CONTINUOUS INFUSION OF TNF ALPHA IN ADIPOSE TISSUE DOES NOT INDUCE THE SAME METABOLIC EFFECTS AS DAILY BOLUS INJECTION IN LACTATING DAIRY COWS

by

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Abstract

Late-lactation Holstein cows (n=9/treatment) were used to evaluate effects of continuous adipose tissue TNFα administration on glucose and fatty acid (FA) metabolism. Cows were blocked by feed intake and milk yield and randomly assigned within block to control or TNFα treatments. Treatments (4 mL saline or 14 μg/kg TNFα in 4 mL saline) were infused continuously over 7 d via 2 osmotic pumps in the adipose layer in the tailhead region. Plasma, milk samples, milk yield, and dry matter intake (DMI) data were collected daily. On d 7, pumps were removed and liver and contralateral tailhead adipose biopsies were collected. Results were modeled with fixed effect of treatment and random effect of block; *P* values > 0.10 were considered non-significant. TNFα did not alter liver TNFα mRNA abundance, plasma TNFα, IL-4, IL-6, or interferon-γ concentrations, DMI, or rectal temperature. Milk fat and lactose concentrations decreased with TNFα (*P* < 0.05*),* but milk yield was unchanged and treatments did not alter the proportion of short vs. long-chain FA in milk on d 7. Treatments did not alter plasma NEFA concentration, liver triglyceride content, or adipose mRNA abundance for hormone-sensitive lipase or perilipin. Plasma glucose turnover rate, as measured by disappearance of U-^{13}C-glucose bolus, was not altered by treatment, nor was liver mRNA abundance for phosphoenolpyruvate carboxykinase or pyruvate carboxylase. However, TNFα tended to decrease adipose TNFα mRNA abundance (*P*=0.09) *a*nd increase liver IL-10 mRNA abundance (*P*=0.05) *c*ompared to controls. Messenger RNA expression of IL-10 in adipose and IL-37 in liver tissue increased significantly in cows treated with TNFα (Figure 1; P = .02 adipose; P < 0.05 liver). This TNFα delivery protocol may have allowed for an adaptive anti-inflammatory

response to suppress systemic inflammation, which may account for the lack of metabolic responses compared with previous responses to daily subcutaneous TNFα injections.

(**Key words**: dairy cattle, gluconeogenesis, TNFα,)

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Dedication

I dedicate this work to my husband and son, they are my world and I will continue to do everything I can to be an amazing wife and mother.

Chapter 1 - Review of Literature

Introduction to Fatty Liver Disease

Hepatic lipidosis or fatty liver disease is an ongoing epidemic on dairy farms, which can account for an estimated $60 million dollars in lost income (Bobe et al., 2004) due to effects resulting in increased incidence of mastitis, metritis, ketosis, reduced reproduction, and ultimately animals being culled. During the transition to lactation, when energy requirements double (Bell, 1995) due to the demand for milk production, the cow can enter lactation in a negative energy balance (NEB) which can lead to fatty liver disease. Fatty liver disease primarily occurs in dairy cows within the first 4 weeks after calving (Grummer, 1993) and recovery can be hampered for the entire time the animal remains in NEB. Negative energy balance results from an inability to consume enough nutrients to meet energy requirements for lactation, growth, and maintenance (Goff and Horst, 1997; Herdt, 2000). As a result of the NEB, the cow must seek out alternative routes to meet her energy requirements. The outcome is mobilization of stored body fat (lipolysis), and delivery of fatty acids to the liver at a rate higher than the liver can oxidize, resulting in storage of excess triglycerides (TG) within the liver cells (Bobe et al., 2004).

This literature review will focus on the role tumor necrosis factor alpha plays in fatty liver disease, including the involvement in gluconeogenesis in the liver and adipose tissue metabolism. Even though research in fatty liver disease over the last decade has greatly advanced, our complete understanding of what mechanisms are regulating the disease remains limited. Research with humans, mice, and rat models in the area of diabetes and non-alcoholic fatty liver disease are helping the animal field better understand the disease.

Etiology of Fatty Liver

Normally the liver does not provide a storage site for fat; instead the liver regulates chemical levels in the blood and secretes bile, which helps to emulsify fats, preparing them for further digestion and absorption (Bobe et al., 2004). When accumulation of fat within the animal's liver occurs, it results in fatty liver syndrome. Cows that develop fatty liver syndrome have a tendency to be obese, over-conditioned cows with high amounts of omental and/or subcutaneous fat. Weight gain typically occurs during late-lactation and the dry period, which can predispose the liver to the disease. It is not during the time the cow increases body condition that she suffers from fatty liver. When a dairy cow is losing body condition during increased energy demands associated with the transition period and/or parturition, the liver has increased fat accumulation.

Fatty liver syndrome causes reduced liver function, elevating urinary ketone concentration (Veenhuizen et al., 1991), and depresses appetite and feed intake (Hippen et al., 1999; Jorritsma et al., 2001). Coppock et al. (1972) found that feed intake could be depressed by as much as 30% in cows with fatty liver, which ultimately affects milk yield in cows already suffering from decreased body condition and energy balance. Cows are placed at higher risk for other diseases such as metritis and mastitis (Bobe et al., 2004), reduced fertility and reproductive performance (Wensing et al., 1997), and severe cases cause ataxia, coma, and death because of liver and kidney failure or cardiac arrest (Rehage et at., 1999). Rukkwamsuk et al. (1999) found cows exposed to greater concentrations of TG within the liver had increased days to first ovulation following calving. This finding helps to strengthening the argument that cows suffering from fatty liver have reduced reproductive performance.

Several studies have found a strong association between the timing of ketosis and fatty liver disease in cattle (Gerloff, 2000; Goff, 2006). The ketotic state is preceded by the increase in fat in the liver. Prior to calving when the cow is entering a NEB state, the animal begins to draw body fat to help meet energy needs for milk production. Excessive NEFA release from body fat overwhelms the capacity of the liver to use fatty acids as fuel (Baird, 1982). As a result, excess NEFA is converted to ketone bodies, acetone, aceto-acetic acid, and β- hydroxybutyrate (BHBA; Goff, 2006) which can be measured with blood plasma testing. In obese cows, NEFA are mobilized from adipose tissue in amounts greater than needed by the cows, thus the total amount transported to the liver in increased to the liver (McNamara, 2000).

Fatty liver has also been shown to have detrimental effects on lipoprotein function. Cows suffering from fatty liver bind more haptoglobin and serum amyloid A to high-density lipoproteins (HDL), which causes a decrease concentration of apolipoprotein A-I and C-III in HDL particles (Katoh, 2002). Lipoprotein transport of TG or other lipids in the blood plasma is affected by fatty liver. As liver TG concentration is increased, researchers have shown it is accompanied by decreased concentrations of structural lipids, like free cholesterol, cholesteryl esters, and phospholipids (Sarrinen and Shaw, 1950, Van den Top et al., 1996), as well as citrate (energy precursors; Mills et al., 1986), and glycogen (energy storage molecules; Johannsen et al., 1992).

Changes in carbohydrate, lipid, and protein metabolism in hepatocytes and adipose tissue occur during fatty liver disease. Increases in the concentration of liver TG is associated with decreased gluconeogenesis (Rukkwamsuk et al., 1999a), variable effects on ketogenesis

(Drackley et al., 1992) and β-oxidation (Grum et al., 1996; Cadórniga-Valiño et al., 1997), and increased lipogenesis in the liver (Grum et al., 1996; Cadórniga-Valiño et al., 1997). Rukkwamsuk et al., (1998) found that cows suffering with fatty liver had a strong association with changes in lipolysis, but no association with adipose tissue lipogenesis (Rukkwamsuk et al., 1999b). Strang et al. (1998) found that increased TG decreases the ability of insulin to increase protein synthesis. Increased concentrations of NEFA, BHBA, and acetoacetate are all associated with fatty liver syndrome and decreased rates of β-oxidation, gluconeogenesis, and changes in the citric acid cycle in hepatocytes (Bobe et al., 2004) can also be associated with fatty liver.

Fatty Liver Disease Characteristics

Fatty liver can be organized into three categories: nutritional, managerial, and genetic, which can then be further divided into four classes: normal, mild, moderate, and severe (Bobe et al., 2004). Cows with severe fatty liver (> 10% liver TAG on wet weight basis), have extreme signs of the syndrome, elevated concentrations of urinary ketones, severe weight loss, decreased feed intake and milk production (Hippen et al., 1999; Jorritsma et al., 2001). Cows with severe cases can have decreased concentrations of glucocorticoids (Morrow et al., 1979) which can affect glucose metabolism. Moderate fatty liver (5 to 10% TAG) and mild fatty liver (1 to 5% liver TG) have elevated concentrations of ketones but not as high as severe cases. Necropsies of cows with fatty liver disease have shown the liver becomes enlarged and swollen with a yellow appearance as the degree of fatty liver increases (Kapp et al., 1979). The degree of fatty degeneration is most prominent in the centrilobular and intermediate areas of hepatic lobules, with a pronounced reticular pattern, on both serosal and cut surfaces. A white

discoloration may be evident in the abdominal fat due to accelerated lipolysis (Bobe et al, 2004).

Adipose Tissue: Metabolic Function and Role in Dairy Cows

Adipose provides energy to the body that allows most tissues to overcome a negative energy balance which can occur during early lactation. Adipose tissue releases free fatty acids and glycerol into circulation to provide organs with energy when dietary intake does not meet the requirements (McNamara, 1991; Van Epps-Fung et al., 1997). The adipose tissue is the major site of lipid synthesis (Kaneko, 2008). During NEB, fatty acids are stored as TG in various adipose tissue depots within the body (Vernon, 2005). However, when blood NEFA levels are elevated for prolonged periods of time, as a result of NEB during lactation and obesity, TG will begin to accumulate in other tissue sites (liver and muscle cells), which can lead to the development of ketosis (Grummer, 1993; Drackley et al., 2001) or type 2 diabetes (Boden & Shulman, 2002; McGarry, 2002).

White adipose tissue (WAT) also has the capability of storing cholesterol and is involved in metabolism of steroid hormones (Trayhurn & Beattie, 2001). Synthesis of steroid hormones *de novo* does not occur directly in WAT, but rather WAT expresses enzymes involved in the conversion of both glucocorticoids and sex hormones, which are subsequently released (Mohamed-Ali et al., 1998). The classical view of adipose tissue function has been to provide a long-term fuel reserve which is mobilized during food deprivation with the release of fatty acids for the oxidation in other organs (Trayhurn & Beattie, 2001). In obese subjects there is a greater accumulation of lipids in WAT.

Adipose tissue can be divided into two fractions; the adipocyte fraction (AF) and the stromal vascular fraction (SVF). Mature adipocyte cells make up the AF and are primarily involved in lipid storage and whole-body energy homeostasis (DiGirolamo et al., 1998). The SVF is composed of numerous cells types (preadipocytes, mesenchymal stem cells, macrophages, endothelial cells and fibroblasts) which play a role in regulating adipose tissue inflammation through the production of cytokines (Nair et al., 2005). The primary cell –type responsible for the production of TNFα are macrophages located in the SVF (Trayhurn and Wood, 2004). Adipose tissue was discovered in the 1990's to be a tissue capable of production of inflammatory cytokines (Hotamisligil et al., 1993).

Importance of Glucose Metabolism in Ruminants

Glucose Metabolism

The presence of ruminal microorganisms has given cattle the ability to efficiently utilize feeds that most animals are incapable of digesting. The rumen is a unique organ allowing cattle the ability to utilize cellulose and other fibrous feeds to derive much of their energy requirements. Prior to amylolytic digestion and absorption in the small intestine, the rumen ensures nonstructural carbohydrates (starches and sugars) are broken down into substrates available to the animal for energy (Kaneko, 2008).

Ruminants are obligate herbivores which rely on gluconeogenesis in the liver and to a lesser extent, kidneys for their tissue glucose requirements (Bell and Bauman, 1997). The physiological state of the cow can control the glucose requirement, and lactating dairy cows' glucose requirement is doubled compared to nonpregnant or nonlactating cows. Cattle

consuming adequate feed utilize propionate as the principal precursor for hepatic gluconeogenesis. Propionate is absorbed via the ruminal epithelium into portal venous blood and almost quantitatively removed by the liver (Elliot, 1980; Brockman, 1993). The rate of ruminal production of propionate and other VFA is directly related to dietary intake of fermentable substrates and highly correlated with digestible energy intake. Diets that favor propionate production have increased fermentation of starches by amylolytic bacteria. As diets change and favor different VFA like acetate and butyrate, propionate supply dwindles, causing other glucogenic substrates, such as lactate, amino acids, and glycerol to increase in importance (Brockman, 1993). Starches and sugars consumed through the diet are fermented by the ruminal microbes preventing glucose from being available to the animal for absorption, thus the animal relies on gluconeogenesis.

Carbohydrates are stored in animals as glycogen and are primarily found in the liver and muscle. Glycogen metabolism is of importance to ruminants, because of its role in blood glucose homeostasis. During glycogenolysis, glycogen is broken down in the liver to glucose by the actions of epinephrine and glucagon (Kaneko, 2008). Together glucagon and epinephrine stimulate adenylate cyclase to form cyclic adenosine monophosphate (cAMP) from ATP. Cyclic adenosine monophosphate (AMP) activates AMP-activated protein kinase, which in turn activates liver phosphorylase to cleave the glucosyl links of glycogen. Glucagon has been found to act only on liver glycogen whereas epinephrine acts on both liver and muscle glycogen (Kaneko, 2008). Glucagon's role in glucose formation and release includes increasing glycogenolysis and decreasing glycogenesis in the liver. Hyperglycemia can be induced during this time as a result of the hydrolysis of glucose-6-phosphate (G-6-P) by glucose-6-phosphatase

(G-6-Pase) causing the release of free glucose. Glucagon can also induce hyperglycemia by stimulating hepatic gluconeogenesis. However, in muscle the absence of G-6-Pase results in the production and release of pyruvate and lactate rather than glucose during glycogen breakdown. Lactate and pyruvate can be transported to the liver where glucose is resynthesized via reverse glycolysis (Kaneko, 2008); because of this, muscle glycogen cannot supply glucose directly to circulation through glycogenolysis, but indirectly via the Cori cycle. Free glucose that is released as a result of liver glycogenolysis can be transported out of the hepatic cell and enters general circulation, contributing directly to the blood glucose pool.

History of Tumor Necrosis Factor

Tumor necrosis factor alpha (TNFα) is a proinflammatory cytokine first discovered during the 19th century (Terlikowski, 2001), as a cachectin associated with chronic wasting disease. A surgeon by the name of William Coley was the first to induce hemorrhagic necrosis of tumors in hospital patients as a therapeutic approach (Beutler, 1992). While working in New York City, Coley administered patients with inoperable neoplastic diseases with gram-positive and gram-negative bacteria with the hope to bring about involution of the tumor. Due to the unhealthy and unacceptable side effects of Coley's treatments, fellow researchers rejected the technique, causing the technique to fall into disrepute. Several investigators felt Coley was on the correct path, so they set forth to isolate the active therapeutic fraction of Coley's toxins. Researchers purified the bacterial polysaccharide from the *Serratia marcescens* organism (Shear et al., 1943ab). Bacterial polysaccharide, commonly known as lipopolysaccharide (LPS), proved to have therapeutic effects similar to that in Coley's original technique, but was shown to induce hemorrhagic necrosis of transplantable tumors in mice.

Lipopolysaccharides or lipoglycans are large molecules of lipids and polysaccharides found in the outer membrane of gram-negative bacteria that are connected by covalent bonds. They act as endotoxins inside the body and have the ability to elicit strong immune responses in animals (Beutler, 1992). It was not until 1962 when O'Malley et al. made a major advancement in the work with TNFα. O'Malley et al. reported observations of an endogenous factor in serum of animals which were treated with LPS, and observed the induction of hemorrhagic necrosis of tumors in animals not exposed to LPS. Tumor necrosis factor alpha was found to induce endotoxic shock after several injections of LPS (Klasing, 1988).

The early 1980's provided more insight into TNFα, with work on cachectin (mediator of wasting, or cachexia in chronic disease). Cachexia occurs most often in animals or humans who are suffering with cancer or various infectious diseases. Patients with cachexia suffer from loss of appetite, weight loss, muscle atrophy, weakness and fatigue, which leads to accelerated consumption of lipid and protein reserves within the body (Beutler, 1992), which are similar to symptoms found in cattle suffering from fatty liver. Since work in TNFα is limited in cattle, researchers have looked for answers using laboratory animals. Rouzer and Cerami (1980) observed in rabbits infected with the *Trypanosoma brucei* organisms that severe cachexia was linked to hypertriglyceridemia resulting from a lipid-clearing defect. The rabbits suffered from suppression of lipoprotein lipase (LPL) activity in both plasma and fatty tissues. Continued work in humans and rats suggests that TNFα affects glucose homeostasis, lipid metabolism, and hormone secretion (Grunfield and Finegold, 1991). New data within the last 10 years has indicated that TNFα is expressed in the liver and adipocytes where it interferes with insulin action (Hotamisligil and Spiegelman, 1996).

Tumor Necrosis Factor and the Liver

The liver is an exceptional organ, because of its metabolic, synthetic, and detoxifying functions within the body. The potential to regenerate after tissue loss, while regulating blood glucose or blood lipids circulating in the body (Tacke et al., 2009) makes the liver a unique organ. Homeostasis is the fundamental function of the liver, while regulating inflammatory responses. The ability of the liver to control homeostasis lies solely on its anatomical location within the body. The unique location allows for continuous blood flow from the gastrointestinal tract through the sinusoids (Tacke et al., 2009). Sinusoids are networks that allow circulating blood cells to contact a variety of intrahepatic cell populations. Inside the intrahepatic cell populations are parenchymal liver cells (hepatocytes), endothelial cells, liver-resident macrophage (Kupffer cell), lymphocytes (natural killer T [NKT] cells), and hepatic stellate cells (Racanelli and Rehermann, 2006).

TNFα is a product of activation of macrophages/monocytes, fibroblasts, mast cells, and some T and NKT cells (Beutler and Cerami, 1989). During infection and inflammation, a series of biochemical changes called acute-phase responses occur in the liver, which activate cytokines. Cytokines help regulate cell to cell communication and regulate hepatic functions within the intrahepatic cell populations. They are messengers secreted by one cell to alter the behavior of the cell itself, a closely related cell, or cells in different organs (Luedde et al., 2002). Regulation is mediated by interactions of cellular receptors signaling internally to the cytosol and the nucleus, resulting in altered transcription, translation, and post-translational modification of regulatory proteins. During embryonic development and organogenesis, cytokines maintain ordered balance between proliferation and controlled cell death

(apoptosis). Once adulthood is reached these functions are preserved; however, disruptions in homeostasis can have deleterious effects. Dysregulation of cytokine activity can occur from liver injury resulting in excessive apoptosis. Excessive apoptosis can be linked to acute and chronic liver diseases, viral and autoimmune hepatitis, cholestatic disease, and alcoholism or drug/toxin-induced liver injury (Tacke et al., 2009).

The liver has been observed to be both a target and a source of TNFα (Ghezzi, 1992). Originally TNFα was identified as a product of activated macrophages. Research has shown Kupffer cells from rats, mice, and rabbits can produce a bioactive TNFα (Magilavy and Rothstein, 1988). Work by Tracey and Cerami (1994) found that TNFα was released from macrophages, lymphocytes, and Kupffer cells when exposed to bacterial toxins, viruses, and inflammatory cytokines.

Relationship to Fatty Liver Disease

Negative energy balance and homeorhetic adaptations during the transition to lactation decrease plasma insulin concentration (Doepel et al. 2002) and decrease the responsiveness of adipose tissue to insulin (Bell and Baumann 1997). The dramatic increase in plasma NEFA leads to the greater flux of FA into the liver which is the primary metabolic factor responsible for inducing fatty liver disease. Fatty liver disease or hepatic lipidosis is a major metabolic disorder in animals (Gruffat et al., 1996; Goff and Horst, 1997). The liver is a major site of fatty acid oxidation and the adipose tissue is the major site of lipid synthesis in ruminants (Kaneko 2008); TNFα is thought to disrupt these pathways. Development of fatty liver disease occurs when hepatic uptake of lipids exceeds the oxidation and secretion of lipids by the liver.

Cows suffering with moderate to severe fatty liver disease were found to have elevated serum TNFα activity (Ohtsuka et al. 2001). A number of studies have investigated the metabolic effects of TNFα administration on cattle and other species, with a large number of responses to TNFα corresponding to those observed in fatty liver disease. Tumor necrosis factor-α in general is observed to promote mobilization of energy stores through impaired insulin sensitivity (Kushibiki et al., 2001), decreased feed intake (Kushibiki et al., 2003), and direct stimulation of lipolysis (Kushibiki et al., 2001; Langin and Arner. 2006), which are all conditions associated with bovine fatty liver disease. Another unique observation about TNFα is the ability to promote TG accumulation once metabolized NEFA reaches the liver (Memon et al., 1998; García-Ruiz et al. 2006; Endo et al. 2007). Work in genetically obese mice showed that administration of antibodies against TNFα prevented or resolved incidence of fatty liver disease in rats fed high-fat diets (Barbuio et al. 2007), mice injected with endotoxins (Endo et al. 2007), and rats receiving total parenteral nutrition (Pappo et al. 1995).

Tumor Necrosis Factor and Obesity

Tumor necrosis factor alpha was linked to obesity in humans and rats about 15 years ago as a result of findings that circulating concentrations and expression during increased weight gain or drastic weight loss (Hotamisligil et al., 1993; Maury and Brichard, 2010). TNFα interacts with two cell surface receptors: TNFα receptor 1 and receptor 2, with the receptor 2 being associated with obesity. Good et al. (2006) found evidence that when body weight increased in obese patients there was an increasing positive correlation with between TNF receptor 2 and TNFα expression. Numerous studies have found that TNFα can regulate apoptosis, adipogenesis, lipid metabolism and insulin signaling within the adipose tissue (Prins

et al., 1997; Galic et al., 2010). In 2004, Trayhurn and Wood gave convincing evidence that macrophages were the primary cell type responsible for the production of TNFα; since that discovery, it has been hypothesized that production of TNFα from macrophages is caused by chemoattractant signals released by dying adipocytes, either from damage or obesity (Prins et al., 1997; Cawthorn et al., 2007). Another speculation is that TNFα could be triggering a signaling cascade which induces cell apoptosis, and in obese subjects this could be one mechanism whereby TNFα regulates adipose tissue mass (Prins et al., 1997; Cawthorn et al., 2007). In obese subjects, TNFα is also thought to work by regulating adiposity by regulation of key transcription factors which control adipogenesis.

When TNFα was administered to rats (Warren et al, 1988) and humans (Van Der Poll et al, 1990), plasma levels of both TG and NEFA increased due to the stimulation of both lipolysis and hepatic lipid synthesis. A similar study conducted in Holstein calves (Kushibiki et al., 2000) resulting in changes in plasma glucose, TG, NEFA, insulin, and GH concentrations after injection of recombinant bovine TNF (rbTNF). Prior to Kushibiki et al. (2000), a study by Kenison et al. (1991) reported that plasma concentrations of TG and NEFA were not affected by rbTNF administration in calves. Contradictory results have also been obtained with respect to how TNFα administration affects GH concentrations. Elsasser et al. (1991) suggested that TNFα could directly inhibit GH secretion from bovine pituitary cells in culture, but Fry et al. (1998) found that TNFα did not influence basal release of GH from cultured sheep pituitary cells. In a more recent study conducted in dairy heifers, a single intravenous injection of rbTNF produced initial hyperglycemia and later hypoglycemia (Kushibiki et al., 2000). In that study glucose changes were similar to those obtained in Kushibiki et al. (2000).

Increasing levels of TNFα promote the secretion of several other pro-inflammatory cytokines and reduce anti-inflammatory cytokines, resulting in an overall proinflammatory state (Wang and Trayhurn, 2006). TNFα shares several pro-inflammatory properties with interleukin 1 (IL-1); they both are capable of inducing fevers. Together, TNFα and IL-1 can induce a fever either through direct or indirect stimulation. Additionally, TNFα, IL-6, and IL-11 share similar properties linked to the induction of acute phase reactant protein production by the liver (Stryjecki and Mutch, 2011). Wang and Trayhurn (2006) showed that treating human adipocytes with TNFα for 24 h caused significant decreases in adiponection expression and increased IL-6 and TNFα expression.

Increases in circulating TNFα in obese subjects have been shown to promote insulin resistance by causing inhibition of the insulin receptor substrate 1 signaling pathway (Stryjecki and Mutch, 2011). Uysal et al. (1997) determined the key role for TNFα in insulin resistance, when it was determined that mice lacking TNFα or TNF receptors had improved insulin sensitivity and glucose tolerance in both dietary and genetic (ob/ob) models of obesity. Hotamisligil et al. (1993) also found that expression of TNFα in obese animals (fa/fa rat and ob/ob mouse) was increased and could regulate insulin action.

TNFα has been discovered to promote insulin resistance in several insulin-responding cells and tissues, by inhibiting tyrosine (tyr) phosphorylation of IRS-1 (Hotamisligil, 2003). Phosphorylation of tyrosine residues of IRS-1 before activation of the insulin receptor is a critical step involved in insulin signaling. Increased production of TNFα during obesity stimulates phosphorylation of IRS-1 on its serine (ser) residues, blocking insulin signaling

(Hotamisligil et al., 1996). This same occurrence is seen in the presence of increased intracellular fatty acids (Shulman 2000).

Conclusions

Fatty liver disease has been shown to be the result of nutritional, managerial, or genetic problems that arise on a farm; however, there are ways to control the incidence rate. When a dairy cow is allowed to gain excessive body fat during late lactation and the dry period, she is predisposed to a number of health related problems, all of which are thought to stem from one common source - excessive accumulation of liver TG. The proinflammatory cytokine TNFα has shown to have a strong connection with cows suffering from fatty liver disease, with increased expression in hepatocytes. Tumor necrosis factor-alpha has been found to mirror the metabolic effects seen in fatty liver disease by promoting the mobilization of energy stores through impaired insulin sensitivity (Kushibiki et al., 2001), decreasing feed intake (Kushibiki et al., 2003), and causing direct stimulation of lipolysis (Kushibiki et al., 2001; Langin and Arner, 2006). Tumor necrosis factor-alpha has also been found to promote TG accumulation once mobilized NEFA reach the liver (Memon et al., 1998; Garcia-Ruiz et al., 2006; Endo et al., 2007). Administration of antibodies against TNFα in obese mice (Li et al., 2003; Garcia-Ruiz et al., 2006) were found to prevent or resolves fatty liver and mice lacking the TNF receptor 1 (Yin et al., 1999) were protected from alcohol-induced fatty liver. Preventing fatty liver disease has proven to be a difficult task, but the ultimate goal is to help reduce the $60 million dollar loss (Bobe et al., 2004) to producers every year.

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Chapter 2 - Continuous infusion of TNF alpha in adipose tissue does not induce the same metabolic effects as daily bolus injections in lactating dairy cows.

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ABSTRACT

Late-lactation Holstein cows (n=9/treatment) were used to evaluate effects of continuous adipose tissue TNFα administration on glucose and fatty acid (FA) metabolism. Cows were blocked by feed intake and milk yield and randomly assigned within block to control or TNFα treatments. Treatments (4 mL saline or 14 μg/kg TNFα in 4 mL saline) were infused continuously over 7 d via 2 osmotic pumps in the adipose layer in the tailhead region. Plasma, milk samples, milk yield, and dry matter intake (DMI) data were collected daily. On d 7, pumps were removed and liver and contralateral tailhead adipose biopsies were collected. Results were modeled with the fixed effect of treatment and the random effect of block; *P* values > 0.10 were considered non-significant. TNFα did not alter liver TNFα mRNA abundance, plasma TNFα, IL-4, IL-6, or interferon-γ concentrations, DMI, or rectal temperature. Milk fat ($P < 0.05$) and lactose ($P < 0.10$) concentrations decreased with TNFα*,* but milk yield was unchanged and treatments did not alter the proportion of short vs. long-chain FA in milk on d 7. Treatments did not alter plasma NEFA concentration, liver triglyceride content, or adipose mRNA abundance for hormone-sensitive lipase or perilipin. Plasma glucose turnover rate, as measured by disappearance of a U-^{13}C-glucose bolus, was not altered by treatment, nor was liver mRNA abundance for phosphoenolpyruvate carboxykinase or pyruvate carboxylase. However, TNFα tended to decrease adipose TNFα mRNA abundance ($P = 0.09$) and increase liver ($P = 0.10$) and adipose ($P = 0.05$) IL-10 mRNA abundance *c*ompared to controls. Messenger RNA abundance of IL-37 in liver tissue decreased significantly in cows treated with TNFα ($P < 0.05$) This TNFα delivery protocol may have allowed for an adaptive anti-inflammatory response to suppress

systemic inflammation, which may account for the lack of metabolic responses compared with previous responses to daily subcutaneous TNFα injections.

(**Key words**: dairy cattle, TNFα, gluconeogenesis)

INTRODUCTION

Tumor necrosis factor alpha (TNFα) is a proinflammatory cytokine first discovered as a cachectin associated with chronic wasting disease (Beutler, 1992). A number of studies have investigated the metabolic effects of TNFα administration on cattle and other species. Sordillo et al. (2009) showed that cows suffering from mastitis and metritis had elevated TNFα, which is in turn linked to the development of fatty liver disease and associated metabolic problems (Bradford et al., 2009). Ohtsuka et al. (2001) found elevated serum TNFα activity in cows suffering from moderate to severe fatty liver disease. A study conducted in Holstein calves (Kushibiki et al. 2000) demonstrated changes in plasma glucose, triglyceride (TG),nonesterified fatty acid (NEFA), insulin, and growth hormone (GH) concentrations after injection of recombinant bovine TNF (rbTNF).

More recently, TNFα has been shown to have a powerful role in insulin resistance, affecting glucose production. It is now widely accepted that TNFα is produced in adipose tissue and that chronic elevations of inflammatory cytokines can interfere with control mechanisms of insulin action (Hotamisligil and Spiegelman, 1994). Tumor necrosis factor-α is observed to promote mobilization of energy stores through impaired insulin sensitivity (Kushibiki et al., 2001), decreased feed intake (Kushibiki et al., 2003), and direct stimulation of lipolysis (Kushibiki et al., 2001; Langin and Arner. 2006), which are all conditions associated with bovine fatty liver disease. Another unique observation about TNFα is the ability to promote TG

accumulation once mobilized NEFA reach the liver (Memon et al., 1998; García-Ruiz et al. 2006; Endo et al. 2007).

Previous work in lactating dairy cattle supported a direct effect of TNFα on triglyceride accumulation and metabolic gene expression in the liver (Bradford et al. 2009). Administration of TNFα (2 μg/kg) to late-lactating dairy cows once daily by subcutaneous injection for 7 d significantly increased liver TG concentrations and significantly decreased abundance of phosphoenolpyruvate carboxykinase (PEPCK) and carnitine palmitoyl transferase (CPT1), transcripts critical to gluconeogenesis (PEPCK) and fatty acid oxidation (CPT1), by 44% and 32% compared to control animals (Bradford et al. 2009). These findings with daily bolus injections of TNFα contributed to the hypothesis of the present study that continuous infusions of TNFα would mimic endogenous release of TNFα and increase liver TG concentration by altering hepatic gene expression. Thus, the objective was to determine whether continuous administration of TNFα, originating in adipose tissue, would cause alterations in systemic nutrient metabolism.

MATERIALS AND METHODS

Experimental procedures were approved by the Institutional Animal Care and Use Committee at Kansas State University.

Animals, treatments, and data and sample collection

Eighteen late-lactation Holstein cows (n=9/treatment) were used to evaluate effects of TNFα administration on glucose and fatty acid (FA) metabolism. Late lactation cows were used to ensure that animals began the experiment without hepatic inflammation and to decrease

animal variability. Cows entered a tie stall facility 7 d prior to treatment to allow for adaption. Cows were offered water ad libitum, fed twice daily and milked 3 times daily in a milking parlor. Following the adaption period, cows were blocked by pre-treatment feed intake and milk production and assigned randomly within block to a control or TNFα treatments. Saline (4 mL) or TNFα (14 μg/kg in 4 mL saline) was infused continuously over 7 d via 2 osmotic pumps (2ML1, Alzet, Cupertion, CA) implanted in the adipose layer in the tail head region. The incision was closed with surgical staples.

Throughout the 7 d treatment period, blood plasma, milk samples, milk yield data, and dry matter intake data were collected daily. Blood samples were collected at the same time each day (0800 h). Two blood samples were collected from the coccygeal vein in evacuated tubes containing potassium EDTA or potassium oxalate with sodium fluoride as a glycolytic inhibitor (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA), centrifuged (2,000 x g for 10 min immediately after collection), and plasma stored at -20°C for plasma analyses. On d 5 of treatment, jugular catheters were placed and at least 18 h of recovery were allowed prior to sample collection via the catheters. On d 6, blood samples were collected every 4 h for 24 h to measure diurnal plasma concentrations of glucose, insulin, and BHBA. On treatment d 7, cows were given a glucose bolus containing U-^{13}C-glucose (99 atom %, Sigma Chemical Co., St. Louis, MO) through jugular catheters, and blood samples were then collected at 10-min intervals for 120 min to determine glucose turnover rate (Schulze et al., 1991). Each cow received approximately 50 mL (1 g of U-^{13}C-glucose in 50 ml of sterile saline); syringes were weighed immediately before and after infusion to determine the exact amount administered. Catheters were flushed with a sterile solution of 3.5% sodium citrate after the labeled glucose bolus (20

mL) and after each blood sample collection (5 mL). Cows were fed every 2 h beginning 6 h before administration of the labeled glucose and continuing through the final sample collection to promote steady-state glucose kinetics. After the glucose turnover test was completed, osmotic pumps were removed and liver and contralateral tailhead adipose tissue samples were collected for analysis of liver TG and mRNA for key transcripts.

Milk analyses

During the treatment period, a single milk sample was collected from each cow at each milking and divided. One sample was analyzed by Heart of America DHIA (Manhattan, KS) to determine concentrations of fat, true protein, and lactose as previously described (Martel et al., 2011). The second sample was used for fatty acid (FA) analysis as described by Sukhija and Palmquist (1988). Prior to FA analysis, samples were thawed, shaken, and 200 μL were aliquoted for lyophilization. Lyophilized samples were resuspended in 1 mL of hexane containing C13:0 as an internal standard and methylated using BF_3-methanol. The resulting FA methyl esters were extracted in hexane and injected onto a Supelco (Bellefonte, PA) SP-2560 capillary GC column (100 m × 0.25 mm × 0.2 μm) for FA profile analysis, with a run time of 67 min.

Plasma analyses

Colorimetric kits were used to quantify glucose, NEFA, and β-hydroxybutyrate (BHBA) concentrations in all plasma samples as previously described (Bradford et al., 2009). Bovine-specific ELISA assays were used to quantify insulin (Bovine Insulin ELISA; Mercodia AB Sweden) and TNFα (Farney et al., 2011) concentrations in samples collected on d 7. Additionally, a multiplexed bovine cytokine ELISA assay (Searchlight, Bovine Cytokine Array; Aushon

Biosystems; Billerica, MA) was used to determine interleukin (IL)-4, IL-6, and interferon-gamma (IFNγ) concentrations in plasma samples collected on d 7. Plasma samples collected on treatment d 7 were also analyzed for IL-10 using a semi-quantitative Western blot assay. For the Western blot, samples (1 μL) were diluted in Laemmli sample buffer, heated at 90°C for 5 min, cooled, vortexed, separated by SDS-PAGE on a 4 to 12% Tris-HCl gel, and dry-transferred onto nitrocellulose membranes (iBlot; Invitrogen, Carlsbad, CA). Membranes were blocked in Tris buffer (pH 7.4) with 5% dry milk powder for 2 h at room temperature and then incubated for 1 h at room temperature with a goat anti-IL10 antibody (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). After washing, membranes were incubated for 1 h at room temperature with secondary antibody (anti-goat IgG, Santa Cruz Biotechnology) diluted 10,000-fold in Tris buffer (pH 7.4). Immunodetection was performed by chemiluminescence (West-Dura; Thermo Scientific, Waltham, MA) using a digital photodocumentation system (ChemiDoc-It Imaging System; UVP Inc., Upland, CA).

Plasma samples collected for the glucose turnover assay were analyzed for U-^{13}C-glucose enrichment (Metabolic Solutions, Inc., Nashua, NH). Glucose was extracted and converted to aldonitrile pentaacetate derivative (Tserng and Kalhan, 1983), and negative chemical ionization GC/MS (Hewlett-Packard 5890) was used to analyze derivatized samples. The isotopic composition of the glucose was determined by monitoring unlabeled (M+0: m/z = 328) versus U-^{13}C-labeled (M+6: m/z = 334) glucose derivatives. This approach, as opposed to oxidation of glucose and measurement of CO_2 enrichment, ensures that results are not biased by carbon recycling via the Cori cycle. Enrichment of plasma glucose for each animal was fitted to an exponential decay curve according to the following equation: $E_t = E_0 \times e^{-kt}$, where t = time

relative to infusion (min), E_t = enrichment of plasma glucose (U-^{13}C-glucose: unlabeled glucose ratio) at time t, E_0 = enrichment at time t=0, and k = rate constant (min^{-1}). After using the best-fit equations to determine k and E_0, the total glucose pool was calculated by the following equation: $G = M \div E_0$, where G = total glucose pool (g) and M = mass of tracer infused (g). Plasma glucose turnover rate (GTR, g/min) was calculated according to the equation $GTR = G \times k$. Samples collected 10 min prior to infusion of U-^{13}C-glucose were also analyzed to verify the lack of natural occurrence of the M+6 isotopomer.

Tissue analyses

Triglyceride content was measured using a method adapted from Starke et al. (2010). Approximately 20 mg of liver was placed in 500 µL of chilled phosphate-buffered saline (pH 7.4) and homogenized. The homogenate was centrifuged at 2000 x *g* for 10 min at 4°C and 100 µL of the supernatant was then removed for free glycerol and total protein analysis. The remaining liver homogenate was incubated with 100 µL of lipase (porcine pancreatic lipase, MP Biomedicals) for 16 h at 37°C, and glycerol content was then determined by an enzymatic glycerol phosphate oxidase method (#F6428, Sigma-Aldrich Co.). Triglyceride content was calculated based on the difference between glycerol concentrations before and after lipase digestion. Total protein content of the original homogenate was analyzed by a Coomassie blue (Bradford, 1976) colorimetric method (kit #23236, Thermo Scientific, Pierce, Rockford, IL). To avoid potential bias introduced by differences in moisture content of liver samples, liver TG concentration was normalized by protein concentration, which is unaltered in fatty liver (Fronk et al, 1980).

Quantitative real-time RT-PCR (qRT-PCR) was used to measure key genes involved in lipolysis, gluconeogenesis, and cytokine pathways. Expression of cytosolic phosphoenolpyruvate carboxykinase 1 (PEPCK) and pyruvate carboxylase (PC) in liver tissue and hormone sensitive lipase (HSL) and perilipin (PLN) in adipose tissue were all measured using quantitative real-time PCR (qRT-PCR). Total RNA was isolated from the liver and adipose tissue using a commercial kit (RNeasy Lipid Tissue Mini Kit, Qiagen, Valencia, CA). Ultraviolet spectroscopy was used to quantify RNA (Nanodrop-1000, Nanodrop Technologies Inc., Wilmington, DE). A high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) was used for synthesis of complementary DNA from 2 μg of total. Quantitative real-time PCR was performed in triplicate with 1/20 of the cDNA product in the presence of 200 nM gene-specific forward and reverse primers using SYBR green fluorescent detection (ABI 7500 Fast, Applied Biosystems). Messenger RNA abundance was quantified using the delta, delta C_t method with RSP9 used to normalize values (Janovick-Guretzky et al., 2007). Primer sequences for TNFα, PEPCK, and PC were previously published (Bradford et al., 2009). The primers for HSL, PLN, IL-10, and IL-37 were designed using the NCBI primer design tool and were as follows: hormone sensitive lipase (NM_001080220.1), forward AGCGTGGCGCACCTCTTTGA (216-235), reverse AAGATGCTGCGGCGGTTGGA (352-333); PLN (NM_001083699.1), forward CAGCGCCAGGAACAGCATCA (485-504), reverse AGCTCGGGTGTTGGCAGCAT (617-598); IL-10 (NM 174088.1), forward GGCTGAGAACCACGGGCCTGA (291-311), reverse AGGGCAGAAAGCGATGACAGC (394-374); and IL-37 (NM_001206735.1), forward GCTCCTCCTGGGCGAAAGCC (2844-2863), reverse ATCTGGGCGTGTGCAGAGCA (2919-2900).

Statistical analysis

Results were modeled with the fixed effects of treatment, day, and treatment by day interaction, and random effects of block and cow within block for repeated measures. Single time point results were modeled with the fixed effect of treatment and the random effect of block. Several variables (liver TNFα, HSL, PLN, IL-10, adipose TNFα, IL-4, IL-6, plasma TNFα and IFNγ) were natural log-transformed for statistical analysis to achieve normal residual distributions, and reported means are back transformed.

RESULTS AND DISCUSSION

Previous work with once daily subcutaneous injections of TNFα to late-lactation dairy cows found that TNFα could increase liver TG concentrations and tended to decrease hepatic mRNA abundance of rate-limiting enzymes for gluconeogenesis and fatty acid oxidation (Bradford et al., 2009). We expected that continuous TNFα administration would better mimic endogenous TNFα release, induce fatty liver disease, alter plasma metabolites used in assessing lipolysis, and alter insulin sensitivity. Analysis of daily plasma samples found that treatments did not alter plasma NEFA, glucose, or insulin concentrations (Table 2-1). Day 7 plasma samples analyzed for TNFα concentration (Table 2-1) were unchanged by infusion treatments. Our results do not correspond with work completed within the last decade, where TNFα was found to promote the mobilization of energy stores by impaired insulin sensitivity (Kushibiki et al., 2001) and direct stimulation of lipolysis (Kushibiki et al., 2001; Langin and Arner, 2006), all conditions associated with bovine fatty liver (Ingvartsen and Andersen, 2000; Bobe et al., 2004; Vernon et al., 2005; Oikawa et al., 2006).

Daily injections of TNFα (2 μg/kg per d) were found to significantly increase liver TG concentrations (Bradford et al., 2009). Endo et al. (2007) and Garcia-Ruiz (2006) also found that TNFα promotes TG accumulation once mobilized NEFA reach the liver. Administration of TNFα through osmotic pumps did not significantly alter liver TG concentration following the 7 d treatment period (Figure 2-1, $P = 0.99$).

Gluconeogenesis is a key process occurring in the liver of dairy cows, allowing the biosynthesis of new glucose (Young, 1977), since very little glucose is absorbed from the intestinal tract following ruminal fermentation of carbohydrates. Within the present study the objective was to determine what effects TNFα could have on key enzymes involved in hepatic gluconeogenesis. Plasma glucose turnover rate, as measured by disappearance of U-^{13}C-glucose bolus, was not altered by treatment (Figure 2-2, $P = 0.26$).

Glucose production via gluconeogenesis involves the expression of PEPCK and PC within the liver. Since administration of TNFα in rodents decreases activity (Hill and McCallum, 1992; Christ and Nath, 1996) and mRNA abundance of PEPCK (Hill and McCallum, 1992; Christ and Nath, 1996), we expected TNFα administration to dairy cows could do the same. However, no differences were observed in liver expression of PEPCK and PC mRNA abundance during the continuous administration of TNFα (Figure 2-3; $P > 0.10$).

Although cytokine infusions can induce direct effects on metabolism, these signals can also be amplified or muted through the endogenous release of pro- or anti-inflammatory cytokines. Several key anti-inflammatory cytokines were analyzed to determine if continuous infusion of TNFα could be affecting the expression. Analysis of the presence of plasma IL-10 through Western Blot found no difference between the treatments of TNFα and control ($P >$

0.50, data not shown). Interleukin-10 has been found to be capable of inhibiting synthesis of pro-inflammatory cytokines like IFN-γ and TNFα (Armstrong et al., 1996). Expression of IFN-γ was also not significantly altered by treatments (Table 2-2). The third plasma cytokine analyzed was IL-6, which can act as both a pro-inflammatory and anti-inflammatory cytokine (Xing et al., 1998); again, IL-6 concentrations were not affected by TNFα treatment (Table 2-2). Interleukin-4 (IL-4) was also observed to have no significant difference in concentration due to the presence of TNFα for 7 d (Table 2-2).

In addition to quantifying circulating cytokines in this study, liver and adipose tissue was analyzed for IL-10 and IL-37 expression, to determine if a possible anti-inflammatory response could be occurring. Messenger RNA abundance of IL-10 in adipose increased significantly, and there was also a tendency for an increase in liver tissue in cows treated with TNFα (Figure 2-3; $P = 0.02$ adipose; $P < 0.10$ liver), suggesting that pro-inflammatory cytokine signaling was being partially counteracted by increased expression of anti-inflammatory cytokines. Conversely, IL-37 mRNA abundance in liver was significantly decreased by TNFα ($P < 0.05$). Expression of IL-37 in macrophages or epithelial cells almost completely suppressed the production of pro-inflammatory cytokines (Nod et al., 2010). No difference was observed in IL-37 expression in adipose tissue ($P = 0.22$; Figure 2-3)

Adipose tissue was also analyzed for mRNA abundance of TNFα, HSL, and PLN. Abundance of TNFα mRNA in adipose tissue tended to be decreased in cows receiving TNFα compared to control cows (Figure 2-3). However, there was no difference observed in TNFα mRNA abundance in liver samples (Figure 2-3). Analysis of mRNA abundance of HSL and PLN in adipose tissue were not observed to have differences between the two treatments (Figure 2-3).

Since the study was conducted in lactating dairy cows, daily milk parameters and dry matter intake was measured and analyzed. Continuous administration of TNFα had no effect on daily dry matter intake; however, milk fat yield and percent decreased ($P < 0.05$) with TNFα, although milk and energy-corrected milk yields were not significantly altered. Zebeli and Ametaji (2009) speculated that LPS-mediated inflammatory responses might affect milk fat synthesis indirectly through the release of proinflammatory cytokines such as TNFα, IL-1, and IL-6. Thus, LPS and/or inflammatory cytokines could be having suppressive effects on key enzymes like FA synthetase and acetyl-CoA carboxylase, which play key roles in de novo FA synthesis. Ultimately these enzymes play a role in clearance of circulating TAG-rich chylomicrons and very low density lipoprotein (VLDL) to decrease LPS toxicity (Feingold et al., 1992) and cause uptake of FA for incorporation into milk fat (Merkel et al., 2002). However, our results revealed a tendency for an increase in milk concentrations of *trans*-10, *cis*-12 CLA and *trans*-10 C18:1 with TNFα (Table 2-3), suggesting that this treatment may have somehow influenced ruminal biohydrogenation. These findings therefore suggest that a more traditional mechanism involving bioactive lipids may underlie the decrease in milk fat yield with TNFα treatment. Treatments did not alter the proportion of short vs. long-chain FA in milk (Table 2-3).

CONCLUSIONS

When the immune system is stimulated by rapid increases in cytokine concentrations, it sets off a cascade of events that lead to activation of inflammatory pathways, which can trigger metabolic changes. It is clear from the lack of treatment effects on inflammatory markers that continuous administration of TNFα did not induce the same responses as when it was delivered

in a single injection once daily. In fact, in this experiment TNFα tended (P = 0.08) to decrease adipose TNFα mRNA abundance and increase (P = 0.08) liver and adipose (P = 0.02) IL-10 mRNA abundance compared with controls. These responses indicate that the TNFα-treated cows had a lesser inflammatory state than the controls, the opposite of the response expected based on previous results. It is possible that such a response was caused by an adaptive, compensatory anti-inflammatory response to the TNFα infusion. The slow, constant administration of the cytokine may have allowed the cows to counteract the inflammatory challenge. These findings indicate that brief, rapid increases in inflammatory cytokines may be more likely to induce liver inflammation than chronic, low-level increases, at least during the course of 7 days. The overall conclusion of this study is that this treatment protocol did not have any clear effects on systemic inflammation or nutrient metabolism, and this may have been due to a compensatory anti-inflammatory response.

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Table 2-1 Effects of continuous infusion of TNFα on plasma metabolites

	Infusion Treatment			
	Con	TNF	SEM	*P* value
BHBA, µmol/L	496.1	475.6	36.6	0.84
NEFA, µEq/L	126.6	134.3	18.9	0.81
Glucose, mg/dl	59.5	60.1	1.82	0.64
Insulin, ng/mL	2.21	2.18	0.39	0.97

Table 2-2 Effects of continuous infusion of TNFα on plasma cytokines

	Infusion Treatment			
	Con	TNF	SEM	*P* value
TNFα, pg/mL	5.91	5.67	3.18	0.95
IFNγ, pg/mL	10.4	13.6	3.73	0.54
IL-4, pg/ml	7.44	5.97	5.89	0.84
IL-6, pg/mL	60.7	134.4	53.2	0.29

All measurements were natural log- transformed for statistical analysis, and reported least square means are from back-transformation.

Table 2-3 Effects of continuous infusion of TNFα on energy balance parameters

	Infusion Treatment			
	Con	TNF	SEM	*P* value
DMI, kg/d	20.9	20.5	0.98	0.63
Milk yield, kg/d	29.9	30.6	1.6	0.74
Milk fat, kg/d	1.21	0.99	0.08	< 0.05
Milk fat, %	4.07	3.22	0.22	< 0.01
Milk protein, kg/d	0.98	0.97	0.05	0.93
Milk protein, %	3.31	3.17	0.11	0.37
Milk lactose, kg/d	1.45	1.51	0.08	0. 58
Milk lactose, %	4.85	4.95	0.05	0.09
Energy-corrected milk, kg/d	23.8	21.2	1.3	0.12
Fatty Acid Profile, g/100 g				
Short & medium chain	21.2	20.6	0.01	0.67
C16	30.3	31.9	0.01	0.09
Long chain	48.5	47.5	0.01	0.56
trans-10 C18:1	1.44	2.78	0.76	0.07
trans-10, *cis*-12 C18:2	0.015	0.024	0.005	0.06

Figure 2-1 : Liver triglyceride concentration during experimental period. Infusion treatments were found to have no significant difference on liver TG (P = 0.99) in late-lactating dairy cows.

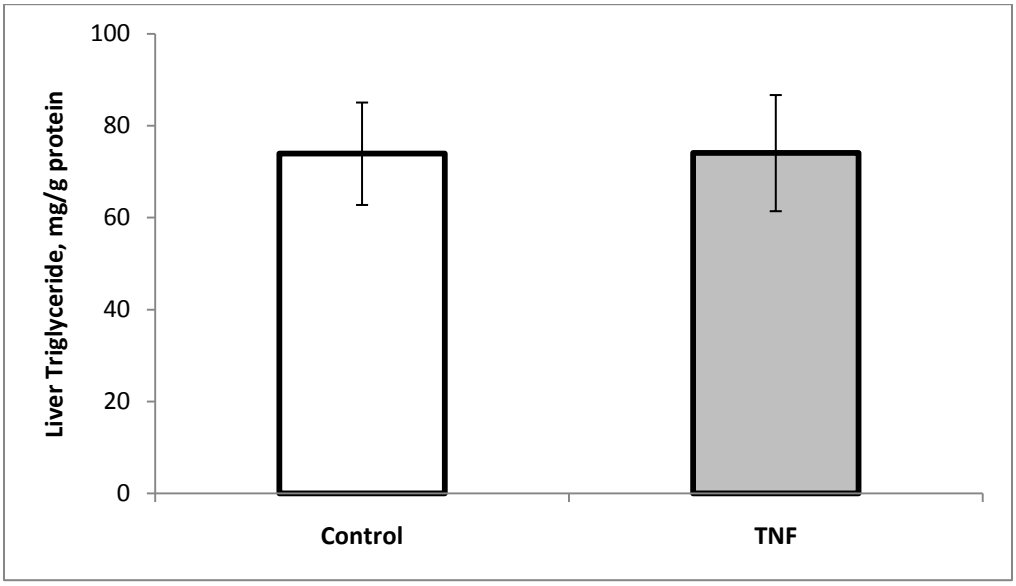


Figure 2-2 Glucose turnover during experimental period. Infusion treatments were found to have no significant difference on glucose turnover (P = 0.26) in late-lactating dairy cows

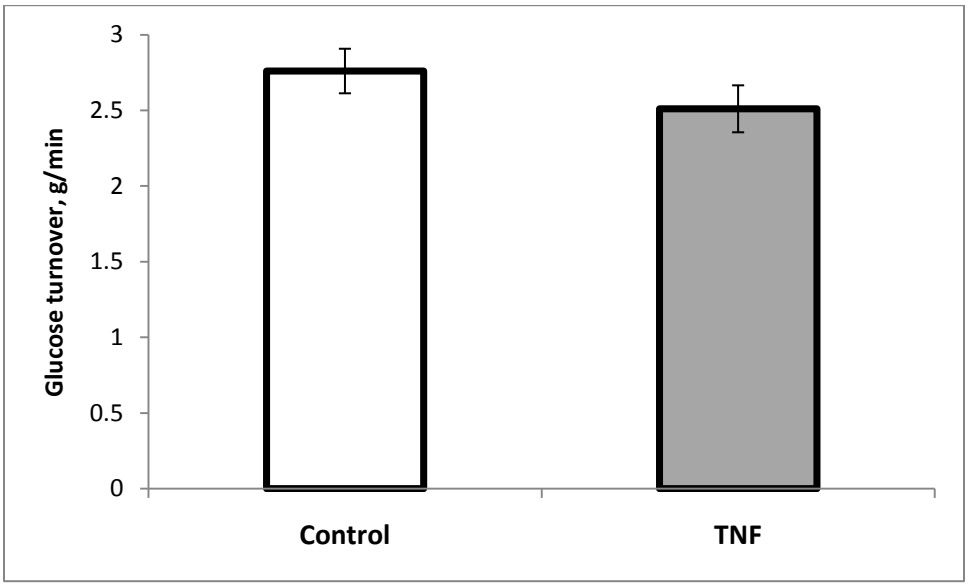
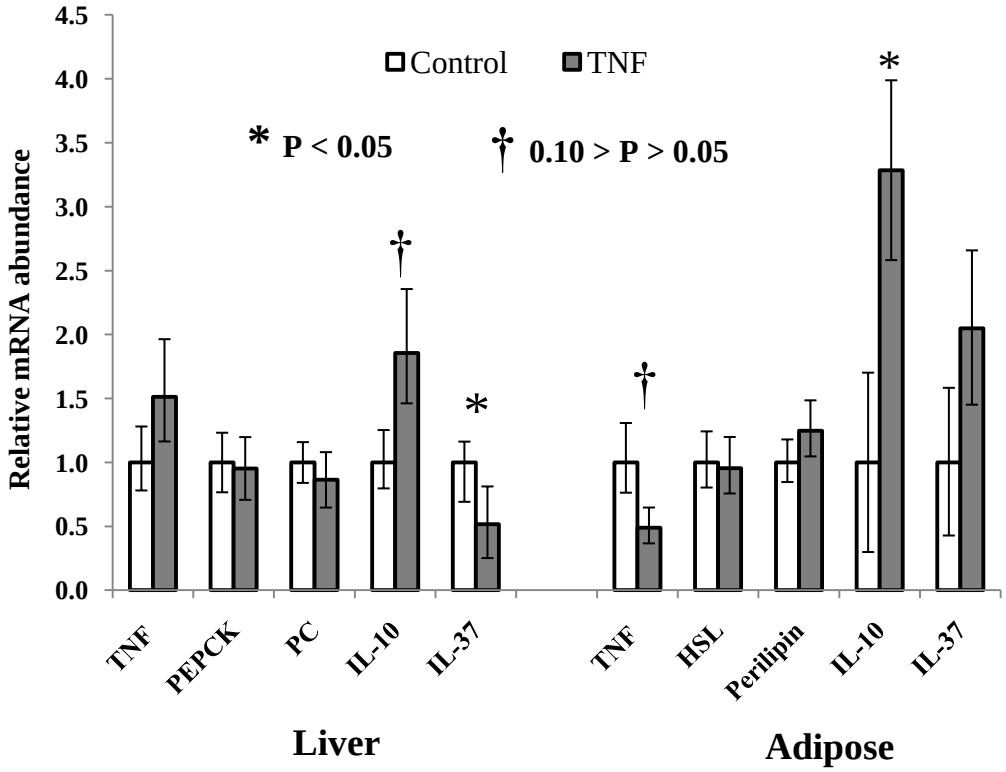


Figure 2-3 : Relative mRNA abundance between control and TNFα treatment in liver and adipose tissue collected at day 7. Natural log-transformation was used for statistical analysis and back-transformed values are shown for liver TNF, IL-10, and Adipose TNF, HSL, and PLN.



Chapter 3 - Effects of TNF receptor blockade during negative energy balance on TNF alpha expression and key plasma metabolites involved in gluconeogenesis and lipolysis.

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ABSTRACT

Non-pregnant dry Holstein cows (n=10) were used to evaluate if metabolic function and liver triglyceride accumulation during feed restriction could be affected by blocking tumor necrosis factor-alpha (TNFα) signaling with a synthesized cyclic peptide (TNF receptor loop peptide; TRLP). Cows were randomly assigned to treatments: control (carrier) or inhibitory peptide (1.7 mg/kg BW TRLP). Treatments were administrated every 4 h for 7 d by subcutaneous injections along the neck. Cows were housed in a tie-stall barn and feed was restricted to 40% of energy maintenance requirements. Daily blood samples were collected and analyzed for glucose, insulin, BHBA, NEFA, and haptoglobin concentrations, with no treatment effects detected. On d 7, 4 h after morning feeding, cows completed a glucose tolerance test (GTT). Cows were administrated a dextrose bolus (50% wt/vol, 300 mg glucose/kg BW) by intra-jugular catheter. Plasma samples were collected prior to infusion and at 10 minute intervals through 120 minutes post-infusion. Samples were analyzed for glucose, insulin, NEFA and reported as area under the curve (AUC). After the last injection of TRLP, liver biopsy samples were collected for liver triglyceride (TG) concentration. Results failed to demonstrate any significant effects of treatment in response to the feed restriction or the GTT. However, plasma and liver analyses were not indicative of dramatic lipolysis or hepatic lipidosis, suggesting that the feed restriction protocol failed to induce the metabolic state of interest.

(**Key words**: dairy, fatty liver, glucose tolerance test, TNFα)

INTRODUCTION

The proinflammatory cytokine, tumor necrosis factor alpha (TNFα) has been implicated in several metabolic disorders, including fatty liver disease in dairy cattle (Ohtsuka et al., 2002). This inflammatory cytokine elicits its effects on tissues and organs after binding one of its receptors (TNFR1 or TNFR2). Activation of TNFα can lead to the activation of caspases (Tran et al., 2009), NF-κB (Osman et al., 1999), and MAP-kinase pathways (Means et al., 2000), which are involved in significant metabolic pathways. If this process does not occur, activation of inflammatory pathways involved in alteration of organ function does not take place. Receptor antagonists and TNFα blocking peptides like TRLP work by interfering with TNFα interactions with its corresponding TNF receptor (TNFR; Takasaki et al., 1997). The inability of TNFα to interact with a receptor prevents signaling and activation. Reducing circulating TNFα levels by inactivation of genes or immunodepletion have beneficial effects on glucose metabolism in obesity (Pamir et al., 2009), which could prove to be effective in cows suffering from energy restriction.

Extensive work has been completed in the area of blocking TNFα activation and signaling in vivo, including administration of antibodies against TNFα (Araujo et al., 2007) and genetic deletion of TNF receptors (Yin et al., 1999). More recent work has been completed in mice to help understand how blocking TNFα could affect bone resorption, rheumatoid arthritis (Kojima et al. 2005), and neurodegenerative disorders (Bartsch et al., 2010), all diseases that lead to chronic inflammation and tissue damage. Tumor necrosis factor alpha has been implicated in many human diseases, many of which that deal with digestive related diseases. A study by Yin and others (2011) studied the effectiveness of a TNF antagonist to treat patients

with Crohn's disease. Yin and others (2011) used a combination of TNFα binding cyclic peptide (TBCP) and TNFR1 binding cyclic peptide (TRBCP) to block TNFα in rats with induced-colitis. This study observed that protein expression of TNFα in rats treated with both TBCP and TRBCP was down-regulated. Another study pertinent to understanding the role of blocking the signaling and activation of TNFα in disease treatment completed by Saito et al. (2007) looked at TNFα involvement in rheumatoid arthritis. Saito and others (2007) set out to block the effects of TNFα with the cyclic peptide TRLP in mice with collagen-induced arthritis. The group found they were able to delay the onset of arthritis longer in mice treated with the cyclic peptide TRLP and inhibit RANKL-induced signaling, which is activated during increased TNFα.

Since little is known about how TNFα could potentially be involved in the development of fatty liver disease in dairy cattle, we proposed the use of a cyclic peptide to block the signaling and activation of TNFα. Blocking signaling and activation could help study its role in development of fatty liver, regulation of plasma glucose concentrations, and triglyceride (TG) metabolism. The TNF receptor loop peptide (TRLP; amino acid sequence YCWSQYLCY) is a synthesized peptide that has been found to be capable of mimicking the most critical TNF recognition loop on TNF receptor I, preventing the interactions of TNFα with its receptor (Saito et al., 2007).

Therefore, the hypothesis was that subcutaneous injections of TRLP would block TNFα signaling and partially prevent the development of insulin resistance and hepatic lipidosis during feed restriction in obese cows. The objectives of the study were to study TRLP's effects

on lipolysis and liver TG accumulation, the rate of glucose clearance after infusion, and expression of genes important for liver FA oxidation and gluconeogenesis.

MATERIAL AND METHODS

Experimental procedures were approved by the Institutional Animal Care and Use Committee at Kansas State University.

Animals and Experimental Design

Ten non-pregnant, non-lactating Holstein cows were randomly assigned one of two treatments control (57% DMSO in PBS) or inhibitory peptide (1.7 mg/kg BW TRLP dissolved in 57% DMSO in PBS) administrated by subcutaneous injections every 4 h for 7 d. Treatments were administrated along the neck, rotating between both the left and right side, to help prevent injection site abscesses. Cows received injections every 4 h to maintain relatively stable TRLP concentrations throughout the day. Cows were housed in a tie-stall facility for 7 d prior to injections allowing for diet and environment adaption. Prior to entering the tie-stall facility all cows were weighed and BCS recorded. During the first 7 d, cows where fed an alfalfa and prairie hay based diet to provide 16.2 Mcal/d NE_L (Table 3-1). One day prior to injection treatments, cows were placed on a restricted diet providing 40% of estimated energy requirements for maintenance; this ration was fed for the 7 d injection period (Table 1). The feed restriction protocol was modeled after that of Cooke et al. (2007), which induced a 10-fold increase in liver TG content. Cows were fed twice per day during both the adaptation and treatment periods, and water was offered ad libitum. During the diet restriction period extra salt (284 g/d) was added to the diet mix as ordered by Kansas State Veterinarian as a recommendation to promote increased water intake to prevent dehydration.

Data and Sample Collection

Blood samples were collected during the 7 d treatment period (1500 h) for analysis of plasma glucose, insulin, BHBA, and NEFA concentrations. On d 5, jugular catheters were surgically placed to prepare for a glucose tolerance test on d 7. Because TNFα administration causes insulin resistance in cattle (Kushibiki et al., 2001), assessment of the effect of TRLP on insulin sensitivity was conducted using a glucose tolerance test. On d 7 (800 h) cows received a bolus sterile solution of 50% dextrose (wt/vol) at a dose of 300 mg glucose/kg of body weight in less than 5 min by intra-jugular catheter (Bradford and Allen, 2007). Plasma samples were collected from the jugular vein 10 min prior to infusion and at 10 min intervals through 120 min post-infusion. Samples from d 7 were analyzed for glucose, NEFA, and insulin concentrations. At the conclusion of the glucose tolerance test, liver biopsies were collected for analysis of liver TG concentration. Sample collection and surgical methods were similar to those described by Morey et al. (2011).

On d 5, a blinded technician scored injection sites on both sides of the neck for degree of tissue inflammation. A scoring system from 0 to 5 was used, with 0 indicating no visible signs of irritation or lumps, and 5 indicating serious irritation (4 + lumps). At the conclusion of the study, all cows were weighed and BCS recorded to determine weight loss during the restriction period.

Sample Analysis

Plasma Analyses

Blood samples were collected at the same time every day (600 h). Two blood samples were collected from the coccygeal vein in evacuated tubes containing potassium EDTA or

potassium oxalate with sodium fluoride as a glycolytic inhibitor (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA), centrifuged (2,000 x *g* for 10 min immediately after collection), and plasma was stored at -20°C. Colorimetric kits were used to quantify glucose, NEFA, and β-hydroxybutyrate (BHBA) concentrations in all plasma samples as previously described (Bradford et al., 2009). Bovine-specific ELISA assays were used to quantify insulin (Bovine Insulin ELISA; Mercodia AB Sweden). Glucose and insulin data from the glucose tolerance test were used to determine the area under the curve by the trapezoidal rule for each variable, using the mean of the -10 and 0 minute time points as a baseline value (Bradford and Allen, 2007). Plasma haptoglobin was measured by bovine-specific ELISA (#2410-7, Life Diagnostics, West Chester, PA, USA) with one modification during the dilution stage; instead of a 2,000-fold dilution, a 3,000-fold dilution was utilized.

Liver Biopsy Analysis

Approximately 20 mg of liver was placed in 500 µL of chilled phosphate-buffered saline (pH 7.4) and homogenized. The homogenate was centrifuged at 2000 x g for 10 min at 4°C and 100 µL of the supernatant was then removed for free glycerol and total protein analysis. Triglyceride content was measured using a method adapted from Starke et al. (2010). The remaining liver homogenate was incubated with 100 µL of lipase (porcine pancreatic lipase, MP Biomedicals) for 16 h at 37ºC, and glycerol content was then determined by an enzymatic glycerol phosphate oxidase method (#F6428, Sigma-Aldrich Co.). Triglyceride content was calculated based on the difference between glycerol concentrations before and after lipase digestion. Total protein content of the original homogenate was analyzed by a Coomassie blue (Bradford, 1976) colorimetric method (kit #23236, Thermo Scientific, Pierce, Rockford, IL). To

avoid potential bias introduced by differences in moisture content of liver samples, liver TG concentration was normalized by protein concentration, which is unaltered in fatty liver (Fronk et al., 1980).

Statistical Analysis.

Results were modeled with the fixed effects of treatment, day, and treatment by day interaction, and random effect of cow for repeated measures. Single time point results were modeled with the fixed effect of treatment. Haptoglobin results were natural log-transformed for statistical analysis to achieve normal residual distributions and reported means were back transformed.

RESULTS AND DISCUSSION

The transition period has been shown to cause great stress on dairy cattle, resulting in increased incidence of metabolic disorders (Bobe et al., 2004), like ketosis and mastitis which could be occurring as a result of pre-exposure to fatty liver disease. During a time when energy requirements can nearly double over the first week of lactation (Bell, 1995), animals have a high tendency to go off feed (Grummer, 1993), resulting in a negative energy balance causing decreased plasma insulin concentration (Bell and Bauman, 1997) and decreased insulin response in adipose tissue (Bell and Bauman, 1997). Dramatic changes during this period can lead to increased plasma NEFA concentration (Ingvartsen and Andersen, 2000), which ultimately leads to greater flux of FA into the liver (Emery et al., 1992), resulting in fatty liver disease.

Determining what involvement TNFα potentially has in fatty liver disease was the goal of this study; in particular, we need a better understanding of how TNFα affects lipolysis and gluconeogenesis during negative energy balance. To accomplish that, we chose to block TNFα signaling with TRLP. Analysis of daily plasma concentrations of insulin, NEFA, glucose, and BHBA was used to study the effects TRLP was having on lipolysis and gluconeogenesis. We determined that the treatment of TRLP had no effect on daily plasma levels of BHBA, glucose, NEFA, or insulin when compared to the control cows (Table 3-2).

The acute phase protein haptoglobin was also analyzed to determine if blocking TNFα signaling could affect its concentration, since haptoglobin is released by the liver during periods of inflammation (Hachenberg et al., 2007) and was found to be increased in cows with fatty liver (Bobe et al., 2004). Plasma haptoglobin concentration was measured during the 7 d treatment period and TRLP treated cows showed a slight increase in the circulating concentration compared to the control cows ($P = 0.16$; Figure 3-1). However, this finding does not coincidence with previous publications; since we were trying to block pro-inflammatory TNFα signaling, we would have expected the control cows have a higher circulating concentration than the TRLP cows.

After 7 d of continuous injections of TRLP, cows were subjected to a glucose tolerance test (GTT) at 800 h. The glucose tolerance test was used to assess the effects of TRLP on insulin sensitivity, because TNFα when administrated is known to increase insulin resistance in cattle (Kushibiki et al., 2001). Thus, if TNFα concentrations are naturally elevated during energy restriction, we hypothesized that blocking its effects would allow us to determine if TNFα plays

a significant role. However, upon analysis of plasma samples collected during the GTT, it was concluded that treatment with TRLP had no significant impact on glucose, insulin, or NEFA dynamics following a glucose challenge (Table 3-3).

Another variable assessed in this study was the visual incidence of injection site irritation. Visual assessment of cows demonstrated a significantly higher degree of injection-site irritation for TRLP vs. control (mean scores of 2.9 vs. 0.1 ± 0.35, $P < 0.001$). This local inflammation suggests that the peptide itself may have had inflammatory effects independent of its ability to block TNFα.

CONCULSIONS

The intentions of this study were to place cows in an energy restricted fatty liver scenario, then determine whether blocking TNFα signaling with TRLP would prevent the metabolic changes that lead to fatty liver disease. However, even though the cows were energy restricted for 7 d, they did not suffer from any signs of fatty liver disease, or even rapid lipolysis. Coupled with observations suggesting a local inflammatory response to the TRLP, the failure of the fatty liver induction protocol limits our ability to reach conclusions regarding the effects of TNFα blockade during negative energy balance in dairy cattle. Therefore, questions remain regarding the necessity of TNFα signaling in the etiology of bovine fatty liver disease.

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Table 3-1 Daily %DM amounts of ingredients and composition of adaptation and restricted diets

Ingredient	Adaptation value	Restriction value
%DM		
Corn silage	31.8	14.6
Alfalfa hay	31.6	17.1
Prairie hay	31.6	52.1
Grain mix[1]	4.95	15.1
Salt		1.01
Predicted Nutrient Value		
DM, % as fed[2]	50.4	49.9
Amount fed, kg DM/d	13.07	4.22±0.8
CP, % DM	13.6	11.9
NDF, % DM	45.9	49.1
NEL, Mcal/d	16.2	4.8±0.6

[1] Grain mix contained corn grain, dicalcium phosphate, Se premix, Sodium bicarbonate, TM salt, Vit. A (30k per g), Vit. D (30k per g), Vit. E (20000).

[2] Water was added to both TMR diets to reduce the DM to 50%.

Table 3-2 Effects of subcutaneous injections of TRLP on plasma metabolites in energy restricted cows.

	Infusion Treatment			
	Con	TRLP	SEM	*P* value
BHBA, µmol/L	232	210	14.9	0.31
NEFA, µEq/L	368	413	28.6	0.28
Glucose, mg/dl	60.0	60.9	1.28	0.63
Insulin, ng/mL	0.562	0.674	0.10	0.44
Haptoglobin[1], µg/mL	3.24	3.80	0.28	0.19

[1] Natural log-transformation was used for statistical analysis, and back-transformation was computed for Haptoglobin.

Table 3-3 Effects of subcutaneous injections of TRLP on glucose tolerance test (GTT) in energy restricted cows.

	Infusion Treatment			
	Con	TRLP	SEM	*P* value
Glucose response, mg/dl x min				
AUC[1]	4023.73	4222.54	462.8	0.77
AUC[2]	5225.95	4932.27	738.5	0.79
Insulin response, ng/mL x min				
AUC[1]	77.322	73.894	32.59	0.94
AUC[2]	151.817	140.896	44.29	0.86
NEFA response, μEq/L x min				
AUC[1]	-4092.35	-2102.76	1080.8	0.25
AUC[2]	-14674.39	-11427.11	2033.6	0.31

[1] Area under the curve at 60 min post-dextrose bolus
[2] Area under the curve at 120 min post-dextrose bolus

Figure 3-1 Liver triglyceride (mg/g protein) concentration in cows administered subcutaneous treatments of TRLP during energy restriction. *P* = 0.76.