ABSTRACT

RADDATZ, JULIA. Measurement of Adiponectin in Lactating Dairy Cows and Adiponectin, Insulin, NEFA, and Glucagon concentrations during an IVGTT and an IVITT in Lactating vs. Non-lactating Holstein Cows. (Under the direction of Dr. Scott Whisnant).

Adipose tissue is now known to be part of the endocrine system as well as an energy storage depot. One of the recently discovered adipose-secreted hormones is adiponectin. In many species, adiponectin concentrations are negatively correlated with adiposity and adiponectin has been reported to increase tissue sensitivity to insulin. No report of adiponectin secretion in cattle or any ruminant species could be found.

In this study, weekly blood samples were taken from Holstein cows (n = 26) over the first 11 weeks of lactation. Plasma adiponectin, insulin and progesterone concentrations were determined and body condition score and milk production data were recorded. Adiponectin concentrations increased from 8.3±1.4ng/ml in the first week to 16.0±2.7ng/ml at week 4 and then declined to remain at 12-13ng/ml for the remainder of the study. Individual cows had consistently high or low adiponectin concentrations. Adiponectin concentrations did not correlate with body condition score, energy-corrected milk yield, lactation number, or progesterone (time to first cycle). Insulin concentrations increased from 4.70±4.79μIU/ml in the first week to 12.35±10.38μIU/ml at week 4. After week 4, insulin concentrations remained at 11-15μIU/ml for the duration of the study. Within individual cows, insulin showed a -0.22 correlation with adiponectin (p=0.12). A human adiponectin kit (HADP-61 HK, Linco, Millipore) was used to measure plasma adiponectin concentrations. Dilution of plasma as recommended by the manufacturer resulted in samples being below the sensitivity of the assay. Using undiluted plasma, adiponectin concentrations ranged from 0-80ng/ml whereas in other species, concentrations are reported in the μg/ml

range. Similarly, samples collected from growing bulls were undetectable when diluted, but ranged from 0-40ng/ml when assayed undiluted. Some equine samples were assayed along with the bovine samples and after dilution (1:500) were in the ng/ml range. After adjusting the equine samples for dilution, a similar range (1-2µg/ml) as reported for horses in the literature was attained. It is uncertain if the human adiponectin kit is able to accurately quantify bovine adiponectin concentrations, but our assays indicate it is adequate for measuring trends.

In the second study, Holstein cows (n=4) were administered an intravenous glucose tolerance test (IVGTT) and intravenous insulin tolerance test (IVITT) at 45.3±3.3 days prepartum and 19.5±3.2 days postpartum. Blood samples were taken -30, -15, 0, 5, 10, 15, 20, 30, 45, 60, 90, 120 and 150 minutes relative to glucose or insulin infusion. Plasma glucose, insulin and adiponectin, and serum non-esterified fatty acid (NEFA) and glucagon concentrations were measured. There was no difference between pre and postpartum glucose time to return to baseline, following the infusion of 0.25g glucose/kg body weight (BW), but prepartum insulin concentrations rose higher ($p<0.0001$), and took longer to return to baseline levels ($p<0.0041$) than postpartum insulin. Area under the curve (AUC) for glucose and insulin during the IVGTT was greater prepartum vs. postpartum. Adiponectin, NEFA, and glucagon were unaffected by the IVGTT. The insulin dose of 0.1IU/kgBW did not result in a decline in circulating glucose, nor did it affect any other hormones. Adiponectin concentrations were significantly higher in prepartum vs. postpartum cows ($p<0.0001$), and individual variability between animals was observed, similar to the preliminary study. There was no difference between pre and postpartum serum NEFA or glucagon concentrations. The lesser rise in, and accelerated time for insulin concentrations to return to pre-injection

levels, in conjunction with the smaller postpartum AUC for insulin during the postpartum IVGTT indicates decreased insulin responsiveness in postpartum cows. The decreased postpartum adiponectin concentrations suggests that low adiponectin levels may help facilitate postpartum insulin resistance which increases the supply of glucose to the mammary gland, and aids in the metabolic support of lactation.

KEY WORDS: adiponectin, insulin, progesterone, NEFA, glucagon, glucose, dairy cows

Measurement of Adiponectin and Insulin in Lactating and Non-lactating Holstein Cows

by
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BIOGRAPHY

Julia Rae Raddatz was born on July 18, 1983 to Ann Kemp and Wes Raddatz in Oshkosh, Wisconsin. Julia and her older sister, Lindsey, attended grade school in rural Wisconsin and enjoyed growing up on their family's dairy farm. In this setting Julia gained a love and fascination for animals that would last a lifetime. When she was 11, Julia's family moved to Green Bay, Wisconsin, where she attended middle school and high school.

In August of 2001, Julia pursued her bachelor's degree in Animal Science at Louisiana State University in Baton Rouge, Louisiana. Throughout college, she kept several jobs that added to her experience working with animals, and volunteered for a therapeutic riding program for disabled children and adults. While a sophomore at LSU, she took a job with the Ornithology department at the Louisiana State University Museum of Natural Science (LSUMNS), preparing ornithological specimens. During her senior year, she was asked to join the director of the museum, Dr. Fred Sheldon, on a month-long collection expedition to Sabah, in Malaysian Borneo. It was in this beautiful country that she decided her life's goal would be to improve upon conservation and endangered species breeding programs.

After completing her bachelor's degree in 2006, Julia moved to Raleigh, North Carolina. Here she entered a graduate program at North Carolina State University and pursued her Master of Science degree in Animal Science, concentrating in reproductive physiology and endocrinology.

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LITERATURE REVIEW

Adiponectin

Chemistry & Secretion

Adiponectin is an adipocyte-derived hormone that plays an important role in lipid metabolism and glucose homeostasis (Lord et al., 2005). Also known as Acrp30, AdipoQ, or GBP28, adiponectin was originally identified independently by four groups using different approaches in the mid 1990's (Kadowaki and Yamauchi, 2005). It is a protein hormone, roughly 245 amino acids in length, which structurally belongs to the complement 1q family and most closely resembles tumor necrosis factor-alpha (TNFα), interleukin-1 (IL-1), and the other adipokines. Adiponectin is composed of four regions: an N-terminal collagen-like sequence, a signal sequence, a short species-specific region, and a C-terminal globular region. It is secreted from adipose tissue directly into the bloodstream, and can exist as a full-length or a smaller, globular fragment; however almost all adiponectin appears to exist as the full-length form in plasma (Kadowaki and Yamauchi, 2005). Adiponectin is very stable in blood, and the half-life for clearance from blood is 32 min (Spranger et al., 2006). It has been reported that adiponectin is found as two forms in serum, as a lower-molecular weight trimer-dimer and as a high molecular weight complex (HMW) (Scherer et al., 1995; Kadowaki and Yamauchi, 2005). These varying forms have more recently been discovered to have differing biological effects and interactions with the adiponectin receptors.

Fat mass may exert direct negative feedback on adiponectin secretion (Gordon et al., 2007). A negative correlation between adiponectin levels and fat mass has been reported in swine (Jacobi et al., 2004; Lord et al., 2005), horses (Kearns et al., 2006), humans (Arita et al., 1999), mice (Kadowaki and Yamauchi, 2005) and rhesus monkeys (Hotta et al., 1998).

Weight reduction was shown to significantly elevate plasma adiponectin levels in diabetic subjects as well as non-diabetic subjects (Hotta et al., 2000). Circulating adiponectin concentrations are reduced in individuals with type 2 diabetes and coronary artery disease (Hotta et al., 2000). Jacobi at al. (2004) reported that incubating pig adipocytes for 6 h with recombinant pig adiponectin resulted in an approximate 30% reduction ($p < 0.05$) in lipogenesis compared with adipocytes under basal concentrations and with those incubated in the presence of insulin. Their findings suggest that adiponectin decreases the incorporation of glucose carbon into lipid in the adipocyte, and provides additional evidence that adiponectin acts as an autocrine regulatory factor to regulate energy metabolism (Jacobi et al., 2004).

At least four studies have demonstrated higher adiponectin levels in women versus men (Cnop et al., 2003; Kern at al., 2003; Silha et al., 2003; Tschritter et al., 2003), but no sexual dimorphism was noted between mares and geldings (Gordon et al., 2007). These results suggest a complexity of other factors, such as reproductive hormones may be involved in the negative feedback system, because women usually have a higher percent body fat than men.

Plasma adiponectin concentrations appear to vary somewhat between species. The range in human subjects is between 1.9 and 17.0 μg/ml, with significantly lower

concentrations found in obese subjects than non-obese subjects (Arita et al., 1999). Wild type mice had plasma adiponectin concentrations ranging from 10 to 30 μg/ml (Combs et al., 2004), and horses ranged between 1.3 and 2.0 μg/ml (Gordon and McKeever, 2005; Gordon et al., 2007). To date, plasma adiponectin levels have not been reported for any ruminants.

Receptors

There are two known, distinct adiponectin receptors, AdipoR1 and AdipoR2, which are 67% homologous. In 2005, Kadowaki and Yamauchi demonstrated that the AdipoR1 receptor was ubiquitously expressed but most abundant in skeletal muscle, whereas AdipoR2 was most abundantly expressed in the mouse liver. Both receptors appear to be integral membrane proteins; the N terminus being internal, and the C terminus being external, which is opposite to the topology of all other reported G protein-coupled receptors. Kadowaki and Yamauchi (2005) used Scatchard plot analysis to reveal that AdipoR1 is a receptor for globular adiponectin, whereas AdipoR2 is a receptor for full-length adiponectin. They were also able to successfully clone both AdipoR1 and AdipoR2.

Although most knowledge of adiponectin receptors has come from research using mice, one study was done to determine the presence and distribution of adiponectin and its receptors in swine (Lord et al., 2005). Lord et al. (2005) also investigated the effects of leptin and tumor necrosis factor-α (TNFα) on pig adiponectin, adipoR1 and adipoR2 gene expression. The results suggest that adiponectin and adipoR2 mRNA levels, but not adipoR1, are modulated by pig visceral fat tissues. Furthermore, the results indicated that TNFα interferes with adiponectin function by downregulation of adipoR2 but not adipoR1

mRNA levels in pigs. They also discovered that adiponectin receptors R1 and R2 are weakly expressed in the pig ovary.

Insulin Sensitizing Effects

The insulin-sensitizing effect of adiponectin was first discovered by three independent groups in 2001, using the murine model (Yamauchi et al., 2001). Genome-wide scans mapped a susceptibility locus for type 2 diabetes and metabolic syndrome to chromosome 3q27, where the gene encoding for adiponectin is located (Yamauchi et al., 2001). Insulin resistance is defined as a clinical state in which a normal or elevated insulin level produces an attenuated biologic response (Cefalu, 2001). It was observed that administration of adiponectin lowers circulating glucose and ameliorates insulin resistance in mice (Yamauchi et al., 2001). Furthermore, adiponectin-deficient mice develop insulin resistance and diabetes (Kubota et al., 2002; Maeda at al., 2002). These studies give reason to believe that adiponectin may be useful in the treatment for insulin resistance associated with type 2 diabetes.

In 2005, Kadowaki and Yamauchi identified three mechanisms of insulin-sensitizing action of adiponectin. The first of which is by reducing tissue triglyceride content in the skeletal muscle and up-regulating insulin signaling. This is achieved by increasing expression of molecules involved in fatty-acid transport such as the integral membrane protein CD36, by combustion of fatty-acids (by increasing acyl-coenzyme A oxidase), and by energy dissipation by increasing the uncoupling of protein 2. The second mechanism is by activating peroxisome proliferator-activated receptor-alpha (PPARα), which as a net result,

decreases triglyceride content in the liver and skeletal muscle and thus coordinately increases in vivo insulin sensitivity. The third mechanism is by stimulating phosphorylation and activation of AMP kinase (which is an important enzyme in many cell signaling pathways) in the skeletal muscle and liver.

The insulin-sensitizing effects of adiponectin have been well documented in humans and mice. However, these effects have only been lightly explored in other domestic animal models: two studies in horses and one in dairy cows. The first study of adiponectin in horses examined Standardbred mares, to test the hypothesis that there is diurnal variation of humoral mediators of peripheral energy balance including active ghrelin, adiponectin, leptin, glucose, insulin, and cortisol (Gordon and McKeever, 2005). That study found that adiponectin concentrations remained stable throughout the 24-hour blood sampling period. The second study tested the hypothesis that grain and intravenous dextrose challenges would alter plasma concentrations of active ghrelin, adiponectin, leptin, glucose, insulin and cortisol (Gordon and McKeever, 2006). The results showed there was no change in plasma adiponectin concentration throughout both the dextrose challenge and in response to the oral grain challenge.

Another study was recently conducted to research gene expression of adiponectin, peroxisome prolipherator-activated receptor gamma 2 (PPARγ2), and insulin-dependent glucose transporter 12 (GLUT12) in peak-, late-, and non-lactating Holstein cows (Komatsu et al., 2007). The results from this study demonstrated that mRNA levels of adiponectin and PPARγ2 in the adipose tissue were greater in non-lactating cows than in peak-lactating cows. In mature adipocytes, PPARγ2 activation induces a number of genes involved in the insulin-

signaling cascade, thereby increasing insulin sensitivity (Maeda et al., 2001). It was also reported that PPARγ2 is required for adiponectin expression during adipogenesis in 3T3-L1 adipocytes (Gustafson et al., 2003). Therefore, in lactating cows, a decrease in PPARγ2 expression in adipose tissue may influence the expression of adiponectin, and as a result, insulin resistance may increase in the whole body (Komatsu et al., 2007). There were no significant differences in the abundance of GLUT12 mRNA in adipose tissue, but the mRNA level of GLUT12 in the mammary gland was greater in non-lactating cows than in peak- or late-lactating cows. Komatsu et al., (2007) proposed that adiponectin and PPARγ2 may have an important function in the adipose tissue during lactation, and GLUT12 may have a possible role in regulating insulin-dependent glucose uptake and development in mammary epithelial cells.

Early Lactation Metabolism in Dairy Cows

At the start of lactation, dry matter intake (DMI) is insufficient to meet the demands of lactation, resulting in a period of negative energy balance (NEB) in the immediate postpartum period, which may last 20 weeks (Beever et al., 2001). Additional substrate required to support milk synthesis is provided through enhanced mobilization of adipose reserves and skeletal muscle (Veerkamp, 1998). Energy balance is not readily calculable on the farm level and is commonly assessed indirectly by monitoring changes in body condition score (BCS) (Pryce et al., 2001). Increased genetic merit for milk yield is associated with a greater degree of BCS loss in early lactation and lower BCS throughout lactation, reflecting a greater degree of NEB (Pryce et al., 2001; Gong, 2002). An antagonistic relationship exists

between genetic merit for milk yield and reproduction, with increased NEB in early lactation being cited as an underlying causal factor (Jorritsma et al., 2003; Pryce et al., 2004). Cows with a BCS of < 2.5 and cows that lose more than 1 unit in BCS after parturition had reduced pregnancy rates after timed artificial insemination (TAI) (Moreira et al., 2000). Stevenson et al. (1999) reported that for every unit increase in BCS conception rate increased 8.6 ± 4%. Santos et al. (2001) reported that cows with moderate BCS (≥2.75) had higher conception rates than those with lower BCS (<2.75) and cows gaining BCS from time of breeding to pregnancy diagnosis had higher conception rates than those maintaining or losing BCS. This supports the premise that poor nutritional status compromises reproductive function (Patton et al., 2007).

It should also be noted that significant metabolic differences exist between cows calving for the first and subsequent times (Meikle et al., 2004; Wathes et al., 2007). For example, in multiparous cows, extended intervals from calving to conception were associated prepartum with greater concentrations of leptin and lesser concentrations of non-esterified fatty acid (NEFA) and urea, and postpartum with reduced insulin-like growth factor-1 at week 2, greater urea at week 7, and greater peak milk yield (Wathes et al., 2007). In primiparous cows, extended intervals from calving to conception were associated with more body condition and more urea prepartum, elevated urea postpartum, and more body condition loss by week 7 (Wathes et al., 2007).

Nutrition, Hormones, and Fertility in Dairy Cows

The interaction between nutrition and reproduction in cattle is very complex, as nutrition can influence multiple sites in the reproductive axis (Gong et al., 2002). Nutrition can influence ruminant fertility directly by the supply of specific nutrients required for the processes of oocyte and spermatozoa development, ovulation, fertilization, embryo survival and the establishment of pregnancy. It can also influence fertility indirectly through its impact on the circulating concentrations of hormones and other nutrient-sensitive metabolites that are required for the success of these processes (Robinson et al., 2006). Increased severity of NEB in early lactation is associated with impaired ovarian function and delayed resumption of estrous cycles (Jolly et al., 1995; Beam & Butler, 1999). Various metabolites and metabolic hormones, including glucose, NEFA, insulin, and insulin-like growth factor-1 (IGF-1), have been implicated as factors affecting ovarian steroidogenesis, follicular dynamics, and in vitro oocyte development (Gong et al., 2002; Leroy et al., 2005). Mitchell et al. (2005) also concluded that the adipokines leptin, adiponectin and resistin produced by adipose tissue and altered with obesity, clearly influenced energy homeostasis and undoubtedly affect female fertility.

Insulin

Insulin is a key metabolic hormone in ruminant and non-ruminant animals (Hart et al., 1979a), although insulin sensitivity in peripheral tissues is much lower in lactating ruminants than in humans (Rose et al., 1997). There is a positive correlation between changes in insulin and body weight (Hart et al., 1979a). Insulin concentrations tend to

decrease in early lactation, particularly in higher yielding cows (Baird et al., 1980; Taylor et al., 2003). Regulation of nutrient partitioning through a change in tissue response to insulin was demonstrated during pregnancy and lactation (Vernon & Saski 1991). It was also concluded that as steers grew older, heavier, and fatter, their peripheral tissues and liver became less responsive to insulin (Eisemann et al., 1997). Although it is difficult to separate independent effects of BW, age, and body composition, Eisemann et al. (1997) states that the decreased sensitivity in older steers was likely due to increased percentage of fat in body tissues, because others have shown similar types of changes in insulin sensitivity as percentage of fat increased in body tissues of sheep (McCann et al., 1986; Bergman et al., 1989) and cattle (McCann and Reimers, 1985).

The hypoinsulinemia of early lactation is part of a series of coordinated changes that occur around the time of parturition in support of lactation. Low plasma insulin concentrations reduce glucose uptake by insulin-responsive tissues (adipose and muscle) and facilitate greater uptake by the mammary gland, a tissue that is not insulin-responsive (Bauman & Elliot, 1983). McDowell et al., (1987) also explains that the increase in glucose supply to the mammary gland during lactation is believed to result from an increase in insulin resistance as well as the decreased insulin-independent glucose uptake in tissues excluding those of the mammary gland. Thus, it is not surprising that genetic advances for milk production have resulted in lower levels of circulating insulin in Holstein cows (Bonczek et al., 1988).

Insulin has actions at all levels of the hypothalamic-pituitary-ovarian axis that likely influence fertility (Beam and Butler, 1999; Lucy et al., 2001). Perhaps NEB acting through

the combined metabolic signaling of low blood glucose and insulin concentrations along with elevated NEFA delays increases in gonadotropin (LH and FSH) pulses necessary for stimulation of ovarian follicles. Low blood insulin concentrations are also responsible for low IGF-1 production from the liver, which together reduces responsiveness of the ovary to gonadotropins (Butler, 2005). When early lactation cows were fed diets (duration of 50 days) designed to maximize the production of glucose precursors (propionate and gluconeogenic amino acids), and therefore increase circulating insulin concentrations, they had a reduced time to first postpartum ovulation, as well as reduced intervals from calving to first service and to conception (Gong, 2002). Gong concluded that feeding dairy cows a diet to increase circulating insulin concentrations during early lactation can improve reproductive performance, without negatively influencing milk yield or energy balance status.

Glucagon

Glucagon influences regulation of hepatic glucose metabolism, principally by accelerating glycogenolysis and gluconeogenesis to increase glucose output from the liver (Bassett, 1975). Insulin, acting directly on the islets of Langerhans of the pancreas, is a potent inhibitor of glucagon secretion (Unger, 1985; De Boer et. al, 1986). However, glucose can suppress pancreatic α cells in the absence of β cells and insulin. Hyperglycemia produces an apparent glucose unresponsiveness of α cells, probably by preempting and/or down-regulating the glucose sensing sites that mediate the inhibition of glucagon secretion after a rise in glucose. (Unger, 1985).

Ruminant dependence on hepatic gluconeogenesis suggests that glucagon may play an important role in glucose metabolism and possibly milk production (Brockman, 1978). Glucagon concentrations were similar throughout lactation (Herbein et. al, 1985; De Boer et. al, 1986), but above average milk production was associated with above average glucagon concentrations (Herbein et. al, 1985). Glucagon concentrations were not affected significantly by feed restriction, but did have large daily diurnal variations (De Boer et. al, 1985; De Boer et. al, 1986).

Insulin-like Growth Factor-1 (IGF-1) & Grown Hormone (GH)

High concentrations of circulating GH and low concentrations of insulin are typical of early lactation, and their respective concentrations facilitate adipose tissue mobilization in support of milk production (Butler et al., 2003). Circulating IGF-1 is produced mainly by the liver in response to GH (Jones & Clemmons, 1995) and this relationship forms the basis of the GH-IGF axis (Butler et al., 2003). During early lactation, when cows are in a period of NEB, the liver becomes refractory to GH (Vicini et al., 1991) and circulating IGF-1 concentrations are significantly reduced. The decline in circulating IGF-1 begins two weeks before parturition and is paralleled by a decline in plasma insulin (Butler et al., 2003) and a rise in plasma GH (Bell et al., 2000).

Adipose tissue contains high levels of IGF-1 mRNA and is responsive to GH (Peter et al., 1993). GH promotes lipolysis by antagonizing the anti-lipolytic activities of adenosine and enhancing the lipolytic response to catecholamines (Houseknecht & Bauman, 1997; Lanna & Bauman, 1999). Butler et. al, (2003) reported that the expression of both GH

receptors and IGF-1 mRNA in adipose tissue was reduced following insulin treatment. Their results indicate that high insulin concentrations are inhibitory to adipose GH receptor expression and thus antagonize the ability of adipose tissue to respond to GH.

Higher plasma insulin and IGF-1 concentrations have been associated with such reproductive measures as; fewer days to first postpartum ovulation, higher basal LH concentrations, LH pulse frequency, and subsequent progesterone concentrations following induced ovulation by GnRH (Gong, 2002). Insulin and IGF-1 are reported to stimulate both proliferation and steroidogenesis of bovine granulosa cells in a dose-dependent manner, but GH did not appear to have a direct effect on bovine ovarian follicles (Gong, 2002). Blood constituents such as NEFA, Beta-hydroxybutyrate (BHB), glucose, urea, and insulin are considered to be more direct indicators of energy balance and metabolic status than IGF-1. However, plasma IGF-1 concentrations may also be useful for predicting performance potential (both productive and reproductive) in dairy cattle (Moyes et al., 2006). One advantage to monitoring IGF-1 instead of other blood constituents is that plasma IGF-1 levels do not show significant diurnal variation, nor undergo rapid fluctuation. IGF-1 also shows greater variation between cows (Moyes et al., 2006)

Non-esterified Fatty Acid (NEFA)

When the concentration of glucose in plasma increases above a background level, insulin is released from the pancreas. Insulin mediates glucose disappearance from the blood by increasing the availability of glucose transporters (GLUT- 4) on the cell surface which enhances the uptake of glucose by tissue (Moate et al., 2007). Although NEFA are not

controlled to a similar intensity as glucose, they are both influenced by insulin (Sechen et al., 1989; Frayn et al., 1995). There are links between NEFA and glucose metabolism because elevated plasma NEFA concentrations have a major role in inhibiting glucose metabolism (Randle et al., 1963) and because glucose and NEFA are known to be reciprocally regulated (Tepperman & Tepperman, 1970; Sumner et al., 2007).

Increased circulating concentrations of NEFA have been observed in such hyperinsulinemic states as obesity, impaired glucose tolerance, type 2 diabetes, and dyslipidemia (Balent et al., 2002). During the immediate prepartum period, depressed DMI and endocrine changes result in increased NEFA in the blood of ruminants (Drackley, 1999). Weusten et. al (2005) reported that in humans, fat ingestion modulates β-cell function and that NEFA is a plausible mediator that acts as a link between fat and glucose metabolism by modulating glucose-stimulated insulin secretion. Under the condition of elevated plasma levels of NEFA, this mechanism may be responsible for hyperinsulinemia, which may lead to peripheral and hepatic insulin resistance (Weusten et al., 2005).

Blood NEFA concentrations may fluctuate considerably in the short term in response to feeding or stress (Frohl & Blum, 1988; Boisclair et al., 1997; Moyes et al., 2006). The principal way in which NEFA concentrations are regulated involves the inhibition of hormone sensitive lipase (HSL) by insulin (Ferrannini et al., 1997). HSL is an enzyme present in adipose tissue that hydrolyses triglycerides allowing release of NEFA and glycerol from adipocytes into the circulation (Frayn et al., 1995; Ferrannini et al., 1997).
Milk fat is composed of approximately 90% triglycerides, and these are assembled in the mammary gland, in part, from long-chain NEFA originating from the circulation (Taylor &

MacGibbons, 2002). More than 90% of the fatty acids in blood are triglycerides, but triglycerides cannot enter the mammary gland. Lipoprotein lipase (LPL), an enzyme bound to capillary endothelium, breaks down circulating triglyceride, releasing NEFA so that they can be absorbed into tissue or escape back into circulation (Frayn et al., 1995, Teusink et al., 2003). Therefore, NEFA are very important for milk fat production (Moate et al., 2007)

The Glucose Tolerance Test

The intravenous glucose tolerance test (IVGTT) is used primarily, in many species, to measure the clearance rate of glucose from the blood. The IVGTT is widely used as a way of detecting diabetes and insulin resistance in humans and other animals. The procedure begins by taking a baseline blood sample, followed by a rapid infusion of glucose. Doses of ~0.25g of glucose/kg body weight (Anderson et al., 2000), 0.30g of glucose/kg body weight (Hove, 1978; Moate et al., 2007) and 0.45g glucose/kg body weight (Sumner et al., 2007) have been administered to mature Holstein bulls, lactating Holstein cows, and growing Holstein heifers respectively. After the glucose administration, frequent blood samples are taken, usually for at least two hours.

In a normal/healthy animal, following a glucose infusion, blood glucose concentrations should rise quickly, then fall steadily, returning to baseline within two hours after the end of the glucose infusion (Anderson et al., 2000; Moate et al., 2007). Insulin concentrations should also peak quickly, but then remain elevated until levels return to baseline within one to two hours after the end of the glucose infusion (Hove, 1978; Moate et al., 2007). These two phases of insulin secretion have been interpreted to represent an initial

release from pancreatic insulin stores, followed by increased insulin production and release (Cerasi, 1967). Animals which are insulin resistant will have a prolonged period of elevated blood glucose, and exhibit a higher peak and prolonged period of elevated insulin concentrations.

Following an IVGTT of 20 growing Holstein heifers, NEFA concentrations increased slightly upon initiation of handling animals due to stress followed by a reduction in NEFA as glucose entry increased after infusion, and eventually ending with an increase of NEFA to basal concentrations as glucose decreased (Sumner et al., 2007; Moate et al., 2007).

The Insulin Tolerance Test (ITT)

Insulin tolerance tests (ITT) are the primary means by which the body's ability to react to a hypoglycemic state can be assessed. In human medicine, ITTs are used frequently to assess the integrity of the hypothalamo-pituitary-adrenal axis, and can help diagnose many diseases and endocrine disorders. An ITT begins by taking a baseline blood sample, followed by an intravenous insulin infusion. The insulin infusion causes hyperinsulinemia, which results in a hypoglycemic state within 45 minutes of the injection (Liew et al., 2004). In a normal/healthy individual, hypoglycemia causes an elevation in GH and cortisol concentrations. A Dose of 1μg insulin/kg of BW/hour using a hyperinsulinemic-euglycemic clamp was used successfully in lactating Holstein cows (Griinari et al., 1997; Butler et al., 2003) and a dose of 0.1IU insulin/kg of BW injection have been successful in growing beef steers (Kegley et al., 2000), and nonlactating, nongestating Holstein cows (Pires et al., 2007; Pires et al., 2008).

In one study, a trend was observed for GH to be lower in insulin-treated Holstein cows than in control cows (Butler et al., 2003). Insulin-treated cows received 1μg insulin/kg body weight/hr via a hyperinsulinemic-euglycemic clamp, for 96 hours, starting on day 10 postpartum. Hepatic expression of GH receptor 1A (GHR 1A) and IGF-1 mRNA was low in control cows, but was significantly increased in insulin-treated cows. The results of this study indicated that insulin is an important metabolic signal for the GH-IGF axis, coordinating the parallel increase in liver GHR 1A and IGF-1 mRNA resulting in the marked elevation in plasma IGF-1 levels (Butler et al., 2003).

Overview

Recent research into diets specifically designed to stimulate insulin secretion by maximizing the production of glucose precursors, increase progesterone production by the corpus luteum and enhance the antiluteolytic mechanism is providing new opportunities for improving dairy cow fertility with associated benefits for suckling beef cows (Robinson et al., 2006). The move to a more mechanistic approach in dealing with nutritional studies of fertility is providing information that can readily be adapted for the formulation of more efficient feeding strategies across a diverse range of ruminant species and production systems (Robinson et al., 2006). Endocrine control of metabolism is characterized by changes in circulating hormone concentrations and the ability of tissues to respond to these changes. In ruminants, as in other animals, alterations in hormonal sensitivity and responsiveness at the tissue level coordinate postabsorptive nutrient utilization in support of growth, pregnancy, and lactation and transitions between those physiological states (Vernon and Sasaki, 1991).

Given our knowledge of the insulin-sensitizing effects of adiponectin, it would be valuable to know what role adiponectin plays during early lactation in conjunction with the period of decreased circulating insulin concentrations. It would also be valuable to know how insulin sensitivity changes pre vs. postpartum, and what hormones and metabolites are involved, in individual cows.

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MEASUREMENT OF ADIPONECTIN IN LACTATING DAIRY COWS

INTRODUCTION

Insulin is a key metabolic hormone in ruminant and non-ruminant animals (Hart et al., 1979). The hypoinsulinemia of early lactation is part of a series of coordinated changes that occur around the time of parturition in support of lactation. Low plasma insulin concentrations reduce glucose uptake by insulin-responsive tissues (adipose and muscle) and facilitate greater uptake by the mammary gland, a tissue that is not insulin-responsive (Bauman & Elliot, 1983). Adiponectin is a recently discovered hormone that has been studied extensively in humans and rodents, but very little is known about adiponectin in cattle. Given that adiponectin has insulin-sensitizing effects in other species, it would be valuable to know if adiponectin plays a role during early lactation in conjunction with the period of negative energy balance (NEB) and decreased circulating insulin concentrations. The primary goals of this preliminary study were to determine if adiponectin could be measured in bovine plasma, and to monitor adiponectin concentrations over the first 11 weeks of lactation in Holstein cows. We also looked for relationships between adiponectin and insulin, milk yield, body condition score (BCS), and progesterone (time to first postpartum estrous cycle).

MATERIALS & METHODS

The North Carolina State University Animal Care and Use Committee approved the experimental procedure and all animals were handled and maintained in accordance with the standards in The Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999). Blood samples were collected weekly from Holstein cows (n = 26) for 11 weeks, beginning the first week of their lactation. All cows calved during November or December, and ranged between first (n = 8), second (n = 7), third (n = 6), fourth (n = 4) and fifth (n = 1) lactations. Weekly blood samples were taken from the tail vein, shortly before the evening milking, at approximately 1630 h, when the cows were being fed, and could easily be caught in headlocks with minimal stress to the cows. Cows were fed a total mixed ration (TMR) (Table 1), and were milked twice daily.

Blood for plasma adiponectin, progesterone and insulin was collected into 10 ml tubes containing sodium heparin (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) and kept on ice until it was centrifuged at 1000xg for 15 minutes at 4°C using an IEC DPR 6000 model centrifuge. Plasma was stored frozen at -20°C until analysis using commercially available radioimmunoassay (RIA) kits. In the absence of purified bovine adiponectin, adiponectin concentrations were measured using human RIA kits (HADP-61 HK, Linco, Millipore). There is no RIA kit available that has been validated for bovine adiponectin, but the Linco kit has been validated for horses (Gordon and McKeever, 2005; Kearns et. al, 2006). Some beef bull (n = 30) and horse samples (n = 5) were also assayed using the Linco

kits for comparison to our cow samples. The sensitivity of the assay was 1ng/ml and the intraassay and interassay CVs were all <10%.

Plasma insulin concentrations were measured using a commercial RIA kit (Coat-a-Count, Siemens Medical Diagnostics, Berkeley, California). The sensitivity of the assay was 1.2 μIU/ml and the intraassay CV was <10%. Plasma progesterone concentrations were measured using commercial RIA kits (Coat-a-Count, Siemens Medical Diagnostics, Berkeley, California). The sensitivity of the assay was 0.02 ng/ml and the intraassay and interassay CVs were all <10%. Body condition scores (BCS) were taken three times throughout the sampling period by the same person; the first near the beginning, the second near the middle, and the third near the end. Milk yield data were collected and energy-corrected milk yield were calculated using the formula energy corrected milk = amount of milk * (0.327 + (7.2 * % milk protein/100 +12.95 * % milk fat/100) found at http://www.thedairycenter.org/applications/calcs/calc.asp?/.

Table 1. Lactating cow total mixed ration (TMR) chemical composition.

Dry Matter Contains:	
Crude Protein	18.1%
Undegradable Intake Protein	7.2%
Soluble Protein	5.4%
Acid Detergent Fiber	20.4%
Neutral Detergent Fiber	32.6%
Non Fiber (Carbohydrate)	36.0%
Net Energy (Mcal)	1.7/kg
Fat	5.9%

Statistical Analyses

The model included cow and week, with week as a repeated measure and weekly means were compared using Tukey's test. All possible weekly comparisons were made. It was decided to compare all subsequent weeks to week zero since most comparisons after the first weeks were not different. After the first analysis cows were grouped according to lactation number (primiparous (n=8) vs. multiparous (n=18), body condition score (<2.5 (n=8) vs. ≥2.5 (n=18)) and milk yield (<36.4 kg per day (n=12) vs. >36.4 kg per day (n=14)) and the effect of those variables was analyzed using PROC GLM. The model included either lactation number, body condition score or milk yield along with week and the means were compared using Tukey's test.

RESULTS

Adiponectin

Data are presented as mean ± SE, both in text and figures, for all hormones. Average plasma adiponectin concentrations increased steadily in the first four weeks of lactation, and nearly doubled from week one (average = 8.22 ± 1.26 ng/ml) to week four (average = 15.79 ± 2.19 ng/ml) (Figure 1). After week four, adiponectin levels declined slightly to remain at 12-13ng/ml for the remainder of the study. Plasma adiponectin concentrations varied greatly among individual cows, but there was no effect of lactation number, BCS or energy corrected milk yield on adiponectin concentrations. The range of measured adiponectin concentrations throughout the experiment, considering all cows, was between 0.0 and 80.0 ng/ml. There was no relationship between concentrations of adiponectin and time to first ovulation.

Insulin

Average plasma insulin concentrations increased steadily from week one (average = 4.70 ± 4.79 μIU/ml) to week four (average = 12.35 ± 10.38 μIU/ml) (Figure 2). After week four, insulin concentrations stabilized and remained between 11 and 15 μIU/ml. Within individual cows, insulin showed a -0.22 correlation with adiponectin ($p < 0.12$). There was no relationship between insulin concentrations and energy-corrected milk yield.

Other Variables

All cows had plasma progesterone concentrations >1 ng/ml (which indicated ovulation) by 10 weeks postpartum. Cows lost an average of 0.2 points of BCS from first to second scoring, then had a slight average gain of 0.06 points of BCS from the second to third scoring. Neither the loss nor gain of BCS was significant. The average BCS at first, second, and third scorings was 2.51 ± 0.07, 2.31 ± 0.06 and 2.37 ± 0.07, respectively. Energy adjusted milk yields averaged 39.2 ± 5.9 kg. As expected, lactation number had an effect on milk yield but after adjusting for mature equivalent there was no relationship between milk yield and adiponectin or insulin concentrations. Mature equivalent adjustments were made using values from the National Dairy Herd Improvement Association (http://www.dhia.org) for mature 305 day lactation adjustments.

DISCUSSION

In the absence of purified bovine adiponectin, a commercially available human RIA kit was used because there were no kits available that had been validated for use with bovine plasma. Dilution of plasma as recommended by the assay manufacturer resulted in samples being below the sensitivity of the assay. Using undiluted plasma, adiponectin concentrations ranged from 0-80 ng/ml. This range is far lower than ranges in other species that have been reported such as humans (1.9-17.0 μg/ml) (Arita et al., 1999), wild-type mice (10-30 μg/ml)(Combs et al., 2004), and horses (1.3-2.0 μg/ml) (Gordon et al., 2007; Gordon and McKeever, 2005). Individual bovine samples were assayed in various dilutions and the results showed parallelism. Plasma samples were also collected from bulls that were in a feed trial study, gaining ~1.4 kg/day. Similar to the cows, adiponectin in the bull samples was undetectable when diluted, but was in the 0-40 ng/ml range when the samples were not diluted. Some equine samples were assayed along with the bull samples and after dilution (1:500), the equine samples were in the ng/ml range, and after adjustment for the dilution, they ranged from 1-2 μg/ml, which agrees with the previously reported plasma adiponectin concentrations in horses (Kearns et. al, 2006). The Linco human RIA kit uses a polyclonal antibody against nearly the entire adiponectin molecule (personal communication). The bovine adiponectin protein sequence, at the amino acid level, shares >85% identity with the mouse, human, and horse sequences (BLAT analysis, Invitrogen Vector NTI Advance 10). With such high homology between species, it is possible that the assay is correctly reading the bovine adiponectin concentrations. However, considering the vast difference between

our bovine adiponectin concentrations and the concentrations that have been reported in other species, it is uncertain if the human kit can accurately measure adiponectin in bovine plasma. It is possible that bovine adiponectin has a lower binding affinity to the antibody in the kit than adiponectin of other species.

From this study, we conclude that adiponectin can be measured in bovine plasma using the Linco kit. However, until an assay can be specifically developed for bovine plasma, that assay kit should only be used to report qualitative trends in bovine adiponectin and not be used as a means of comparing concentrations quantitatively, against other species.

Average plasma adiponectin concentrations increased significantly during the first four weeks of lactation. At four weeks postpartum, adiponectin peaked, and nearly doubled in comparison to concentrations in week one. After week four, adiponectin decreased slightly and maintained average concentrations between 12 and 13ng/ml for the remainder of the study. These data suggest that adiponectin may play a role during the period of extreme metabolic change, when NEB and milk production are increasing. It was also observed that individual cows' adiponectin concentrations varied considerably from each other. Cows with higher adiponectin concentrations overall tended to have wider ranges of values, and cows that had lesser concentrations had narrower ranges over the course of the study. This may suggest that adiponectin is regulated in a somewhat chronic manner versus a more acute manner, and that adiponectin concentrations may vary genetically to a certain extent.

Although the -.22 correlation between adiponectin and insulin within individual cows was just at a p-value of 0.12, it may suggest a trend. Because adiponectin increases tissue sensitivity to insulin, higher adiponectin concentrations may facilitate a biological response

to lower concentrations of insulin. This relationship seems to be independent of the increasing trends in average adiponectin and insulin concentrations that were observed during the first 4 weeks of lactation. Further exploration into the role of adiponectin in ruminant metabolism would be beneficial to gain a better understanding of its functional importance.

The cows in our study did not lose as much body condition as some cows do during early lactation, and level of milk production did not correlate with BCS loss. More directly aimed studies may be able to show a negative correlation between adiponectin and adiposity in cattle, similar to what is reported in many other species. The small amount of BCS loss in our cows may also explain why average insulin concentrations increased over the first four weeks of lactation, instead of decreased, as we had expected based on previous literature (Bauman & Elliot, 1983).

It does not appear that adiponectin has a direct relation to time to the first postpartum ovulation as indicated by progesterone levels.

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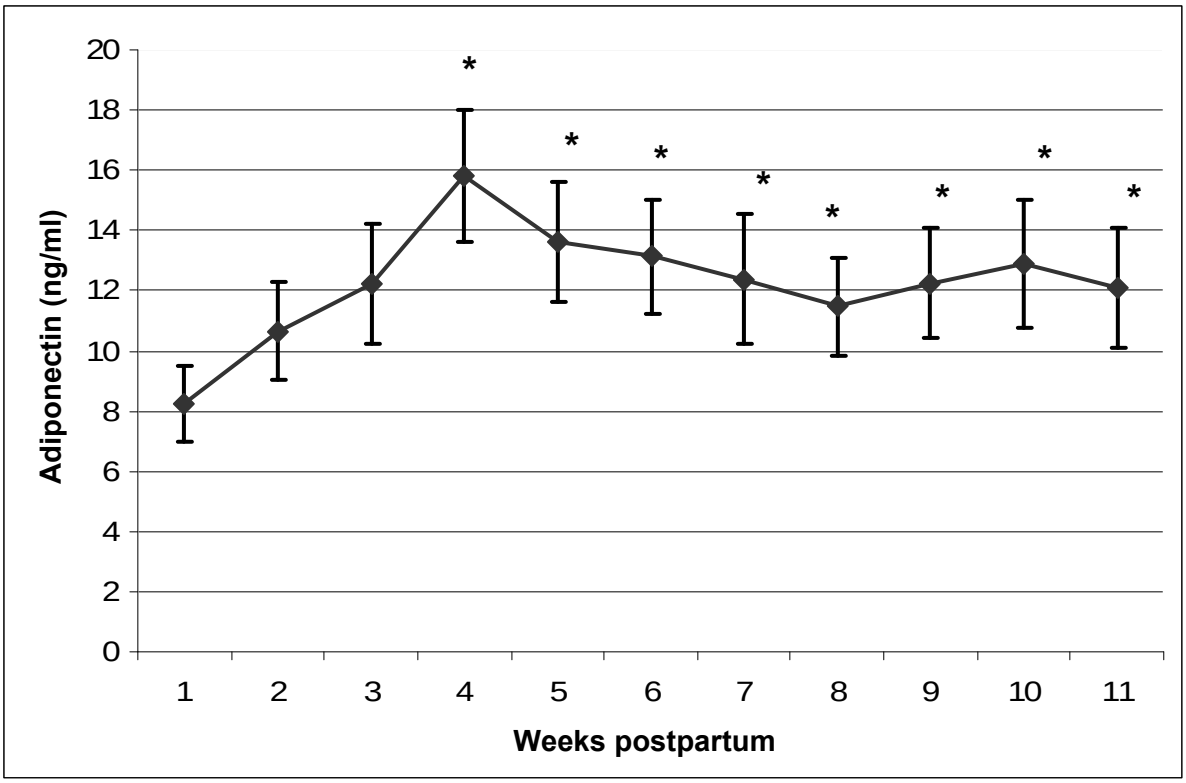
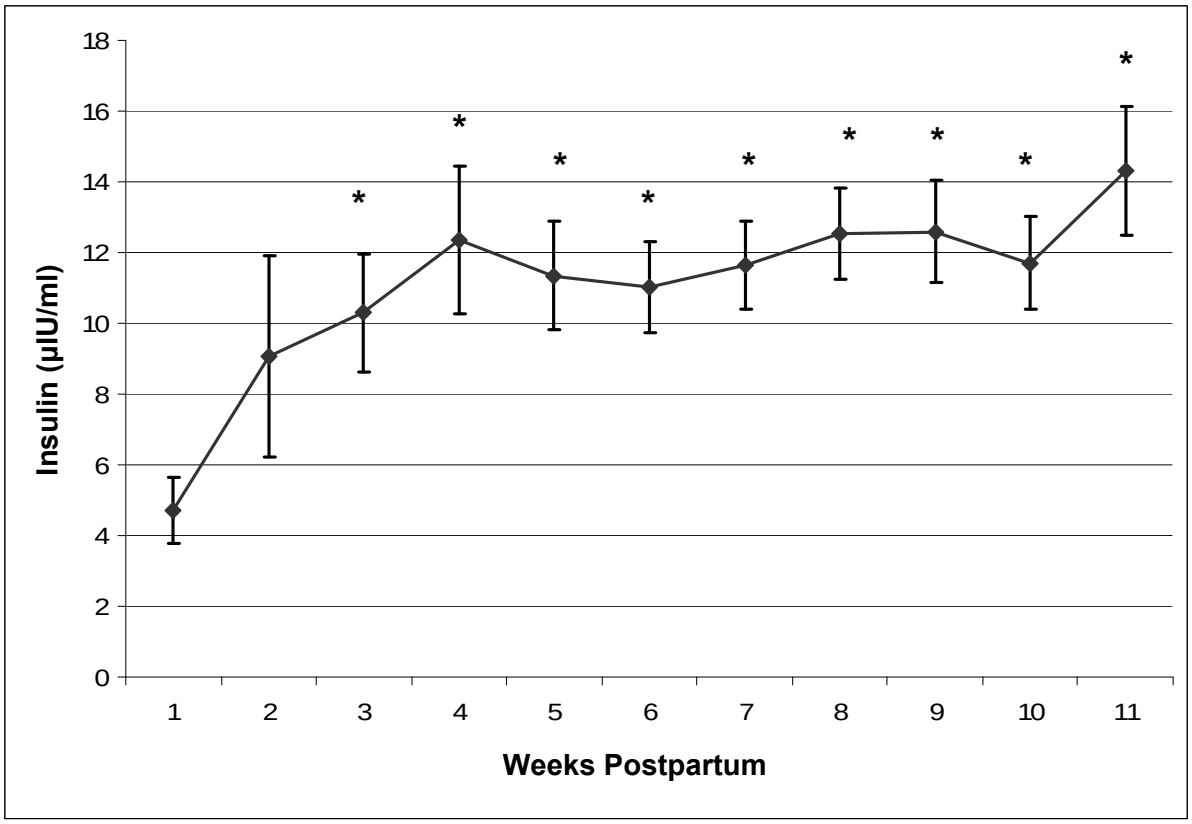


*** = Weeks differing from week one (P ≤ 0.05) (n = 26)**

Figure 1. Mean plasma adiponectin concentrations over the first 11 weeks of lactation in 26 Holstein cows sampled weekly.



*** = Weeks differing from week one (P ≤ 0.05) (n = 26)**

Figure 2. Mean plasma insulin concentrations over the first 11 weeks of lactation in 26 Holstein cows sampled weekly.

ADIPONECTIN, INSULIN, NEFA, AND GLUCAGON CONCENTRATIONS DURING AN IVGTT AND AN IVITT IN LACTATING VS. NON-LACTATING HOLSTEIN COWS

INTRODUCTION

Insulin is a key metabolic hormone in ruminant and non-ruminant animals (Hart et al., 1979). The hypoinsulinemia of early lactation is part of a series of coordinated changes that occur around the time of parturition in support of lactation. Low plasma insulin concentrations reduce glucose uptake by insulin-responsive tissues (adipose and muscle) and facilitate greater uptake by the mammary gland, a tissue that is not insulin-responsive (Bauman & Elliot, 1983). The insulin-sensitizing effects of the adipose-secreted hormone, adiponectin were discovered in 2001, and have since been well described in humans and rodents, but very little is known about adiponectin in cattle.

Komatsu et al. (2007) reported that adiponectin mRNA in adipose tissue was greater in non-lactating cows than in cows in peak lactation. In our preliminary study, we discovered that adiponectin could be measured in bovine plasma using a human radioimmunoassay (RIA) kit (HADP-61 HK, Linco, Millipore). We hypothesized that plasma adiponectin concentrations would be higher in non-lactating cows than in lactating cows, and that low levels of adiponectin may mediate the insulin resistance needed for the support of lactation. The goal of this study was to observe hormone and metabolite responses to an intravenous glucose tolerance test (IVGTT) and an intravenous insulin tolerance test (IVITT) during late pregnancy compared to early lactation. Plasma glucose, insulin and adiponectin, and serum non-esterified fatty acid (NEFA) and glucagon concentrations were measured.

MATERIALS & METHODS

The North Carolina State University Animal Care and Use Committee approved of the experimental procedure and all animals were handled and maintained in accordance with the standards in The Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999). Four, multiparous, Holstein cows of third (n = 3) and fourth (n = 1) lactations, and at 44.25 ± 3.3 days prepartum, were weighed and fitted with indwelling jugular catheters on day 1. The cows were kept in a dry lot before cannulation and were fed hay ad-libitum, and were given the lactating cow total mixed ration (TMR) (Table 1) during blood sampling. At the time of catheterization, the body weight (BW) of the cows was 721.8kg ± 46.5kg. Cows were returned to the outdoor dry lot enclosure and allowed access to hay and water overnight. The next day (day 2), the cows were locked in stanchions, and were allowed the lactating cow TMR during the duration of restraint. All catheters were checked and flushed with ~10 ml of 3.5% sodium citrate solution to ensure that all cows had functional catheters. The cows were then given an intravenous glucose tolerance test (IVGTT) by infusing 0.25g of glucose/kg BW (dextrose 50% wt./vol., Fisher; Raleigh, NC) dissolved in 0.9% sterile saline. This dose was chosen because it has been successfully used in nonlactating, nonpregnant Holstein cows (Pires et al., 2007; Pires et al., 2008) and mature Holstein bulls (Anderson et al., 2000). Blood samples were obtained before (-30 and -15) and 0, 5, 10, 15, 20, 30, 45, 60, 90, 120, and 150 minutes after each cows' infusion. Infusions did not take longer than 3 minutes. The 0-samples were taken immediately prior to the infusions and the 5-minute samples were taken 5 minutes after

approximately half of the glucose solution had been infused. Catheters were flushed with ~5 ml sodium citrate solution after each sample was taken. After the last blood samples were taken, catheters were again flushed with ~10 ml sodium citrate solution and the cows were returned to their dry lot enclosure. On day 3, the cows were again locked into stanchions, allowed the lactating cow TMR, and their catheters were checked and flushed. Each cow was then given an intravenous insulin tolerance test (IVITT) by administering a single intravenous injection of 0.1 IU bovine insulin/kg BW dissolved in saline (Sigma Chemical; St. Louis, MO). This insulin dose was chosen because it has been successfully used in nonlactating, nongestating Holstein cows (Pires et al., 2007; Pires et al., 2008) and growing beef steers (Kegley et al., 2000). The insulin preparation contained 27 IU/mg and the insulin was diluted so that injection volumes were 3-4ml. Blood samples were obtained at the same time points as the IVGTT. After sampling was complete, catheters were removed and the cows were returned to the dry cow herd.

After parturition, cows were moved into the lactating herd and the above procedure was repeated when the cows were 19.5 ± 3.2 days postpartum. The cows were then being fed the lactating cow TMR (Table 1), and were milked twice daily. The cows were also offered the TMR during blood sampling. On the day of catheterization, the cows weighed 673.4 ± 38.2 kg.

Blood samples for plasma glucose, insulin, and adiponectin were collected into 10-ml tubes containing sodium heparin (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) and kept on ice until they were centrifuged at 1000xg for 15 minutes at 4°C using an IEC DRP 6000 model centrifuge. Plasma was stored frozen at -20°C until analysis using commercially

available radio immuno assay (RIA) kits. In the absence of purified bovine adiponectin, adiponectin concentrations were measured using human RIA kits (HADP-61 HK, Linco, Millipore). There is no RIA kit available that has been validated for bovine adiponectin, but the Linco kit has been validated for horses (Gordon and McKeever, 2005; Kearns et. al, 2006). Sensitivity of the assay was 1 ng/ml and the intraassay and interassay CVs were all <10%. Plasma insulin concentrations were measured in a single assay, using a commercially available insulin RIA kit (Coat-a-Count, Siemens Medical Diagnostics, Berkeley, California). The intraassay CV was <10%, and the sensitivity of the assay was 1.2 μIU/ml. Plasma glucose concentrations were measured using a commercially available colorimetric enzymatic test (Mutarotase- glucose oxidase (GOD)) and spectrophotometer (at 505nm) (Wako Chemicals; Richmond, VA). When saline is assayed using this method, the absorbance is not more than 0.07.

Blood for serum non-esterified fatty acid (NEFA) and glucagon was collected into 7-ml tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ), allowed to clot, and kept on ice before serum was collected after centrifugation. Serum was stored frozen at -20°C until analysis. Serum NEFA concentrations were measured using a commercially available enzymatic colorimetric microtiter technique and a microplate reader (Wako Chemicals; Richmond, VA). The minimum detectable concentration using this method is estimated to be 0.0014 mEq/L. Serum glucagon was measured using a commercially available RIA kit (GL-32K, Linco, Millipore). The intraassay and interassay CVs were all <10%, and the sensitivity of the assay was 20 pg/ml.

Milk yield for the cows averaged 48.8 ±5.4 kg per day during the week before the postpartum blood sampling and was not affected by blood sampling on either day.

Statistical Analyses

Hormone concentrations were analyzed for the effect of cow and prepartum or postpartum status using PROC GLM (SAS 9.1, SAS, Cary, NC) for analysis of variance using repeated measures. The model included cow, treatment (prepartum vs. postpartum), time (blood sample) and the treatment by time interaction. Means were compared using Tukey's test when main effects were significant. Area under the curve for glucose and insulin after the glucose tolerance test was calculated from time zero until 90 minutes after glucose infusion using the trapezoid method using SAS 9.1 as described by Yeh (www2.sas.com/sugi27/p229-27.pdf posted on April 14, 2002). The times from zero to 90 were chosen because it appeared that concentrations had returned to preinfusion levels by then. Areas under the curve were compared between prepartum and postpartum times using PROC TTEST (SAS 9.1).

RESULTS

Due to a severe mastitis infection, cow number 5285 was omitted from the postpartum data because her milk production dropped to < 10 kg/day and she developed an abnormal hormone profile. When cow number 5285 was removed from the prepartum data, it did not change the significance of any of our results, but it did change the values for the area under the curve (AUC), so both values are reported. Data are presented as mean ± SE, both in text and figures, for all hormones.

Glucose

Following the prepartum and postpartum glucose infusions, there was a significant increase in plasma glucose concentrations in the 5 to 30 minute samples compared to time 0 ($p \leq 0.05$) (Figure 3). Glucose returned to basal concentrations in the 45-minute samples, both pre and postpartum (Figure 3). The area under the curve (AUC) for glucose from time zero until 90 minutes after glucose infusion was greater (1827.6 ± 125 mg/dL (including 5285)) (1737.5 ± 102.4 mg/dL (excluding 5285)) than postpartum (1438.2 ± 93.7 mg/dL) ($p < 0.01$). No change in glucose was observed following the insulin injections pre or postpartum (Figure 4).

Insulin

A significant rise in circulating insulin concentrations occurred in response to the pre and postpartum glucose infusions. Insulin concentrations rose significantly higher prepartum versus postpartum ($p < 0.0001$), and insulin concentrations returned to basal levels significantly faster postpartum (by the 15-minute sample) versus prepartum (by the 45-minute sample) ($p < 0.01$) (Figure 5). The AUC for insulin from time zero until 90 minutes after glucose infusion prepartum (2841.3 ± 236.9 μIU/mL (including 5285)) (2696.1 ± 188.5 μIU/mL (excluding 5285)) was greater than the AUC for insulin postpartum (1619 ± 135.7 μIU/mL) ($p < 0.01$). The insulin dose of 0.1 IU/kg BW caused circulating insulin to rise, but there was no difference between pre and postpartum time to return to basal concentrations, nor did it cause a reduction in pre or postpartum plasma glucose concentrations (Figure 4). The insulin injection also had no effect on adiponectin concentrations (Figures 8 and 9).

Adiponectin

Average plasma adiponectin concentrations were significantly higher in the prepartum cows (average = 75.26 ± 1.74) than in the postpartum cows (average = 10.23 ± 1.44) ($p < 0.0001$) (Figure 7). Individual variation among animals was observed, similar to our preliminary study (Figures 8 and 9). Animals with the highest prepartum adiponectin concentrations had the lowest concentrations postpartum, and vice versa. Plasma adiponectin concentrations remained stable throughout both the IVGTT, and the IVITT (Figures 8 and 9).

Non-esterified fatty acid (NEFA)

NEFA tended to decrease over the course of sampling on some days, but remained stable on others. The decreasing trends were not in response to the glucose infusions or insulin injections, as the declines began before the treatments were administered (Figure 10). There was no significant difference between overall average prepartum and postpartum NEFA concentrations which were 69.6 ± 24.61 and 66.17 ± 23.39, respectively.

Glucagon

Glucagon was unaffected by both the pre and postpartum IVGTT and IVITT (Figure 11). There was no significant difference between overall prepartum and postpartum glucagon concentrations.

DISCUSSION

Insulin

During lactation, the pancreas reduces insulin secretion and becomes less responsive to insulinotropic agents such as glucose (Lomax et. al, 1979). In the present study, both the pre and postpartum glucose infusions elicited a rise in circulating insulin concentrations, but the rise in insulin was significantly higher prepartum than postpartum. These data support the findings of Lomax et. al, (1979). However, it should be noted that basal insulin levels varied greatly from day to day, and the differences among basal insulin concentrations may have had a small effect on the overall insulin responses. Other studies using nonlactating, nonpregnant Holstein cows have reported average basal insulin concentrations of 10.2μIU/ml (Pires et al., 2007) and 28.7μIU/ml (Pires et al., 2008), but the average daily basal insulin concentrations in our cows ranged between 2.7 and 62.2μIU/ml. Based on data from our preliminary study, this degree of daily fluctuation in insulin concentrations does seem unusually high, although not completely impractical, as insulin concentrations in our preliminary study ranged between 0 and 80μIU/ml. The small sample size of cows in the present study may be a reason for this observation.

Postpartum insulin concentrations returned to basal levels approximately 30 minutes sooner than prepartum insulin during the IVGTT. An enhanced insulin metabolic clearance rate in lactating cows may be facilitated by an up-regulation of insulinases in the liver and kidneys, and has been previously documented (Sano et. al, 1993). The lesser rise in, and accelerated time it took for insulin concentrations to return to pre-injection levels during the postpartum IVGTT indicates a reduced insulin responsiveness in postpartum cows. Also, the

smaller postpartum AUC of insulin during the IVGTT provides further evidence that the postpartum cows had reduced insulin responsiveness. This reduced insulin responsiveness during early lactation spares glucose from peripheral tissues, thus shunting glucose toward lactose synthesis (Subiyatno et. al, 1996). This metabolic phenomenon has been reported by many other studies (Lomax et. al, 1979; Sartin et. al, 1985; Sartin et. al, 1988; Sano et. al, 1993).

The smaller AUC for postpartum glucose during the IVGTT suggests that glucose utilization was increased in postpartum cows, which is likely due to the increased demand for glucose during lactation. There was no glucose response to either the pre or postpartum insulin injections. Because our insulin dose did not elicit a reduction in circulating glucose, but a rise in insulin concentrations did occur, this alone may be an indicator of insulin resistance in both the pre and postpartum cows. The injection dose of 0.1 IU insulin/kg BW has been used successfully in growing beef steers (Kegley et. al, 2000) and nonlactating, nongestating Holstein cows (Pires et al., 2008), but in each of those studies, circulating insulin concentrations rose much higher (~500μIU in growing beef steers and ~1,200-1,500μIU in nonlactating, nongestating Holstein cows) during the ITTs than it did in the present study. Based on rough calculations of the blood volume in the cows used in the present study, it would be expected that such a dose of insulin would result in concentrations peaking at approximately 1200μIU shortly after the insulin injections, but our results showed peaks of <250μIU. This suggests that there may have been a problem with the insulin we used, or an error in either our dose calculation, or in the administration/saline dilution of our injections.

Adiponectin

Plasma adiponectin concentrations were unaffected by the IVGTT, which is consistent with a previous study in horses (Gordon and McKeever, 2006). Individual variation was observed, similar to the data gathered in our preliminary study. It was also noted that the cows with the highest prepartum adiponectin concentrations, had the lowest concentrations postpartum, and vice versa. This suggests that adiponectin fluctuates more in some cows than it does in others, and that adiponectin secretion and responsiveness to changes in metabolic state may vary genetically, between animals. The bovine adiponectin gene is located on chromosome 1, in the ClQ domain, which codes for the collagen superfamily. Alterations in the human adiponectin gene have been shown to cause insulin resistance and diabetes (Yamouchi et. al, 2001; Kadowaki and Yamouchi, 2005). If there is genetic variation in adiponectin secretion in dairy cows, it may be valuable to learn more about its effects.

Decreased insulin responsiveness in lactating dairy cows has been linked to energy balance (Terashima et. al, 1991), obesity (McCann et. al, 1989), age (McClary et. al, 1988), and concentrations of sex steroids (Sartin et. al, 1988; McCann et. al, 1989). Adiponectin is shown to have multiple mechanisms of action that increase insulin sensitivity in humans and mice (Kadowaki & Yamouchi, 2005). In this study, we found that plasma adiponectin concentrations were significantly higher in prepartum cows than in postpartum cows. These data parallel that of Komatsu et al. (2007), who reported that mRNA levels of adiponectin in adipose tissue were greater in non-lactating cows than in cows at peak lactation. These data suggest the possibility that low adiponectin concentrations help facilitate postpartum insulin

resistance which increases the supply of glucose to the mammary gland, and aids in the metabolic support of lactation.

NEFA

During our experiments, NEFA tended to decrease over the course of sampling on some days, but remained stable on others. The decreasing trend was not in response to the glucose infusions or insulin injections, as the decline began before the treatments were administered. These trends were probably due to feeding schedules, as blood NEFA concentrations may fluctuate considerably in the short term in response to feeding or stress (Frohl & Blum, 1988; Boisclair et al., 1997; Moyes et al., 2006). The NEFA levels that were observed in the present study were similar to those reported by Chung et al, (2007) for lactating Holsteins in the first 21 days postpartum.

Glucagon

Glucagon did not respond to the glucose infusion or the insulin injection prepartum or postpartum, which was not surprising because it has been shown that hyperglycemia produces an apparent glucose unresponsiveness of α cells, probably by preempting and/or down-regulating the glucose sensing sites that mediate the inhibition of glucagon secretion after a rise in glucose. (Unger, 1985). Because there was no decline in plasma glucose concentrations after the pre or postpartum IVITT, hypoglycemia could not have stimulated an increase in glucagon secretion. Furthermore, there was no significant difference between overall prepartum and postpartum glucagon concentrations. The glucagon concentrations

observed in the present study were within the range that has been previously reported for dairy cows in early lactation (Herbein et al., 1985; DeBoer et al., 1986).

Conclusions

Increased severity of negative energy balance (NEB) in early lactation is associated with impaired ovarian function and delayed resumption of estrous cycles (Jolly et al., 1995; Beam & Butler, 1999). Insulin has actions at all levels of the hypothalamic-pituitary-ovarian axis that likely influence fertility (Beam and Butler, 1999; Lucy et al., 2001). A NEB may act through the combined metabolic signaling of low blood glucose and insulin concentrations along with elevated NEFA to delay increases in gonadotropin (LH and FSH) pulses necessary for stimulation of ovarian follicles. Thus, it is not surprising that genetic advances for milk production have led to a higher degree of postpartum NEB, resulting in lower concentrations of circulating insulin in Holstein cows (Bonczek et al., 1988).

Our research suggests that adiponectin could have a role in the facilitation of postpartum insulin resistance in dairy cows. Lower adiponectin concentrations appear to be beneficial in support of lactation. Although a certain level of insulin resistance (and reduced circulating adiponectin) may be beneficial for supporting lactation, extreme insulin resistance may be a culprit in the growing percentage of reproductively inefficient, high-producing dairy cows. It may be valuable to determine what effects adiponectin administration has on reproductively inefficient dairy cows, and if it ameliorates insulin resistance, as it does in mice (Yamauchi et al., 2001). If administration of adiponectin was able to temporarily sensitize anestrous cows to insulin, it may be enough to allow for the required increase of

gonadotropins to result in an ovulation. Another possibility is that adiponectin may be useful for preventing extreme loss of body condition in cows that may have started lactation underweight, or in cows that become sick during lactation.

Further exploration into the role of adiponectin in ruminants is needed in order to fully understand this hormone's possibilities as a means of health and/or reproductive management. Genetic variability in adiponectin may also be avenue worth exploration.

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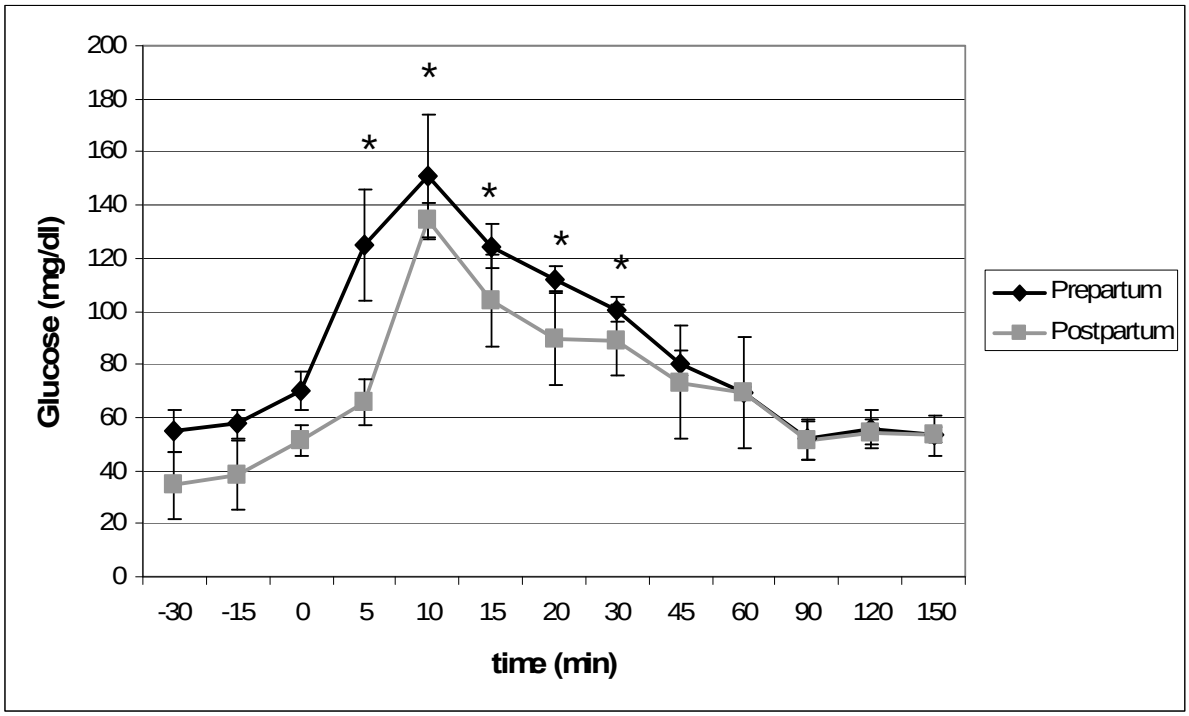
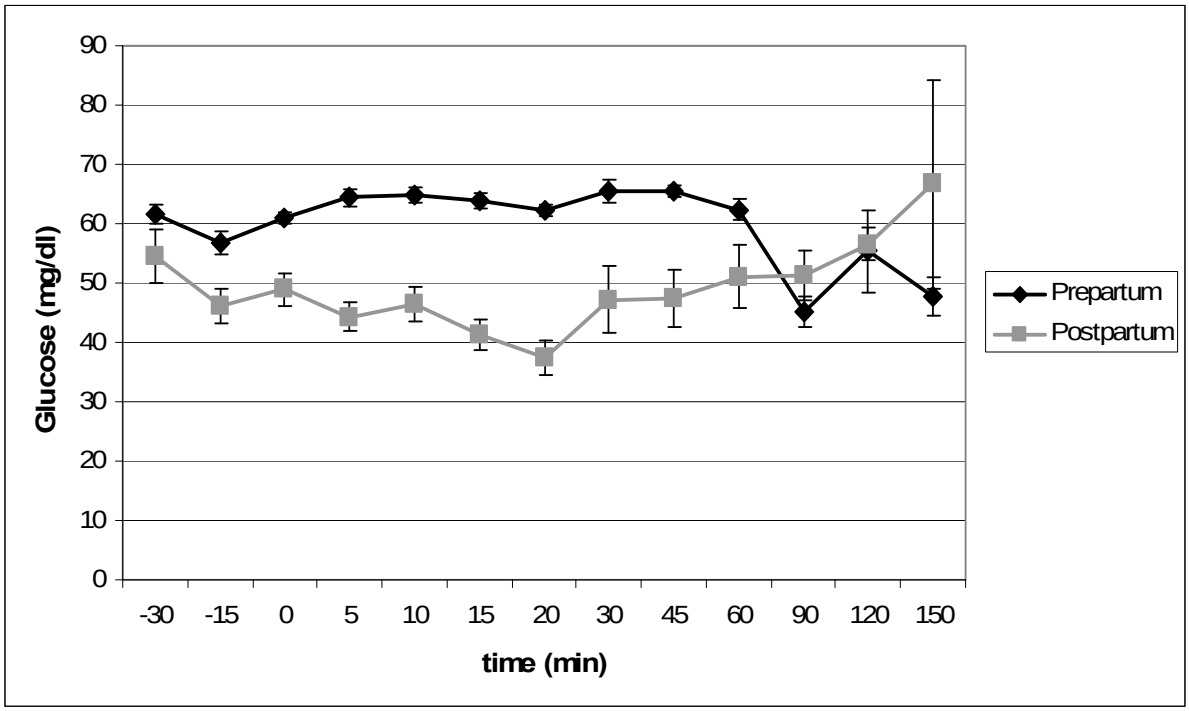


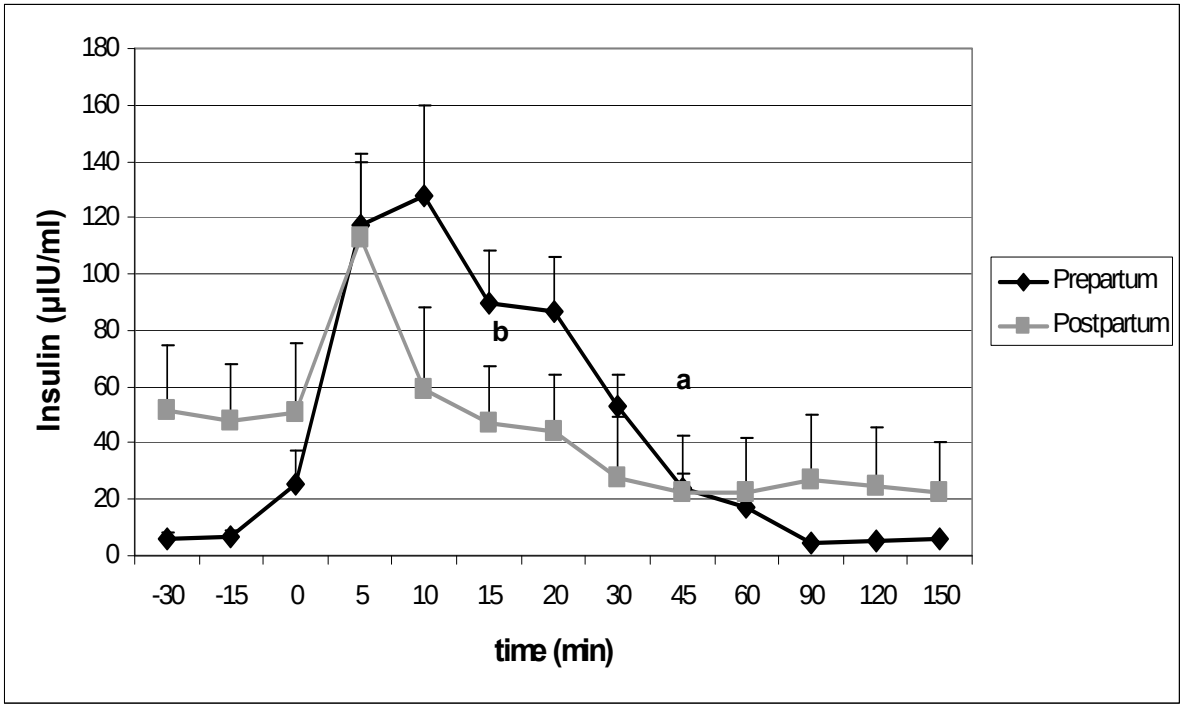
*** = pre and postpartum times differing from time 0 ($p \leq 0.05$)**
n = 4 prepartum
n = 3 postpartum

Figure 3. Mean pre and postpartum plasma glucose concentrations before and after an intravenous injection of 0.25g of glucose/kg BW.



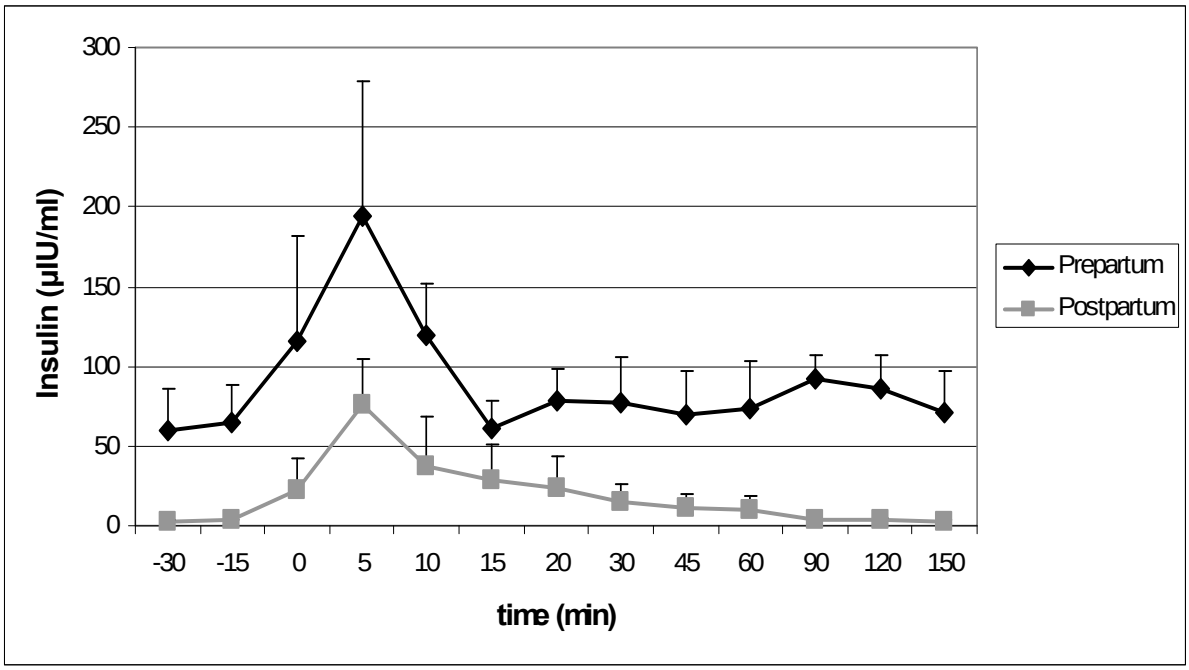
n = 4 prepartum
n = 3 postpartum

Figure 4. Mean pre and postpartum plasma glucose concentrations before and after an intravenous injection of 0.1 IU insulin/kg BW.



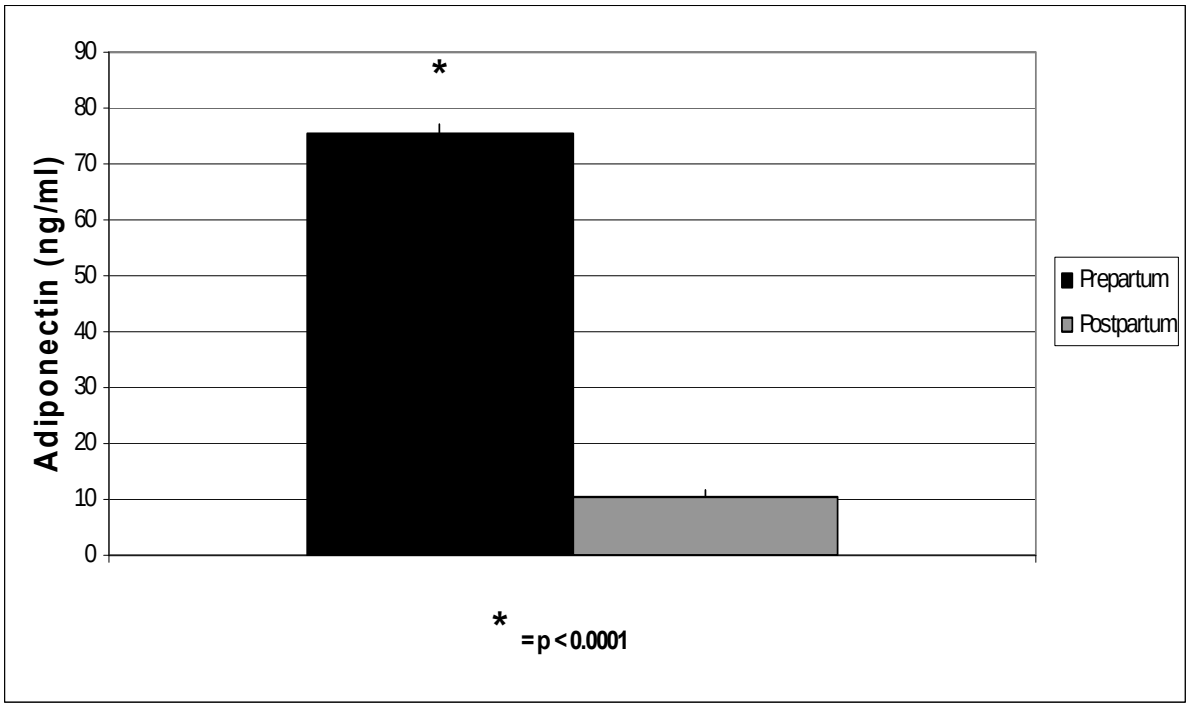
a = prepartum insulin return to basal concentrations ($p \leq 0.05$) (n = 4)
b = postpartum insulin return to basal concentrations ($p \leq 0.05$) (n = 3)

Figure 5. Mean pre and postpartum plasma insulin concentrations before and after an intravenous injection of 0.25g of glucose/kg BW.



n = 4 prepartum
n = 3 postpartum

Figure 6. Mean pre and postpartum plasma insulin concentrations before and after an intravenous injection of 0.1 IU insulin/kg BW.



n = 4 prepartum (repeated sampling)
n = 3 postpartum (repeated sampling)

Figure 7. Mean pre and postpartum plasma adiponectin concentrations over 2 days of sampling.

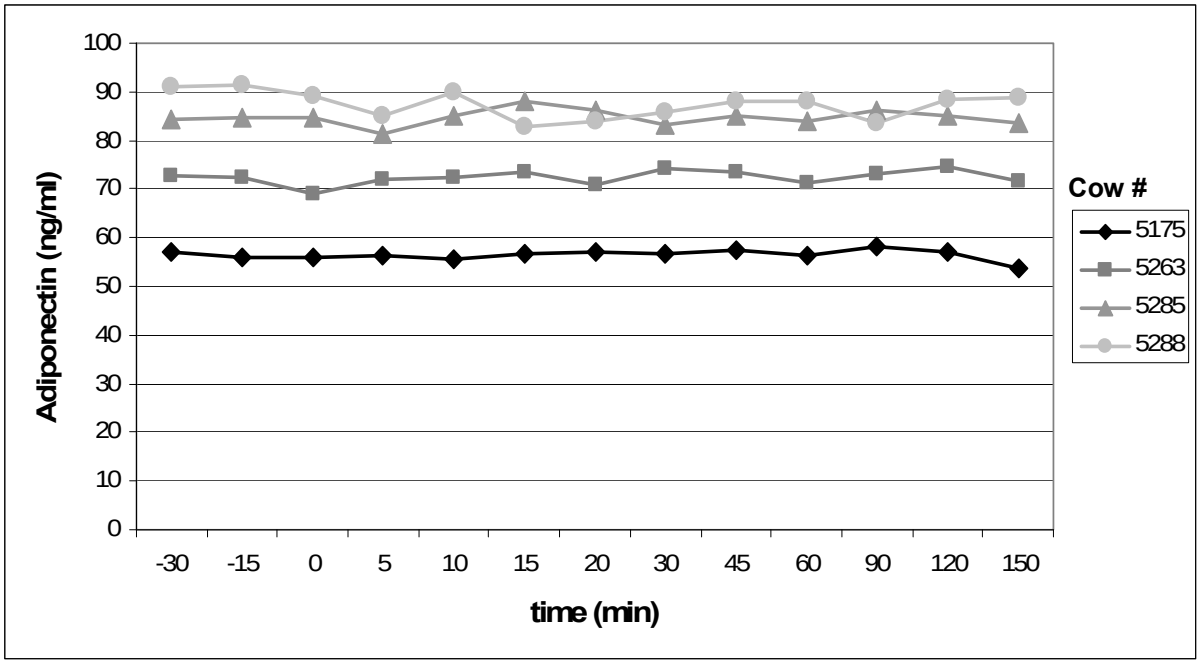


Figure 8. Mean prepartum plasma adiponectin concentrations for individual cows over 2 days of repeated sampling.

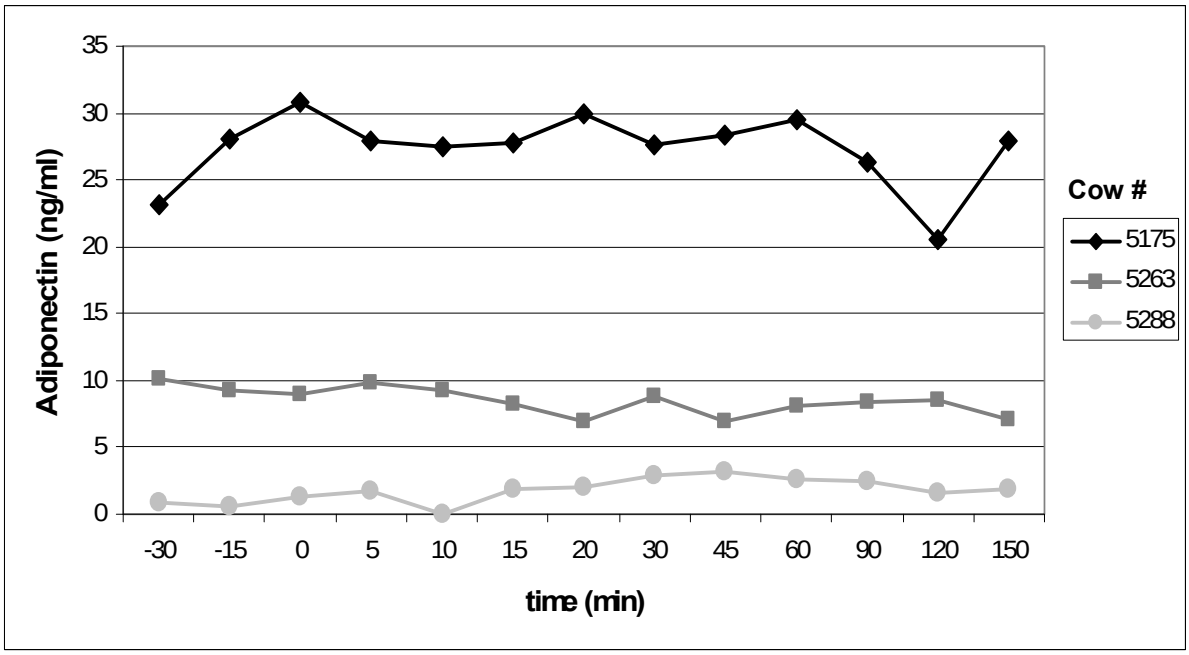
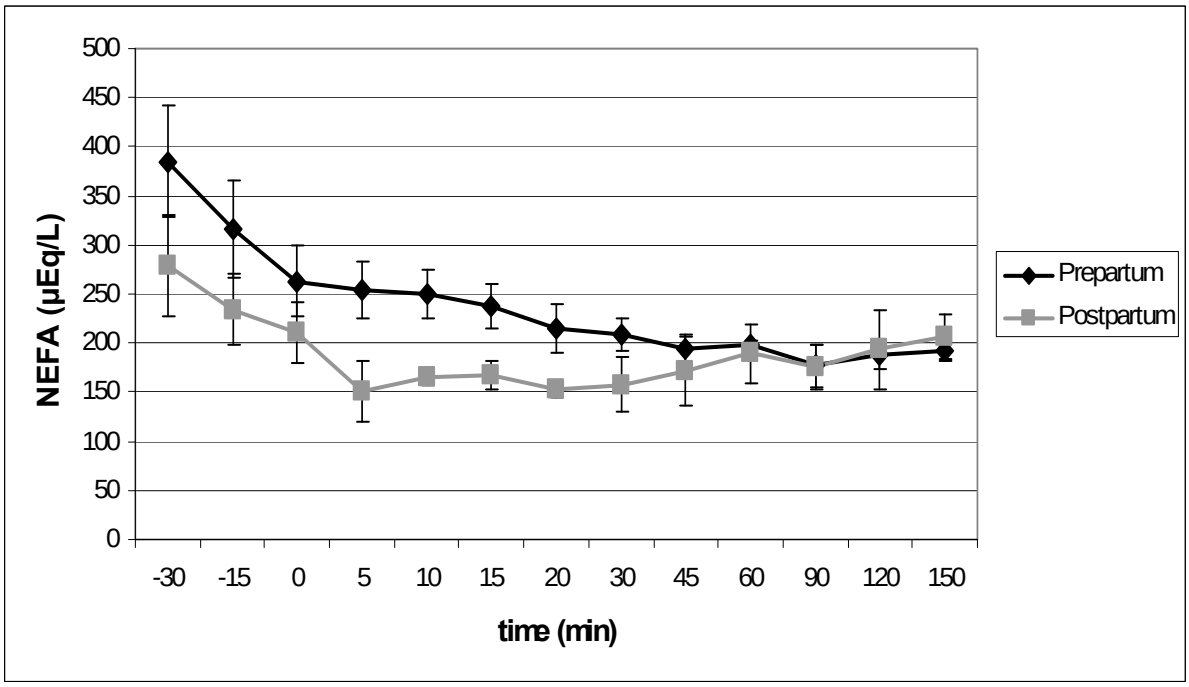
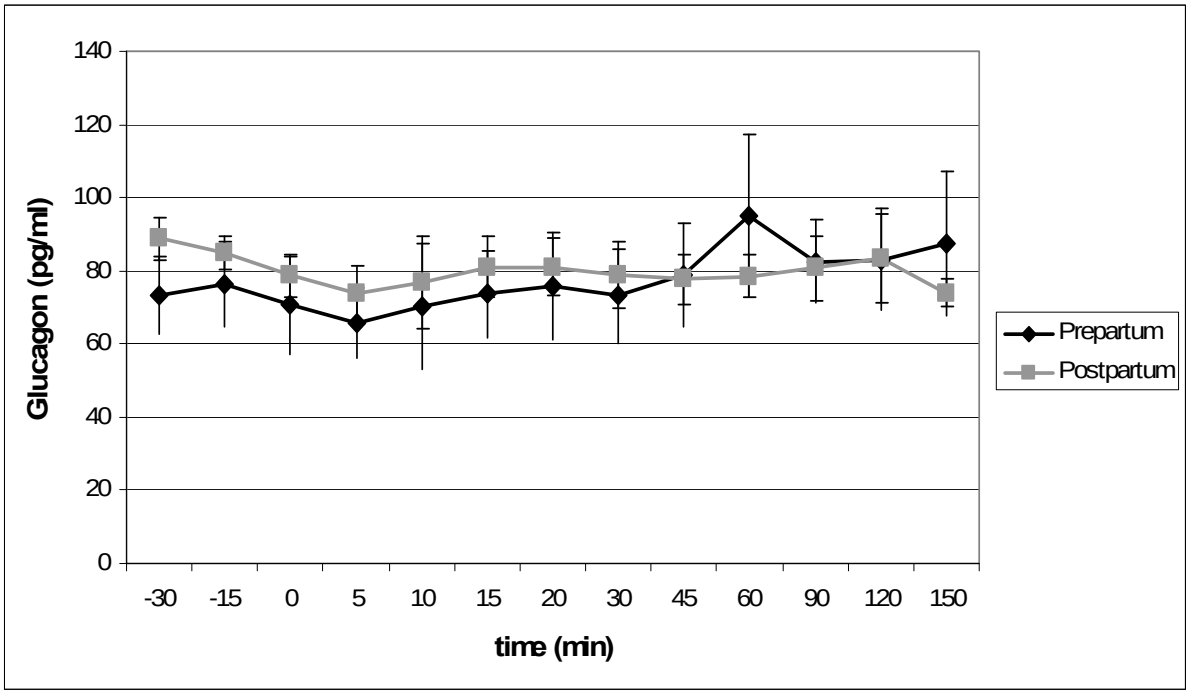


Figure 9. Mean postpartum plasma adiponectin concentrations for individual cows over 2 days of repeated sampling.



n = 4 prepartum (repeated sampling)
n = 3 postpartum (repeated sampling)

Figure 10. Mean pre and postpartum serum NEFA concentrations over 2 days of sampling.



n = 4 prepartum (repeated sampling)
n = 3 postpartum (repeated sampling)

Figure 11. Mean pre and postpartum serum glucagon concentrations over 2 days of sampling.

SUMMARY OF CONCLUSIONS

The primary goal of our preliminary study was to determine if adiponectin could be measured in bovine plasma. We used a commercially available radioimmunoassay (RIA) kit (HADP-61 HK, Linco, Millipore) designed for human use, to measure plasma adiponectin concentrations in Holstein cows because there is no assay kit validated for use in cattle. Dilution of plasma as recommended by the manufacturer resulted in samples being below the sensitivity of the assay. Using undiluted plasma, adiponectin concentrations ranged from 0-80ng/ml, whereas in other species, concentrations are reported to be in the μg/ml range. Some bull samples were assayed in the same manner, and the samples ranged from 0-40ng/ml. Individual bovine samples were assayed in various dilutions and the results showed parallelism. When equine samples were assayed, results ranged from 1-2μg/ml, which agrees with previously reported concentrations in the literature. The Linco human RIA kit uses a polyclonal antibody against nearly the entire adiponectin molecule. The bovine adiponectin protein sequence is highly homologous (>85%) to that of humans, mice, and horses, but considering the vast differences between our bovine adiponectin concentrations and the concentrations that have been reported in other species, it is uncertain if the human kit can accurately measure adiponectin in bovine plasma. It was concluded that until an assay can be validated for bovine plasma, the Linco kit should only be used to report qualitative trends in bovine adiponectin and not be used as a means of comparing concentrations quantitatively, against other species. However, measurements of plasma adiponectin in other ruminant

species such as sheep and goats using the Linco kit could help determine if ruminants in general might have consistently lower adiponectin concentrations than nonruminants.

The secondary goal of our preliminary study was to observe plasma adiponectin concentrations over the first 11 weeks of lactation, and look for any relationships between adiponectin and insulin, body condition score (BCS), lactation number, milk yield, or progesterone (time to first cycle). Adiponectin concentrations increased from 8.3 ± 1.4 ng/ml in the first week to 16.0 ± 2.7 ng/ml at week 4 postpartum and then declined to remain at 12-13 ng/ml for the remainder of the study. Individual variation among animals existed. From these data, it was hypothesized that adiponectin may play a role during the period of extreme metabolic change, when negative energy balance (NEB) and lactation are increasing, and that adiponectin concentrations may (to a certain extent) vary genetically.

Adiponectin concentrations did not correlate with body condition score, energy-corrected milk yield, lactation number, or progesterone (time to first estrous cycle). Within individual cows, insulin showed a -0.22 correlation with adiponectin ($p < 0.12$). It was concluded that further exploration into the role of adiponectin in ruminant metabolism would be beneficial to gain a better understanding of its relationship with insulin and functional importance, which became the goal of our second study.

In the second study, Holstein cows were administered a prepartum and postpartum intravenous glucose tolerance test (IVGTT) and intravenous insulin tolerance test (IVITT) and their glucose, insulin, adiponectin, non-esterified fatty acid (NEFA) and glucagon concentrations were analyzed. There was no difference in the time it took for pre and

postpartum glucose concentrations to return to baseline levels, following the infusion of 0.25g of glucose/kg body weight (BW), but prepartum insulin levels rose higher, and took longer to return to pre-injection levels than postpartum insulin. AUC for insulin during the IVGTT was greater prepartum vs. postpartum. This indicated decreased insulin responsiveness in postpartum cows. Adiponectin, NEFA, and glucagon concentrations were unaffected by the IVGTT. Because the insulin dose of 0.1 IU/kg BW did not result in the expected decline in circulating glucose or have an effect on any of the other hormones, it was concluded that there may have been an error made in the calculation of the insulin dose, or in the administration/saline dilution of the injections.

Adiponectin concentrations were significantly higher in prepartum cows than in postpartum cows, and individual variability between animals was observed, similar to what was seen in our preliminary study. The large difference between pre and postpartum adiponectin concentrations suggests that lactation may suppress adiponectin production in agreement with data on adiponectin mRNA in lactating cows and that low adiponectin levels help facilitate postpartum insulin resistance which increases the supply of glucose to the mammary gland, and aids in the metabolic support of lactation. The individual variation among animals again leads to the hypothesis that adiponectin secretion may vary genetically.

Our research suggests that adiponectin has a role in the facilitation of postpartum insulin resistance in dairy cows. Lower adiponectin levels appear to be beneficial in support of lactation. Although a certain level of insulin resistance (and reduced circulating adiponectin) may be beneficial for supporting lactation, extreme insulin resistance may be a culprit of the growing percentage of reproductively inefficient, high-producing dairy cows.

Further exploration into the role of adiponectin in ruminants is needed in order to fully understand this hormone's possibilities as a means of health and/or reproductive management. Genetic variability in adiponectin may also be avenue worth exploration.