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Effects of glucagon, glycerol, and glucagon plus glycerol on gluconeogenesis, lipogenesis, and lipolysis in periparturient Holstein cows

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Effects of glucagon, glycerol, and glucagon plus glycerol on gluconeogenesis, lipogenesis, and lipolysis in periparturient Holstein cows

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To

My Mother

To

Ghada

Ali, Sarah, and Hassan

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ABBREVIATIONS

Acetyl-CoA carboxylase (ACC)

Acyl-CoA synthetase (ACS)

Adipose triglyceride lipase (ATGL)

AMP-activated protein kinase (AMPK)

ATP-citrate lyase (ACL)

Beta-hydroxybutyric acid (BHBA)

Carbohydrate response transcription factor (ChoRF)

Comparative gene identification-58 (CGI-58)

Conjugated linoleic acid (CLA)

Diacylglyceriols (DAG)

Dry matter intake (DMI)

Extracellular signal-related kinase (ERK)-2

Fatty acid synthase (FAS)

Fatty liver syndrome (FLS)

Fatty acid transport protein (FATP)

Fork head transcription factor (FOXO1)

Fructose-1,6-bisphosphatase (FBPase)

Glucocorticoid response unit (GRU)

Glucose 6-phosphatase (G6Pase)

Glucose response element (GRE)

Glycerolphosphate acyltransferase (GPAT)

Hepatocyte nuclear factor 4-alpha (HNF4-α)

Hormone-sensitive lipase (HSL)

Inhibitor kappa beta kinase beta (IKKβ)

Insulin response element (IRE)

Insulin receptor substrate 1 (IRS-1)

Interferon gamma (IFNγ)

Interleukin (IL)

Jun-N-terminal kinase (JNK)

Lipoprotein lipase (LPL)

Negative energy balance (NEB)

Nonalcoholic fatty liver disease (NAFLD)

Nonsterified fatty acids (NEFA)

Nuclear factor kappa B (NFκB)

p38 mitogen-activated protein kinase (MAPK)

Peroxisome proliferator activated receptor gamma (PPARγ)

Phosphodiesterase type 3B (PDE3B)

Phosphoenolpyruvate carboxykinase (PEPCK)

Polyunsaturated fatty acids (PUFA)

Profilerative receptor-gamma co-activator 1 (PGC-1)

Propionyl-CoA carboxylase (PCC)

Protein kinase A (PKA)

Pyruvate carboxylase (PC)

Pyruvate kinase (PK)

Sterol regulator element (SRE)

Sterol regulatory element binding protein-1 (SREBP-1)

Transforming growth factor beta (TGFβ)

Triacylglycerols (TAG)

Tumor necrosis factor-alpha (TNF-α)

CHAPTER 1: GENERAL INTRODUCTION

Thesis Organization

This thesis is presented as a manuscript prepared for the submission to the Journal of Dairy Science. It is prepared from research to fulfill the requirements for the Master of Science degree. This paper is complete by itself; it contains an abstract, introduction, materials and methods, results, discussion, conclusion, and references. The paper is entitled “Effects of glucagon, glycerol, and glucagon plus glycerol on gluconeogenesis, lipogenesis, and lipolysis in periparturient Holstein cows” and indicates the potential use of glucagon as a preventive agent of the fatty liver disease in transition dairy cows. The paper is preceded by a general literature review and followed by general conclusions, discussion, and recommendations for further future research. Finally, it is concluded by a list of references.

Introduction

With a prevalence up to 54% of a dairy herd (Jorritsma et al., 2001) and a morbidity and mortality up to 90% and 25%, respectively (Raoofi et al., 2001), fatty liver (hepatic lipidosis) causes a major economic loss for U.S dairy farmers (Littledike et al., 1981). Fatty liver is a metabolic disorder and develops during early lactation of dairy cows. Fatty liver disease affects the health status, productivity, and reproductive performance (Gerloffe and Herdt, 1984; Jorritsma et al., 2001). Cows with fatty liver also can develop many other side conditions such as ketosis, milk fever, udder edema, displaced abomasum, retained placenta, metritis, and mastitis

(Morrow, 1976; Drackley, 1999; Katoh, 2002; van Winden et al., 2003). Bobe et al. (2004) extensively reviewed the epidemiology, pathology, and etiology of fatty liver in dairy cattle. The molecular basis of the development of the disease was reviewed in detail (Nafikov et al., 2006).

This review will focus on the molecular and cellular changes that affect the three basic metabolic pathways involved in the development of fatty liver disease: gluconeogenesis, lipogenesis, and lipolysis. Then, it summarizes what is known about the involvement of tumor necrosis factor-alpha (TNF-α) in the development of fatty liver and in the control of the different metabolic pathways. Finally, the review will focus on the metabolic and cellular changes caused by glucagon in alleviating and preventing fatty liver development.

Gluconeogenesis

Suppression of gluconeogenesis is one of the major metabolic changes that occur during fatty liver condition in dairy cattle (Bobe et al., 2004). Activities of several hepatic enzymes were lower in fatty liver cows compared with control cows during the prepartal period (Murondoti et al., 2004; Rukkwamsuk et al., 1999a; Graber et al., 2010). Both phosphoenolpyruvate carboxykinase (PEPCK) and propionyl-CoA carboxylase (PCC) activities remain low in fatty liver cows after parturition (Rukkwamsuk et al., 1999a; Murondoti et al., 2004). As for pyruvate carboxylase (PC), its activity increased after calving for both control and fatty liver cows (Murondoti et al., 2004). Glucose 6-phosphatase (G6Pase) activity, on the other hand, showed a tendency to be higher in fatty liver cows than in control cows

(Murondoti et al., 2004). The authors suggested that rapid breakdown of stored liver glycogen of fatty liver cow is the reason for such high activity of G6Pase during high energy demanding postpartal period. Fructose-1,6-bisphosphatase (FBPase) activity showed no significant difference between controls and fatty liver cows (Murondoti et al., 2004). Others, however, have shown a tendency in FBPase activity to increase in the postpartal period (Rukkwamsuk et al., 1999a).

During the development of the fatty liver condition, the different hormones and nutrients that control gluconeogenesis including insulin, glucagon, TNF-α, glucose, and fatty acids; however, glucagon and TNF-α will be discussed in later section of this literature review.

Insulin. Because of its direct suppressant effect on PEPCK gene transcription as shown in H4IIE hepatoma cells (Granner et al., 1983; Beale et al., 1986), high insulin concentrations during the prepartal period could be a major player in the suppression of gluconeogenesis in postpartal fatty liver cows. Velez and Donkin (2005) showed that PEPCK mRNA concentrations increased significantly in the livers of cows experiencing low insulin concentrations as a result of feed restriction. The same study, however, showed no effect of feed restriction on PC mRNA concentrations. The molecular mechanism by which insulin suppresses PEPCK activity is better understood in nonruminant than ruminant animals. In rats, insulin suppresses PEPCK transcription through an insulin response element (IRE), which is a part of the glucocorticoid response unit (GRU) in the third region of the PEPCK promoter of the H4IIE hepatoma cells (O'Brien et al., 1990; Chakravarty and Hanson, 2007; Yabaluri and Bashyam, 2010). Insulin down-regulates PC expression in livers of diabetic rats

(Weinberg and Utler, 1980). Although the mechanism is not clearly understood, there is evidence that insulin promotes PC gene expression through IRE in the proximal promoter of the gene (Jitrapakdee et al., 1997). Insulin is also a potent suppressor of G6Pase; it lowers both the G6Pase mRNA concentrations and activity in rat livers (Argaud et al., 1996; Massillon et al., 1996).

Glucose. Another potential suppressant of gluconeogenic enzymes is the end product of the process itself, i.e., glucose. Glucose expresses an inhibitory effect on the transcription of PEPCK gene in rat hepatocytes through the first 490-bp region of the PEPCK gene promoter (Cournarie et al., 1999). This effect, however, can only take place if glucose is phosphorylated by glucokinase (Cournarie et al., 1999).

An insulin–independent effect of glucose on gluconeogenesis also is exerted through glucose activation of G6Pase gene expression in rat liver (Lange et al., 1994) (Massillon et al., 1996); in this case too, glucose phosphorylation by glucokinase is a condition for this effect to take place (Arguad et al., 1997).

Fatty Acids. Accumulating lipids in liver could be another factor contributing to the suppression of gluconeogenesis during the fatty liver condition in dairy cows (Cadórniga-Valiño et al., 1997). Aiello and Armentano (1988) provided evidence that rates of propionic acid conversion to glucose in goat hepatocytes increased in the presence of oleic acid compared with control of no oleic acid. The same study, however, showed that oleic acid has no effect on gluconeogenesis in calf hepatocytes. In another study, oleic acid previously or concurrently incubated with calf hepatocytes decreased propionic acid conversion to glucose by 24% compared with that in controls with no fatty acid treatment (Cadórniga-Valiño et al., 1997). A third

study showed that concurrent exposure of bovine hepatocytes to oleic acid has no effect on rates of gluconeogenesis from propionic acid (Strang et al., 1998). Finally, in a more recent study, Mashek et al. (2002) found that bovine hepatocytes treated with oleic acid have higher gluconeogenesis rates from propionic acid. On the basis of previous data, the effect of oleic acid on conversion of propionic acid to glucose seems relatively weak and is subject to different experimental conditions (Mashek et al., 2002). The effect of fatty acids other than long-chain monosaturated fatty acids on gluconeogenesis were studied too. Polyunsaturated fatty acids (PUFA) such as arachidonic acid (C22:6) inhibited propionic acid conversion to glucose or to cellular glycogen in bovine hepatocytes (Mashek and Grummer, 2004). The same group found that different conjugated linoleic acid (CLA) isomers have no effect on rates of gluconeogenesis from propionic acid in bovine hepatocytes (Mashek and Grummer, 2004). In nonruminants, rats fed high fat diets showed more FBPase protein concentrations and greater rates of alanine conversion to glucose in liver (Song et al., 2001). Authors suggested that elevated hepatic lipid perooxidation rates activated the nuclear factor kappa B (NFκB), which is a regulator of hepatic FBPase gene expression (Fong et al., 2000). As for PEPCK, oleic acid was found to be a potent stimulator of PEPCK gene expression in rat adipocytes (Antras-Ferry et al., 1995). Free fatty acid stimulating effect on PEPCK is mediated by p38 mitogen-activated protein kinase (MAPK) (Collins et al., 2006). G6Pase gene expression also is up-regulated by fatty acids (Massillon et al., 1997; Charelain et al., 1998). In vitro, PUFA suppressed G6Pase transcription in HepG2 hepatoma cells by inhibiting the hepatocyte nuclear factor 4-alpha (HNF4-α), which is an activator of the promoter for

the G6Pase gene (Rajas et al., 2002). Short-chain fatty acids induced G6Pase gene expression in H4IIE hepatoma cells by activating HNF4-α (Massillon et al., 2003). On the other hand, NEFA seem to regulate PC mRNA expression through specific activation of PC promoter 1 (White et al. 2010).

Lipogenesis

Although esterification of plasma nonsterified fatty acids (NEFA) contributes up to 60% of the triacylglycerols (TAG) accumulating in livers of human patients with nonalcoholic fatty liver disease (NAFLD), de novo lipogenesis, on the other hand, is not unimportant; it accounts for about 26% of the TAG in livers of patients with NAFLD (Donnelly et al., 2005). In normal healthy human livers, the contribution of de novo lipogenesis to TAG synthesis did not exceed 5% (Timlin et al., 2005); however, patients with both insulin resistance conditions and NAFLD showed a five-fold increase in lipogenesis compared with that of normal subjects. This comparison indicates that greater rates of lipogenesis contribute to the fatty liver condition (Schwarz et al., 2003). As for adipose tissue, the other major anatomical site of lipogenesis, despite its active lipogenesis, its contribution to TAG storage in liver is relatively minor compared with hepatic de novo lipogenesis (Diraison et al., 2003b). Hepatic lipogenesis significantly increases with high insulin plasma concentrations associated with high carbohydrate diets (Diraison et al., 2003a).

In ruminants, lipogenesis mostly takes place in adipose tissue rather than in liver; both liver and adipose tissues are significant sites of lipogenesis in nonruminants (Bauman, 1976). However, de novo lipogenesis in adipose contributes

only to about 30-35% of adipose deposition in ruminants (Vernon, 1983); the reminder of stored TAG is derived from the diet. The contribution of de novo lipogenesis to fatty liver development in ruminants has not yet been studied. Recently, it was shown that the expression of genes coding for lipogeneic enzymes decreases in adipose tissue between 30 d prepartum and 14 d in milk in first-lactation dairy cows (Sumner-Thomson et al., 2011). The rate of fatty acid esterification in adipose tissue of overfed cows significantly increases before parturition (Rukkwamsuk et al., 1999b). In addition, unrestricted feeding increases hepatic esterification rates of fatty acids by raising glycerolphosphate acyltransferase (GPAT) activity in the prepartal period of dairy cows (van den Top et al., 1996); lipogenesis in adipose tissue, however, is suppressed significantly after calving (van den Top et al., 1996; Rukkwamsuk et al., 1999b; Murondoti et al., 2004). Integration of the activation of lipogenesis by high carbohydrate diets and high insulin concentrations in nonruminants (Tamura and Shimomura, 2005) in addition to the increased esterification of fatty acids in adipose tissue and livers of ruminants (van den Top et al., 1996; Rukkwamsuk et al., 1999b) could shed some light on the contribution of de novo lipogenesis in adipose tissue to development of fatty liver in cattle.

Lipogenesis in adipose tissue is controlled by different nutritional and hormonal factors; the next four parts will cover regulation by insulin, leptin, glucose, and PUFA. Effect of glucagon and TNF-α on lipogenesis will be discussed under later separate topics.

Insulin. Insulin stimulates lipogenesis in liver and adipose tissue of both ruminants and nonruminants (Vernon, 1983). High energy, unrestricted diets during the prepartal

period of dairy cows increases plasma insulin concentrations, and, as a consequence, lipogenesis rates increase during that particular period (Rukkwamsuk et al., 1999b). Insulin activates lipogenesis by two different mechanisms that explain short-term and long-term effects (Kresten, 2001). Acetyl-CoA carboxylase (ACC), a key enzyme in lipogenesis pathway, is activated indirectly by insulin (Naka and Accili, 1999). Insulin inhibits both cAMP-dependent protein kinase A (PKA) and AMP-activated protein kinase (AMPK), which are two enzymes responsible for phosphorylation and thus activation of ACC (Allerd and Reily, 1997; Munday, 2002). As for the long-term effects, insulin activates the expression of ACC by activating several transcription factors, depending on the specific tissue (Kresten, 2001). Transcription factor called the sterol regulatory element binding protein-1 (SREBP-1) is the most well defined transcription factor that is activated by insulin in both hepatocytes (Kim et al., 1998; Zhao et al., 2010) and adipocytes (Fortez et al., 1999) in both ruminants and nonruminants (Travers et al., 1997). In turn, activated SREBP-1 activates ACC gene transcription (Azzout-Marniche et al., 2000). Several mechanisms have been proposed to explain how insulin activates SREBP-1. Azzout-Marniche et al. (2000) suggested that SREBP-1 mRNA expression is activated by insulin through a phosphatidylinositol 3-kinase-mediated pathway. Direct phosphorylation and activation of SREBP-1 by AMPK in the course of insulin signal transduction is a second suggested mechanism (Roth et al., 2000). A third mechanism proposed by Horton et al. (1998) suggests that the insulin signal activates the proteolytic cleavage of membrane-bound SREBP-1 and thus increases the active free form of SREBP-1. Up-stream stimulatory factors (USFs) are other transcriptional factors activated by

insulin signal and, in turn, stimulate ACC gene expression (Kresten, 2001). In ovine adipose tissue, USF bind to the E-box motif, which is a part of the proximal promoter of ACC-α gene, and thus activates ACC gene expression (Travers et al., 1997). In adipose tissue, a third transcription factor involved in adipocyte differentiation also can be activated by insulin signal. That factor is the peroxisome proliferator-activated receptor gamma (PPARγ) (Vidal-Puig et al., 1997). PPARγ controls the expression of several fatty acid metabolism-related enzymes such as lipoprotein lipase (LPL), fatty acid transport protein (FATP), acyl-CoA synthetase (ACS), and PEPCK (Yoon et al., 2000). In addition, only small fat deposition in the mutant PPARγ mice fed a high carbohydrate diet also suggests a lipogenic role of PPARγ (Kubota et al., 1999; Miles et al., 2000).

ATP-citrate lyase (ACL) also is controlled by short- and long-term effects of insulin (Park et al., 1994). As mediated by AMPK, insulin activates ACL by increasing phosphorylation of its tryptic peptide A and by decreasing phosphorylation of its peptide B (Ramakrishna et al., 1989). Insulin also stimulates ACL gene expression through the -104 to -20 bp region of the ACL promoter (Fukuda et al., 1996).

Fatty acid synthase (FAS) is another lipogenic enzyme controlled by insulin (Fukuda et al., 1999). Unlike ACC and ACL, insulin does not have a short-term effect on FAS; instead, insulin controls FAS at the transcriptional level (Sul and Wang, 1998). Both USFs and SREBP-1 are involved in insulin stimulation of FAS gene expression (Griffin and Sul, 2004). An E-box at -65 was identified in the proximal

promoter region of FAS gene (Sawadogo and Roeder, 1985). This E-box was found to be associated with insulin activation of FAS expression in mice (Wang and Sul, 1995). As for SREBP-1, this protein stimulates FAS gene expression by binding to sterol regulator element (SRE) at -150 of the proximal promoter region of the FAS gene (Kim et al., 1998).

High concentration of insulin also activates GPAT, the enzyme responsible for esterification (Shin et al., 1991). Insulin action on GPAT could be mediated by SREBP because SREBP binding sites are located in the promoter of the GPAT gene (Ericsson et al., 1997). The identification of an E-box at the -320 bp region of the GPAT promoter suggests involvement of USFs in GPAT gene expression in response to the insulin signal (Jerkins et al., 1995).

Leptin. Leptin, a hormone produced by adipocytes (Friedman and Halaas, 1998), inhibits lipogenesis in porcine (Ramsay, 2003) and ovine adipocytes (Newby et al., 2001). In their micro-array study, Soukas et al. (2000) showed that many of the lipogenic-related genes in mice white adipose tissue such as the FAS, ACL, and SREBP-1 genes are all suppressed by leptin. Suppression by leptin suggests the involvement of SREBP-1 in leptin suppression of FAS gene expression (Kakuma et al., 2000; Nogalska et al., 2005). Recent studies showed that leptin plasma concentrations decrease significantly on day 1 postpartum in dairy cows (Sadri et al., 2010). Decreased leptin concentrations in plasma is accompanied by expression of ACC and FAS.

Glucose. Glucose stimulates lipogenesis by different ways (Kresten, 2001). Glucose is a precursor of acetyl-CoA, the primary substrate for the lipogenic pathway; thus,

high glucose concentrations stimulate lipogenesis through greater substrate availability (Vernon, 1983). Furthermore, high plasma glucose concentrations are associated with high insulin concentrations that ultimately stimulate lipogenesis as discussed previously (Kresten, 2001). Glucose, however, can stimulate gene expression of some lipogenic enzymes in an insulin-dependent manner by recruiting the carbohydrate response transcription factor (ChoRF), which binds to the glucose response element (GRE) (Foufelle et al., 1996). GRE, a lipogenesis related nuclear protein, was identified in the -1601 to -1395 region of the S14 protein gene promoter in rat hepatocytes (Shih and Towle, 1994). A homologous sequence to GRE that can interact with glucose also was identified in both FAS and pyruvate kinase (PK) genes (Foufelle et al., 1996; Koo and Towle, 2000).

PUFA. PUFA suppress the transcription of several different lipogenic enzymes (Jump et al., 1994). The expression of ACC (Fukuda et al., 1992), ACL (Fukuda et al., 1996), and FAS (Blake and Clarke, 1990) are all suppressed by PUFA. PUFA control ACL gene expression through a PUFA-responsive region in the proximal part of ACL gene promoter (Fukuda et al., 1996). As for FAS, PUFA destabilizes the FAS mRNA and thus the decay of SREBP-1 mRNA in the cell; this response results in less activation of lipogenesis by SREBP-1 (Xu et al., 2001; Howell et al., 2009).

Lipolysis

To meet the high energy requirement of milk production and especially during the pospartal negative energy balance, adipose tissue accelerates the rates of lipolysis just prior to parturition and even more so during early lactation period (McNamara

and Hillers, 1986) because of the increased activity of hormone-sensitive lipase (HSL) during this peripartal period (McNamara, 1988).Overfeeding cows during the dry period increases lipid deposition in adipose tissue. This response to overfeeding provides more substrate for lipolytic enzymes during the early postpartal period, and thus more NEFA per unit time will be released into the blood (van den Top et al., 1995). In liver, NEFA are esterified to form TAG, which then are usually released into blood in the form of very low density lipoproteins (VLDL) (Gruffat et al., 1996). Unusually low release of VLDL results in the accumulation of TAG in liver and development of fatty liver disease (Bobe et al., 2004). On the other hand, high concentrations of NEFA in blood also result in high concentrations of β-hydroxybutyrate, a potent inhibitor of lipolysis (Metz et al., 1972). In addition, overfeeding increases the concentrations of insulin (Bobe et al., 2004). Insulin is a potent inhibitor of HSL. Thus, insulin could be a major for control of lipolysis in the prepartal period (Rukkwamsuk et al., 1998).

Insulin and catecholamines are the primary hormones available for control of lipolysis (Hayrili et al., 2002). Other factors and hormones that affect lipolysis, such as glucagon and TNF-α, will be reviewed in a later section of this review of literature. Holm et al. (2003) reviewed extensively the regulation of adipocyte lipolysis by catecholamines and insulin. Binding of catecholamines like epinephrine and norpeinephrine to membrane receptors on the adipocytes stimulate positive coupling of Gs protein with adenylyl cyclase for greater production of cAMP. High concentrations of cAMP activate PKA, which is responsible for the phosphorylation and thus activation of both the HSL and the perilipins (Holm, 2003). Perilipins form a

barrier layer by binding to the surface of the lipid droplet; this barrier protects the droplet from breakdown by lipases (Londos et al., 1996). However, when phosphorylated, the perilipins barrier becomes less tight and allows for initiation of the activated HSL to pass through to the surface of the lipid droplet and start the TAG hydrolysis action (Souza et al., 1998). Phosphorylated perilipins increase in early lactation stage in dairy cows (Elkins and Spurlock, 2009). Insulin, on the other hand, inhibits lipolysis by inducing phosphorylation and activation of phosphodiesterase type 3B (PDE3B), which can degrade cAMP to 5′ AMP. The diminishing cAMP concentrations decrease the PKA activity and, in turn, slow the lipolytic rates (Degerman et al., 1997).

Adipose triglyceride lipase (ATGL), a recently discovered enzyme that is responsible for TAG breakdown to diacylglyceriols (DAG) in mammalian adipose tissue, was extensively reviewed (Zechner et al., 2009). Similar to HSL, β-adrenergic stimulation of lipolysis is not accompanied by increased gene expression to ATGL. Increased cAMP cellular concentration in response to β-adrenergic stimulation results in phosphorylation of perilipin A on fat droplet. Comparative gene identification-58 (CGI-58) dissociates from phosphorylated perilipin A and thus activates ATGL. Like HSL, insulin down regulates ATGL gene expression; however, the mechanism remains unclear.

Glucagon Treatment Effect

Because of its ability to increase glucose concentrations in the blood of dairy cattle that, in turn, increase plasma insulin concentrations, which ultimately inhibit

lipolysis in adipose tissue, glucagon makes a good candidate to treat or prevent fatty liver in dairy cattle (De Boer et al., 1986). Glucagon increases plasma glucose concentrations by stimulating hepatic gluconeogenesis, glycogenolysis, amino acid uptake, and ureagenesis (Flakoll et al., 1994; Donkin and Armentano, 1995). Continuous intravenous infusions of glucagon for 14 days to normal lactating dairy cattle increased both insulin and glucose concentrations in blood; however, NEFA, BHBA, and urea concentrations did not change(She et al., 1999a). According to these results, the researchers concluded that glucagon enhanced both glycogenolysis and gluconeogenesis but not lipolysis. On the other hand, intravenous infusion of the same amount of glucagon for 14 days into cows with the fatty liver condition increased glucose concentrations but did not affect the already high blood insulin concentrations. The infused glucagon also decreased the glycogen content in the liver of those cows (Hippen et al., 1999). Also, glucagon infusions decreased NEFA and TAG release into plasma. These results lead to the conclusion that glucagon injections alleviate the fatty liver condition by decreasing lipolysis rates (Hippen et al., 1999). In the same study, She et al. (1999b) showed that the 14-day glucagon injections into normal cows increased plasma insulin; the high insulin, in turn, decreased the PEPCK mRNA concentrations within one week. This decrease in PEPCK mRNA, however, was not manifested in dairy cows with fatty liver.

Because glucagon intravenous infusions are difficult to apply in field conditions, Bobe et al. (2003a) investigated the use of subcutaneous glucagon injections as an alternative to treat fatty liver condition in dairy cattle. Three equally timed subcutaneous glucagon injections for 14 days beginning on day 8 postpartum

increased both glucose and insulin concentrations in plasma and decreased plasma NEFA concentrations. Liver TAG concentration also was decreased (Bobe et al., 2003a; Bobe et al., 2007). Also, the glucagon injection protocol tended to increase phospholipids and free cholesterol concentrations in liver when the ambient temperature below 35°C but not above 35°. Concentrations of VLDL-TAG, high-density lipoprotein$_1$- phospholipids, and high-density lipoprotein$_2$-free cholesterol all tended to decrease in response to glucagon injections during ambient temperature above 35°C. However, during ambient temperature below 35°C, only VLDL-TAG tended to decrease (Bobe et al., 2003a; Bobe et al., 2007).

The previous research suggested that the use of glucagon could be extended from treatment of the fatty liver condition to prevention of the disease in highly susceptible dairy cattle. In a later study, Nafikov et al. (2006) investigated the effect of subcutaneous injections of glucagon for 14 days to dairy cow, starting the injections at day 2 postpartum rather than at day 8 postpartum. The early postpartal glucagon injections prevented TAG accumulation in the livers of dairy cows that have been subjected to a prepartal fatty liver-promoting diet. Accumulation of TAG occurred with untreated cows given the same prepartal diet. In addition, both glucose and insulin concentrations in plasma increased in response to the injection, whereas NEFA concentrations decreased. This study revealed the effectiveness of subcutaneous glucagon injections in preventing fatty liver development in transition dairy cattle (Nafikov et al., 2006). Overall, the alleviation or prevention of fatty liver in transition dairy cows seems based on particularly gluconeogenesis, lipogenesis, and lipolysis in liver and/or adipose tissue.

In his recent study, Osman et al. (2010) investigated the chronic effects of subcutaneous injections of glucagon oral administration of glycerol for 14 days to dairy cows starting as early as calving rather than at day 2 postpartum. The early postpartal glucagon injections increased plasma glucose and plasma glucagon concentrations and decreased plasma NEFA concentrations but did not affect plasma TAG concentrations. Similar results were recorded when cows treated with both glucagon injections and glycerol administration. Oral glycerol decreased plasma glucagon concentration; however, it did not affect plasma glucose, NEFA, or TAG concentrations. It was concluded that glycerol actually potentiates the glucagon effect, and thus glucagon plus glycerol is a more efficient treatment than glucagon injection alone. In addition, acute effects of glucagon injections and glycerol administration also were measured (Osman et al., 2010) Serial blood samples were collected during 8-hr period after starting glucagon and/or glycerol treatment. The early postpartal glucagon injections increased plasma glucose and plasma glucagon concentration and decreased plasma NEFA concentration and increased plasma glucose concentration; however, plasma glucagon concentration decreased. It was suggested that glucagon and glycerol have additive effects when used together to decrease the incidence of diseases developed as a result of high energy status of the cow.

Gluconeogenesis. Glucagon can increase glucose concentrations in plasma by directly activating key enzymes for regulation of gluconeogenesis pathway. Jianag and Zhang (2003) extensively reviewed the role of glucagon in regulation of glucose metabolism. Glucagon increases the concentrations of cellular cAMP. This change leads to the activation of PKA, which, in turn, phosphorylates the cAMP-response

element-binding (CREB) protein. This phosphorylated and active form of CREB binds to the cAMP-response element in the promoter region of peroxisome profilerative receptor-gamma co-activator 1 (PGC-1) gene and thus produces more PGC-1 (Herzig et al., 2001). The PGC-1, in turn, recruits the nuclear transcription factor hepatocyte nuclear factor-4 (HNF-4) (Yoon et al., 2001) and fork head transcription factor (FOXO1) to bind to the PEPCK promoter and induce the PEPCK gene expression (Puigserver et al., 2003). In addition, Hod and Hanson (1988) provided evidence that high concentrations of cAMP induced by glucagon actually stabilizes the mRNA of PEPCK and thus increases its translation in gluconeogenic cells.

A cAMP response element was identified in the G6Pase hydrolytic subunit gene promoter in H411E hepatoma cells (Schmoll et al., 1999). Furthermore, Thiel et al. (2005) indicated the involvement of CREB in the activation of G6Pase in response to cAMP. These actions suggest that glucagon regulates both PEPCK and G6Pase in a similar pattern. FBPase1, on the other hand, is activated indirectly by glucagon. Glucagon activates PKA that, in turn, phosphorylates and inactivates the FPK2/FBPase2, resulting in alleviation of the inhibitory effect of fructose (2,6) bisphosphate on FBPase1 (Pilkis et al., 1982).

Lipid Metabolism. Glucagon also is involved in regulation of lipogenesis (Beynen et al., 1996). Glucagon activates AMPK, which, in turn, phosphorylates and inactivates ACC (Holland et al., 1985; Swenson, 1985). On the other hand, glucagon down regulates ACC gene transcription initiated from both promoters 1 and 2 (Hillgartner et al., 1997; Yin et al., 2000). As for lipolysis, lipolytic effects of glucagon are more

controversial (Vernon, 1983). Positive lipolytic effects of glucagon in rats were demonstrated clearly; physiological concentrations of glucagon, however, showed no lipolytic effects in ruminants. A minor lipolytic response was noticed in caprine adipose tissue exposed to supra-physiological concentrations of glucagon (Vernon, 1983). In humans, glucagon infusions in healthy males increased plasma NEFA concentrations (Carlson et al., 1993). However, by using the in situ microdialysis technique of glucagon administration, other researchers demonstrated that glucagon did not affect the lipolysis rates in either human abdominal adipose tissue (Gravholt et al., 2001) or human subcutaneous adipose tissue (Bertin et al., 2001).

Lipolytic effects of glucagon were reported in different studies by using rat (Slavin et al., 1994) and chicken adipocytes (Oscar, 1991). These lipolytic effects were mediated by increased intracellular concentrations of cAMP in response to glucagon and the subsequent change in the phosphorylation status of HSL and ultimately its activation (Slavin et al., 1994). Finally, Slavin et al. (1994) found an evidence that glucagon can directly affect the expression of the HSL gene.

Tumor Necrosis Factor-alpha

TNF-α is a 26 kDa plasma membrane-bound monomeric protein (Kriegler et al., 1988). The TNF-α is cleaved and secreted by the activity of TNF-α converting enzyme (TACE) (Black et al., 1997). TNF-α has two distinct receptors called 1 and 2 (Loetscher et al., 1990). Both receptors complex with cytoplasmic adapter proteins, which, in turn, activate stress-related kinases such as Jun-N-terminal kinase (JNK), inhibitor kappa beta kinase beta (IKKβ), MAPK, and protein kinase B/AKT. These

proteins control the activity of other transcription factors such as transcription NFκB and insulin receptor substrate 1 and 2 (IRS-1 and IRS-2) (Wajant et al., 2003).

TNF-α is regulated by different factors like adiponectin produced by adipocytes (Fernandez-Real et al., 2003). Vassalli (1992) and Tracey and Cerami (1993) reviewed TNF-α interactions with other cytokines. Different cytokines have different effects on TNF-α. Interleukin 10 (IL-10), IL-5, and transforming growth factor beta (TGFβ) all down regulate TNF-α, whereas interferon gamma (IFNγ) and IL-12 activate TNF-α (Vassalli, 1992; Tracey and Cerami, 1993). Elenlkov et al. (2000) reviewed TNF-α regulation by neuroendocrine secretions. Norepinephrine, epinephrine (van den Poll et al., 1996), histamine (Vannier et al., 1991), and adenosine (Prabhakar et al., 1995) all inhibit the production of TNF-α (Elenkov et al., 2000).

In their study, Sordillo et al. (1995) showed that mononuclear cells isolated from peripheral blood and supramammary lymph nodes of periparturient dairy cows produced more TNF-α than did those cells isolated from mid to late lactating dairy cows. Other studies showed that TNF-α concentrations significantly increase in the weeks close to parturition (Rontved et al., 2005; Ametaj et al., 2003). On the other hand, Kushibiki et al. (2000) established an insulin resistance effect of TNF-α in dairy heifers. A high correlation between serum TNF-α concentrations and insulin resistance was calculated in dairy cows with fatty livers. This correlation suggests a role of TNF-α in the development of insulin-resistance and subsequent fatty liver condition in dairy cattle (Ohtsuka et al., 2001).

Insulin Resistance. In their reviews, Diehl (2004) and Nieto-Vazquez et al. (2008) summarized the molecular mechanisms that TNF-α uses to initiate insulin resistance that leads to NAFLD in humans and animals. Briefly, TNF-α activates several stress-related protein kinases such as JNK, p38 MAPK, IKKβ, protein kinase B/AKT, and rapomycin-dependent kinase that control serine phosphorylation of IRS-1 and IRS-2. After phosphorylation, the IRSs become less responsive to insulin signal, leading to insulin resistance and ultimately NAFLD (Ruan et al., 2003). IKKβ, activated by TNF-α, is a complex of several factors; one of them is NFκB that activates TNF-α gene transcription (Pauli, 1994). Furthermore, NFκB interacts with and inhibits PPARγ; PPARγ is vital for adipocyte differentiation. This inhibition of PPARγ action will decrease the differentiation of adipocytes. As a result, adiponectin release decreases, and thus its antagonistic activity against TNF-α diminish and cellular TNF-α concentrations remain high enough to induce insulin resistance (Ruan et al., 2003). Adiponectin, on the other hand, inhibits fatty acid uptake in hepatocytes and activates lipoprotein export from liver (Yamauchi et al., 2002). Inhibition of adiponectin by NFκB induced by TNF-α ultimately leads to more TAG accumulation in the liver (Arner, 2003). In a different mechanism, TNF-α activates PTP1B (Nieto-Vazquez et al., 2008). PTP1B down regulates IRS-1.

Glucose and Lipid Metabolism. In addition to its direct effect on insulin action, TNF-α contributes to insulin resistance indirectly by activating lipolysis and increasing the concentrations of plasma NEFA (Green et al., 2004). TNF-α stimulates lipolysis by different biochemical mechanisms. It increases the intracellular

concentrations of cAMP. Higher concentrations of cAMP activate PKA, which, in turn, phosphorylates perilipins. This phosphorylation will weaken their attachment to fat droplet and increase the accessibility for fat droplet to HSL (Souza et al., 2002). In rats, TNF-α down regulates the expression of the antilipolytic GTP-binding membrane protein $G\alpha_i$. Decreasing $G\alpha_i$ protein concentrations alleviate their inhibitory effect on adenylyl cyclase and ultimately increases the intracellular cAMP concentrations (Gasic et al., 1999). In humans, TNF-α uses a different pathway to increase cellular cAMP concentrations. TNF-α activates the MAPK including the extracellular signal related kinase (ERK)-2 (p42/p44) (Wallach et al., 1999). ERK-2, in turn, decreases the expression of cyclic nucleotide phosphodiesterase 3B (PDE3B), which decreases cAMP conversion to 5′AMP. This response will increase intracellular concentrations of cAMP (Zhang et al., 2002).

In 313-L1 cells, TNF-α is shown to use different cellular mechanism to stimulate lipolysis. TNF-α directly inhibits perilipin gene expression and thus gives HSL more accessibility to fat droplets (Souza, et al., 1998; Souza, et al., 2003). A third mechanism that TNF-α uses to increase lipolysis is by directly activating HSL. Greenberg et al. (2001) showed that TNF-α signal passes through MAPK and p44/42 pathway in 313-L1 adipocytes cells to phosphorylate and thereby activate HSL. TNF-α seems to down regulate ATGL mRNA through a PPARγ inhibition mediated mechanism (Zechner et al., 2009).

As for lipogenesis, hepatic and adipocytic lipogenesis show opposite response to TNF-α. Both ACC and FAS gene expression in adipocytes are down regulated by

TNF-α (Doerrler et al., 1994). This down regulation will stop the differentiation of pre-adipocytes to fully mature fat cells (Pape and Kim, 1988; Pape and Kim, 1989). Hepatic lipogenesis, on the other hand, is stimulated by TNF-α (Grunfeld et al., 1990; Grunfeld et al., 1991). TNF-α raises the concentrations of hepatic citrate, which, in turn, activates ACC (Grunfeld et al., 1988). Recent studies (Cawthorn and Sethi, 2008) should that TNF-α down regulates expression of FFA transport protein (FATP) and FFA translocase (FAT). FATP and FAT down regulation result in peroxisomal β-oxidation inhibition and lipogenesis stimulation (Chen et al., 2009).

Finally, the rate of gluconeogensis decreases in response to TNF-α during septic shocks (Hill and McCallum, 1992). Daily injection of TNF-α to lactating dairy cows increased transcript abundance of G6Pase and PEPCK (Bradford et al., 2009). PEPCK gene expression in rat hepatocytes is down regulated when incubated with TNF-α (Goto et al., 2001). TNF-α attenuates insulin suppression effect on PEPCK gene expression by altering cellular localization of forkhead nuclear transcription factor Foxa2 (Pandey et al., 2009). G6Pase gene expression also is decreased in H4IIE hepatoma cells when incubated with TNF-α. This response suggests that NFκB activation by TNF-α suppresses G6Pase gene expression (Grempler et al., 2004).

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CHAPTER 2. EFFECTS OF GLUCAGON, GLYCEROL, AND GLUCAGON PLUS GLYCEROL ON GLUCONEOGENESIS, LIPOGENESIS, AND LIPOLYSIS IN PERIPARTURIENT HOLSTEIN COWS[1]

A paper to be submitted to the Journal of Dairy Science

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Abstract

The objective of this study was to investigate the effect of daily glucagon subcutaneous injections of 15 mg and oral administration of 400 mL glycerol during the first 14 d postpartum on the rates of gluconeogenesis in liver and lipogenesis and lipolysis in adipose tissues obtained from dairy cows with fatty liver syndrome (FLS). To induce FLS, 16 multiparous cows with body condition score of ≥ 3.5 (1-5 scale) cows were daily fed dry-cow ration plus 6 kg of cracked corn during the last 6 weeks

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of dry period. Cows were assigned equally and randomly to 4 treatment groups: saline, glycerol, glucagon, and glucagon plus glycerol. Liver and adipose tissue slices were incubated with 10 nM glucagon and 1 nM tumor necrosis factor-alpha (TNF-α). Glucose synthesis rates from propionic acid and alanine were measured in liver, and glycerol release rates and fatty acid synthesis rates were measured in adipose tissues. Glucagon injections and glycerol oral administration did not affect the rates of gluconeogensis or lipolysis. Lipogenesis rates tended to decrease with glucagon and glycerol treatments. Incubation of adipose and liver tissues with TNF-α and glucagon did not affect glucose synthesis rates or fatty acid breakdown or synthesis in adipose tissue. We conclude that glucagon and glycerol treatments alleviate the symptoms of FLS by long-term effects rather than direct effects on liver or adipose metabolic pathways.

Introduction

FLS is a metabolic disorder that affects dairy cows during the transition stage between late prepartal and early postpartal periods (Drackley, 1999). With a prevalence up to 54% (Jorritsma et al., 2001) and a morbidity and mortality up to 90% and 25%, respectively (Raoofi et al., 2001), FLS causes a major economic loss for U.S dairy farmers (Littledike et al., 1981). FLS increases the risks of other periparturient diseases including ketosis, endometritis, retained fetal membranes, displaced abomasums, mastitis, and milk fever (Katoh, 2002; van Winden et al., 2003). Decreased reproductive success (Jorritsma et al., 2000) and suppressed immune functions (Wentink et al., 1999) are long-term effects of FLS. Between low

dry matter intake (DMI), 70% of normal, and increased demand for glucose at the onset of lactation, transition dairy cows develop negative energy balance (NEB) (Herdt, 2000). To meet nutrient and energy requirements under NEB status, the dairy cow mobilizes fatty acids reserves in adipose tissue, which results in NEFA accumulation in blood (Grummer, 1993; Drackley, 1999). Blood NEFA are mainly taken up by the liver where they are either oxidized in mitochondria to produce energy or esterified into triacylglycerol (TAG). Triacylglycerol is either directly stored in liver or released in blood in the form of TAG-rich very low density lipoproteins. When the rate of TAG storage in liver is higher than rates of NEFA oxidation to CO_2 and to ketone bodies and rates of TAG-rich very low density lipoprotein release, then FLS develops (Grummer, 1993).

TNF-α concentrations significantly increase before parturition (Rontved et al., 2005; Ametaj et al., 2003). Serum TNF-α concentrations are highly correlated with insulin resistance in dairy cows with fatty livers (Kushibiki et al., 2000). This correlation suggests a role of TNF-α in the development of insulin resistance and subsequent fatty liver condition in dairy cattle (Ohtsuka et al., 2001). High TNF-α concentration increases insulin resistance by triggering insulin receptor substrates (IRSs) phosphorylation (Ruan et al., 2003) and stimulates lipolysis by increasing hormone-sensitive lipase (HSL) accessibility to fat droplet (Souza et al., 2002). Adipose tissue is the major site of lipogenesis in ruminants (Bauman, 1976); de novo lipogenesis in adipose contributes only to about 30-35% of adipose deposition in ruminants (Vernon, 1983); the remainder of stored triacylglycerol (TAG) is derived

from the diet. The contribution of de novo lipogenesis to fatty liver development in ruminants, however, remains unclear.

Feeding high energy diets, feeding glucogenic precursors or slow-release insulin treatments do not alleviate FLS conditions (Hayrili et al., 2002; Douglas et al., 2004; Hoedemaker et al., 2004). Multiple glucagon intravenous infusions (Hippen et al., 1999) or subcutaneous injections (Bobe et al., 2003a) could treat or prevent (Nafikov et al., 2006) experimentally induced FLS by up regulating gluconeogenesis (She et al., 1999) and down regulating adipose tissue lipolysis (Hippen et al., 1999). Glucagon injections induce gene expression of phosphoenolpyruvate carboxykinase and pyruvate kinase in early lactating cows (Bobe et a., 2009). When co-administered with glucagon subcutaneous injections, oral glycerol potentiates glucagon preventive action (Osman et al., 2008; Osman et al., 2010). Orally administered glycerol is metabolized in the rumen and liver to VFA and in liver to intermediates of gluconeogenesis and glycolysis (Remond et al., 1993; Goff and Horst, 2001; Osman et al., 2008).

The effect of glucagon on gluconeogenesis, lipogenesis, and lipolysis was determined by measuring blood concentrations of glucose, NEFA, TAG, and BHBA. The effect of glucagon injections on the rates of these pathways, however, is still to be determined. Therefore, we hypothesized that subcutaneous injection of glucagon alleviates FLS symptoms by affecting the rates of gluconeogenesis in liver and the rates of lipolysis and lipogenesis in adipose tissue directly. The objective of this study is to measure the rates of the three metabolic pathways before and after glucagon treatment and under different hormonal factors such as TNF-α and epinephrine.

Materials and Methods

Experimental Design

The experimental design was described previously (Osman et al., 2008). Briefly, 16 multiparous Holstein cows with a BCS ≥3.5 were selected from the Iowa State University dairy herd. Experimental FLS was induced by giving cows ad libitum access to a dry-cow TMR supplemented with 6 kg of cracked corn during the last 6 wk of the dry period. Right after parturition, experimental cows were housed in a sand-bedded tie stall and offered ad libitum access to an NRC (2001) recommended TMR for high-producing dairy cows twice a day. Four hours after parturition, 16 cows were injected with 60 mL of 0.9 M NaCl solution (pH 10.25) that contained either 0 (control: n = 4; glycerol: n = 4) or 5 (glucagon: n = 4; glucagon plus glycerol: n = 4) mg of glucagon. Cows were injected s.c every 8 h for 13 d and 16 h apart on d 14 and received a single injection on d 15 postpartum. Glycerol-treated cows received orally 400 mL of pure glycerol (West Central Inc., Ralston, IA) diluted with 100 mL of water once a day at 0600 h for 14 d. The presented results are from 14 cows that had prepartal liver samples. The remaining 6 cows in the study calved before they were expected, and thus no prepartal liver sample had been collected. All the experimental and surgical procedures used in this study were approved by the Iowa State University Institutional Animal Care and Use Committee.

Liver and Adipose Samples

Liver samples were collected 4 days before calving and 9 days after calving. Cows were anesthetized locally with lidocaine and a biopsy needle was inserted

through a 12 to 15 mm incision made through the skin at either the 11^{th} or 12^{th} intercostal space. Liver biopsy was monitored by ultrasound (Aloka Micrus 500, Corometrics Inc., Wallingford, CT) to avoid puncture of internal organs and any major blood vessel. About two grams of liver tissue were collected at each entry of the body. About two grams were frozen in liquid nitrogen (-80 °C) for DNA quantitation. The liver sample used for incubation was stored on ice in a 150 mM sodium chloride, 150 mM sodium phosphate (pH 7.2) solution. Adipose tissue was collected from the tail head region after local anesthesia with lidocaine 7 days before calving and 4 days after calving and stored in phosphate-buffered saline at 37 °C for less than 30 min until incubations.

Incubation Procedures

Incubation procedures to measure gluconeogenesis rates have been previously described (Mills et al., 1986). Briefly, thin slices of liver tissue (80-150 mg) were incubated for 2 hr at 37 °C in 3 mL of calcium-free Krebs-Henseleit buffer (pH 7.4) containing 30 μmol of alanine or propionic acid and at least 1 μCi of L[1,3-^{14}C] alanine or L[3-^{14}C] propionic acid . Treatments were added either 1 nM rec-TNF-α or 10 nM glucagon. Reactions were stopped by 1 M perchloric acid. Incubations continued for another hour to collect CO_2 on filter papers treated with 10% NaOH. After CO_2 collection, media were neutralized with 2 M potassium carbonate. About 0.1 μCi of [6-^{3}H] glucose was added to media as internal standard. Glucose was isolated by ion-exchange chromatography (Mills et al., 1986). Finally, radioactivity in both CO_2 collection filter papers and glucose containing fractions were determined

with a scintillation spectrophotometer.

To measure lipogenesis rates, sections of adipose tissue (80-150 mg) were incubated for 2 hr at 37 °C in 3 mL of calcium-free Krebs-Henseleit buffer (pH 7.4) (Whitehurst et al., 1978). In addition to 1 nM rec-TNF-α or 10 nM glucagon treatments, 5 mM glucose, 25 mM sodium acetate, and 1 μCi of acetic acid-1-^{14}C also were added to the incubation mixture. Incubations were stopped by 1 M sulfuric acid, and CO_2 was collected on filter papers soaked in 10% NaOH. Adipose tissues were separated from media by filtration, and then lipids were extracted from tissue (Folch et al., 1957). Radioactivity in extracts was determined by a scintillation spectrophotometer.

To measure lipolyis rates, sections of adipose tissues (80-150 mg) were incubated in 3 mL calcium-free Krebs-Henseleit buffer (pH 7.4) with 5 mM glucose and 3% bovine serum albumin for 2 hours at 37 °C. Three treatments were added to separate flasks: 1 nM rec-TNF-α, 10 nM glucagon, and 1.5 μM of epinephrine. Reactions were stopped by placing the reaction media on ice (DiMarco et al., 1986). Lipolysis rates were determined by quantifying the free glycerol in media (kit number F 6428, Sigma, St. Louis, MO).

Statistical Analysis

Data were analyzed as repeated measures by using the mixed model procedure (PROC MIXED) in the SAS Version 9.1.3 (SAS Institute Inc., Cary, NC). Effects of different cow treatments on rates of gluconeogenesis, lipogenesis, and lipolysis were evaluated by comparing each group with saline (control) group. Separate comparisons

were used for data obtained during the prenatal and postnatal periods of treatment. Least square means were calculated, and significance was declared at $P<0.05$.

Results

Gluconeogenesis

To study acute effects of TNF-α and glucagon within cow treatment groups on gluconeogenesis, livers were incubated with either propionic acid or alanine as glucose precursors. Liver tissues incubated with propionic acid produced significantly more glucose than did those incubated with alanine (Table 1). This substrate effect was observed in all cow treatment groups and tissue treatment subgroups ($p < 0.0001$). Livers from control cows and cows treated with glycerol have faster glucose synthesis rates from propionic acid in post-calving stage than those of pre-calving stage. This result was not the case for cows treated with glucagon or glucagon plus glycerol. Parturition did not affect glucose synthesis rates from alanine. Incubating liver tissues with TNF-α or glucagon did not affect glucose synthesis rates when propionic acid ($p \leq 0.85$) or alanine ($p \leq 0.36$) was used as precursor. This effect is true for all cow treatment groups in the pre- and post-calving periods.

Oxidative capacity of liver was determined by measuring the rates of oxidation of propionic acid and alanine to CO_2. Opposite to substrate specificities for glucose production rates, tissues incubated with alanine as substrate released more CO_2 than did those incubated with propionic acid ($p < 0.0001$) (Table 2). This effect is true for all cow treatment groups and substrate treatment subgroups. Glucagon or glucagon plus glycerol treatments did not affect the rates of propionic acid ($p \leq 0.11$)

or alanine ($p \leq 0.75$) oxidation to CO_2. Propionic acid and alanine oxidation to CO_2 tend to increase after parturition. This tendency was significant for control cows, but not statistically significant ($P > 0.05$), for glycerol treatment cows when propionic acid was used as a substrate. Treatment of liver tissues with TNF-α or glucagon did not affect the rate of oxidation of either substrate to CO_2 (propionic acid, $p \leq 0.68$, and alanine, $p \leq 0.96$). This effect was true for all cow treatment groups.

Despite the increased glucose production rates after calving, ratio of rate of propionic acid or alanine oxidation to CO_2 to rate of propionic acid or alanine conversion to glucose was not significantly affected by calving ($p \leq 0.45$) , cow treatment ($p \leq 0.55$), or TNF-α and glucagon treatments ($p \leq 0.32$) (Table 3). In general, rates of propionic acid oxidation to CO_2 were 6.4% of rates of propionic acid conversion to glucose. Rates of alanine oxidation to CO_2 were 160% of rate of alanine conversion to glucose.

Lipolysis

Acute effects of TNF-α and glucagon on lipolysis were measured in the presence and absence of epinephrine as a stimulant of maximal lipolysis of adipose tissue. Epinephrine stimulated glycerol release from adipose tissue obtained from different cows subjected to different treatments ($p < 0.0001$) (Table 4). For all cow and adipose tissue treatments, epinephrine resulted in a 1.6-fold increase in rates of lipolysis. During the pre-calving period or the post-calving period, there was no significant difference in the rate of glycerol release among the four cow treatment groups whether epinephrine was present ($p \leq 0.19$) or absent ($p \leq 0.38$). Generally,

Table 1. Rates of glucose synthesis from propionic acid and alanine in liver (nmol glucose /hr*mg DNA ± SE)[1]

Cow treatment	Liver tissue treatment	Propionic acid				Alanine			
		Pre-calving	Post-calving	SEM[2]	*P*[3]	Pre-calving	Post-calving	SEM[2]	*P*[3]
Saline	Control	1212 [a]	2849 [b]	704	0.02	40 [a]	94 [b]	308	0.73
	TNF-α	941 [a]	2450 [b]	704	0.03	43 [a]	68 [b]	308	0.94
	Glucagon	1087 [a]	2526 [b]	704	0.04	43 [a]	69 [b]	308	0.93
Glycerol	Control	722 [a]	2379 [b]	787	0.04	30 [a]	66 [b]	308	0.91
	TNF-α	587 [a]	2531 [b]	787	0.02	24 [a]	79 [b]	308	0.86
	Glucagon	590 [a]	2977 [b]	787	0.003	27 [a]	100 [b]	308	0.81
Glucagon	Control	1658 [a]	1715 [b]	787	0.94	57 [a]	68 [b]	275	0.97
	TNF-α	1544 [a]	1598 [b]	787	0.95	71 [a]	76 [b]	275	0.99
	Glucagon	1688 [a]	1063 [b]	787	0.43	67 [a]	104 [b]	275	0.91
Glucagon and glycerol	Control	1103 [a]	1792 [b]	643	0.29	36 [a]	87 [b]	275	0.85
	TNF-α	747 [a]	1980 [b]	643	0.06	33 [a]	102 [b]	275	0.80
	Glucagon	812 [a]	1681 [b]	643	0.18	39 [a]	99 [b]	275	0.83

[1]Liver was obtained from cows treated with saline, glycerol, glucagon, or glucagon plus glycerol. Tissues were either controls or treated with 1 nM rec-TNF-α or 10 nM glucagon.
[2] SEM represents the standard error for the difference between the post-calving means within combinations of cow and liver treatment.
[3]*P* Represents the p-value for the t-test of the null hypothesis of no difference between pre and post-calving means within combination of cow and liver treatments.
[a-b] Within a column, means with different superscripts differ ($P<0.05$).

Table 2. Rates of oxidation of propionic acid and alanine to CO_2 in liver (nmol CO_2 /hr*mg DNA ± SE)[1]

Cow treatment	Liver tissue treatment	Propionic acid				Alanine			
		Pre-calving	Post-calving	SEM[2]	*P*[3]	Pre-calving	Post-calving	SEM[2]	*P*[3]
Saline	Control	14 [a]	427 [b]	117	0.001	296 [a]	1056 [b]	218	0.001
	TNF-α	12 [a]	92 [b]	117	0.50	340 [a]	707 [b]	218	0.10
	Glucagon	18 [a]	272 [b]	117	0.03	377 [a]	1113 [b]	218	0.001
Glycerol	Control	26 [a]	165 [b]	130	0.29	363 [a]	523 [b]	218	0.47
	TNF-α	31 [a]	223 [b]	130	0.14	530 [a]	480 [b]	218	0.82
	Glucagon	18 [a]	204 [b]	130	0.16	366 [a]	386 [b]	218	0.93
Glucagon	Control	32 [a]	109 [b]	107	0.56	527 [a]	642 [b]	195	0.60
	TNF-α	33 [a]	96 [b]	107	0.63	716 [a]	380 [b]	195	0.13
	Glucagon	34 [a]	64 [b]	107	0.81	621 [a]	398 [b]	195	0.31
Glucagon and glycerol	Control	11 [a]	60 [b]	107	0.65	274 [a]	537 [b]	195	0.18
	TNF-α	11 [a]	45 [b]	107	0.75	323 [a]	576 [b]	195	0.20
	Glucagon	13 [a]	62 [b]	107	0.65	162 [a]	663 [b]	195	0.01

[1]Liver was obtained from cows treated with saline, glycerol, glucagon, or glucagon plus glycerol. Tissues were either controls or treated with 1 nM rec-TNF-α or 10 nM glucagon.
[2] SEM represents the standard error for the difference between the post-calving means within combinations of cow and liver treatment.
[3]*P* Represents the p-value for the t-test of the null hypothesis of no difference between pre and post-calving means within combination of cow and liver treatments.
[a-b] Within a column, means with different superscripts differ ($P<0.05$).

Table 3. Ratio of oxidation of propionic acid or alanine to CO_2 to rates of glucose synthesis from propionic acid or alanine in liver.[1]

Cow treatment	Liver tissue treatment	Propionic acid				Alanine			
		Pre-calving	Post-calving	SEM[2]	*P*[3]	Pre-calving	Post-calving	SEM[2]	*P*[3]
Saline	Control	0.02 [a]	0.27 [b]	0.15	0.11	13 [a]	7 [b]	6.0	0.81
	TNF-α	0.02 [a]	0.04 [b]	0.15	0.13	14 [a]	11 [b]	6.0	0.47
	Glucagon	0.03 [a]	0.14 [b]	0.15	0.93	17 [a]	25 [b]	6.0	0.26
Glycerol	Control	0.04 [a]	0.10 [b]	0.10	0.32	19 [a]	18 [b]	6.0	0.82
	TNF-α	0.06 [a]	0.11 [b]	0.10	0.92	29 [a]	22 [b]	6.0	0.54
	Glucagon	0.03 [a]	0.08 [b]	0.10	0.26	19 [a]	24 [b]	6.0	0.41
Glucagon	Control	0.02 [a]	0.06 [b]	0.17	0.52	19 [a]	14 [b]	8.0	0.56
	TNF-α	0.03 [a]	0.06 [b]	0.17	0.47	14 [a]	14 [b]	8.0	0.83
	Glucagon	0.02 [a]	0.07 [b]	0.17	0.16	17 [a]	14 [b]	8.0	0.44
Glucagon and glycerol	Control	0.03 [a]	0.07 [b]	0.13	0.41	12 [a]	12 [b]	8.0	0.56
	TNF-α	0.03 [a]	0.06 [b]	0.13	0.51	16 [a]	15 [b]	8.0	0.68
	Glucagon	0.03 [a]	0.11 [b]	0.13	0.87	10 [a]	10 [b]	8.0	0.91

[1]Liver was obtained from cows treated with saline, glycerol, glucagon, or glucagon plus glycerol. Tissues were either controls or treated with 1 nM rec-TNF-α or 10 nM glucagon.
[2] SEM represents the standard error for the difference between the post-calving means within combinations of cow and liver treatment.
[3]*P* Represents the p-value for the t-test of the null hypothesis of no difference between pre and post-calving means within combination of cow and liver treatments.
[a-b] Within a column, means with different superscripts differ ($P<0.05$).

Table 4. Rates of glycerol release from adipose tissues (nmol glycerol /hr*g tissue ± SE)[1]

Cow treatment	Liver tissue treatment	Epinephrine (-)[2]				Epinephrine (+)[3]			
		Pre-calving	Post-calving	SEM[4]	*P*[5]	Pre-calving	Post-calving	SEM[4]	*P*[5]
Saline	Control	204 [a]	166 [b]	91	0.56	478 [a]	314 [b]	87	0.07
	TNF-α	216 [a]	164 [b]	96	0.47	339 [a]	306 [b]	87	0.71
	Glucagon	334 [a]	170 [b]	96	0.51	503 [a]	261 [b]	87	0.01
Glycerol	Control	283 [a]	211 [b]	91	0.44	420 [a]	281 [b]	84	0.09
	TNF-α	167 [a]	178 [b]	91	0.91	446 [a]	229 [b]	84	0.01
	Glucagon	288 [a]	171 [b]	91	0.20	409 [a]	252 [b]	79	0.05
Glucagon	Control	137 [a]	184 [b]	111	0.68	279 [a]	200 [b]	97	0.42
	TNF-α	137 [a]	162 [b]	111	0.83	274 [a]	274 [b]	97	0.55
	Glucagon	130 [a]	157 [b]	111	0.81	243 [a]	205 [b]	97	0.70
Glucagon and glycerol	Control	240 [a]	223 [b]	128	0.82	377 [a]	232 [b]	97	0.12
	TNF-α	257 [a]	178 [b]	128	0.54	422 [a]	217 [b]	97	0.04
	Glucagon	236 [a]	161 [b]	146	0.56	364 [a]	175 [b]	97	0.06

[1]Adipose was obtained from cows treated with saline, glycerol, glucagon, or glucagon plus glycerol. Tissues were either controls or treated with 1 nM rec-TNF-α or 10 nM Glucagon.

[2-3] (-): No epinephrine was added to the incubations; (+):1.5 μM of epinephrine was added to the incubations

[4] SEM represents the standard error for the difference between the post-calving means within combinations of cow and adipose treatment.

[5]*P* Represents the p-value for the t-test of the null hypothesis of no difference between pre and post-calving means within combination of cow and adipose treatments.

[a-b] Within a column, means with different superscripts differ ($P<0.05$).

glycerol release decreased after calving; this effect, however, was usually significant only ($p < 0.05$) in the presence of epinephrine stimulation. Incubating adipose tissues from all cows with either TNF-α or glucagon did not affect glycerol release rates; this lack of effect of TNF-α and glucagon was observed in either presence ($p \leq 0.84$) or absence ($p \leq 0.73$) of epinephrine.

Lipogenesis

To study acute effects of TNF-α and glucagon on de novo lipogenesis, adipose tissues were incubated with acetate as a fatty acid precursor. During the pre-calving period or post-calving period, there was no significant difference ($p > 0.05$) in the rate of lipogenesis among the four cow treatment groups ($p \leq 0.80$) (Table 5). The rates of lipogenesis in adipose tissue of pre-parturient cows were greater than those of post-parturient cow. In general, rates were 6.5 times greater before calving. This difference was more pronounced in tissues obtained from cows treated with glycerol or glucagon plus glycerol. Incubating adipose tissues from all cows with either TNF-α or glucagon did not affect palmitate synthesis rates ($p \leq 0.76$).

To study acute effects of TNF-α and glucagon on oxidative capacity of adipose tissue, the rates of oxidation of acetate to CO_2 were quantified. Cow treatments, whether during the pre-calving period or post-calving period, did not affect CO_2 release when acetate was used as a substrate ($p \leq 0.74$) (Table 6). In addition, TNF-α or glucagon treatment of adipose tissues did not affect the rate of acetate oxidation to CO_2 ($p \leq 0.19$). The ratio of acetate oxidation to CO_2 to rate of

Table 5. Rates of acetate conversion to long-chain fatty acids in adipose tissue (nmol acetate converted to fatty acids /hr*g tissue ± SE)[1]

Cow treatment	Adipose tissue treatment	Lipogenesis			
		Pre-calving	Post-calving	SEM[2]	*P*[3]
Saline	Control	78 [a]	15 [b]	41	0.08
	TNF-α	75 [a]	17 [b]	36	0.11
	Glucagon	53 [a]	15 [b]	36	0.24
Glycerol	Control	99 [a]	19 [b]	32	0.02
	TNF-α	101 [a]	17 [b]	32	0.01
	Glucagon	108 [a]	21 [b]	32	0.01
Glucagon	Control	86 [a]	12 [b]	40	0.07
	TNF-α	89 [a]	11 [b]	40	0.06
	Glucagon	71 [a]	12 [b]	40	0.15
Glucagon and glycerol	Control	122 [a]	14 [b]	42	0.01
	TNF-α	160 [a]	13 [b]	42	0.001
	Glucagon	112 [a]	12 [b]	42	0.01

[1]Adipose was obtained from cows treated with saline, glycerol, glucagon, or glucagon plus glycerol. Tissues were either controls or treated with 1 nM rec-TNF-α or 10 nM glucagon.
[2] SEM represents the standard error for the difference between the post-calving means within combinations of cow and adipose treatment.
[3]*P* Represents the p-value for the t-test of the null hypothesis of no difference between pre and post-calving means within combination of cow and adipose treatments.
[a-b] Within a column, means with different superscripts differ ($P<0.05$).

acetate conversion to long-chain fatty acids significantly increased in the post-calving cows treated with glucagon only. The ratios were not affected by TNF-α or glucagon treatment of adipose tissues ($p \leq 0.44$). The average ratio for pre-calving cows was 2.05, and that for post-calving cows was 8.94. This 4.4-fold difference, however, was not significant.

Discussion

The objective of the current study was to measure the effect of subcutaneously administered glucagon for 14 days, starting at day 2 after calving, to dairy cows highly susceptible to fatty liver development on the rates of different metabolic pathways, specifically, gluconeogenesis, lipolysis, and lipogenesis, under the in vitro effect of different hormonal and substrate treatments. Propionic acid conversion to glucose is 10 to 35 times faster than that for alanine. These ratios agree with those observed in previous reports (Mills et al., 1986; Overton et al., 1999; Wladron et al., 2003). In these reports propionic acid conversion to glucose is shown to be 5 to 25 more than that of alanine. Propionic acid and alanine oxidation rates to CO_2 were in the same ranges of those reported previously (Mills et al., 1986). In agreement with previous studies, the ratios of propionic acid oxidation to CO_2 to glucose synthesis rates are much smaller that those of alanine (Mills et al., 1986; Veenhuizen et al., 1991; Donkin and Armentano, 1994; Piepenbrink et al., 2004).

Based on the increased blood glucose concentrations (Bobe et al., 2003b; Nafikov et al., 2006; Osman et al., 2008), and increased expression of gluconeogenic enzymes (Bobe et al., 2009) after glucagon injections, glucagon injections in early

Table 6. Rates of acetate oxidation to CO_2 (nmol CO_2 nmol/hr*g tissue ± SE) and ratio of rate of acetate oxidation to CO_2 to rate of acetate conversion to long-chain fatty acids in adipose tissue.[1]

Cow treatment	Adipose tissue treatment	Oxidation to CO_2				Oxidation to CO_2/lipogenesis			
		Pre-calving	Post-calving	SEM[2]	*P*[3]	Pre-calving	Post-calving	SEM[2]	*P*[3]
Saline	Control	74 [a]	53 [b]	28	0.41	1.5 [a]	4.1 [b]	5.4	0.60
	TNF-α	83 [a]	96 [b]	25	0.60	1.9 [a]	7.6 [b]	4.8	0.23
	Glucagon	72 [a]	62 [b]	25	0.98	2.4 [a]	4.3 [b]	4.8	0.59
Glycerol	Control	81 [a]	104 [b]	23	0.30	2.5 [a]	6.3 [b]	4.4	0.38
	TNF-α	101 [a]	115 [b]	23	0.53	2.8 [a]	5.3 [b]	4.4	0.36
	Glucagon	122 [a]	85 [b]	23	0.11	2.5 [a]	5.2 [b]	4.4	0.54
Glucagon	Control	90 [a]	133 [b]	28	0.13	2.5 [a]	23 [b]	5.8	0.0003
	TNF-α	139 [a]	115 [b]	28	0.40	2.9 [a]	17 [b]	5.8	0.01
	Glucagon	84 [a]	85 [b]	28	0.97	2.0 [a]	18 [b]	5.8	0.04
Glucagon and glycerol	Control	104 [a]	81 [b]	31	0.50	1.3 [a]	5.3 [b]	5.8	0.41
	TNF-α	88 [a]	88 [b]	31	0.94	1.1 [a]	5.8 [b]	5.8	0.35
	Glucagon	76 [a]	108 [b]	31	0.26	1.2 [a]	7.9 [b]	5.8	0.20

[1]Adipose was obtained from cows treated with saline, glycerol, glucagon, or glucagon plus glycerol. Tissues were either controls or treated with 1 nM rec-TNF-α or 10 nM glucagon.
[2] SEM represents the standard error for the difference between the post-calving means within combinations of cow and adipose treatment.
[3]*P* Represents the p-value for the t-test of the null hypothesis of no difference between pre and post-calving means within combination of cow and adipose treatments.
[a-b] Within a column, means with different superscripts differ ($P<0.05$).

lactation period are predicted to activate gluconeogenesis. Contrary to previous studies, gluconeogenesis rates measured by propionic acid and alanine conversion to glucose were not affected by glucagon injections. Furthermore, livers obtained from cows that did not receive glucagon treatment utilized propionic acid at greater rates than those who received glucagon injections. High individual variations among cows in addition to the low number of cows per cow treatment group make it difficult to draw final conclusions about the effect of glucagon injections on the rates of substrates conversion to glucose.

Nevertheless, the possibility of gluconeogenesis inhibition by elevated insulin concentrations in glucagon-treated cows can not be excluded. Glucagon subcutaneous injections result in increased insulin concentrations in plasma (Bobe et al., 2003b; Nafikov et al., 2006; Osman, et al., 2008). Insulin suppresses PEPCK activity in hepatoma cells (Beale et al., 1986; Granner et al., 1983). Insulin specifically decreases glucose synthesis rates from propionic acid versus lactate in neonatal bovine livers (Donkin and Armentano, 1994). Low plasma insulin concentrations in the post-parturition period of glycerol and control group (Osman et al., 2008) decreases the potential insulin inhibitory effect on gluconeogenesis in livers obtained from cows in these of two groups. Weak inhibitory effect of insulin on gluconeogenic enzymes in these two groups may explain higher propionic acid conversion rates compared with those in cows treated with glucagon or glucagon plus glycerol. This observation is consistent with previous findings that PEPCK mRNA concentrations increased significantly in the livers of cows experiencing low insulin concentrations as a result of feed restriction (Velez and Donkin, 2005). In accordance with previous

observations, increased oxidation rates in control and glycerol treatment cows can also be explained by the absence of insulin inhibitory effect on gluconeogenesis in these two groups. Propionic acid or alanine carbon repartitioning between gluconeogenesis and oxidation through TCA cycle was not affected by glucagon injections.

Addition of 10 nM glucagon to liver slices did not increase glucose synthesis rates from propionic acid or alanine. This observation is consistent with previous reports of ineffectiveness of glucagon to increase glucose synthesis rates from propionic increase significantly in ovine (Looney et al., 1987) or bovine (Donkin and Armentano, 1993) hepatocyte cultures. It was suggested, however, that hepatocyte isolation procedure could have resulted in an impaired signal transduction system (Donkin and Armentano, 1993). In another study, glucagon concentration of 1 nM increased the Vmax of propionic acid conversion to glucose when added to cultures of monolayer of neonatal bovine hepatocytes (Donkin and Armentano, 1994). Short-term administration of glucagon to midlactation dairy cows did not increase expression of gluconeogenic enzymes or increase rates of propionic acid conversion to glucose (Williams et al., 2006). Less accessibility of glucagon to incubated liver tissues is another possible factor that can result in lowered effects of glucagon. Similarly, the lack of TNF-α on gluconeogensis can be explained by less responsive or inaccessible signal transduction system in directly obtained liver tissues. Adding 1.2 nM TNF-α to rat hepatocyte cultures did not decrease glucose synthesis rates from alanine or lactate (Rofe et al., 1987).

To measure effects of glucagon subcutaneous injections of glucagon on

adipose tissue lipolysis, glycerol release rates were determined by in vitro experiments. Glycerol release rates reported in current study are either in agreement or are 2 to 5 fold lower than the rates cited in several previous reports (Vernon, 1983; McNamara and Hillers, 1986a; McNamara and Hillers, 1986c; McNamara, 1988; McNamara, 1989; DiMarco et al., 1991; McNamara et al., 1992; Rukkwamsuk et al., 1998; Lanna and Bauman, 1999; McNamara and Valdez, 2005). Relatively lower lipolysis rates in this study can be explained by prolonged inhibition of lipolysis by relatively higher than normal insulin concentrations in plasma (Dann et al., 2006). Feeding cows high energy diets during dry period is usually associated with relatively higher insulin concentrations than normally fed cows (van den Top et al., 1995; Rukkwamsuk et al., 1998; de Feu et al., 2009). Insulin inhibits lipolysis by decreasing cAMP cellular concentrations by inhibiting adenylyl cyclase (Lambert and Jacquemin, 1979), activating phosphodiesterase (Kono et al., 1975), or both (Vernon, 1983). Decreasing active phosphorylated HSL concentrations by activating protein phosphatase is another suggested mechanism explains insulin inhibitory effect on lipolysis (Strålfors and Honnor, 1989). Although epinephrine impact on in vitro glycerol release rates was significant in the current study, this effect is lower than that in previous reports (Bauman, 1976). Relatively high insulin concentrations because of high energy feeding can explain the attenuated glycerol release rates in response to adipose tissue treatment with epinephrine. Previous in vitro studies (Yang and Baldwin, 1973) showing that insulin decreased epinephrine-stimulated lipolysis rates by 10-25% are consistent with the attenuated lipolysis response to epinephrine observed in our experiment.

Parturition effect on lipolysis was not statistically significant for all cow and adipose tissues treatments; however, when stimulated by epinephrine, lipolysis rates tended to decrease in the post-calving stage in saline-treated or control cows. Lower glycerol release rates in postpartal versus prepartal stage can be attributed to the low responsiveness of adipose tissue to epinephrine in late pregnancy and early postpartum compared with mid-lactation times (McNamara, 1988). On the other hand, elevated BHBA concentrations in plasma in the first pospartal week in saline group (Osman et al., 2010) may inhibit lipolysis in the pospartal stage. In vitro incubation of bovine adipose tissue with BHBA significantly decreased glycerol release rate (Metz et al., 1974). Increased plasma BHBA concentration because of rumen glycerol fermentation (DeFrain et al., 2004; Osman, et al., 2010) explains the lowered lipolysis rates adipose tissues obtained from cows treated with glycerol or glycerol plus glucagon in the postpartal stage. Postpartal glucagon injections decreased BHBA plasma concentrations significantly in cows treated with glucagon only. Glucagon alleviated BHBA inhibitory effect on lipolysis in adipose tissues obtained from cows treated with glucagon only.

Short incubation time (2 hr) of adipose tissue with 1 nM TNF-α can explain the lack of response to TNF-α. Incubating isolated rat adipocytes with 1.2 nM TNF-α for a time range from 30 to 120 min did not affect glycerol release (Rofe et al., 1987); however, incubating isolated rat adipocytes with 4 nM TNF-α for 24 hr decreased glycerol rate significantly (Porter et al., 2002). Low sensitivity to TNF-α also may explain adipose tissue response. TNF-α gene expression (Karcher et al., 2008) and its

concentrations in plasma (Rontved et al., 2005; Winkelman et al., 2007) of dairy cows increases significantly one week before calving and drops at calving day. Then, TNF-α expression and its concentration in plasma increase again in the first two weeks after calving. Although not measured, the probability of high TNF-α concentrations desensitizing adipose tissue to TNF-α may not be ruled out. This observation could be supported by previous reports of increased endogenous expression of TNF-α in adipose tissue (Hube and Hauner, 1999). Glucagon ineffectiveness to affect adipose tissue glycerol release rates is consistent with previous reports indicating no effect of glucagon on in vitro measured glycerol rates in bovine adipose tissues (Etherton et al., 1977). Other studies observed that glucagon decreased in vitro glycerol rates of caprine adipose tissues but only at high concentrations (~300 nM) (Bartos et al., 1975).

To measure effect of subcutaneous glucagon injections and acute effects of TNF-α and glucagon on adipose tissue lipogenesis, rates of acetic acid incorporation in fatty acids were determined by in vitro experiments. Lipogenesis rates are in agreement with those of previous studies reports (Whitehurst et al.,1978; McNamara and Hillers, 1986a; McNamara and Hillers, 1986b; McNamara, 1989; McNamara et al., 1995; Lake et al., 2006). Other studies, however, reported almost 10 fold higher lipogenesis rates (Whitehurst et al., 1981; Mills et al., 1986; McNamara and Valdez, 2005). The tendency of lipogenesis rates to decrease in the post-parturition stage in all cow groups agrees with other several studies showing that lipogenesis rates significantly decrease in the post parturition period (Mills et al., 1986; McNamara and

Hillers, 1986a; McNamara and Hillers, 1986b; McNamara et al., 1995; McNamara and Valdez, 2005). Esterification (Rukkwamsuk et al., 1999) and lipogeneic enzymes activities in liver (Murondoti et al., 2004) also decrease in the post-parturition period. Decreased lipogenesis rates in control and treatment cows can be explained by the low insulin plasma concentrations in the early post-parturition stage (Osman et al., 2010). Insulin activates the key enzymes of fatty acid synthesis (Kresten, 2001). Low insulin concentration after calving may results in less gene expression of ACC and FAS. The pronounced tendency of lipogenesis rates to decrease after calving in glucagon-treated cows can be explained inhibition of ACC by glucagon (Holland et al., 1985; Yin et al., 2000).

Glycerol treatment significantly decreased lipogenesis rates. The mechanism of glycerol action on lipogenesis remains unclear. Acetate is produced as a result of glycerol ruminal fermentation (DeFrain et al., 2004). In the presence of rumen-originated acetate, acetyl-CoA and palmitoyl-CoA intermediates will accumulate in adipose tissue because of the activity of acetyl-CoA synthetase, the enzyme responsible for acetyl-CoA synthesis from acetate in ruminant adipose tissue (Hanson and Ballard, 1967). The inhibitory effect of palmitoyl-CoA on ACC may explain the negative impact of glycerol on lipogenesis rates. Neither parturition nor cow treatment affected acetic acid oxidation to CO_2 , however, ratio of acetic acid oxidation to CO_2 to lipogenesis rate significantly increased after calving for cows treated with glucagon alone. This ratio increase is consistent with glucagon inhibition of ACC. Glucagon inhibits ACC but does not inhibit acetyl-CoA incorporation in TCA cycle. Subcutaneously injected glucagon and orally administered glycerol may

have an inhibitory action on lipogenesis that results in lower NEFA synthesis and ultimately lower TAG accumulation in the livers of cows susceptible to FLS.

Despite the reported inhibitory effect of TNF-α on lipogenesis (Cawthorn and Sethi, 2008), incubation of adipose tissues obtained from control and treatment cows for 2 hr with 1 nM TNF-α did not affect lipogenesis rates. In their study, Doerrler et al. (1994) reported that TNF-α required 16 hr incubation to inhibit ACC and FAS activity and expression in 3T3-L1 and 3T3-F442A adipocyte cell lines. In another study, ACC activity decreased significantly in cells from 30A-5 preadipocyte cell line after a 72 hr exposure to TNF-α (Pape and Kim, 1988). Short incubation time (2 hr) can explain lack of response in our study. Low sensitivity because of elevated TNF-α concentration in plasma around parturition (Rontved et al., 2005; Winkelman et al., 2007) also may explain lack of response in adipose tissue. In agreement with previous studies, adipose tissue incubation with 100 nM glucagon for 2 hr did not affect lipogenesis rates (Holland et al., 1985).

Conclusion

This is the first study to measure the effect of subcutaneous glucagon injections used to treat FLS symptoms on the rates gluconeogenesis, lipolysis, and lipogenesis. Greater conversion rates of propionic acid to glucose than those observed for alanine. Epinephrine stimulated glycerol release rates for all cow and sample treatments. Lipogenesis rates significantly dropped in the post-calving period. The results presented in the current study suggest that the alleviating effect of subcutaneous glucagon injection on some FLS symptoms is not achieved by a direct

action on rates of gluconeogenesis in liver or lipolysis rates in adipose tissue but probably rather by long-term effects. Glucagon and glycerol inhibitory may inhibit lipogenesis rates and decrease NEFA and TAG synthesis and accumulation in the liver of dairy cows. Short incubation times and less absorption of in vitro regulators by tissue biopsies could have weakened the direct effects of glucagon and TNF-α on liver and adipose tissue. Therefore, longer incubation times with monolayer hepatocytes and adipocytes could be more effective to test the effect of cow treatment with glucagon subcutaneous injections on the rates of gluconeogenesis and lipolysis and lipogenesis in different tissues.

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CHAPTER 3: GENERAL CONCLUSION

General Discussion

Different studies emphasized the effect of subcutaneous injections of glucagon for 14 days after calving on different metabolic processes in both normal cows and cows with fatty liver. The objective of the present study is to measure the effect of subcutaneously administered glucagon for 14 days, starting at day 2 after calving, to dairy cows highly susceptible to fatty liver development on the rates of different metabolic pathways, specifically, gluconeogenesis, lipolysis, and lipogenesis, under the effect of different hormonal and substrate treatments. The hypothesis of this study is that subcutaneous injection of glucagon directly affects the turnover rates of gluconeogenesis in liver and the rates of lipolysis and lipogenesis in adipose tissue. Appropriate changes in these metabolic rates may result in relieving FLS symptoms. This study was designed to measure the rates of the three metabolic pathways before and after in vivo glucagon treatment and under different in vitro substrate and hormonal treatments such as TNF-α and epinephrine.

Liver tissues incubated with propionic acid produced significantly more glucose than did those incubated with alanine. This observation agrees with previous reports. In agreement with other studies, the ratios of propionic acid oxidation to CO_2 to glucose synthesis rates are much smaller that those of alanine. Gluconeogenesis rates measured were not affected by in vivo glucagon injections. Livers obtained from control and glycerol-treated cow utilized propionic acid at greater rates than did those from cows who received glucagon and glucagon plus glycerol treatments. Individual

cow variations and the low number of cows per treatment group make final conclusions about the effect of glucagon injections on the rates of substrates conversion to glucose difficult to draw. Nevertheless, the possibility of gluconeogenesis inhibition by elevated insulin concentrations right after glucagon injections into cows can explain the lower glucose synthesis rates in glucagon and glucagon plus glycerol treatment cows compared with those cows of the other two groups. Incubating liver tissues with TNF-α or glucagon did not affect glucose synthesis rates when propionic acid or alanine was used as precursor. TNF-α and glucagon did not affect propionic acid or alanine oxidation too. Impaired signal transduction of hepatocytes because of isolation procedure, low absorption of glucagon and TNF-α by liver tissues, and short incubation times are all probable explanations for the lack of effect of glucagon and TNF-α on gluconeogenesis rates.

Glycerol release rates reported in the current study are relatively lower than some previous reports. Feeding cows with high energy diets during dry period is usually accompanied by relatively high insulin plasma. Insulin inhibits lipolysis by decreasing cAMP cellular concentrations by either inhibiting adenylyl cyclase or activating phosphodiesterase or both. Insulin can also inhibit lipolysis by activating protein phosphatases and, in turn, decreasing active HSL concentrations. Relatively high insulin concentrations because of high energy feeding can explain the attenuated glycerol release rates in response to adipose tissue treatment with epinephrine. When stimulated by epinephrine, lipolysis rates tended to decrease in the post-calving stage in saline, glycerol- and glucagon plus glycerol-treated cows. Lower glycerol release

rates in postpartal versus prepartal stage can be attributed to the low responsiveness of adipose tissue to epinephrine in late pregnancy and early postpartum. It also can be explained by lipolysis inhibition by the usually elevated BHBA concentrations in plasma in the first pospartal week. Glucagon antagonism of BHBA inhibitory can explain less affected lipolysis rates in adipose tissues obtained from cows treated with glucagon only. Short incubation times may explain the neutral effects of TNF-α and glucagon on glycerol release rates.

The tendency of lipogenesis rates to decrease in the post-parturition stage in all cow groups agrees with results of other studies. Low insulin concentration after calving may result in decreased lipogenesis rates in control treatment. The pronounced tendency of lipogenesis rates to decrease after calving in glucagon-treated cows can be explained by glucagon inhibition of ACC. In vivo glycerol treatment significantly decreased lipogenesis rates. The mechanism of inhibitory effect of glycerol on lipogenesis is not clear. Accumulating palmitoyl-CoA, synthesized from excess acetate, inhibits ACC. The ratio of acetic acid oxidation to lipogenesis rate significantly increased after calving for cows treated with glucagon alone. This result is consistent with glucagon inhibition of ACC but not acetyl-CoA incorporation in TCA cycle. Subcutaneously injected glucagon and orally administered glycerol may have an inhibitory action on lipogenesis that results in lower NEFA synthesis and ultimately lower TAG accumulation in the livers cows susceptible to FLS.

Recommendation for Future Research

This study shed light on the direct effects of subcutaneous injections of glucagon on three major metabolic pathways, i.e. gluconeogenesis in liver tissues and lipolysis and lipogenesis in adipose tissues during the course of FLS development during the periparturient period of dairy cattle. The current study conducted a comparison between the rates of these metabolic pathways in the late prepartal and early postpartal stages in cows with relatively moderate fatty liver cases. Previous studies conducted in this research group using the dietary supplementation of 1,3-butanediol were more effective in inducing FLS in transition cow. Inducing FLS with this compound and then measuring the rates of different metabolic pathways could give a clearer and more definitive answer about the severity of the disease.

The effect of subcutaneous injections of glucagon on the expression of different genes is another aspect that could be investigated in future research. Expression of genes of key gluconeogenic enzymes and lipid metabolic enzymes in addition to expressions of genes of major metabolic hormones such as glucagon and insulin also could be investigated under normal and fatty liver conditions. Measuring gene expression of these enzymes and hormones under normal and fatty liver conditions at different times after parturition could give a clear answer about the differences between short-term and long-term changes during the development of the disease.

More studies need to be done to investigate tissue incubations under different conditions. Further studies using monolayer hepatocytes or adipocytes drawn from

liver and adipose tissues obtained from different treatment cows can be more useful than directly using the original biopsied tissues. In these cell culture studies, tissue quantity and regulatory factor availability to all cells are not a limiting factor. With this factor being avoided, then, incubation times, incubations conditions, and effective factor concentrations are optimized more easily.

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