile	(I)	2.5%	Pyl	(O)	0.0%
Leu	(L)	10.7%	Sec	(U)	0.0%

Figure 3.4 Synthesized bovine ANGPTL4.
3.4.1 The image on the left is pET-15b-ANGPTL4 and the image on the right is the pT7CFE-Chis vector; both were cut with restriction enzymes Nde I and BamH I and the cleavage products were detected with gel electrophoresis. We collected the 1245-bp ANGPTL4 and 3627-bp pT7CFE-Chis fragments by gel extraction. The 2 fragments were then combined using T4 ligase to form pT7CFE-Chis-ANGPTL4.



3.4.2 Detection of synthesized bovine ANGPTL4. Bovine ANGPTL4 was synthesized with an in vitro cell-free glycoprotein expression system and detected by Western blot. Lane1 is synthesized ANGPTL4 labeled with a 6-His tag and with L-photo-leucine incorporated. Lane 2 is the protein molecular weight marker. The functional ANGPTL4, presumably with varying degrees of glycosylation, is likely located between the 38 to 49 kDa standards.

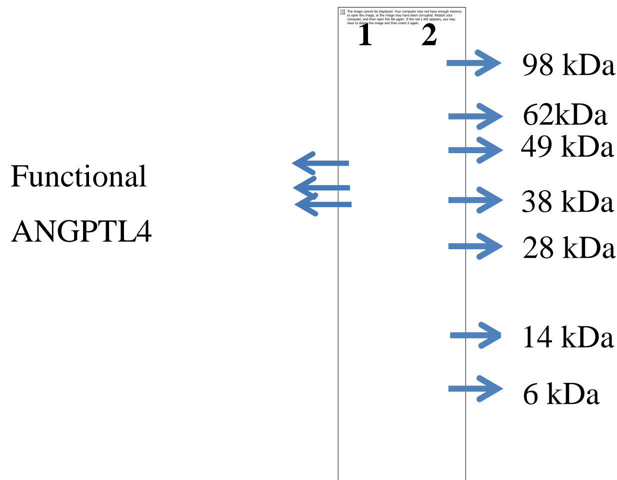
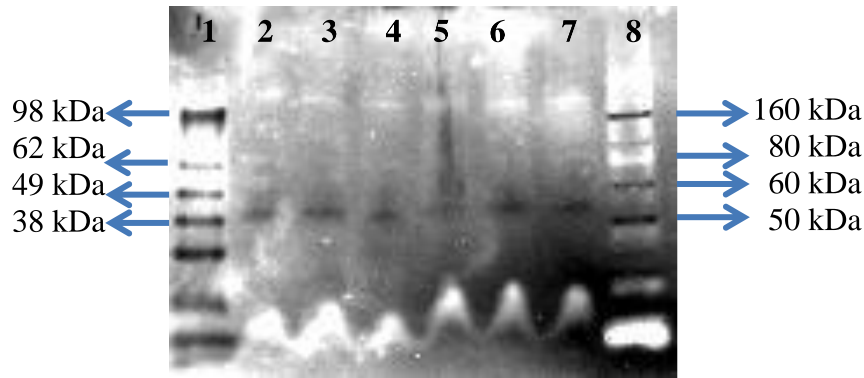


Figure 3.5 UV activated ANGPTL4 with bovine muscle satellite cell and adipose tissue.
3.5.1 UV activated ANGPTL4 with bovine muscle satellite cell. Homogenized and denatured protein was separated by SDS-PAGE and ANGPTL4 detected by Western blot. Lanes 1 and 8 show the protein standard. Lane 2 is the control bovine muscle satellite cells without UV exposure and without ANGPTL4 treatment. Lane 3 is bovine muscle satellite cells without UV exposure and treated with ANGPTL4. Lane 4 is bovine muscle satellite cells with UV exposure and without ANGPTL4. Lanes 5 to 7 are muscle satellite cells treated with ANGPTL4 with 1 h of UV exposure. The image did not show the expected protein complex at the higher molecular weight.



(Bovine muscle satellite cell)

3.5.2 UV activated ANGPTL4 with bovine adipose tissue. Homogenized and denatured protein was separated by SDS-PAGE and ANGPTL4 detected by Western blot. Lanes 1 and 11 are the protein standards. Lanes 2 to 4 are negative controls which were adipose tissue incubated in 37°C for 1 h without UV and ANGPTL4 treatment. Lanes 5 to 7 are negative controls which were adipose tissue exposed to UV for 1 h without ANGPTL4 treatment. Lanes 8 to 10 are adipose tissue exposed to UV for 1 h and treated with ANGPTL4. The image did not show the expected protein complex at the higher molecular weight.