Control of Hepatic Gluconeogenesis During the Transition Period

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Introduction

The importance of gluconeogenesis, a metabolic pathway that results in formation of glucose from non-carbohydrate carbon substrates, is underscored in dairy cattle by to the lack of intestinal glucose absorption that occurs as a consequence of the extensive fermentation of free dietary carbohydrate in the rumen. In early lactation greater than 90 % of whole animal glucose requirements are met through endogenous glucose production and liver is the primary site of synthesis for glucose that is available for metabolism and mammary lactose synthesis. Lactation and gestation impose the greatest demands on glucose economy of ruminants and ketosis and pregnancy toxemia are commonly linked to gluconeogenic insufficiency. Transition to lactation in dairy cattle represents one of the most dramatic changes in glucose metabolism. Understanding of the control of gluconeogenesis in transition cows has the potential to yield management strategies to increase glucose supply for optimal milk production and to alleviate clinical diseases linked to glucose insufficiency. This review will provide background information on control of gluconeogenesis with a primary focus on emerging information on control of gluconeogenesis in transition dairy cows.

What Is Gluconeogenesis and Why Is It Important?

Gluconeogenesis is the process of formation of new glucose in the body. This process occurs primarily in liver and to a lesser extent in kidney and serves to assemble small carbon-containing compounds into a six carbon glucose molecule. The resulting glucose is then available for distribution to other tissues in the body for immediate metabolism, lactose synthesis in the case of mammary tissue, or for storage. Because milk lactose is derived from blood glucose the capacity for milk production is directly determined by the capacity for gluconeogenesis in liver and the ability of other tissues to spare glucose use during lactation and make it available to the mammary gland. However the supply of glucose to several tissues is essential for their normal function. Red blood cells require glucose as an energy source, the brain and central nervous tissue oxidize glucose (Mayes, 1996), and in ruminants the source of the glycerol part of the triglyceride molecule in adipose tissue and milk is derived from glucose.

How Much Glucose is Needed by Dairy Cows?

Blood glucose concentrations are controlled within fairly narrow limits under normal physiological conditions. Whole body glucose metabolism is characterized by

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the appearance of glucose in blood from either intestinal absorption or gluconeogenesis and the removal of glucose by peripheral tissues. Because of the appreciable fermentation of starch and free sugars in the rumen there is little glucose absorbed form the diet of dairy cattle. The concentration of glucose plasma is function of the entry of glucose from gluconeogenesis (and other sources) and the removal of glucose by extrahepatic tissues (Figure 1). Consequently changes in blood glucose concertation can reflect any of the possible combinations of contribution to, or pull form, the circulating glucose pool. Although feed processing may affect starch escaping form the rumen and availability for absorption as glucose numerous studies indicate that the net flux of glucose from the portal drained viscera (**PDV**; gut, pancreas, spleen and associated adipose tissue) is negligible (Reynolds, 2006).

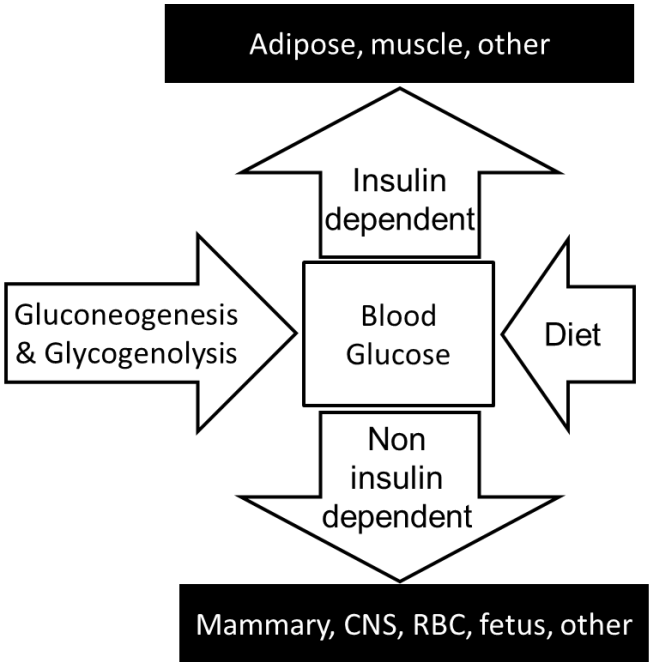

Figure 1. Factors controlling blood glucose concentrations. The appearance of glucose in blood is a function of glucose adsorption from glucose in the diet and gluconeogenesis. The removal of glucose from blood to tissues can be either insulin mediated (as per adipose tissue, muscle and other tissues) or non-insulin dependent (as per mammary tissue, central nervous system (**CNS**), red blood cells (**RBC**) and the developing fetus). The concentration of glucose in blood is the combined result of these physiological events.

Estimates of glucose needs for maintenance functions for dairy cows range between 200 g/d based on the data of Bickerstaff et al. (1974) to more than 400 g/d based on data from steers to estimate maintenance needs of cattle (Reynolds et al, 1991). During the transition to lactation the needs for gluconeogenesis increase abruptly from 1200 g/d at 21 days prior to calving to approximately 3 kg/d at 3 weeks postpartum as milk production increases to 80 lbs/d (36 kg/d) (Reynolds et al., 2003; Aschenbach et al., 2010). Estimates of glucose needs are derived from estimates of glucose needed for maintenance and the need for lactose synthesis as well as needs for oxidation by

mammary tissue. The latter has been estimate using the quantity of lactose produced per day multiplied by 1.5 to account for glucose use by mammary tissue other metabolism that does not directly yield milk lactose (Hanigan et al., 1992).

Adequate feed intake is needed to provide the precursors for gluconeogenesis from rumen fermentation. Consequently the capacity for glucose production is closely matched by the capacity for energy intake and in particular for energy sources that provide glucogenic precursors, particularly propionate. Because transition cows often voluntarily self-impose feed intake limitations there is a need to adapt liver metabolism to use alternatives sources of glucose carbon such as lactate and amino acids. This capacity is achieved through changes at the cellular and molecular levels in liver cells (hepatocytes) in response to signals received from extrahepatic tissues including increased circulating fatty acids, changes in other metabolites or hormones such as insulin, glucagon and glucocorticoids.

Substrates for Gluconeogenesis and Primary Control Points and Processes

The main substrates for glucose synthesis in fed ruminants are lactate, propionate and amino acids. Glycerol, from adipose tissue, can also contribute carbon for glucose synthesis during feed restriction and energy deficiency. Propionate contributes approximately 50% of the carbon for gluconeogenesis while lactate or amino acids contribute 10-15% each (Huntington, 1990). In addition the use of amino acids for glucose synthesis may be of considerable importance when intake is depressed in late gestation (Bell et al., 2000).

Pyruvate is a common entry point in the gluconeogenic pathway for lactate, alanine or other gluconeogenic amino acids. Alanine accounts for approximately 24% of the net portal appearance of amino acid nitrogen in fed ruminants (Reynolds et al., 1991). A lack of increase in hepatic glucose output in response to mesenteric vein infusions of alanine in cattle is accompanied by decreased liver extraction of lactate (Reynolds et al., 1991) and suggests a common point of regulation for lactate and alanine metabolism to glucose.

During gluconeogenesis pyruvate formed from lactate, alanine and other amino acids is transported into the mitochondria and carboxylated to oxaloacetate by pyruvate carboxylase (**PC**; Figure 2). In contrast, propionate is metabolized through part of the TCA cycle to oxaloacetate. Oxaloacetate can be metabolized to phosphoenolpyruvate (**PEP**) by phosphoenolpyruvate carboxykinase (**PEPCK**) or metabolized in the tricarboxylic acid (**TCA**) cycle. In turn, PEP carbon can be metabolized to glucose or recycled to pyruvate via pyruvate kinase (**PK**). In order for lactate carbon to be metabolized to glucose, the flux through PEPCK and PC must exceed the PK flux, whereas net flux of propionate carbon requires only a greater flux through PEPCK relative to PK and PC.

Hormonal control of metabolism is determined by changes in hormone concentrations and the ability of tissues to respond to those changes. The rates of

glucose production and utilization are regulated by insulin, glucagon and glucocorticoids in ruminants (reviewed by Brockman, 1986). Blood insulin concentrations are responsive to diet (Jenny and Polan, 1975), stage of lactation (Herbein et al., 1985), and dietary differences within developmental state (Breier et al., 1988). Developmental differences have been identified for the acute responsiveness of hepatocytes to insulin and glucagon (Donkin and Armentano, 1993; Donkin and Armentano, 1995). Data indicate that direct insulin and glucagon directly regulate in gluconeogenesis in ruminants, and insulin opposes the effects of glucagon and suggests PEPCK as a major regulatory site in this regard.

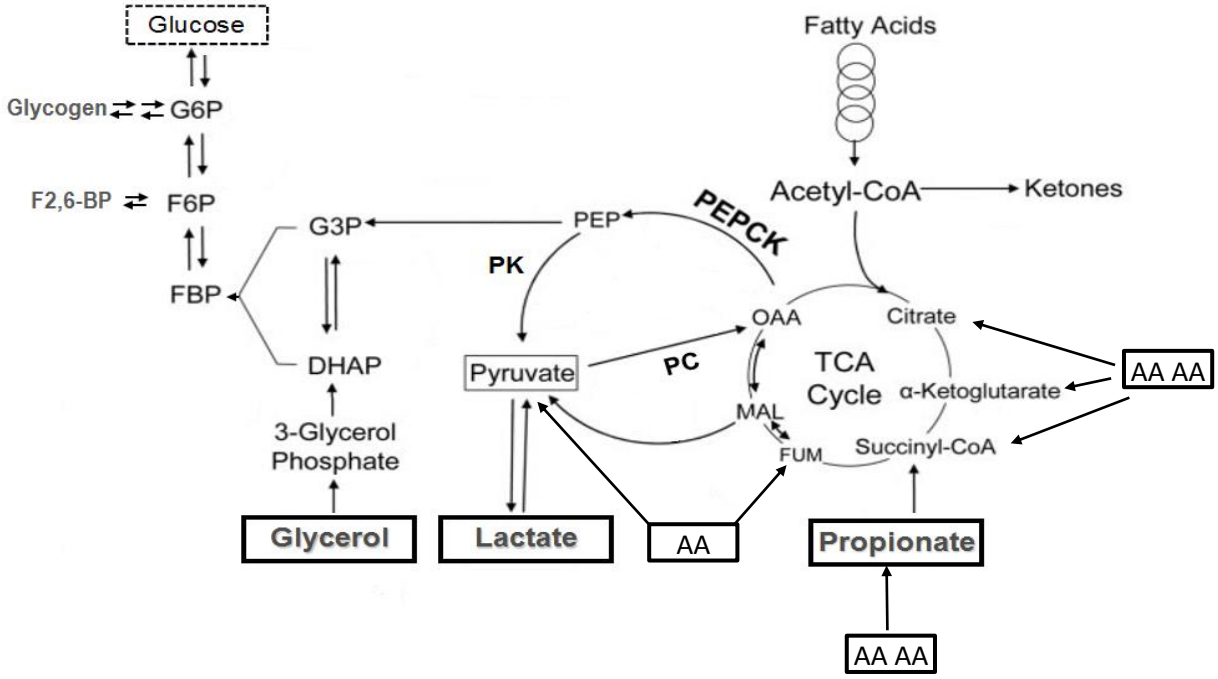

Figure 2. Metabolism of propionate, lactate, pyruvate, amino acids and glycerol to glucose in bovine liver and relationship to fatty acid oxidation. Abbreviations: Glucose-6-phosphate (**G6P**), Fructose-6-phosphate (**F6P**), Fructose-2, 6-bisphosphate (**F 2,6-BP**), Fructose-1,6, bisphosphate (**FBP**), dihydroxyacetone phosphate (**DHAP**), glycerol 3 phosphate (**G3P**), phosphoenolpyruvate (**PEP**) oxaloacetate (**OAA**) fumarate (**FUM**), malate (**MAL**), pyruvate kinase (**PK**), pyruvate carboxylase (**PC**), phosphoenolpyruvate carboxykinase (**PEPCK**), Amino acids (**AA**). Solid boxes indicate precursors for glucose synthesis.

Control of gluconeogenesis in non-ruminants occurs mainly in three distinct segments of the gluconeogenic pathway. First through the combined actions of PEPCK and PC, described above, which to oppose the actions of PK. These reactions generate PEP which is subject to further metabolism to dihydroxyacetone phosphate (**DHAP**) or glycerol-3 phosphate (**G3P**) and are substrates for aldolase in the formation of fructose 1,6, bisphosphate. The opposing reactions of fructose 1,6, bisphosphatase to form fructose-6-phosphate and phophofructokinase-1 to form fructose 1,6, bisphosphate

determine the relative flux of carbon to gluconeogenesis or glycolysis respectively. After subsequent isomerization of fructose-6-phosphate to glucose-6-phosphate the relative activities of glucokinase and glucose-6-phosphatase (**G6Pase**) determine the availability of free glucose for release from liver. The resulting free glucose in the endoplasmic reticulum is released from the hepatocyte through the action of facilitated glucose transporter 2. The combined flux through the three reaction loci that result in formation of PEP, fructose 1,6, bisphosphate and free glucose and their opposing reactions determines the net flux of non-carbohydrate precursors to glucose. Control of gluconeogenesis, like most metabolic processes, occurs through a combination of substrate availability, short term hormonal regulation, allosteric regulation, and regulation involving changes in gene expression. Overall homeostatic and homeorhetic control of gluconeogenesis occurs through combinations of these primary modes of metabolic control and action on the three reaction that distinguish gluconeogenesis and glycolysis. Although these reactions and their control have been broadly explored across species there a need for additional specific information for ruminants on all levels of control of gluconeogenesis.

Glucose precursors are supplied to liver through absorption across the rumen wall and other portions of the gastrointestinal tract into the hepatic portal vein. The provision of glucogenic precursors is critical for hepatic glucose production. In vitro experiments using bovine hepatocytes indicate maximal gluconeogenesis from propionate, lactate, and glycerol that occurs between 2 and 4 mM (Donkin and Armentano, 1994) which correspond to concentrations that are well above physiological levels for the hepatic portal vein (Reynolds et al., 1988a). Despite additional capacity for gluconeogenic precursor metabolism with increased substrate supply it appears that this is not a primary regulator of glucose output in vivo as short term infusion of alanine into the mesenteric vein in beef heifers (Reynolds and Tyrrell 1991) or propionate in mid-lactation dairy cows (Casse et al. 1994) had little impact on hepatic glucose output. In contrast, prolonged changes in supply of glucose precursors appear to alter the capacity for gluconeogenesis through changes in expression of key genes in gluconeogenesis (Karcher et al., 2007).

Gluconeogenesis is also controlled allosterically. Acetyl-CoA, is a known allosteric activator of PC and ruminant PC has served as a model to characterize these effects of acetyl-CoA on PC activity (Easterbrook-Smith et al., 1979). This allosteric activation and the increased non esterified fatty acid release from adipose tissue with feed restriction and transition to lactation (Greenfield et al. 2000; Velez and Donkin, 2005) and subsequent metabolism through β –oxidation in liver to acetyl CoA may be instrumental in amplifying changes in PC expression for increased gluconeogenesis from lactate and amino acids. In addition acetyl CoA acts as a allosteric inhibitor of pyruvate kinase. Additional potential for allosteric control of gluconeogenesis occurs through the repression of phosphofructokinase -1 (**PFK-1**) exerted by ATP and citrate and repression of hexokinase by glucose-6-phosphate. The allosteric repression of these reactions diminishes glycolysis and favors gluconeogenesis. One of the most potent allosteric regulators of gluconeogenesis is fructose 2,6 bisphosphate, a metabolite that simultaneously allosterically activates PFK-1 to stimulate glycolysis and inhibits fructose 1,6-bisphosphatase (**FBPase-1**) to reduce gluconeogenesis. The

accumulation of fructose 2,6-bisphosphate in liver is favored by high glucose and insulin concentrations whereas elevated glucagon leads to the reduction of fructose 2,6-bisphosphate to favor gluconeogenesis. The abundance of fructose 2,6-bisphosphate in liver is controlled by the phosphorylation state of the bifunctional enzyme phosphofructokinase 2/fructose-2,6-bisphosphatase (**PFK-2/FBPase-2**). Although the role of PFK-2/FBPase-2 regulation has been well described for nonruminants (Rider et al., 2004) the role of this enzyme in regulating gluconeogenesis in ruminants remains largely unexplored.

Gene Expression and Control of Gluconeogenesis

Long-term regulation of gluconeogenesis in nonruminants has been characterized by changes in the expression of genes encoding glucoregulatory enzymes, mainly PEPCK and pyruvate kinase (Pilkis and Claus, 1991). It is well established that insulin represses PEPCK whereas glucagon (or cAMP) and glucocorticoids induce the activity of the PEPCK enzyme by directly regulating expression of the gene through transcriptional modulation and mRNA stability (reviewed in O'Brien and Granner, 1990). Control of lactate use for glucose synthesis is distributed between pyruvate kinase and the reactions involving PC and PEPCK (Sistare and Haynes, 1985). Glucocorticoids have little effect on flux through pyruvate kinase; therefore, an increase in gluconeogenesis from lactate in response to glucocorticoid is mainly due to the combined increases in flux through reactions catalyzed by PC and PEPCK (Jones et al., 1993).

In cattle and sheep the activity of PC is responsive to nutritional and physiological states that impose the greatest demands for endogenous glucose production such as lactation and feed deprivation (Smith and Walsh, 1982). In contrast, the activity of PEPCK is relatively invariant between different nutritional and physiological states in ruminants. The expression and activity of PC is highest in liver, kidney, adipose, brain, adrenal gland, and lactating mammary tissue (Barritt, 1985). Short-term allosteric regulation of PC activity by acetyl-CoA has been well characterized and is likely under these conditions; however, sustained changes in the activity of the PC enzyme require parallel increases in PC mRNA (Zhang et al., 1995). Changes in PC abundance, through alteration in rates of synthesis, represent long-term regulation of lactate metabolism (Barritt, 1985). Data from our laboratory (Greenfield et al., 2000) indicate that expression of PC mRNA is dramatically increased across the transition to lactation whereas PEPCK is relatively unchanged during this period. In bovine liver, the capacity for gluconeogenesis from lactate appears to be directly related to the expression of PC mRNA (Velez and Donkin, 2005). The activity and mRNA abundance of PC are closely linked in transition dairy cows (Greenfield et al., 2000). Taken together these data indicate that the capacity for gluconeogenesis in transition cows is regulated at the level of PC mRNA abundance.

Pyruvate Carboxylase During Transition to Calving and Feed Restriction

The transition to lactation underscores the importance of gluconeogenesis in ruminants as hypoglycemia, ketosis, and related metabolic disorders are often observed when gluconeogenic capacity fails to adapt to the increased demands for glucose to support lactose synthesis and mammary metabolism. The importance of appropriate adaptation to the increased demands for glucose in the periparturient cow is highlighted by reports that incidence of ketosis in commercial dairy herds is 17 to 26 % (Dohoo and Martin, 1984; Melendez et al., 2006). The impact of proper nutritional management of the lactating cow in minimizing these disorders has been recognized previously (Zamet et al., 1979); the implications of appropriate management of the dairy cow during late gestation (Bell, 1995, Grummer 1995; Jouany, 2006) have become well recognized over the past two decades. Additional efforts are still needed to define and meet the nutritional requirements of the transition dairy cow in order to optimize animal health, production in the ensuing lactation, overall longevity, and animal well-being (NRC, 2001).

Because obligatory requirements exist for glucose as a substrate for brain, erythrocytes, kidney medulla and mammary tissue (Mayes, 1996) ruminants have evolved adaptations to reduced supply propionate from rumen fermentation such as during feed restriction. Under these conditions there is increased gluconeogenesis from lactate, amino acids and glycerol to meet glucose needs (Baird et al., 1980). An increase in PC mRNA coupled with increased metabolism of lactate to glucose in response to partial feed restriction in dairy cows (Velez et al., 2005) and a lack of effect on PEPCK expression suggests a critical role of PC in regulation of hepatic metabolism in ruminants especially during nutrient insufficiency.

We examined the mRNA abundance for PC and PEPCK in transition cows (Greenfield et al., 2000; Hartwell et al., 2001) and determined that PC responds to the onset of calving and that PEPCK expression is elevated after lactation is established. The activities of both enzymes are reflected by changes in mRNA abundance at calving (Greenfield et al., 2000; Agca et al., 2002). We have cloned and sequenced the promoter region of bovine PEPCK-C (Zhang et al., 2014). Comparisons between the promoter regions of rat and bovine PEPCK revealed no similarities in the overall nucleotide sequence. We have identified the transcription start site, linked the promoter to a luciferase reporter sequence, and generated a family of 5' promoter truncations. When expressed in liver cell cultures the bovine PEPCK promoter is responsive to propionate (Zhang et al., 2014), which suggests feed forward control of gluconeogenesis in bovine that is linked to rumen propionate production.

We have further explored control of PC and PEPCK with feed restriction and bST administration and determined that PC, but not PEPCK, is elevated during feed restriction (Velez and Donkin, 2005) and PEPCK, but not PC, is elevated with bST (Velez and Donkin, 2004). These data highlight the unique aspects of bovine metabolism with regard to control of glucose synthesis. When these data are considered collectively our current understanding of the major control points for gluconeogenesis in ruminants shows that PC plays a pivotal role in determining the rate of gluconeogenesis when intake is compromised such as during the transition to

calving, whereas PEPCK-C is linked to control of gluconeogenesis when feed intake is not constrained.

Additional Roles of Pyruvate Carboxylase in Liver Metabolism

The two possible fates of pyruvate in liver are conversion to acetyl CoA by pyruvate dehydrogenase followed by further metabolism through the TCA cycle, or conversion to oxaloacetate via PC as an intermediate in the synthesis of glucose. Likewise, the abundance of non-esterified fatty acids (**NEFA**), released from adipose tissue at calving, may be oxidized to acetyl CoA and further metabolized in the TCA cycle or alternatively, can be partially oxidized to ketones. Increased PC mRNA abundance and activity on the day of calving may provide an adaptive mechanism which allows pyruvate carbon to be channeled through oxaloacetate to maintain hepatic glucose output and simultaneously minimize ketogenesis from non-lipid precursors. The direct allosteric activation of PC by acetyl CoA (Mayes, 1996) would serve to further augment the impact of increased PC mRNA expression in the periparturient dairy cow.

An additional role for increased PC at calving and during feed restriction may be to generate mitochondrial oxaloacetate as a substrate in TCA cycle oxidation of NEFA. Although the pathogenesis of ketosis is not clearly understood, it is generally thought to be initiated by inadequate availability of endogenous glucose. Secondary signs of the disorder result in fatty liver and further impairments in gluconeogenesis and ammonia detoxification (Grummer, 1995). Increases in PC mRNA which coincide with increased clearance of lipid from liver in dairy cattle given glucagon infusions (Hippen et al., 1999; She et al., 1999) support a role for PC in this process.

Propionate and PEPCK Expression in Bovine

The effects of short chain (volatile) fatty acids to stimulate growth and differentiation of rumen epithelium have been recognized for some time (Van Soest, 1994). Recently the effects of short chain fatty acids, produced by colonic bacteria, have been recognized as beneficial in suppressing proliferation of colon cancers and regulating immune function (Sanderson et al., 2001). More recently propionate, acetate and butyrate have been identified as repressors (Tran et al., 1998) and as activators of gene expression (Drozdowski et al., 2002). The potency of volatile fatty acids to regulate gene expression is highlighted by striking example that the developmental switching of the globin gene can be halted and even reversed by infusion of butyrate in the sheep fetus (Perrine et al., 1990).

Expression of PEPCK was elevated up to 5 fold when rat hepatoma cells (H4IIE cells) were incubated for 4 h in the presence of 2.5 mM of short chain volatile fatty aicds (Massillon et al., 2003). Incubation of rat hepatoma (**H4IIE**) cells with acetate, propionate, caproate and valerate also increased glucose-6 phosphatase (**Gluc-6-Pase**) mRNA (Massillon et al., 2003). Promoter analysis indicates that these effects are mediated through specific transcription factors expressed in liver, intestine and kidney. These data indicate that short chain volatile fatty acids can affect gluconeogenesis

directly by genes that control the fate of glucose precursors (Van Schaftingen and Gerin, 2002).

Recent data from our laboratory supports an induction of PEPCK in liver in response to increased propionate supply to liver. Feeding monensin, a feed additive that causes a shift in rumen fermentation to favor increased propionate production. The data indicate an increase in abundance of PEPCK-C mRNA with prepartum monensin feeding. Prepartum feed intake did not differ for the monensin and control cows and PEPCK transcript abundance observed for monensin fed cows at -14 and +1 days relative to calving was similar to the expression observed during lactation when feed intake is much greater than the prepartum period. These data suggest that the effect of propionate on PEPCK expression is independent of level of feed intake but linked to the end products of rumen fermentation. Experiments outlined in this proposal will determine the effects of propionate in this regard.

Expression of PEPCK mRNA that is sensitive to propionate supply would serve to increase the gluconeogenesis from propionate and also gluconeogenesis from other substrates including lactate and alanine. Induction of PEPCK in bovine and increased capacity for gluconeogenesis from propionate represents a novel control mechanism for gluconeogenesis. The action of propionate to promote PEPCK expression would serve to couple the availability of the primary substrate for gluconeogenesis in ruminants to a novel feed-forward induction of gluconeogenic capacity.

Recent experiments in our laboratory have explored the potential for propionate to directly control PEPCK expression and gluconeogenesis. Postruminal propionate infusions over an 8-h period to augment the daily supply of propionate by 25% resulted in an increase in liver expression of PEPCK and flux of propionate to glucose despite elevated blood insulin concentrations. Separate experiments using the bovine PEPCK gene promoter revealed that propionate is a potent and direct activator of PEPCK gene expression. The effects of propionate suggest primary control that is dominant to glucagon, dexamethasone, and insulin in this regard. Follow up experiment have identified a specific region within the PPECK gene promoter responsible for this control. The mechanisms by which propionate acts to control expression of PEPCK, and consequently the capacity for gluconeogenesis, are the subject on ongoing investigations.

Strategies to Improve Glucose Economy of Transition Dairy Cows

Many of the feeding strategies that are already currently employed for transition cows are successful because they impact the ability of the cow to produce glucose in liver and supply it to other tissues. These include maintaining feed intake and increasing intake as soon after parturition as possible. Because energy intake and glucose production are closely correlated the inclusion of rumen fermentable feeds that increase in supply of propionate serve to promote glucose supply as long as overall intake is not compromised in response to the higher energy density ration. Feeds, feed additives, and feeding management strategies that promote maximal daily rumen propionate

production and absorption will directly impact the production of glucose by the transition cow. Our current work indicates that glucose production becomes more efficient with greater propionate supply. Feed additives, including ionophores, that shift fermentation pattern to promote greater propionate production per unit of feed intake have a direct positive effect on glucose status through promoting increased capacity for gluconeogenesis. Feeding management strategies that encourage more consistent and frequent feeding are likely to promote this process as well. Provision of alternative precursors for glucose including lactate, certain amino acids, and glycerol may be beneficial during the immediate postpartum interval as ability of the cow to metabolize these nutrients is elevated. Care should be exercised not to supply these at the expense of normal rumen fermentation or feed intake.

Summary, Conclusions, and Future Directions

Robust and appropriate responses and controls for gluconeogenesis are critical for optimal growth, lactation and well-being of ruminants used in production agriculture. Propionate, lactate, amino acid sand glycerol contribute to whole animal glucose metabolism although their contributions vary with physiological state and their supply. Features of ruminant metabolism results in aspects of the control points for gluconeogenesis in ruminants that appear to be unique compared with nonruminants particularly with respect to the response to reduced feed intake and to supply of gluconeogenic precursors, namely propionate. The adaptations to nutrient insufficiency associated with the transition to lactation results in increased capacity for lactate metabolism though changes in the PC gene and the adaptation to increased feed intake and greater ruminal propionate production appear to involve specific changes in PEPCK. These coordinated responses appear to be essential in maintaining tissue needs for glucose and involve responses at the level of their respective gene promoters. Although recent research has expanded knowledge of gluconeogenesis in ruminants to include the molecular control additional work needed to integrate the impact of genetics, breed type, pre- and postnatal nutrition and environment to control of this critical process and to permit application of this knowledge to precision management and feeding programs that optimize productivity, health, and profitability for ruminant production systems.

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SESSION NOTES