GLUCOSE FLUX IN MONOCYTES OF PERIPARTURIENT DAIRY COWS

By

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ABSTRACT

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The incidence of periparturient disease in dairy cows continues to remain unacceptably high. This persists despite an industry research focus into both managemental and nutritional intervention over the last few decades. The intense lipomobilization and glycemic dysregulation during early lactation have drawn parallels with human metabolic conditions and states of immune dysfunction. The pronounced glucose demand by the mammary gland for milk synthesis leads to substantial metabolic change. Little is known how glucose uptake in key immune cells such as the monocyte may be affected. The objective of this study was to identify and examine the expression of glucose transporters (GLUTs) in bovine monocytes and how they may alter as a consequence of lactogenesis and upon stimulation with endotoxin. Sampling points were refined to select 10 cows at 28-35d before expected calving and at 5 ± 2d after calving. Monocytes were isolated from total peripheral blood mononuclear cells, GLUT isoforms 1,3 and 4 were assessed for mRNA and protein expression following endotoxin stimulation. GLUT isoforms were found to change expression as a consequence of lactogenesis and endotoxin stimulation. This finding warrants further investigation into energy utilization of immune cells during the periparturient period and how it may contribute to immune dysfunction.

DEDICATION

With Love to Katherine, Clóda, Fintan and Brídín.

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CHAPTER 1

Nutrition of the Periparturient Dairy Cow

by

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CHAPTER 1

Nutrition of the Periparturient Dairy Cow

From a physiological standpoint, the transition period - that is, the last three weeks before and the first three weeks after parturition - is a pivotal time in a dairy cow's lactation. Despite a strong industry and research focus in this area over the past 20 years, approximately half of all high-yielding dairy cows fail to make the transition without some form of periparturient disease. This is a sobering statistic, which is unique among mammalian species. This article reviews the conjecture behind the failings of the transition period in cows and examines some new nutritional approaches being investigated to reduce the levels of disease in these animals.

CHANGING DEMANDS OF THE PERIPARTURIENT COW

Cows experience many metabolic and hormonal challenges in the periparturient period, including rapid growth of the fetus, calving and initiation of lactation. This is further confounded by the genetic drive to produce vast quantities of milk. During the catabolic phase of early lactation, a dairy cow's energy requirements can increase by up to 300 percent. The principal demand is for glucose and, at the onset of lactation, virtually all the glucose is directed toward the mammary gland for milk synthesis. Glucose is the precursor of lactose, which dictates milk volume via osmotic regulation.

Most herbivores and omnivores derive their main fuel from dietary carbohydrates; however, ruminants undergo a relatively inefficient process that involves breaking down dietary carbohydrates during fermentation. Of the three major volatile fatty acids derived from ruminal fermentation, prop ionic acid, in the form of propionate, is the only one that contributes directly to glucose synthesis (gluconeogenesis) via the Krebs cycle.

Gluconeogenic amino acids, lactate and glycerol play a relatively minor part in the dairy cow experiencing a state of positive energy balance. However, the extraordinary demand for milk production renders the early lactating dairy cow in a state of negative energy balance, as dietary intake is inadequate to meet energy demands. Consequently, the role of other gluconeogenic substrates increases and body reserves are called upon.

MOBILIZATION OF BODY FAT

Adipose tissue is the main energy reserve called upon during negative energy balance. Non-esterified fatty acids (NEFAs) are released from adipose tissue to provide a source of energy after parturition and are processed by the liver in various ways. They can be:

- Re-esterified and exported into the circulation as very low-density lipoproteins;
- Oxidized for fuel;
- Converted to ketone compounds.

When NEFAs are present in a large excess, they also can be converted back into triglycerides and stored as fat droplets in hepatocytes.

Ruminants have less capacity to export fat stores from the liver than other species and this can lead to a vicious cycle of events. As NEFA levels rise, there is an increase in fatty acid uptake and esterification, as well as triglyceride storage in the liver. The buildup of fat in the liver compromises hepatic function, including gluconeogenesis, which causes further breakdown of adipose tissue and the creation of major NEFAs; thus, the cycle continues. This increase in NEFAs also is associated with immunosuppression.

Additional metabolic stress results from a buildup of triglycerides in the liver before calving. In the last two to three weeks before parturition, triglyceride levels can increase to

between four and five times the normal amount. This further exacerbates the situation at calving when liver function is paramount.

FEED INTAKE DEPRESSION

As well as the mobilization of the body fat, dairy cows undergo a periparturient dip in feed intake. This is not unique to ruminants but occurs in a variety of species and is a normal response to the metabolic adaptations taking place at this time (Ingvartsen and Andersen 2000). Intake depression does not just occur around calving; it starts in late pregnancy and continues into early lactation (see the graph below).

Intake regulation is a complex arrangement involving hormones, nutrients, metabolites, gut peptides, neuro-peptides and cytokines (Ingvartsen and Andersen 2000). Most research into the intricacies of intake regulation is extrapolated from human and laboratory animals and there is little information describing how this affects ruminants.

OVERCOMING THE ENERGY DEFICIT

Various strategies are used to overcome the energy deficit, including:

- Maximizing dry matter intake.
- Increasing the available energy of the prepartum diet;
- A short dry cow period.

Traditional strategies involve feeding a high-energy diet and maximizing the dry matter intake of the transition cow. These seem like logical steps and that have been reinforced by numerous studies. However, translation to the field has been very disappointing and this approach is currently being re-evaluated.

MAXIMIZING DRY MATTER INTAKE

It has long been thought that the slump in feed intake is due to a reduction in ruminal capacity resulting from a growing fetus. Increasing the amount of concentrate in the feed has therefore been a popular method to counteract this drop. However, through force-feeding and intake trails, it has been established that cows with greater intakes prepartum continue to have higher intakes in lactation and have lower hepatic triglyceride levels (Bertics and others 1992). This has led the dairy industry to focus on maximizing the dry matter intake around this time, and recent research has revealed that the role of the growing fetus may have been overemphasized.

A higher concentrate forage ratio has been shown to result in a larger decline in late pregnancy intakes, so the optimum diet is currently being reassessed (see section on high-energy diets). Attempting to increase the dry matter intake when it is declining is extremely challenging, and it is possible that the amount and duration of the intake drop are the more important factors than actual intake in the dairy cow. Previous strategies may have focused too much on maximizing feed intake rather than minimizing intake depression (Grummer 2006).

Maximizing dry matter intake could be achieved through better understanding of the nutritional physiology of the cow, as well as minimizing disease and social stressors. Good feed delivery and pen management are vital to ensure the best possible chance of not limiting intakes (Cook 2007).

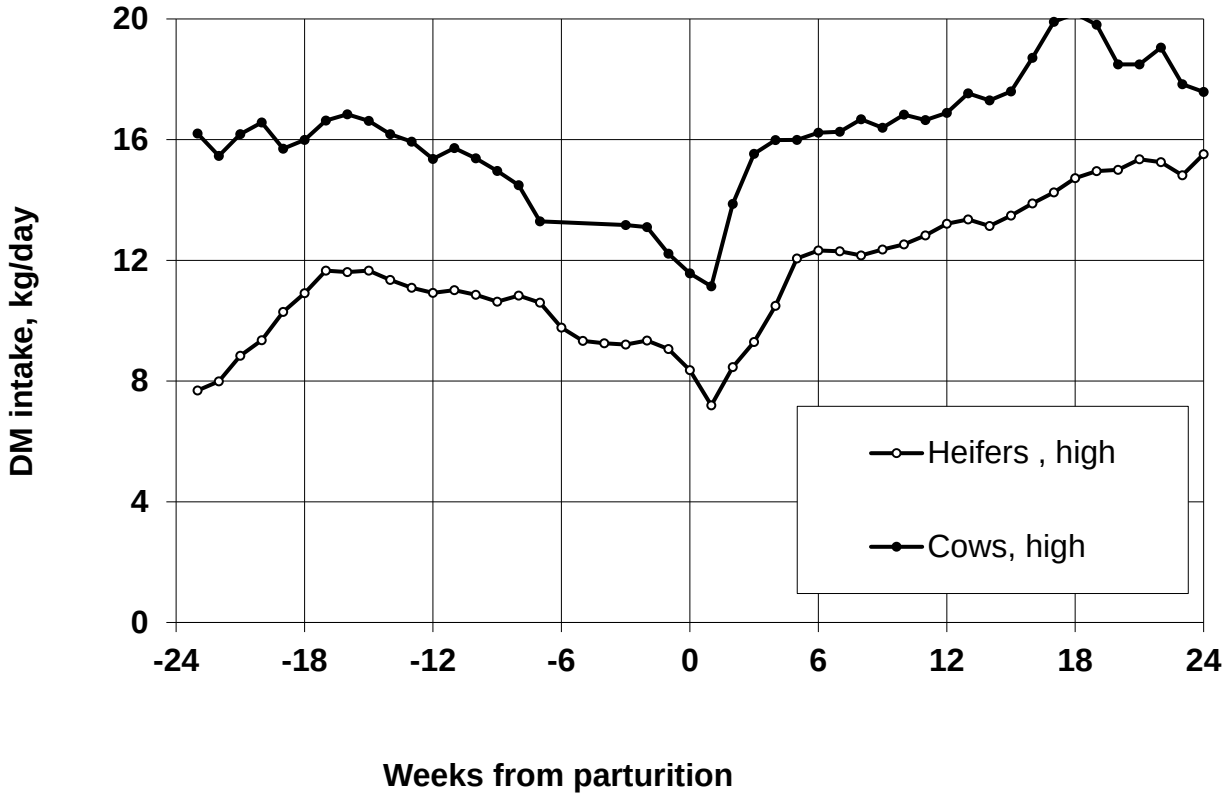

Figure 1. Dry matter intake of dairy cows before and after parturition. Reproduced, with permission, from Ingvartsen and Andersen (2000).

INCREASING THE AVAILABLE ENERGY OF THE PREPARTUM DIET

Feed Additives

Feed additives can help to alleviate an energy-deficit problem and many are employed as a 'Band-Aid®' solution. Propylene glycol is one of the most successfully used energy sources and glucose precursors, but its toxicity is a concern; it is therefore recommended that cows do not receive more than 500 g per day (Nielsen and Ingvartsen 2004). Other additives, used to reduce fatty acid mobilization (e.g.; niacin) or to increase fact export out of the liver (e.g.: choline, inositol and methionine) have shown promise but have given inconsistent results. The use of fatty acids such a linoleic and linoleic acids that have a reduced capacity to accumulate in

fact cells is currently being investigated.

Monensin sodium, an ionophore, is now licensed and popular in the USA for dairy cows. One of its modes of action is to influence the ratio of volatile fatty acids, thus increasing the amount of propionate available for gluconeogenesis.

High-energy diets

The traditional dry cow period involves cows being placed in two groups: a 'far-off' group for cows leaving the lactation string, and a 'close-up' group for cows approximately three weeks prepartum.

It is logical to assume (particularly for close-up cows) that increasing the energy density of the feed will improve intake and compensate for any shortfall. Adding concentrates to diets relatively high in forage will always increase feed intakes and is widely used in dairy cow rations. The belief has also been that the rumen papillae and microbial populations need time to adapt to a high-energy diet in preparation for early lactation. The use of a 'steaming-up' diet with a higher level of concentrate has been the accepted practice to achieve this. However, the significance of microbial adaptation and rumen papillae elongation on a total mixed ration transition is unknown. This concept of steaming-up is currently being challenged due to a perceived lack of success in the field. Overfeeding in both far-off and close-up groups is suspected as a cause of failure.

The dangers of overfeeding are well known, and overfed dry cows have a lower ability to re-esterify fatty acids within adipose tissue or to esterify mobilized fatty acids. Combined with a higher rate of lipolysis postpartum, this paves the way for elevated concentrations of circulating NEFAs, leading to fatty liver disease. Dann and others (2005) reported that allowing cows to

consume more energy than required during the dry period resulted in a response typical of over fat cows, even though the cows appeared to be in optimum condition.

The energy density of the diet postpartum is arguably more important than that prepartum. This is when cows face their largest challenge in meeting nutrient needs. The postpartum period is challenging from a research standpoint, as there is a lot more individual variability. The two diets are intertwined because the physical and physiological consequences of the prepartum diet greatly influence the intake and use of the postpartum diet (Allen and Bradford 2008). Increasing energy density by including dietary fat and higher concentrate diets has been commonplace but this, too, is a topic of discussion and debate.

RETHINKING HIGH-ENERGY DIETS: THE GOLDILOCKS DIET

A high-forage dry cow diet is not a new concept. Since the late 1990s, practitioners in the USA (in particular Dr. G. Jones in Wisconsin), have implemented a high-fiber diet on many dairies, with great anecdotal success in reducing post calving problems.

A group at the University of Illinois, led by Professor James Drackley, has investigated the feeding of this high-fiber, low-energy diet under trial conditions to establish the underlying basis and guidelines for the concept of high-energy diets (Drackley and others 2007). Overfeeding of the dry cow is extremely common even at ‘safe’ energy levels, so the group formulated a diet based on a relatively low-energy density and high-forage component. The energy density is diluted mainly with cereal straw (wheat straw anecdotally works best), and up to 4 to 5 kg/cow/day can be fed. This has the added advantage that the cows have to eat a large amount in order to meet their energy requirements, which may also contribute to preventing the intake depression late in pregnancy and early lactation.

High-energy diets are formulated to ensure all dietary requirements are met and that neither too much nor too little energy is provided, hence the term “Goldilocks diet”. This approach has been advocated in the UK by Professor Beever at Keenan Ltd, who has collected field evidence that shows an improvement in periparturient cow health.

Professor Drackley’s group speculates that by not overfeeding energy, the problem of fat reserve build-up is reduced, thereby lowering insulin resistance and fat mobilization at calving.

Points to consider

The Goldilocks diet does not work on all farms, possibly reflecting the insulin resistance and the condition of the cows coming into the program, (T. Herdt, personal communication). It is important to stress that this is not a low-input-diet - as much attention to detail, if not more, is needed than for the lactating cow ration. It is vital that there is no overcrowding at feeding, with cows having optimum access to trough space as they need to eat a great deal to meet energy demands. The wheat straw must be fed in a total mixed ration, processed to a length of about 4 to 6 cm to reduce sorting by the cows, and dry matters of the forages should be checked regularly.

It may be unwise to advocate this ration on a farm where facilities, feed delivery and trough management are at the root of the initial problem, as it may not receive the attention required for proper execution. It is also of paramount importance that the diet is not suddenly introduced to close-up cows (two to three weeks prepartum) that have not had access to it in the far-off period, as a sudden change in diet together with calving date variability could reduce the dry matter intake, with very damaging consequences.

SHORT DRY COW PERIOD

Lowering the recommended dry cow period from 60 to 30 days has received a lot of attention of late. It has many attractions: milk can be harvested for an extra 30 days, and one dry group enables fewer pen and feed changes, thus easing management and providing less social stress for the cow, resulting in fewer transition problems. However, it is also possible that the cows have less time to be overfed and succumb to the metabolic consequences. It is work emphasizing that the dry period is not the optimal time to manipulate body condition - this should be controlled in late lactation.

Although there is evidence that a shortened period is detrimental to primiparous animals (Annen and others 2003), some studies have shown that multiparous cows have transitioned with fewer problems with shortened or zero dry periods (Rastani and others 2003). Retrospective analysis of cows with an unintentional 30-day dry period may reflect a biased group (e.g.: twins, wrong breeding dates, abortions). A reevaluation of the optimum dry cow period is therefore underway.

The assumption that there is an economic advantage from the extra 30 days' milk may be flawed (Eicker and others 2006). Milk volume achieved from extending the lactation of an eight-month pregnant cow compared with the average herd member will always be below average and, therefore, the dairy will run below capacity.

Until more research has been undertaken, it may be more prudent to shorten the dry cow period only on an individual basis and keep the majority of the herd in the 45- to 60-day bracket.

REASONS FOR TRANSITION FAILURE

It may be argued that many practices, such as short dry cow period, the use of bovine somatotrophine, delayed breeding, multiple milking in early lactation, taking temperatures post-calving and drenching fresh cows, to receive attention in the UK from across the Atlantic, are an attempt to compensate for a lack of success in the transition period (G. Jones, personal communication).

Milk fever, mastitis, metritis, retained placenta, fatty liver disease, ketosis and displaced abomasum are the main periparturient problems that affect high-yielding dairy cow operations, and are collectively thought of as production diseases, that is, fundamentally diseases of domestication. Although each condition can have its own etiological factors (e.g.; milk fever [Husband 2005]), it has been acknowledged that they are all interrelated. They all have a well-established effect on fertility and are an obvious drain on both the economical and welfare components of the dairy industry. Ketosis and fatty liver disease have been suggested as underlying factors behind these conditions.

TYPE II KETOSIS

Although a large amount of overlap exists in type, degree and timing of ketosis, type II is now the most familiar form of the condition found in high-yielding systems. Named after its similarities to type II diabetes in humans, it is linked to fatty liver infiltration and insulin resistance. It is more refractory to treatment than the classic type I ketosis and greatly impairs immune function. Cook (2007) describes the different types of ketosis and includes an excellent review on monitoring techniques for periparturient disease.

Obesity is the primary risk factor for type II ketosis, and avoiding over conditioned cows

entering the dry period has been the cornerstone of prevention. It is now thought that overfeeding in the dry period produces similar effects and is a large risk factor.

METABOLIC SYNDROME

In humans, obesity-related diseases are a major strain on global health and welfare. The term metabolic or insulin-resistance syndrome is used to describe a state characterized by central obesity, high blood pressure, high triglyceride concentrations, cholesterol disparity and insulin resistance. This syndrome leaves the sufferer at an increased risk of conditions such as heart disease, stroke and diabetes.

The path physiology behind metabolic syndrome is still to be fully determined, although oxidant stress, pro-inflammatory cytokines and insulin resistance are recognized as main contributors and have been implicated as etiological factors in all the common periparturient diseases of the dairy cow.

OXIDANT STRESS

Reactive oxidant species (ROS) are very small molecules that are highly reactive due to the presence of unpaired valence shell electrons. They are a normal by-product of cellular oxygen metabolism but, under stress, the number of ROS generated can escalate. If ROS overwhelm the antioxidant defense, they can affect biological harm. ROS occur during increased metabolism or inflammation, thus contributing to disease and cell damage and enhancing inflammation.

PROINFLAMMATORY CYTOKINES

Cytokines are polypeptide hormones that can bind to specific membrane receptors and alter gene expression. They have a role in the mediation and regulation of immunity, inflammation and hematopoiesis. These inflammatory markers can be produced from clinical or sub clinical disorders, and promote hypophagia. Those that are involved in the amplification of immune reactions are known as pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α which can promote a pro-inflammatory state and oxidant stress. It also plays a role in insulin resistance by blocking insulin-signaling transduction.

INSULIN RESISTANCE

In a healthy animal, insulin is a regulator of blood glucose by making the liver and muscle cells take up glucose, where it is converted to glycogen, and causing fat cells to convert blood lipids into triglycerides. Insulin resistance is a condition whereby normal amounts of insulin are not enough to produce a typical response from the liver as well as fat and muscle cells. In fat cells, this results in hydrolysis and hence there is an increase in the concentration of free fatty acids. Glucose uptake is therefore reduced in muscle tissue, as is glucose storage in the liver, with both effects serving to elevate the level of glucose in the blood.

DAIRY COW/HUMAN PARALLELS

Insulin and Insulin resistance

Insulin resistance is a normal part of mammalian gestation as the mother partitions resources to the offspring. The high-yielding dairy cow remains somewhat insulin resistant after parturition because the mammary gland exhausts glucose demands. The levels of growth

hormone (somatotrophin) and insulin are paradoxically linked (see graph below), and although the dairy cow draws parallels in terms of endocrine physiology in early lactation with both type 1 and type 2 diabetes in humans, it has both low insulin and insulin resistance.

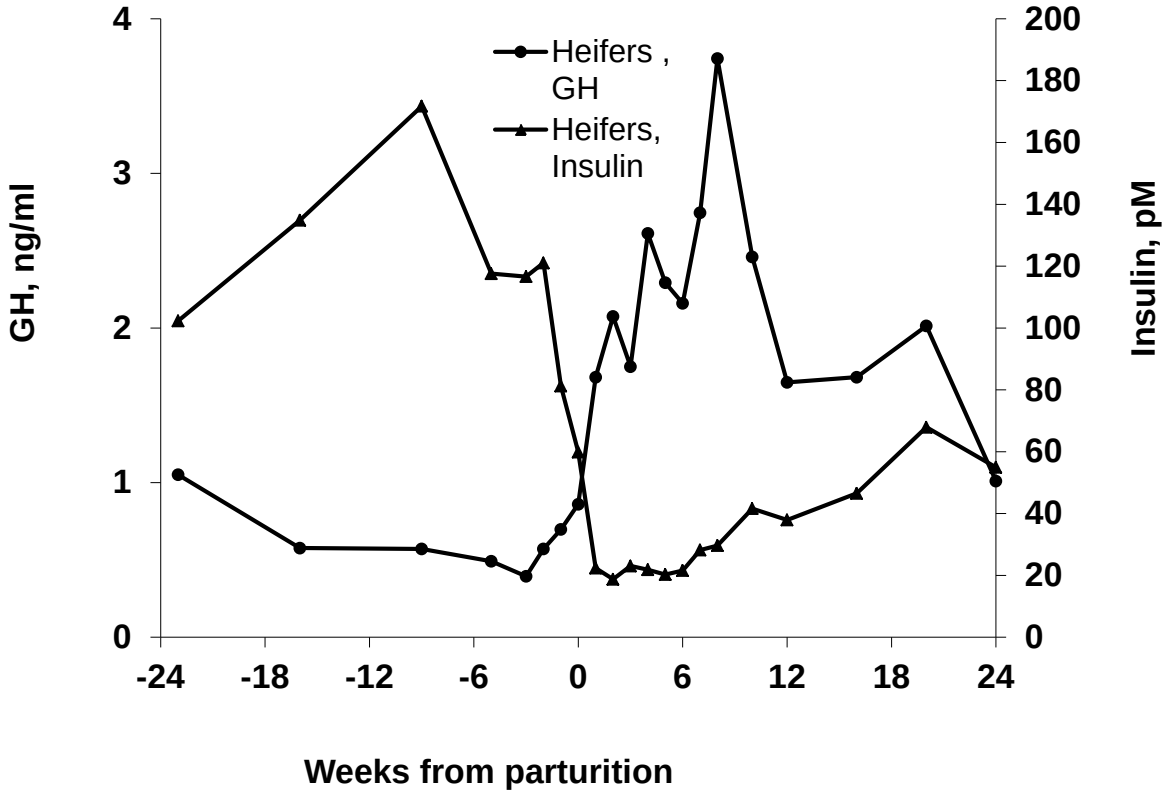

Figure 2. Growth hormone and insulin levels in heifers before and after parturition. Reproduced, with permission, from Ingvartsen and Andersen (2000).

Fatty Liver Disease

Non-alcoholic fatty liver disease is an ever recognized consequence of metabolic syndrome in humans, and has striking similarities with bovine fatty liver aberrations. There are two stages to its etiology: first, insulin resistance and then the presence of fatty acids, cytokines

and oxidative stress.

Obesity

An investigation into the relationship between body condition score, redox status and tumor necrosis factor (TNF)-α (O'Boyle and others 2006) revealed that obese cows have a decreased antioxidant potential without an increase in non-esterified fatty acids, together with a significantly higher expression of TNF-α. This illustrates that there is a similar relationship between humans and cows with regard to obesity, oxidant stress and TNF-α, and thus a potential destructive synergy between adiposity and infection. Further work is needed to establish underlying pathways.

OBESITY-RELATED DISEASES

A research group at Harvard University has achieved many breakthroughs in the understanding of obesity-related diseases. Initially, it identified the adipocyte as more than just a dormant storage cell (Hotamisligil and others 1993) and illustrated how these cells act collectively as an endocrine organ, interacting with inflammatory mediators and possibly causing an inflammatory state. To explore the relationship with immunity, they investigated the fruit fly *Drosophila melanogaster*.

This fly contains a fat body, which is homologous to adipose tissue, the mammalian liver and a hemopoietic immune organ. In mammals, the adiposity, hepatocytes and immune cells (e.g.: macrophages) share many cellular and functional pathways. Hotamisligil (2006) proposed that an evolutionary relationship enables the cells to facilitate fighting of disease and inflammation in times of food storage at the cellular level. When food is abundant, however, this relationship becomes a hindrance and leads to metabolic and immune system dysfunction.

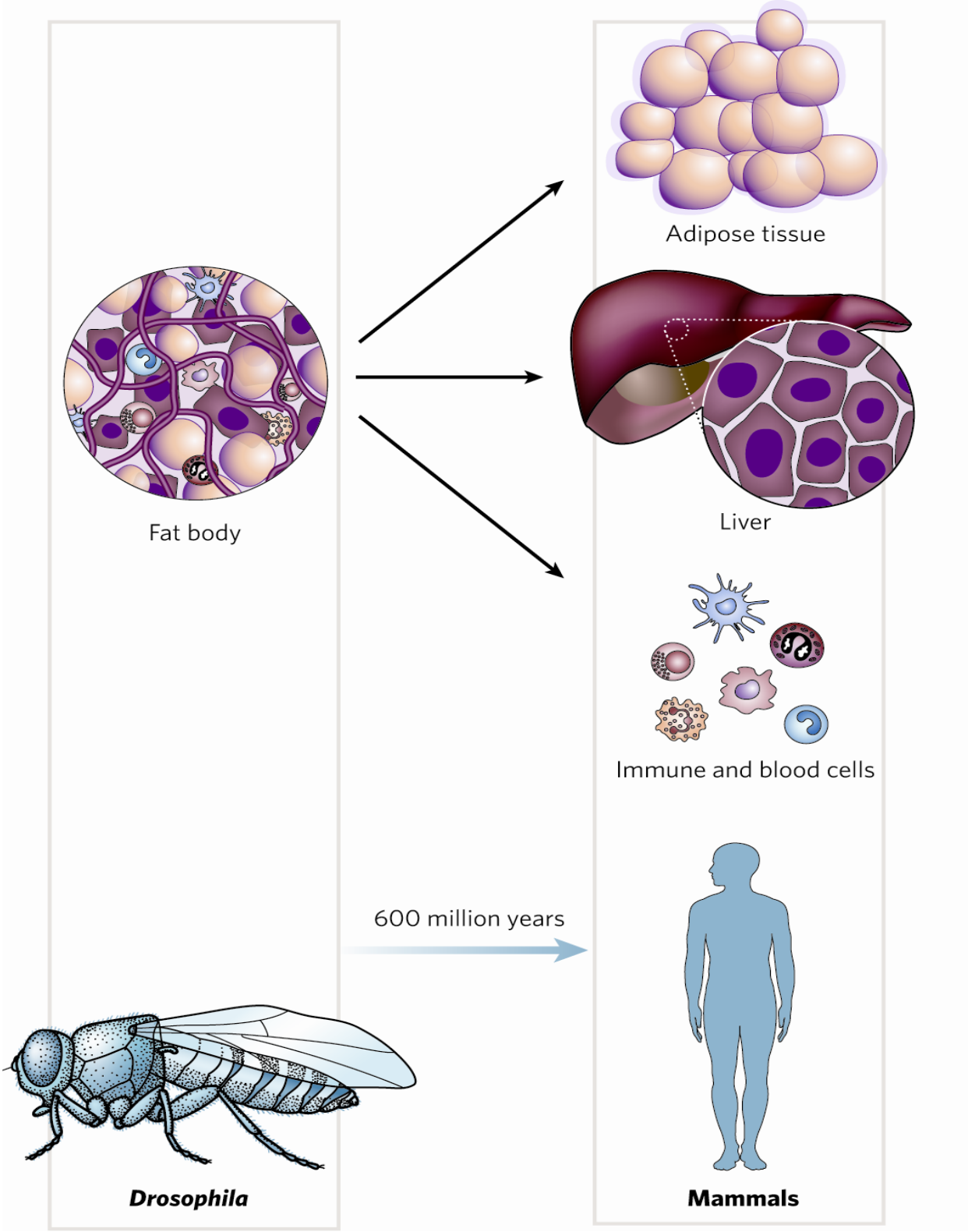

Figure 3. Obesity related diseases. Reproduced, with permission, from Hotamisligil. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

DISEASES OF OVERNUTRITION

Eight percent of human fat is subcutaneous, yet visceral fat has a much stronger correlation with metabolic dysfunction. The composition and proximity of visceral fat to the portal vein lends itself to faster mobilization and hormonal control because it is nearer the site of action.

It is known that differing genetics, dietary compositions and stages of lactation alter the state of visceral adiposity in ruminants. Condition scoring in dairy cattle is an excellent tool, but there is little work to correlate the quantity and dynamics of visceral fat with subcutaneous fat in the dairy cow. New research has indicated that substantial internal fat disposition can occur in the dairy cow with only modest over consumption of energy in the absence of a change in body condition score (Nikkhah and others 2008).

SUMMARY

Despite decades of research, disease levels in high-yielding transition cows remain unacceptable. Improved cow comfort and adequate trough space are areas that have clearly improved the animals' wellbeing and continue to do so, however, nutritional approaches are not so well defined. Previous strategies are being challenged, with the energy requirement of the dry cow receiving much attention. Over-nutrition may also play a large role in disease susceptibility, as it does in humans. A better understanding of genetics, diet, fat deposition and mobilization, and their interaction with the immune system, is being sought to fight the escalating problem of obesity-related diseases in humans. This area may warrant further exploration in order to reduce the unacceptable levels of disease in high-yielding dairy cows.

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CHAPTER 2

GLUCOSE TRANSPORTER EXPRESSION IN BOVINE MONOCYTES

by

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ABSTRACT

The transition from involution of the mammary gland to lactation exerts substantial glucose demands on the dairy cow to support copious milk synthesis and secretion. Immune dysfunction also occurs at this time with a concurrent high incidence of periparturient disorders. Macrophages are known to increase during the periparturient period and have enhanced inflammatory properties. Little is known, however, about how the prioritization of glucose towards the mammary gland affects glucose uptake by macrophages. The objective of this study was to identify glucose transporter expression (GLUT) in bovine monocytes and examine how they alter during lactogenesis and following stimulation with endotoxin. Blood samples from 10 dairy cows were collected 28-35d before expected calving and at 5 ± 2d after calving. Monocytes were isolated from total peripheral blood mononuclear cells and GLUT isoforms 1,3 and 4 were assessed for mRNA and protein expression following endotoxin stimulation. The expression of GLUT isoforms changed as a result of lactogenesis and stimulation with endotoxin. Although differences were observed between isoforms, the onset of lactation generally served to decrease overall GLUT expression. This study identified for the first time the presence of GLUT isoforms in bovine monocytes. The changes in GLUT expression with respect to lactation stage warrant further investigation to ascertain how glucose may be utilized by monocytes during the periparturient period and how energy utilization may contribute to periparturient immune dysfunction.

Key Words: GLUT, glucose, transition period, macrophage

INTRODUCTION

Almost half of all dairy cows in North America succumb to periparturient disorders (Goff, 206), representing a huge financial loss to the dairy industry and causing animal welfare concerns. Increased incidence of disease during the periparturient period is directly related to diminished host defense mechanisms (Sordillo and Streicher, 2002). The composition of peripheral blood mononuclear cell (PBMCs) populations' change and shifts in leukocyte trafficking during this time is correlated with enhanced inflammatory responses of monocytes (Kayoko, et al, 2002; Sordillo, et al, 1995). This coincides with a tremendous metabolic adaptation as the mammary gland requires large amounts of glucose for copious milk synthesis and secretion as glucose is the precursor of lactose (Bickerstaffe, et al, 1974). The monocyte's heightened response also demands energy. Human literature indicates glycemia is inextricably linked with the inflammatory state and immune function, (Collier, et al, 2008).

Although the mammary gland is enabled to prioritize glucose for milk production, little is known about the monocyte's capacity to sequester energy in the periparturient dairy cow.

Glucose is the primary energy source for most mammalian cells, including immune cells. The mammary Glucose uptake is controlled, in part, by the cell-surface expression of a family of glucose transporters (GLUTs). These cell-membrane proteins have several isoforms each unique in their expression, cellular dynamics and behavior in different physiological circumstances. This diversity ensures a complex, but efficient mechanism to maintain homeostasis or exert precedence to specific tissues. The ubiquitous GLUT 1 is the predominant glucose transporter in the bovine mammary gland. The expression of GLUT1 increases in mammary epithelial cells during lactogenesis but is diminished in the non-lactation period (Zhao and Keating, 2007). GLUT1 is recognized as responsive to cellular stress (Wertheimer, et al. 1991). In fact GLUT1

known to be redistributed and increase in expression upon activation of human monocyte-derived macrophages (Fu, et al., 2004; Malide, et al, 1998; Wertheimer, et al, 1991). GLUT3 is thought to be restricted to tissues with a high dependency on glucose as a fuel source as it is a high affinity transporter (Haber, et al, 1993). Moreover, macrophages are thought to gain an advantage in the immune response to obtain cellular energy by increasing expression of GLUT3 (Fu, et al 2004). The insulin responsive glucose transporter GLUT4 is consistent with other species where it is widely expressed in adipose tissue and muscle. However, it is thought the bovine differs in GLUT4 expression compared to other species in both fat and muscle tissue, believed to enable the pronounced partitioning of glucose towards either milk production or muscle synthesis (Bonnet et al, 2004).

Immunity is a finely regulated and complex process. Imbalances in energy sources can change the direction and outcome of inflammatory and immune responses. To date the presence of GLUTs is not described in bovine leukocytes. It is plausible that immune cells, such as the monocyte, utilize glucose differently during the periparturient period, as glucose is prioritized to the mammary gland. One approach to better understand the diminished immune response in the periparturient cow may be to elucidate how glucose uptake is regulated in cells such as the monocyte. The hypothesis of this study was that certain GLUTs on monocytes would increase in expression both as a function of lactation stage and to an endotoxin challenge. A greater understanding of the metabolic capabilities of the bovine monocyte in different physiological environments may identify new mechanisms related to the incidence and severity of periparturient disease.

MATERIALS AND METHODS

Animals and Diets

All animal procedures were approved by the Michigan State University Animal Care and Use Committee. Ten healthy, mature, multiparous, Holstein cows were selected at the moment of dry-off from a large commercial Michigan dairy herd. Animals were chosen based on the following criteria: more than 210 d of gestation, SCC less than 250,000 cells/mL, and a body condition score of 3.5 to 3.75. During the trial, cows were monitored for health status and exhibited no lameness or other symptoms of disease. Animals were housed in free stalls and fed 2 different rations based on lactation status including transition and lactation diets. The ration composition of each diet is shown in Table 1. Samples were collected 28-35 d before expected calving and at 5 ± 2d after calving.

Monocyte Isolation and Flow Cytometry

Samples were collected in the morning between 0500 and 0600 h after feed was delivered. Blood (450 mL) was obtained by jugular venipuncture and immediately mixed with a 45 mL 40 mM EDTA solution containing ascorbic acid (5 g/L). The PBMC were isolated by differential gradient using Ficoll-Paque Plus (GE Healthcare, Upsala, Sweden), collected at the interface after centrifugation (456 g for 30 min), and washed 3 times with Hank's balanced salt solution. A sub-sample of PBMC was collected to determine cellular populations using flow cytometry as described by (Shafer-Weaver, et al., 1999). Cells were incubated with each of the following lineage-specific bovine monoclonal antibodies (VMRD, Pullman, WA): CD2 (T lymphocytes, BAQ95A at 1:100 vol/vol), CD3 (T-cell receptor, MM1A at 1:100 vol/vol), CD4 (T-helper, CACT83B at 1:160 vol/vol), B-B2 (B-lymphocytes, BAQ44A at 1:150 vol/vol), MG (monocyte/granulocyte, CD172a DH59B at 1:100 vol/vol), and CD14 (monocyte, M-M8 at 1:50

vol/vol).

PBMC were cultured for 2 h at 37°C in RPMI media (Hyclone Laboratory, Logan, UT) supplemented with 10% FBS. After incubation, culture dishes were washed 3 times with RPMI media to remove non-adherent cells. Adherent cells were then incubated in RPMI 10%FBS for 1 h either added with F12K media or stimulated with endotoxin (lipopolysaccharide (LPS) (100 ng/mL)). Media was then removed and monocytes (adherent cells) were harvested by adding 10 mL of RPMI 10%FBS and 10 mL of 10 mM EDTA in 1XPBS. A subset of adherent cells was used to assess monocyte isolation by using flow cytometry. Cells were incubated for 30 min with each of the following lineage-specific monoclonal antibodies: GLUT1 (Abcam, at 1:1000), GLUT3 (Santa Cruz Biotechnology, at 1:100), and GLUT4 (Santa Cruz Biotechnology, at 1:200). After 3 washes with FACS solution, mononuclear cells were incubated with the secondary antibody GLUT1 (goat anti-rabbit Pierce l:5000), GLUT3 (goat anti-mouse Pierce 1:5000) and GLUT4 (rabbit anti-goat Pierce 1:5000). Cytometric analysis was performed using the FACSdiva software (BD).

Quantitative Real Time Polymerase Chain Reaction (qPCR)

Monocyte RNA was extracted for quantification of GLUT1, GLUT3, GLUT4, and β-actin mRNA transcript expression by qPCR as formerly described (Corl, et al, 2010) Briefly, total RNA was extracted utilizing the Qiagen RNeasy Plus Mini Kit (Qiagen, Valencia, CA). This kit includes a step to remove genomic DNA. The purity of RNA was determined using a NonoDrop 100 spectrophotometer. All samples were found to have a 260/280 reading between 1.95 and 2.1. Purified RNA was converted to cDNA using the Applied Biosystems High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). All qPCR assays were conducted utilizing Custom TaqMan gene expression assays from Applied Biosystems. TaqMan

primer and probe sets (Table 2) were designed from bovine sequences with the Applied Biosystems Pipeline software and synthesized by Applied Biosystems. Efficiency tests were run on each custom TaqMan gene expression assay. Each assay was found to be 100% efficient. In house testing also showed b-actin to be very stable for monocyte cDNA, therefore it was chosen as the reference gene. Samples were assayed in triplicate with 100 ng of cDNA per reaction along with 10 μL of TaqMan Fast Universal PCR Master Mix (2X), and 1 μL of the appropriate TaqMan Gene Expression Assay Mix (20X) on the Applied Biosystems 7500 Fast Real-Time PCR System. The relative quantification of each gene was calculated utilizing the 7500 Fast SDS Software (version 1.3.1) (Livak, 2001). Data were calculated based on the comparative Ct method ($2^{-\Delta\Delta Ct}$) of relative quantification using β-actin as the reference gene (Steibel, et al, 2009). The calibrator was set as the pre-calving unstimulated sample.

Western Blot Analyses

Protein quantification was performed by protein blot analysis as previously described (Corl et al., 2010). After monocyte collection, whole cell lysates were harvested in M-Per reagent (Pierce, Rockford, IL) and centrifuged at 10,000 × g for 10 min at 4°C to remove membrane fractions. Supernatant were collected and total protein was quantified using the Coomassie brilliant blue method. Equal amounts of protein (30 μg) were electrophoresed on a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The Millipore Snap i.d. Protein Detection System (Millipore, Billerica, MA) was utilized to carry out the remaining steps. The membrane was blocked in 0.5% dry milk in Tris-buffered saline (TBS) with 0.01% Tween-20 and washed three times with TBS. Membranes were incubated for 10 min with anti-bovine GLUT1 (1:400 dilution) (Abcam Inc, Cambridge, MA), anti-human GLUT3 (1:200 dilution), and anti-human GLUT4 (1:250) (Santa Cruz Biotechnologies, Santa Cruz, CA)

in 1% bovine serum albumin in TBS with 0.01% Tween-20. Following 3 washes with TBS, membranes were incubated with the correspondent anti-host IgG secondary antibody labeled with horseradish peroxidase (HRP; 1:3,000 dilution in 0.5% dry milk, Pierce) for 10 minutes at room temperature, washed 3 times with TBS, exposed to HRP substrate (Pierce), and visualized by chemiluminescence using the ChemiDoc™ XRS (Bio-Rad, Hercules, CA) and Quantity One® software (Bio-Rad, Hercules, CA). Anti-human actin (1:3,000 dilution, Millipore) served as the loading control. Density of the bands was quantified by the Quantity One® software. The ratio of specific antibodies: actin was calculated and the values were expressed as a fold change over the precalving unstimulated monocytes sample.

Statistical Analysis

Variables were analyzed as repeated measures using a mixed model procedure (PROC MIXED; SAS Inst. Inc., Cary NC). The following model was used to estimate the sampling day effect on each of the measured variables:

$$Y_{ij} = \mu + S_i + e_{ij}.$$

Where Y_{ij} was the dependent variable for cow_i in $sample_j$ relative to calving; μ is the overall mean of the population; S_i is the fixed effect of time as the repeated factor; and e_{ij} is the random error, assumed to be correlated. Least square means were calculated and adjusted using the Tukey-Kramer method.

RESULTS

Evaluation of PBMC Populations

Changes in leukocyte populations were observed when comparing phenotype distributions during the late dry period to immediately post-calving (Figure 4). Monocyte populations were characterized in the present study by the expression of the specific monocyte/granulocyte CD172a marker and the monocyte specific CD14 involved in endotoxin - induced monocyte activation. As described in previous reports (Kimura et al, 1999; Shafer-Weaver et al, 1999), the proportion of monocyte populations (CD172a) increased significantly as a percent of total of PBMC just after calving (26.41%±5.2) when compared to the dry period (16.2%±5). In contrast, the T cell subpopulation characterized by the CD3 T-cell receptor significantly decreased at calving (36.66±4.2%) when compared to the gestating non-lactating stage (43.2±4.2%). As for the monocyte specific co-receptor CD14, no differences were observed between pre calving (18.56% ±7.9) and post calving samples (21.94% ±7.1).

The efficiency of isolating monocytes from the total PBMC population for in vitro assay also was determined by flow cytometric analyses using phenotype specific cell markers CD172a and CD14. The specific cell markers verified the monocytes to be isolated to at least 80% of the cell population.

Monocyte GLUT Expression

GLUT1 mRNA abundance significantly decreased post-calving compared to pre-calving gene expression (Figure 5A). Protein expression was assessed using blot densitometry and statistical comparison was calculated using the glucose transporter to actin ratios. Reflecting the gene transcription findings; GLUT1 protein expression decreased significantly during post-

calving compared to pre-calving (Figure 5B). Fluorescence intensity values were used to characterize changes in glucose transporter cell-surface protein expression. GLUT1 protein expression did not significantly differ from pre-calving to post calving (Figure 5C)

GLUT3 gene expression did not significantly change over sampling time points (Figure 6A). GLUT3 protein expression remained unchanged when relative to both sampling time points (Figure 6B). Significant changes in cell-surface protein expression were observed for GLUT3 that decreased significantly from pre-calving (357.89±38.3) to post-calving (230.50± 24.3) (Figure 6C).

GLUT4 protein expression decreased significantly from pre-calving values when compared to post-calving values (Figure 7B). GLUT4 surface expression also decreased significantly from pre-calving (652.29±38.3) to post-calving (415.86±69.5) (Figure 7C).

Endotoxin stimulation alters monocyte GLUT expression.

The effect of stimulating isolated bovine monocytes with endotoxin on GLUT expression was established (Figure 8). At the transcription level, there was a significant increase in the expression of GLUT3 and GLUT4 when monocytes were exposed to endotoxin. In contrast, there were no changes in the gene expression of GLUT 1. When analyzing protein expression of GLUTs 1, 3, and 4 no effects were observed after exposure to endotoxin.

Lactation Stage and Endotoxin stimulation effects on GLUT expression.

The effects of the interaction between lactation stage and endotoxin stimulation where analyzed (Table 4). No effects were observed for GLUT 1 and GLUT 3 expression. However GLUT 4 gene expression in response to endotoxin was significantly higher before parturition than after calving. No differences were observed after calving.

GLUT and pro-inflammatory cytokine expression.

We further analyzed the correlations between the expression of different GLUT and specific pro-inflammatory cytokines (Table 3). Monocyte TNFα expression was positively correlated with the expression of GLUT 1,3 and 4. As for IL-1β, its expression was strongly correlated to the gene expression of GLUT3.

DISCUSSION

This study sought out to identify the expression of GLUT isoforms within bovine monocytes and additionally identify shifts in GLUT expression both as a result of lactogenesis and following stimulation with endotoxin. The hypothesis indicated GLUT expression would increase during times of high metabolic demands and that GLUT expression would correlate to changes in monocyte pro-inflammatory responses. The rationale for investigating GLUT expression on monocytes was based on previous studies that demonstrated increased percentages and pro-inflammatory responses of monocytes and macrophages in dairy cows during periparturition (Sordillo, et al, 1995). Indeed, the results from the present study are consistent with the findings of others that showed percentages of monocytes increase as a proportion of total PBMC in the blood of dairy cows (Figure 1), when cows are also known to express increased markers of oxidative stress and inflammation (Sordillo et al, 2009). The increase and enhanced inflammatory capabilities of macrophages during the periparturient period may be influenced by the competition for limited energy sources such as glucose.

GLUT1 is known as the ubiquitous glucose transporter. Despite being the first GLUT identified and most intensively studied, its behavior is not fully understood (Carruthers et al, 2009). GLUT1generally serves to produce glycemic equilibrium; expression typically decreases

in hyperglycemia and increases in hypoglycemia (Duelli et al, 1998; Duelli et al, 2000; Smoak and Branch, 2000). Our observations suggest monocyte GLUT1 expression appears to diminish after calving, demonstrated by decreased protein and mRNA expression (Figure 5A, Figure 5B). This is in contrast with the theme of our hypothesis which expected the increased period of metabolism and cellular stress post-partum to increase GLUT1 expression. It is known in across other species monocytes utilize other fuels besides glucose particularly glutamine and oleate, as well as other fatty acids and ketone bodies (Newsholme, et al., 1996). The bovine monocyte may adapt similar to other tissues and rely further on alternative fuels. The periparturient dairy cow initially maintains a high level of glutamine postpartum, likely due to muscle catabolism, though this does decrease as lactation progresses (Zhu et al, 2000). Moreover (although not the preferred substrate) glutamine is required in higher amounts than glucose to accomplish metabolic requirements in cultured rat monocytes, (Pithon-Curi, et al., 2004). The GLUT1 expression described also contrasts with the bovine mammary gland where GLUT1 expression increases substantially as lactation ensues and is recognized as the predominant glucose transporter (Zhao et al 2007). Although conjecture, the contrast in GLUT1 expression between circulating monocytes and the mammary gland could be explained by the localized physiological environment of mammary tissue which may serve to increase GLUT1 expression e.g. hypoxia (Bashan et al, 1992; Rodriguez-Enriquez et al 2010), hyperosmolarity (Hwang and Ismail-Beigi, 2001). Regulation of GLUT1 via lactogenic hormones that favor milk synthesis also was proposed (Zhao and Keating 2007). Our descriptive study did not look at actual glucose uptake, though it could be speculated it is decreased in monocytes as glucose is prioritized to the mammary gland during lactogenesis. Further studies exploring monocyte function, GLUT1 expression and actual glucose uptake in different physiological conditions such as hypoxia are

warranted. Also the behavior of bovine monocytes with differing amounts of alternative fuels (particularly glutamine) may elucidate further information.

GLUT3 is a high affinity isoform typically located in tissues which require rapid bursts of metabolic energy such as developing blastocyst (Pantaleon et al, 1997), neural tissue, sperm cells (Haber et al, 1993) and white blood cells (Maratou et al, 2007). A previous study indicated GLUT3 mRNA was only present at very low levels in bovine tissue (Zhao et al, 1988). In our findings, GLUT3 expression was present on bovine monocytes and observed to be less abundant after calving with significant decreases in cell surface protein expression (Figure 6C). GLUT 3 expression was observed in human monocytes where it mainly exists in an intracellular role and the thought is to be initiate the sudden surge of energy needed upon activation (Malide et al, 1988a). Insulin and IGF-1 are known to positively affect expression and uptake of glucose via GLUT3 in human monocytes (Estrada et al, 1994). Both insulin and IGF-1 are known to decrease postpartum in the dairy cow (Llewellyn et al, 2007; Lucy et al, 2001). Further observations of GLUT3 expression and function on bovine monocytes with differing concentrations of insulin and/or IGF-1 are warranted.

Glucose transporter 4 is the most insulin responsive isoform of the GLUT family. The proposed mechanism involves the translocation of GLUT4 to the membrane as a result of insulin exposure rather than glucose concentration (Hansen et al, 1998). In fact, the increase in cell surface expression was shown to be dose dependent in adipocytes (Govers et al, 2004). Our study revealed GLUT4 significantly decreased post calving in both protein and cell surface expression (Figures 7B and 7C). Previous reports have not detected GLUT4 in the bovine mammary gland in dry or lactating stages (Zhao et al, 1996). Although insulin was not measured, our findings concur with a decreased translocation perhaps via decreased insulin

common to lactogenesis in the dairy cow (Lomax et al, 1979). The decline in insulin is thought to reflect the homeorhetic drive for milk production and preparation for colostrum synthesis (Bonczek et al, 1988; Ingvartsen et al, 2003). In fact, hypoinsulinemia is the most positively correlated change amongst hormones or metabolites with regard to differences in milk yield (Ingvartsen and Friggens, 2005). A similar behavior was observed in human monocytes with insulin and this interaction was reviewed as a potential insulin resistance marker and assessment of metabolic health (George et al, 2005; Yorgi et al, 2009). The possibility that insulin levels could affect GLUT4 behavior within the bovine monocyte should be investigated further based on our initial descriptive studies.

The prominent correlation with GLUTs and cytokine expression was observed with GLUT3 which was highly correlated with TNF-α and IL-1β (Table 3), whilst GLUTs 1 and 4 also showed correlation to TNF-α. There was significant increase in GLUT3 and GLUT4 mRNA expression upon stimulation with endotoxin though not with protein expression (Figure 8). Although a temporal description was not observed in our study, it concurs with GLUT3's ability to respond quickly to activation; in the human monocyte GLUT3 has been known to show play a major role in the early stages of activation (Malide et al, 1998). Whilst there was no increase in GLUT1 upon endotoxin stimulation which contrasts with previous studies in other species (Fukuzumi, et al, 1996), this may reflect the shorter time we observed the cells after activation. A more temporal description of monocyte GLUT behavior in the bovine monocyte is required, but these initial observations suggest that GLUT expression may be involved in controlling monocyte inflammatory responses during the postpartum period.

The metabolic switch during the onset of lactogenesis is recognized to have consequences for immune competence (Sordillo et al, 2009). Due to an overwhelming demand for milk

synthesis the mammary gland dominates glucose utilization after calving. Glucose is a primary fuel for immune cells (Calder et al, 2007). To date there has been scant information on how immune cells such as the monocyte may utilize glucose during the periparturient period. Overall GLUT expression showed a dampened response in early lactation. This was in contrast to our hypothesis where we expected to see a general increase in GLUT expression, particularly GLUT1. We postulated the heightened inflammatory response may reflect an up-regulation to sequester glucose for energy, the change in proportion of other fuels may also have a large impact on macrophage function (Newsholme, et al, 1996; Yaqoob and Calder, 1998). In fact in dairy cows lipomobilization leads to shifts in fatty acid profiles, thought to contribute to altered leukocyte function (Contreras, et al, 2010).

Further work to examine factors common to the postpartum period which influence GLUTs may be applicable, such as hypoxic markers, insulin, and IGF-1. A recent study recognized a disturbed expression of GLUT isoforms in leukocytes of diabetics, with a reduction in activity of GLUTs 3 and 4. They postulated the disturbance may diminish activation and factor in the decreased immune response and predisposition to infection seen in diabetes (Kipmen-Korgun et al, 2009). Our results indicate that GLUTs are present on bovine monocytes and isoforms alter in expression dependent on lactogenesis and stimulation with endotoxin. Although the experiment was descriptive and did not measure glucose uptake or utilization, it provides novel information to further explore the high incidence of periparturient disease.

SUMMARY

At the onset of lactation, the dairy cow's mammary gland demands huge quantities of glucose for milk production. This coincides with a high incidence of periparturient disorders. The objective of this study was to identify specific glucose transporters and investigate changes in expression on monocytes as a consequence of lactation and stimulation with endotoxin. Results demonstrated that glucose transporter expression changed as a result of lactogenesis and endotoxin stimulation. Alterations in the glucose transporting ability may influence monocyte function during times of increased susceptibility to disease.

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Table 1. Ingredient composition of pre-calving and lactation diets

INGREDIENT	DIET	
	Transition	Lactation
Alfalfa haylage[1]	0	6.52
Corn Silage[2]	14.27	23.53
Wheat Straw	4.24	0.46
Wet Corn Gluten	1.23	1.14
Dry Corn Gluten	0	1.28
Bakery byproduct	0	1.23
Canola Meal	0	1.00
Corn Gran Medium Grind	0	1.78
Soybean meal solvent	0	2.01
Dried Citrus Pulp	0	1.28
Wet Beet Pulp	0	3.97
High moisture corn	0	2.22
Supplements and mineral mix:	0.55	1.60
Vitamin A D E mix[3]	0.04	0.02
Trace Mineral Mix[4]	0	0.02
Selenium blend[5]	0.05	0
Vitamin E[6]	0.005	0
Sodium Selenate	0.003	0
Sodium Sesquicarbonate	0	0.52
Calcium Carbonate	0.1314	0.47
Sodium Chloride	0.078	0.26
Ground Soya Bean Hulls	0.096	0
Wheat Midds	0	1.2
Magnesium Sulfate	0.187	0
Calcium Sulfate	0.311	0
Magnesium Oxide	0.062	0.067
Blood Meal	0.187	0.552
Fishmeal	0.062	0.247
Biotin 1%	0.005	0.004
Mepron[7]	0	0.01
Tallow	0.024	0.1
Rumensin 80	0.004	0.003
Chemical analysis, % of DM		
NDF	50.9	29.2
ADF	29.9	16.89
Ether Extract	3.08	3.9
NEI MJ/Kg of DM[8]	5.49	7.27

TABLE 1 continued

Values expressed in kg of dry matter
[1]Corn silage 31% DM (as fed)
[2]Alfalfa haylage 42% DM (as fed)
[3]Vitamin ADE mixture contained (g/kg) 10.8 retinyl acetate, 0.18 cholecalciferol, and 0.047 DL-α-tocopherol.
[4]Trace mineral mix contained (g/100g): 13.0 calcium, 0.3 magnesium, 2.0 copper, 8.8 magnesium, 12.0 sulfur, 10.5 zinc, 0.3 manganese, 0.25 cobalt and 0.19 iodine.
[5]Selenium blend contains 0.006% sodium selenite.
[6]Vitamin E contained 68.0 g/kg of DL-α-tocopherol
[7]Rumen-protected methionine; Evonik Industries AG, Essen, Germany.
[8]Net energy for lactation.

Table 2. Gene targets and primer sequences for qPCR

TARGET	Accession #		Sequence (5' to 3')
GLUT1	NM_174602	Forward	CGGCTGCCCTGGATGTC
		Reverse	GCCTGGGCCCACTTCAAA
		Probe	ATGGCCACAATGCTCA
GLUT3	XM_001256170.1	Forward	CAAGTCACAGTGCTAGAGTCTTTC
		Reverse	GGAGAGCTGGAGCATGATAGAGAT
		Probe	CCGGCAACCCATCATT
GLUT4	BC114082.1	Forward	GTCAACACAGTCTTCACCTTAGTCT
		Reverse	CCAGGCCCAGGAGATGGA
		Probe	CCCAGCCCGTTCCAC
TNFα	NM_173966	Forward	GCCCCCAGAGGGAAGAG
		Reverse	CCAGAGGGCTGTTGATGGA
		Probe	CCCCAGGTGGCCCC
B-actin	NM-173979	Forward	CCGCCCCGCTAGCA
		Reverse	AACTGGTTGCGGTGTCGA
		Probe	CCTTCGCCGCTCCGC

Table 3. Pearson correlation Pearson correlation coefficients for glucose transporter and cytokine gene expression from transition dairy cows (n=6)

		Cytokines	
GLUTs		TNFα	IL-1β
GLUT1	*R*	0.505	0.216
	p	*0.0119*	*NS*
GLUT3	*R*	0.763	0.740
	p	*<.0001*	*<.0001*
GLUT4	*R*	0.421	0.102
	p	*0.041*	*NS*

[1]*NS* (*P*>0.05)

Table 4. –ΔCt values, for GLUT4 mRNA expression comparing the interaction between sampling time-point and endotoxin stimulation (n=6)

Date	**Treatment**	GLUT1	GLUT3	GLUT4	
Pre-calving	Control	0.4001 ± 0.5067	0.4209 ± 0.1027	0.4330 ± 0.1102	**a**
Pre-calving	LPS	0.5639 ± 0.5067	0.08358 ± 0.09376	-0.3277 ± 0.07793	**b**
Post-calving	Control	2.8864 ± 0.4309	0.5285 ± 0.03925	-0.1983 ± 0.1401	**ab**
Post-calving	LPS	2.5172 ± 0.4309	0.2486 ± 0.04300	-0.08823 ± 0.1566	**ab**

abc Means in a column without a common letter differ, *P*< 0.05

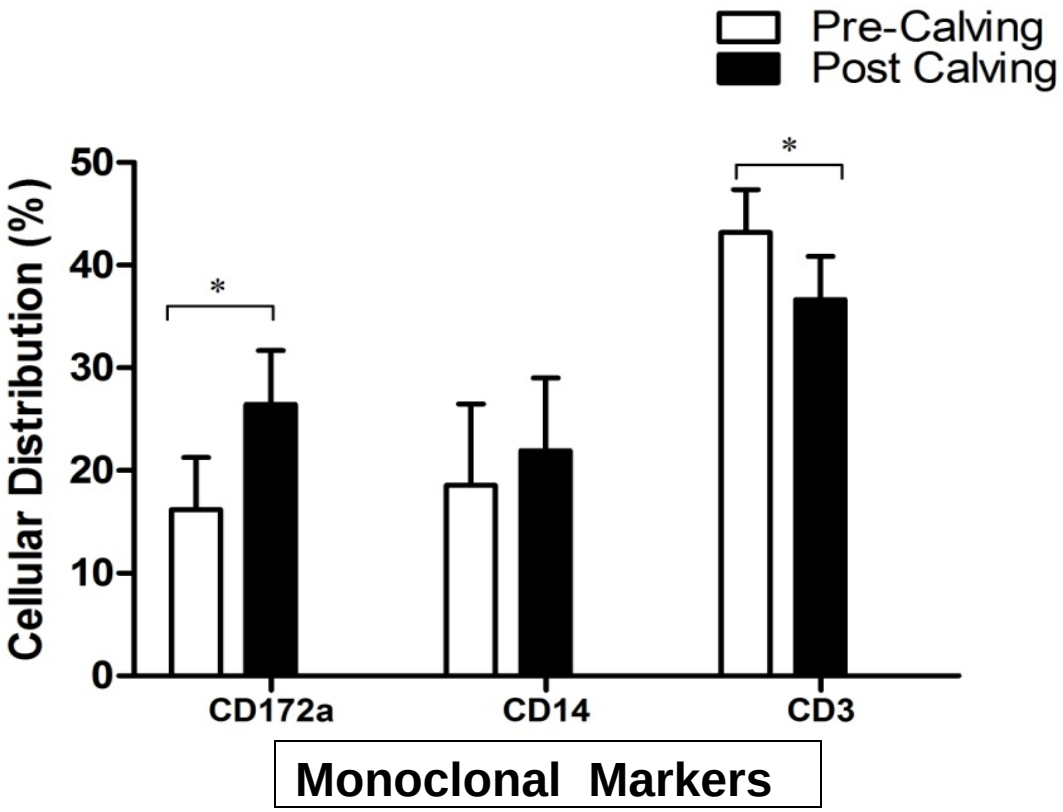
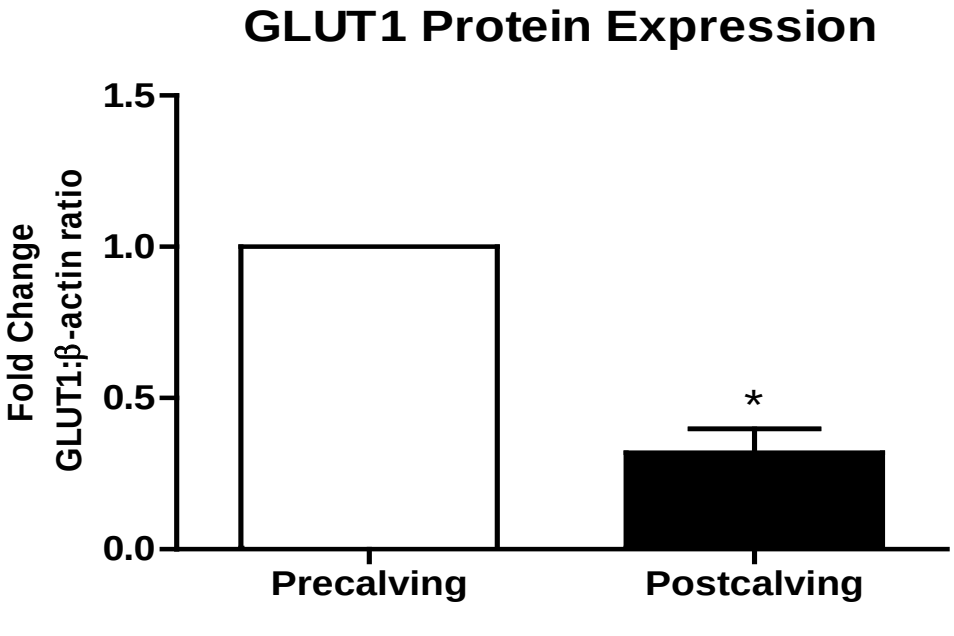


Figure 4. Peripheral blood mononuclear cells (PBMC) phenotype distribution changes relative to calving date in periparturient dairy cows (n =9). Values are percentage of the population ± SEM as measured by flow cytometry using specific bovine monoclonal antibodies. Phenotypes were characterized by the expression of CD3 for lymphocytes and CD172a for monocytes. CD14 is a co-receptor that also identifies monocytes in the total PBMC population. Means with an asterisk differ, $P < 0.05$.

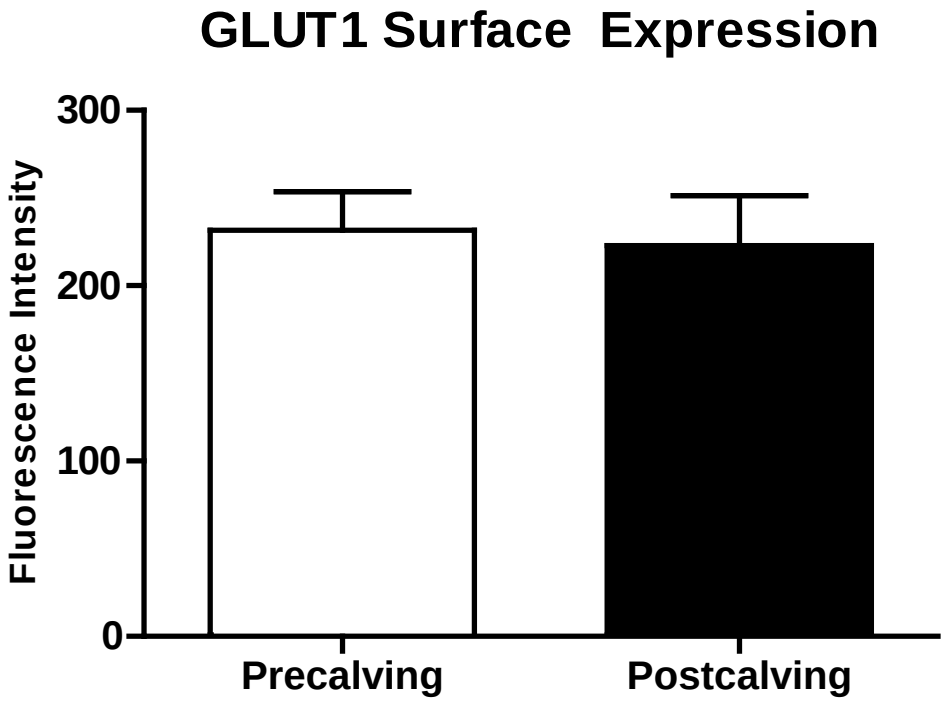
Figure 5. Analysis of GLUT1 (a) mRNA expression (n=6), (b) protein expression (n=7) and (c) cell surface expression on monocytes pre- and post-calving (n=7). (A) Data reported as least square means ± SEM. Significant differences (P<0.05) are represented with an asterisk. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method with Pre-calving as the reference expression point. (B) Data reported as a ratio of GLUT: β-actin ± SEM. Significant differences (P<0.05) are represented with an asterisk. Data were analyzed by the fold-change in protein expression using Pre-calving as the calibrator. (C) Values are expressed as fluorescence intensity of positive monocytes ± SEM as measured by flow cytometry. Means with an asterisk differ, P < 0.05.

A.



B.

FIGURE 5 continued

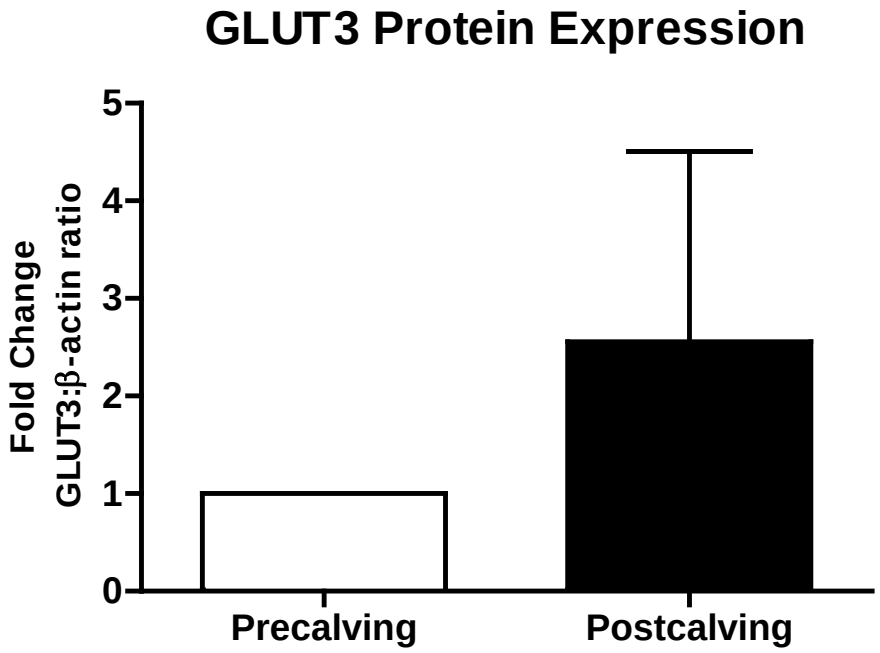


C

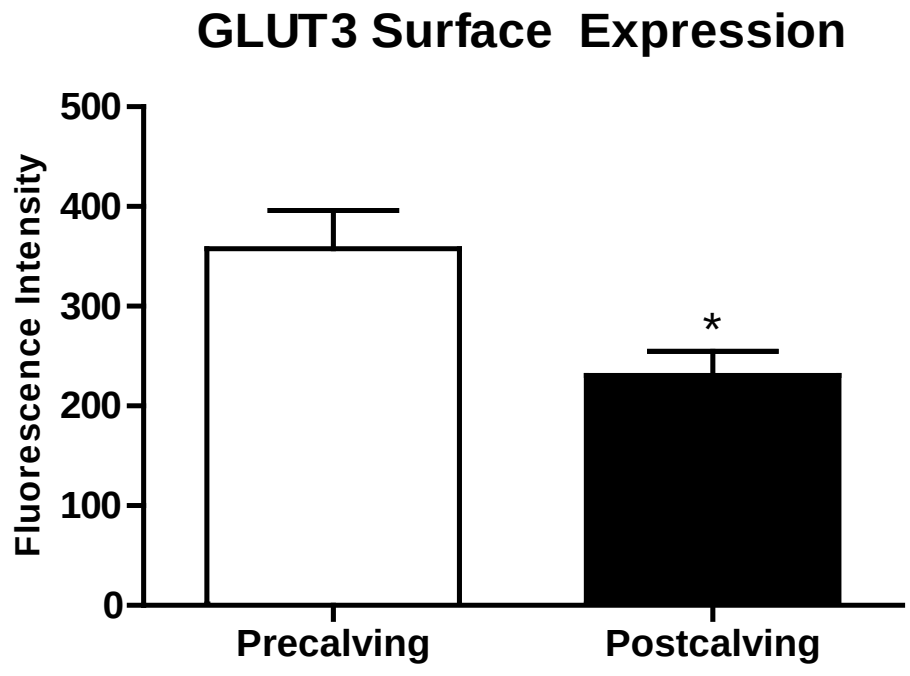
Figure 6. Analysis of GLUT3 (a) mRNA expression (n=6), (b) protein expression (n=7) and (c) cell surface expression on monocytes pre- and post-calving (n=7). (A) Data reported as least square means ± SEM. Significant differences (P<0.05) are represented with an asterisk. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method with Pre-calving as the reference expression point. (B) Data reported as a ratio of GLUT: β-actin ± SEM. Significant differences (P<0.05) are represented with an asterisk. Data were analyzed by the fold-change in protein expression using Pre-calving as the calibrator. (C) Values are expressed as fluorescence intensity of positive monocytes ± SEM as measured by flow cytometry. Means with an asterisk differ, P < 0.05.

A.

Figure 6 continued



B



C

Figure 7. Analysis of GLUT4 (a) mRNA expression (n=6), (b) protein expression (n=7) and (c) cell surface expression on monocytes pre- and post-calving (n=7). (A) Data reported as least square means ± SEM. Significant differences (P<0.05) are represented with an asterisk. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method with Pre-calving as the reference expression point. (B) Data reported as a ratio of GLUT: β-actin ± SEM. Significant differences (P<0.05) are represented with an asterisk. Data were analyzed by the fold-change in protein expression using Pre-calving as the calibrator. (C) Values are expressed as fluorescence intensity of positive monocytes ± SEM as measured by flow cytometry. Means with an asterisk differ, P < 0.05.

A.

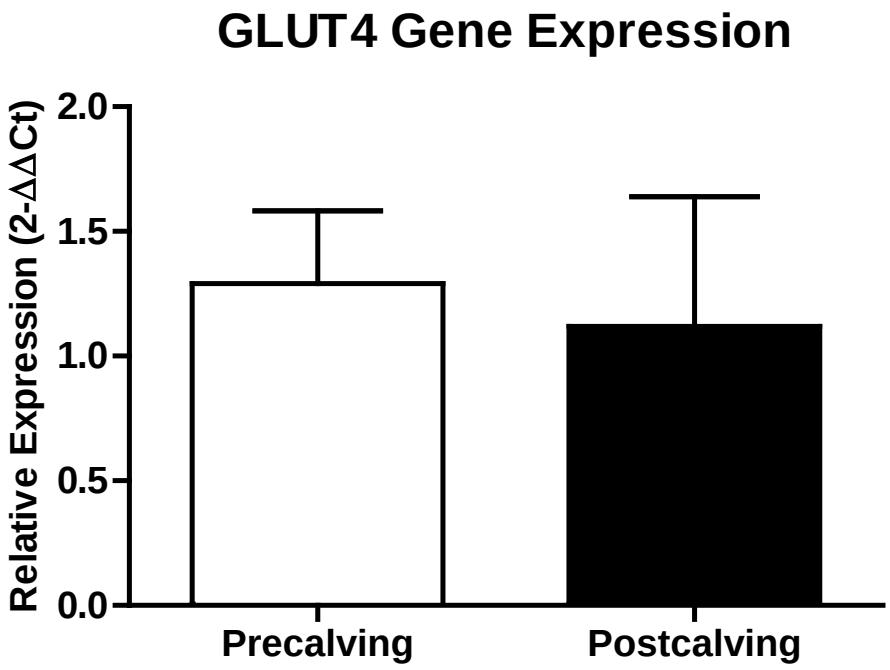
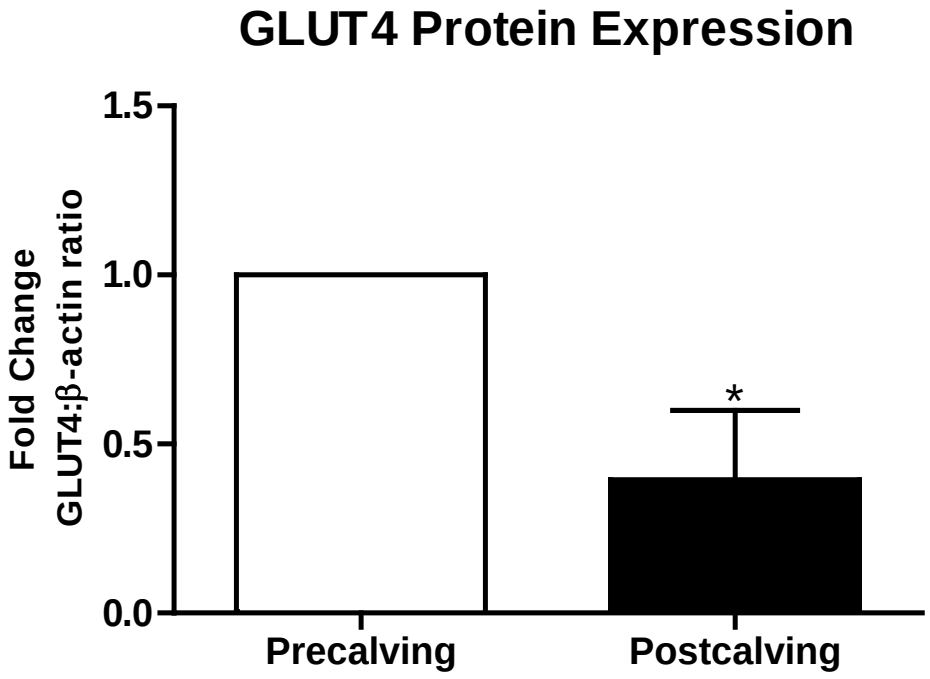


Figure 7 continued

B.



C.

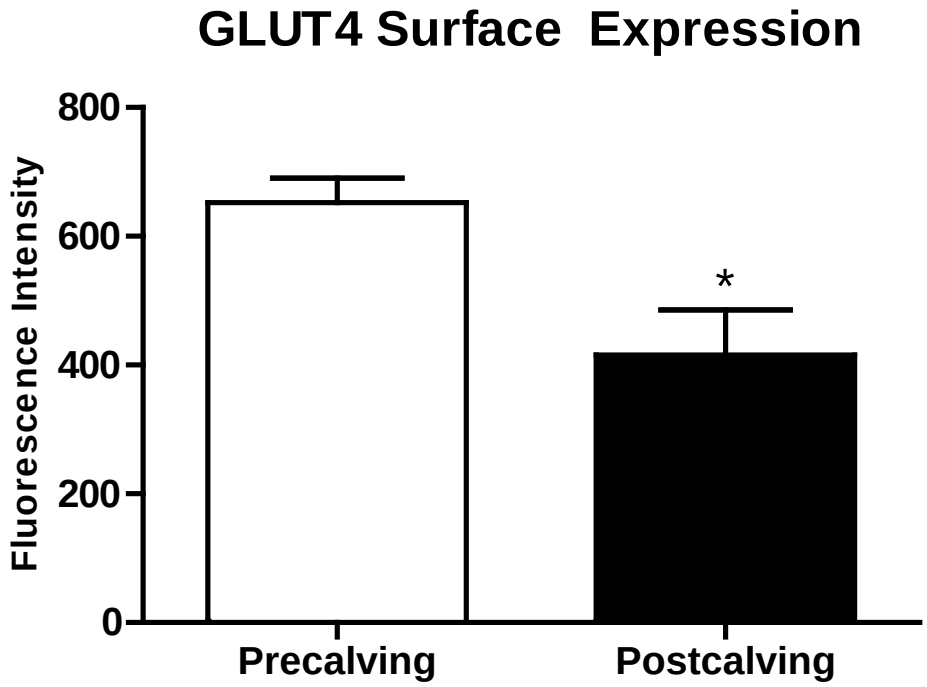
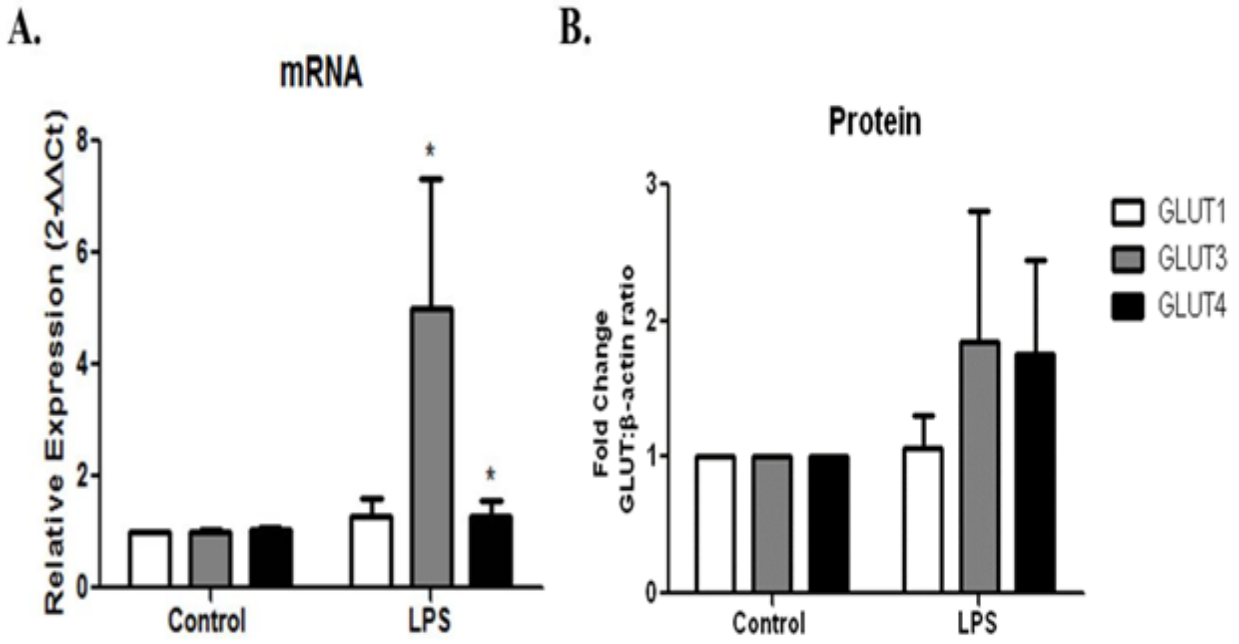


Figure 8. Effect of stimulation with LPS on (A) mRNA expression (N=6) and (B) protein expression (n=7) of glucose transporters. (A) Data reported as least square means ± SEM. Significant differences (P<0.05) are represented with an asterisk. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method with Control as the reference expression point. (B) Data reported as a ratio of GLUT: β-actin ±SEM. Significant differences (P<0.05) are represented with an asterisk. Data were analyzed by the fold-change in protein expression using Control as the calibrator.



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