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Purdue University

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Control of bovine Pyruvate Carboxylase Expression by Saturated and Unsaturated Fatty Acids and Impact on Fatty Acid Metabolism

For the degree of Doctor of Philosophy

Is approved by the final examining committee:

Shawn Donkin

Chair

Kola Ajuwon

Shihuan Kuang

Dorothy Teegarden

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Head of the Departmental Graduate Program

12/2/2015

Date

CONTROL OF BOVINE PYRUVATE CARBOXYLASE EXPRESSION BY
SATURATED AND UNSATURATED FATTY ACIDS AND IMPACT ON
FATTY ACID METABOLISM

A Dissertation

Submitted to the Faculty

of

Purdue University

by

Katherine E. Boesche

In Partial Fulfillment of the

Requirements for the Degree

of

Doctor of Philosophy

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Purdue University

West Lafayette, Indiana

For my family

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Co-Authorship Declaration

I hereby declare that this dissertation incorporates material that is a result of joint research as follows: the entire dissertation is an outcome of joint effort with Dr. Shawn S. Donkin and Stephanie L. Koser. In addition, the in silico promoter binding site analysis reported in Chapter 4 of the dissertation is the result of the PROMO virtual laboratory of ALGGEN, which utilizes TRANSFAC version 8.3 provided by Messeguer et al., 2002 and Farré et al., 2003. In all other cases, the key ideas, primary contributions, experimental designs, data analysis and interpretation were performed by the author and the contribution of co-authors was primarily through the provision of technical assistance.

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LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CD36	Scavenger receptor; fatty acid translocase
ChREBP	Carbohydrate-responsive element-binding protein
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DMEM	Dulbecco's Modified Eagle's medium
ER	Endoplasmic reticulum
F1,6BPase	Fructose-1,6-bisphosphatase
FABP	Fatty acid-binding protein
FATP	Fatty acid transport protein
FBS	Fetal bovine serum
G6Pase	Glucose-6-phosphatase
H4IIE	Rat hepatoma cells
HNF	Hepatocyte nuclear factor
HSL	Hormone-sensitive lipase
LDL	Low density lipoprotein

LPL	Lipoprotein lipase
LXR	Liver X receptor
MDBK	Madin-Darby bovine kidney cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NEFA	Nonesterified fatty acid
NF	Nuclear factor
OAA	Oxaloacetate
P1	Bovine pyruvate carboxylase promoter 1
P2	Bovine pyruvate carboxylase promoter 2
P3	Bovine pyruvate carboxylase promoter 3
PC	Pyruvate carboxylase
PCC	Propionyl-CoA carboxylase
PCK	Phosphoenolpyruvate carboxykinase
PCK1	Cytosolic phosphoenolpyruvate carboxykinase
PCK2	Mitochondrial phosphoenolpyruvate carboxykinase
PCR	Polymerase chain reaction
PDC	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PDP	Pyruvate dehydrogenase phosphatase
PEP	Phosphoenolpyruvate
PPAR	Peroxisome proliferator-activated receptor

PPRE	PPAR response element
PUFA	Polyunsaturated fatty acid
RXR	Retinoid X receptor
SCAP	SREBP cleavage-activating proteins
SREBP	Sterol regulatory element binding protein
TCA	Tricarboxylic acid
TSS	Transcription start site
UTR	Untranslated region
VFA	Volatile fatty acid
VLDL	Very low density lipoprotein

ABSTRACT

Boesche, Katherine E. Ph.D., Purdue University, December 2015. Control of bovine pyruvate carboxylase expression by saturated and unsaturated fatty acids and impact on fatty acid metabolism. Major Professor: Shawn S Donkin.

Pyruvate carboxylase (PC; EC 6.4.1.1) ostensibly links carbohydrate and lipid metabolism by supplying oxaloacetate (OAA) from the carboxylation of pyruvate. The OAA pool is necessary in maintaining the anaplerotic supply of carbons to the TCA cycle, and to promote the complete oxidation, to CO₂, of acetyl-CoA. The actions of PC in supplying OAA are critical during periods of increased cellular lipid load, including the negative energy balance experienced by periparturient dairy cows. The bovine PC gene contains three promoter sequences, with products of the proximal promoter (PCP1) being specific to glucogenic and lipogenic tissues. Previous work links control of bovine PC mRNA expression to nonesterified fatty acids (NEFA), but the direct effects of saturated and unsaturated fatty acids on PC mRNA expression and subsequent fatty acid metabolism were unclear. The central hypothesis of this dissertation is that the ratio of the most prevalent circulating saturated and unsaturated fatty acids in dairy cows regulates PC flux and subsequent fatty acid metabolism. The objectives of this dissertation were, first, to evaluate the response of PC mRNA to the copresence of the most abundant circulating saturated and unsaturated fatty acids in periparturient dairy cows. Second, to determine the direct effects of these saturated and unsaturated fatty

acids, and their combinations, on the cellular oxidation of fatty acids, and the potential relationship to PC, and third, to examine the effects of the copresence of saturated and unsaturated fatty acids on the transcriptional activity of bovine PCP1.

Bovine PC mRNA expression was first examined, in response to copresence of saturated and unsaturated fatty acids, in a bovine kidney cell line and in bovine primary hepatocytes. Both C16:0 or C18:0 alone significantly depressed PC mRNA in bovine kidney cells. Inclusion of unsaturated C18:3n-3 *cis* restored PC expression to levels similar to control in bovine kidney cells. Data from bovine primary hepatocytes displayed a similar numerical pattern in response to fatty acid combinations, although means did not differ. However, incubation with C18:3n-3 *cis* alone resulted in nearly two-fold increase in PC mRNA expression, compared to PC mRNA expression after control or saturated fatty acid exposure. Treatment with monounsaturated C18:1n-9 *cis* did not alter PC mRNA expression in either cell model. Results indicate that C18:3n-3 *cis* is a potent alleviator of saturated fatty acid-induced depression of PC mRNA expression.

To determine the impact of alterations in PC mRNA expression on cellular metabolism of fatty acids, the flux of radiolabeled C16:0 to CO₂ or to acid-soluble metabolites, including ketone bodies, was analyzed after pretreatment of bovine kidney cells with combinations of saturated and unsaturated fatty acids which are most prevalent in periparturient dairy cows. Prior exposure to either C16:0 or C18:0 alone significantly depressed both complete and partial oxidation compared to exposure to C18:3n-3 alone. Data for complete oxidation of radiolabeled C16:0 to CO₂ were correlated ($r = 0.63$; $P < 0.05$) with PC mRNA expression, suggesting a potential relationship between PC flux and subsequent fatty acid metabolism. Oxidation of radiolabeled C16:0 to CO₂ was also

correlated ($r = 0.80$; $P < 0.05$) to expression of cytosolic phosphoenolpyruvate carboxykinase (PCK1). The data indicate that although PC is regulated by fatty acids and in turn modulates the oxidation of fatty acids, the concomitant increase in PCK1 may oppose these effects by pulling carbon from the OAA pool toward gluconeogenesis. Factors remain to be identified that permit increased PC flux while maintaining, but not enhancing, PCK1 flux.

The role of bovine PCP1 in regulating the expression of PC mRNA in response to the copresence of saturated and unsaturated fatty acids prevalent in periparturient dairy cows was evaluated using bovine PCP1 ligated to a Firefly luciferase reporter and transiently transfected into bovine kidney cells. Bovine PCP1 activity increased in response to either C16:0 or C18:3n-3 *cis*, and combinations of those fatty acids, indicating a dominant role of C18:3n-3 *cis* in controlling bovine PCP1 activity. Truncations of bovine PCP1 indicate a region responsive to fatty acids located between -773 to -494 bp (PCP1_(-773_+3)) relative to transcription start site (TSS), as activity in response to fatty acid greatly diminished with truncation of this region. In silico analysis of bovine PCP1 indicates several transcription factor binding site consensus sequences, including peroxisome proliferator activated receptor (PPAR) α and sterol regulatory element binding proteins (SREBP) sites, that are candidate response elements for control by C18:3n-3 *cis* and C16:0 or C18:0. Results indicate a dominant role of C18:3n-3 *cis* to signal through bovine PCP1_(-773_+3) containing a PPAR α binding site. A SREBP binding site within bovine PCP1_(-773_+3) is a candidate for saturated fatty acid responsiveness within this promoter region.

Together, these data indicate control by saturated and unsaturated fatty acids prevalent in periparturient bovine on PC mRNA expression, regulated through the PC proximal promoter, and in determining subsequent cellular fatty acid metabolism. Responsiveness of bovine PCP1 to saturated and unsaturated fatty acids may be mediated through identified putative binding sites. The cellular oxidative capacity, dependent upon the pool of OAA generated by PC, can help determine the response to physiological factors, including fatty acids, which increase during metabolically stressful periods in dairy cows.

CHAPTER 1. LITERATURE REVIEW

INTRODUCTION

First discovered for its role in gluconeogenesis in chicken liver (Utter and Keech, 1960), pyruvate carboxylase (PC; EC 6.4.1.1) is a mitochondrial enzyme central to several metabolic pathways. Pyruvate carboxylase is responsible for the irreversible, ATP-dependent carboxylation of pyruvate to oxaloacetate (OAA; Utter and Keech, 1960). Oxaloacetate supply by PC is inextricably linked to the activity of the tricarboxylic acid (TCA) cycle, which functions to oxidize acetyl-CoA to CO₂ and provide intermediates for several other biological pathways. The flux of carbons into, and maintenance of the TCA cycle biological intermediates, is referred to as anaplerosis (Kornberg, 1966; Owen et al., 2002). The anaplerotic role of PC in catalyzing OAA synthesis from pyruvate, is therefore evident. The reciprocal pull of carbons away from the TCA cycle, or cataplerosis, links products of the TCA cycle to several biosynthetic pathways, including fatty acid synthesis, gluconeogenesis, and glyceroneogenesis (Owen et al., 2002). Because the TCA cycle cannot serve as a carbon sink, cataplerotic enzymes, including phosphoenolpyruvate carboxykinase (PCK; EC 4.1.1.32), become especially critical during periods of increased energy intake (Owen et al., 2002). Without increased

cataplerotic pull, the anaplerotic action of PC could function to increase the oxidative capacity of the TCA cycle, increasing the oxidation of acetyl-CoA.

Regulation of PC by circulating fatty acids has been extensively explored in both human and murine models (Jitrapakdee et al., 2008; Gray et al., 2014), and recently explored in bovine (White et al. 2011b,c; White et al., 2012). A direct relationship between PC activity and influences of specific fatty acids has yet to be fully elucidated. This review will focus on the regulation and anaplerotic roles of PC, examine the potential influences of circulating fatty acids, and stress the significance of these roles in ruminant animals.

PYRUVATE CARBOXYLASE

Enzyme structure

The mammalian PC enzyme, found in the mitochondrial matrix, consists of four identical subunits, each of approximately 120 – 130 kDa. These subunits are arranged as dimers of dimers in a homotetrameric structure. The PC enzyme is only active in this tetrameric structure (Jitrapakdee et al., 2008; Gray et al., 2014). Similar to other biotin-dependent enzymes, PC contains three functional domains: an N-terminal domain with a biotin carboxylation site, a central domain with a carboxyltransferase site, and a C-terminus domain containing the biotin carboxyl carrier protein unit (St. Maurice et al., 2007). A prosthetic biotin group is covalently bound to a specific lysine residue within the carrier protein unit (Jitrapakdee and Wallace, 1999). Pyruvate carboxylase shows a

high degree of structural similarity to other biotin-dependent enzymes, including propionyl-CoA carboxylase (PCC; EC 6.4.1.3), and acetyl-CoA carboxylase (ACC; EC 6.4.1.2; Jitrapakdee et al., 2008).

Enzyme function

Conversion of pyruvate to OAA by PC occurs over two spatially separate enzyme subsites. This reaction is catalyzed in two steps with biotin acting as the carboxyl carrier. The first, ATP-dependent step involves carboxylation of biotin with a CO_2 from HCO_3^- , via the formation of a carboxyphosphate intermediate (Attwood, 1995; Jitrapakdee and Wallace, 1999; Gray et al., 2014). The carboxybiotin group is then transferred to the PC carboxyltransferase site where the carboxyl group is transferred from carboxybiotin to pyruvate, resulting in OAA and a regeneration of the biotin cofactor (Jitrapakdee and Wallace, 1999; Gray et al., 2014). Requirements for ATP, acetyl-CoA, and Mg^{2+} , for the reaction to proceed were identified very early on (Utter and Keech, 1960). The role of Mg^{2+} as an essential cofactor was later confirmed through several kinetic studies to identify Mg^{2+} binding sites (Attwood and Graneri, 1992). Necessity of acetyl-CoA as an allosteric activator of PC is now well established (review by Adina-Zada et al., 2012). Binding of acetyl-CoA drastically alters both the structure and catalytic activity of PC through stabilization of the tetrameric structure of the enzyme and enhancement of substrate binding to HCO_3^- (Adina-Zada et al., 2012). The major influence of acetyl-CoA is only the first partial reaction carried out by PC, and the allosteric binding site of acetyl-CoA between the different domain sites has been identified (St. Maurice et al., 2007).

ROLE OF PYRUVATE CARBOXYLASE IN METABOLISM

Anaplerotic Roles of Pyruvate Carboxylase

Pyruvate carboxylase was initially identified, along with fructose-1,6-bisphosphatase (F1,6BPase; EC 3.1.3.11), glucose-6-phosphatase (G6Pase; EC 3.1.3.9), and PCK, as one of the four primary gluconeogenic enzymes (Utter and Keech, 1960). More recently, PC has been described as the “archetypical anaplerotic enzyme” for its fundamental role in replenishing OAA supply to the TCA cycle (Owen et al., 2002). The limiting factor for TCA cycle capacity is the pool of OAA, and while this pool is utilized for several biological endpoints, discussed below, the pull of carbon intermediates out of the TCA cycle must be concurrently coupled with OAA supply. Any biochemical pathway that begins with an intermediate of the TCA cycle relies on the anaplerotic replenishment of TCA cycle intermediates, in part by PC, to promote flow through the pathway (Figure 1.1).

Overview of Tricarboxylic acid cycle

The TCA cycle is common to all aerobic organisms and generates energy through the oxidation of carbon fuel sources to generate reducing equivalents, which will in turn be utilized to generate ATP through oxidative phosphorylation. The essentiality of the TCA cycle in many biochemical pathways across a variety of organisms illustrates its conservation through evolution. In eukaryotes, acetyl-CoA, produced from the catabolism of amino acid carbons, carbohydrates, and lipids, is oxidized in the mitochondria, yielding three equivalents of NADH, one equivalent of FADH₂, and one equivalent of

GTP per acetyl-CoA equivalent. The NADH and FADH₂ produced are subsequently utilized, through oxidative phosphorylation, to generate ATP. The initial reaction of the TCA cycle involves the condensation of 4-carbon OAA with 2-carbon acetyl-CoA and is catalyzed by citrate synthase (EC 2.3.3.1; Remington, 1992). This is the first of three exergonic reactions of the TCA cycle. Binding of OAA to citrate synthase induces a conformational change in the enzyme, only then exposing the binding site for acetyl-CoA (Remington, 1992). The need for the pool of OAA to drive the reaction is again emphasized here. Subsequent dehydrogenation and decarboxylation reactions yield the byproducts of two CO₂ molecules, and the regeneration of OAA (Figure 1.1; Berg et al., 2002).

Figure 1.1. Anaplerotic and cataplerotic pull of oxaloacetate (OAA) by pyruvate carboxylase (PC) and phosphoenolpyruvate carboxykinase (PCK). Adapted from Burgess et al., 2004. The oxidative capacity of the tricarboxylic acid (TCA) cycle is dependent upon the ratio of acetyl-CoA to oxaloacetate (OAA). Activation of PC promotes anaplerotic pull of carbons to OAA synthesis and promotes TCA oxidative capacity. Dihydroxyacetone phosphate, DHAP; fructose-6-phosphatase, F6P; fructose 1, 6-bisphosphate, FBP; glyceraldehyde-3-phosphate, G3P; glucose-6-phosphatase, G6P; phosphoenolpyruvate, PEP.

Regulation of Tricarboxylic acid cycle

Both entry into, and rate of, the TCA cycle are tightly controlled through the actions of several enzymes which are regulated, in large part, by the cellular concentrations of ATP and NADH. The first of three exergonic steps of the TCA cycle, catalyzed by citrate synthase, was described above. The remaining exergonic steps involve the enzymes isocitrate dehydrogenase (EC 1.1.1.42) and α -ketoglutarate dehydrogenase (EC 1.2.4.2; Berg et al., 2002). Substrate availability for citrate synthase of either OAA or acetyl-CoA can vary under different metabolic circumstances. Increases in the ratio of the concentrations of NADH and NAD^+ can lead to negative feedback of both isocitrate dehydrogenase and α -ketoglutarate dehydrogenase, as can increases in ATP levels (Williamson and Cooper, 1980; Berg et al., 2002). An increased NADH to NAD^+ ratio would also indicate a decreased concentration of OAA, decreasing flux into the initial cycle reaction. Additionally, ADP acts as an allosteric activator of isocitrate dehydrogenase by enhancing substrate affinity (Berg et al., 2002). Such tight allosteric regulations by reaction products allows for acute and rapid response to energy status changes within the cell. Deficiency of α -ketoglutarate dehydrogenase is a very rare autosomal recessive trait that presents with hypotonia, encephalopathy, and failure to thrive; no specific treatment is available (Pithukpakorn, 2005). Such dramatic alterations to metabolism illustrate the central role of this enzyme and the TCA cycle. The role of PC in several aberrant metabolic systems is discussed in depth in subsequent sections.

Substrates of Tricarboxylic acid cycle

Substrate flow into the TCA cycle increases during the fed state when metabolism of glucose to pyruvate is increased. During the postprandial period, glucose enters hepatocytes via the GLUT2 glucose transporter and is immediately phosphorylated by glucokinase to glucose-6-phosphate. Gluconeogenesis and the cataplerotic role of pyruvate carboxylase will be discussed in detail below. In brief, glucose-6-phosphate can either be stored as glycogen or metabolized to pyruvate, potentially supplying this substrate to PC for carboxylation to OAA (Rui, 2014). Linking cytosolic pyruvate metabolism to the TCA cycle, the mitochondrial pyruvate carrier functions to transport pyruvate from the mitochondrial intermembrane space to the mitochondrial matrix (Gray et al., 2014; Patel et al., 2014). During the fed state, at least in nonruminants, most pyruvate (with NAD^+) is converted by the pyruvate dehydrogenase complex (PDC) to acetyl-CoA, NADH, and CO_2 , with generated acetyl-CoA subsequently entering the TCA cycle (Berg et al., 2002). Acetyl-CoA may also be utilized in other biochemical reactions, including generation of acetylcholine, cholesterol formation, or lipogenesis. The PDC is very tightly regulated by the energy status by the cell and is inhibited during high energy status, when acetyl-CoA, ATP, or NADH levels increase (Gray et al., 2014). Regulation of PDC occurs through transcriptional abundance of the proteins comprising the complex, or acutely through phosphorylation and dephosphorylation by pyruvate dehydrogenase kinases (PDK) and pyruvate dehydrogenase phosphatases (PDP), respectively. Phosphorylation of specific residues on PDC subunits by PDK allow for rapid downregulation of PDC in response to cellular energy levels. For example, during fasting, activity of PDC can be repressed to promote the flux of pyruvate to other cellular

fates. Pyruvate dehydrogenase phosphatase functions to dephosphorylate and activate PDC to increase the flux of acetyl-CoA to the TCA cycle. Pyruvate dehydrogenase phosphatase is activated by insulin and AMP, and inhibited by acetyl-CoA, ATP, and NADH (Gray et al., 2014).

Because the supply of both acetyl-CoA and OAA regulate substrate availability for PC, both pools must be taken into consideration when investigating the anaplerotic or cataplerotic roles of PC. The anaplerotic activity of PC, through the generation of OAA from pyruvate, serves to offset the cataplerotic pull of carbons from the TCA cycle. The ratio of acetyl-CoA to OAA must remain equal if activity of the TCA cycle to completely oxidize acetyl-CoA is to be maintained (White, 2015). The circulating fatty acid profile both supplies acetyl-CoA from fatty acid β -oxidation, and serves as signaling molecules to regulate both gene expression and cellular metabolism. Increases in the oxidative capacity of the TCA cycle, through increased PC flux would allow lactating dairy cows to better cope with the increased plasma nonesterified fatty acid (NEFA) load experienced at calving.

Cataplerotic Roles of Pyruvate Carboxylase

A primary role of PC involves supply of OAA for conversion to phosphoenolpyruvate (PEP) by PCK (Figure 1.1). This cataplerotic pull of carbons away from the TCA cycle and into other biochemical pathways is necessary to avoid the excessive accumulation of ions in the mitochondrial matrix. Primary cataplerotic pathways in which PC is critical include gluconeogenesis and synthesis of fatty acids.

Gluconeogenesis: Overview and Regulation

Gluconeogenesis denotes the production of glucose, *de novo*, from non-carbohydrate precursors, including glucogenic amino acids, glycerol, lactate, and propionate. Gluconeogenesis is critical in providing glucose to tissue with an obligatory requirement for glucose, including the brain, erythrocytes, and mammary gland (Aschenbach et al., 2010). Glucose supply from gluconeogenesis is critical during periods when both dietary glucose and glycogen stores are expended, such as fasting or intense exercise, in nonruminants, or at all times in ruminant animals (Aschenbach et al., 2010). Liver and kidney are the major gluconeogenic tissues (Exton, 1972; Berg et al., 2002), with recent research revealing small intestine as a third site of gluconeogenesis with propionate, glycerol, and glutamine serving as gluconeogenic substrates (Croset et al., 2011; De Vadder et al., 2014). The relative contribution of small intestine to whole-body glucose production is still nominal in comparison to liver and kidney production. While the process of gluconeogenesis does share the same pathway with glycolysis, the thermodynamic barriers of irreversible reactions in glycolysis necessitate a separate set of enzymes and leads to more than just a simple reversal of the steps to glycolysis.

Cellular transport systems allow entry of gluconeogenic precursors, namely alanine, glycine, lactate, or serine, into the cytosol for conversion to pyruvate. In what is the most widely known role for PC, pyruvate is converted to OAA in the mitochondria (Berg et al., 2002), the first step in the conversion of pyruvate to PEP. This irreversible step to gluconeogenesis requires two enzymes; the first, PC, and the second, PCK. The decarboxylation of OAA to PEP by PCK requires GTP and is an endothermic process (Berg et al., 2002). There are two isoforms of PCK; PCK1 is compartmentalized to the

cytosol and PCK2 to the mitochondria (Hanson and Garber, 1972). The two PCK isoforms are encoded by separate genes, but have highly similar kinetic characteristics and in most species, including ruminants, activity of the two forms is equal (Hanson and Garber, 1972; Hanson and Reshef, 1997). For conversion of OAA to PEP by PCK1, OAA must first be transported to the cytosol. Because OAA cannot cross the mitochondrial membrane, it is first converted to malate and aspartate. Malate and aspartate are transported across the membrane of the mitochondrion and converted back to OAA once in the cytosol. These reactions require NADH within the mitochondria and NAD^+ in the cytosol (Exton, 1972; Berg et al., 2002). Cytosolic OAA can then serve as a substrate for PCK1 conversion to PEP. Conversion of OAA to PEP by PCK2 occurs in the mitochondria (Berg et al., 2002). Deficiency of PCK1, even with maintained PCK2 activity, results in hepatic steatosis, hypoglycemia, and death before early childhood (Vidnes and Sovik, 1976), illustrating the central metabolic role of the enzyme.

Subsequent reactions of gluconeogenesis require enzymes to overcome the kinetic barriers of the glycolysis pathway. The actions of phosphofructokinase in glycolysis are circumvented by conversion of fructose 1,6-bisphosphate to fructose 6-phosphate by fructose 1,6-bisphosphatase. Finally, glucose-6-phosphatase catalyzes the conversion of glucose 6-phosphate to glucose, allowing for release of glucose to the blood stream (Berg et al., 2002). The rate-limiting enzymes for gluconeogenesis are therefore PC, PCK, fructose 1,6-bisphosphatase, and glucose-6-phosphatase. It is important to note that glucose-6-phosphatase is only expressed in liver and kidney, and not muscle or adipose tissue. This prevents substrate release from these glucose-utilizing, non-gluconeogenic tissues (Exton, 1972). Furthermore, ruminant liver expresses hexokinase, rather than

glucokinase. Hexokinase has a comparatively lower K_m for glucose (Raggi et al., 1963; Hayirli, 2006). This allows for the important distinction that glucose is not extracted, but released from ruminant liver.

Gluconeogenesis in Ruminants

Nonruminants are supplied during the postprandial state through intestinal absorption with large quantities of glucose. The main obstacle to overcome in nonruminant glucose metabolism is maintaining glycogen stores after absorption, and mobilization from these stores (Woerle et al., 2003). Gluconeogenesis from *de novo* sources is only necessitated during fasting or under periods of increased glucose demand, such as exercise. Microbial fermentation of fiber carbohydrates in the forestomach, or rumen, of ruminant animals allows for the utilization of feedstuffs indigestible by nonruminants. In functional ruminants, dietary carbohydrates are fermented to volatile fatty acids (VFA), including propionate, leaving minimal amounts of exogenous glucose available (Ballard et al., 1969; Reynolds et al., 1988). Very little glucose is therefore presented for intestinal absorption, and ruminant glucose needs must be met through *de novo* gluconeogenesis. In ruminants, propionate serves as the principal substrate for gluconeogenesis (Reynolds et al., 2003). Furthermore, intestinal absorption of any glucose present is extremely limited in ruminants (Mills et al., 1999). Therefore, by necessity, over 90% of glucose requirements in ruminant animals is met through *de novo* gluconeogenesis (Aschenbach et al., 2010).

The requirement for *do novo* gluconeogenesis is drastically augmented with the onset of milk production during lactation in dairy cows. Lactose, the major milk osmole,

depends on the availability of glucose as its major precursor during milk synthesis (Barry, 1964; Smith, 1971). Between 60-85% of total glucose requirements in lactating ruminants can be attributed to lactose synthesis in mammary gland (Annison and Linzell, 1964). Splanchnic measurements have determined that the difference in glucose uptake by mammary gland in lactating ruminants cannot be accounted for simply by increases in glucose absorption across the portal-drained viscera (Doepel et al., 2009). Additionally, studies utilizing isotope tracers have found that the rate of *de novo* glucose synthesis and milk production in lactating dairy cows are positively correlated (Aschenbach et al., 2010).

Gluconeogenic Precursors

Gluconeogenic amino acids, glycerol, lactate, and propionate serve as the precursors for *de novo* gluconeogenesis. Ruminant gluconeogenesis is, in stark contrast to gluconeogenesis in nonruminants, highest during the postprandial period when both the production, and absorption, of propionate are increased (Reynolds et al., 2003). Propionate serves as the primary precursor for *do novo* gluconeogenesis during the postprandial state when the contribution from propionate is up to 60-80% (Annison and Bryden, 1999; Aschenbach et al., 2010). Approximately 95% of propionate that is absorbed from ruminal fermentation is subsequently converted to glucose, making this process exceptionally efficient in ruminant animals (Steinhour and Bauman, 1988). In contrast, *de novo* gluconeogenesis from propionate is dramatically decreased during fasting, or during periods of imposed feed restriction (Lomax and Baird, 1983). After a 24 h fast, utilization of propionate by liver was decreased 80% and net glucose output

decreased by 45% (Lomax and Baird, 1983). This lack of gluconeogenic precursors is emphasized during the postpartum period when drastic glucose demands, accompanying lactogenesis and lactose synthesis, are not met by feed intake (Bell, 1995). In turn, gluconeogenesis from other precursors must increase to meet metabolic demands. Contributions to *de novo* gluconeogenesis from lactate, gluconeogenic amino acids, and glycerol range between 16-26%, 11-16%, and 0.5-3%, respectively (Aschenbach et al., 2010). Mobilization of body fat reserves during periods of severe negative energy balance, including the periparturient period in dairy cows, increase the availability of fatty acids for metabolism in liver. Triglyceride degradation in liver supplies glycerol for *de novo* gluconeogenesis. Approximately 15-20% of glucose requirements during these periods can be met through mobilization of adipose tissue reserves (Bell, 1995).

Fatty Acid Synthesis

Synthesis of fatty acids, *de novo*, requires a carbon source to serve as the glycerol backbone. Cytosolic citrate lyase (EC 4.1.3.8) catalyzes the cleavage of citrate, in the presence of Coenzyme A and ATP, to OAA and acetyl-CoA. Fatty acid biosynthesis utilizes this citrate cleavage as the initial step in fatty acid generation from acetyl-CoA and malonyl-CoA precursors (Ballard and Hanson, 1967). Citrate formation from OAA is dependent upon the pool of OAA generated by PC. For utilization in other biochemical reactions, regenerated cytosolic OAA from the citrate lyase reaction would first need to be converted to malate by malate dehydrogenase (EC 1.1.1.40), and then to pyruvate to permit translocation into the mitochondria.

In contrast, adipose tissue is the primary site of fatty acid synthesis in ruminants (Ingle et al., 1972), where acetate utilization, compared to glucose substrate utilization, is increased ten times over that in liver, adipose, and mammary gland of ruminants, compared to nonruminants (Hanson and Ballard, 1967). Dramatically decreased abundance of citrate lyase in ruminant liver prevents the citrate cleavage reaction to OAA and acetyl-CoA (Hanson and Ballard, 1967; Ingle et al., 1972). Ruminant fatty acid biosynthesis is supported, in large part, by acetyl-CoA synthase (EC 6.2.1.1), which catalyzes the formation of acetate to acetyl-CoA (Hanson and Ballard, 1967). A discrepancy currently exists over the activity of citrate lyase in dairy cows, particularly during the peripartum period, with some findings to indicate that citrate lyase activity increases postpartum in control cows, but remains lower in dairy cows with fatty liver (Murondoti et al., 2004), with a similar study finding increased citrate lyase levels at 4 wk postpartum, compared to 3 wk prepartum, but a decreased level at 13 wk postpartum (Graber et al., 2010). In opposition to these findings, no differences were seen in citrate lyase in dairy cows with high or low BHBA levels during the peripartum period (van Dorland et al., 2009) or during feed restriction postpartum (Gross et al., 2013). Acetyl-CoA has also been shown to inhibit citrate lyase, further depressing enzyme activity (Chesworth and Smith, 1971).

REGULATION OF PYRUVATE CARBOXYLASE

Pyruvate Carboxylase Gene Promoters

Short-term regulation of PC is largely influenced by availability of the allosteric activator, acetyl-CoA, as discussed above. Increases in acetyl-CoA supply from the metabolism of mobilized fatty acids can enhance OAA production by PC and the cataplerotic supply of carbons to gluconeogenesis. Long-term regulation of PC occurs through transcriptional regulation of PC gene promoters by both nutritional and physiological cues. Regulation of PC mRNA expression in mammals is complex. Studies in rat and mouse models (Jitrapakdee et al., 1996; Jitrapakdee et al., 1997; Jitrapakdee et al., 2001), and on human PC regulation (Carbone et al., 1998; Thonpho et al., 2013) have revealed regulation by multiple promoters. Two alternative promoters, the proximal (P1) and distal (P2), have been identified in rat (Jitrapakdee et al., 1996). Through alternative splicing, these promoters yield five different mRNA variants, differing only in their 5' untranslated regions (UTR), produced from different 5' UTR exons. Illustrating an added level of genomic complexity, bovine PC gene contains three unique promoter regions (P3, P2, P1 from 5' to 3') which yield six unique 5' UTR variants (Hazelton et al., 2008b). Similar to human and rat, tissue-specific PC mRNA expression is seen in bovine where 5' UTR A, B, C, and F (arising from promoter 1) are specific to glucogenic and lipogenic tissues and 5' UTR D and E (arising from promoter 3 and promoter 2, respectively) are expressed in several different tissues (Agca et al., 2004; Hazelton et al., 2008b). In rat, 5'UTR products of P1 are expressed in gluconeogenic and lipogenic tissues, while 5'UTR products of P2 are more ubiquitous (Jitrapakdee et al., 1996;

Jitrapakdee et al., 1997). Expression of tissue-specific variants is likely due to the presence of tissue-specific transcription factors that may interact with specific response elements of the unique promoters (Jitrapakdee et al., 1999).

Different tissue-specific PC promoters also exhibit different translational efficiencies. In rats, transcripts from 5' UTR C or E exhibit similar translational efficiencies, which are both higher than translational efficiency from 5' UTR D (Jitrapakdee et al., 1998). Transcript D was shown to potentially develop a secondary structure blocking access for translation (Jitrapakdee et al., 1998). Similarly, in bovine, translation efficiencies of PC mRNA variants A, B, D, and F did not differ (Hazelton et al., 2008a). Translation efficiency from bovine PC 5' UTR D was greater than synthesis from PC 5' UTR C or E for bovine PC (Hazelton et al., 2008a). The pattern of PC 5' UTR variants expressed may therefore influence the total rate of PC enzyme synthesis. Response of species 5' UTR variants to physiological cues is then of specific interest to determine the complete pattern of transcriptional regulation of PC. The products of bovine PC promoter 1, UTR variants A, B, C, and F, exhibit increased expression of 4-fold in samples from feed restricted cows and 6-fold during parturition (White et al., 2011a).

Nutritional Control of Pyruvate Carboxylase

The activity of hepatic PC in rats after a period of starvation dramatically increases, up to three-fold, compared to activity in control rats (Wimhurst and Manchester, 1970). This parallel between increased PC activity and decreased caloric intake is seen in several other species, including bovine (Ballard et al., 1968), guinea pig

(Söling et al., 1970), and sheep (Taylor et al., 1971; Lemons et al., 1986). Increased expression of PC mRNA during feed restriction has been reported in mid-lactation dairy cows (Velez and Donkin, 2005). This increase in PC was concurrent with an increase, of nearly four times control levels, of lactate converted to glucose (Velez and Donkin, 2005). During this change in the capacity for gluconeogenesis from lactate, plasma NEFA levels were simultaneously increased in the feed restriction group, compared to control (Velez and Donkin, 2005). Potential regulation of PC gene expression by fatty acids is discussed in the following section.

Increased plasma NEFA levels during a period of caloric deprivation result from the mobilization of adipose tissue reserves. This homeorhetic response to the increased nutritional demands due to the onset of lactation and demands of the gravid fetus is heightened during the transition period in dairy cows, the time from 3 wk prior to parturition until 3 wk after parturition. During this period of dramatic increase in energy requirement, PC mRNA levels also increase. Hepatic PC mRNA levels are elevated at calving, and 7 d postpartum (Greenfield et al., 2000). Other gluconeogenic enzymes, including PCK, do not show this same pattern. Increases in PCK mRNA expression do not occur until 28 d postpartum (Greenfield et al., 2000; Agca et al 2002).

Dietary fat has been shown to alter whole body metabolism in both rats and mice (Kraegen et al., 1991; Bizeau et al., 2001; Hagopian et al., 2004). A high saturated fat diet promotes insulin resistance and obesity with concurrent increases in serum triglyceride levels in rodents (Kraegen et al., 1991). A study in rats comparing the effects of high fat, sucrose, or starch in the diet found that increased gluconeogenesis observed in the high fat and sucrose diets was due to increased flux through PC (Bizeau et al., 2001).

In dairy cows, diets fed prepartum have been shown to influence the concentration and profile of circulating fatty acid postpartum (Rukkwamsuk et al., 2000). Taken together with the concurrent increase in PC postpartum (Greenfield et al., 2000), the role for potential influence of circulating fatty acids on PC flux becomes of particular interest.

Transcriptional regulation of Pyruvate Carboxylase by long-chain fatty acids

Fatty acids have been shown to alter signal transduction cascades, regulate activity of G-protein-linked membrane receptors, and, along with their acyl-CoA derivatives, can bind to several transcription factors, including peroxisome proliferator-activated receptors (PPAR), sterol regulatory element binding protein (SREBP), carbohydrate-responsive element-binding protein (ChREBP), liver X receptor (LXR), farnesoid X receptor (FXR), toll-like receptor 4 (TLR4), hepatic nuclear factor-4 α (HNF-4 α), and nuclear factor (NF) κ B, to regulate gene transcription. Circulating long chain nonesterified fatty acids (NEFA) are transported through blood bound to albumin. While blood fatty acids also include esterified fatty acids in triglycerides, lipoproteins, and chylomicrons, these fatty acids, arising from dietary sources, are largely unavailable to liver as adult liver lacks lipoprotein lipase required to free esterified fatty acids (Semenkovich et al., 1989; Goldberg et al., 2009). Furthermore, mature ruminant liver contains essentially no lipoprotein lipase or hepatic triglyceride lipase (Cordle et al., 1983; Liesman et al., 1984) to allow for lipoprotein utilization. This review will therefore focus solely on NEFA uptake and utilization.

For cellular uptake, over 90% of cellular long chain fatty acid movement into cells is through protein-mediated uptake (Kazantzis and Stahl, 2012). Specifically,

plasma membrane fatty acid-binding protein (FABP), fatty acid translocase (CD36), and fatty acid transport proteins (FATP), are responsible for the majority of cellular fatty acid uptake. In addition to their role in cellular fatty acid uptake, it remains unclear if FATP play a direct role fatty acyl-CoA synthesis or transport within the cell (Jump et al., 2005; Kazantzis and Stahl, 2012). Inside the cell, fatty acids can serve as signaling molecules through their influence on cellular receptor systems, including steroid hormones, retinoic acid, and other hydrophobic molecules (Chawla et al., 2001). Research in several species has illustrated the role of fatty acids in regulation of PC (Jitrapakdee et al., 2008). Research on the direct action of fatty acids to control gene expression, especially in bovine has, thus far, been limited. Responsiveness of other gluconeogenic genes to fatty acids has been studied in several species, and may provide insight to regulation of PC by fatty acids and associated intracellular systems in bovine.

Regulation by PPAR

Peroxisome proliferator-activated receptors are nuclear hormone receptors which function as ligand-dependent transcription receptors, with ligands including small lipophilic molecules such as fatty acids, peroxisome proliferators, antidiabetic, and hypolipidemic drugs (Schoonjans et al., 1996). Ligand binding to PPAR elicits heterodimerization of PPAR with retinoid X receptor (RXR) and subsequent binding to response elements and recruitment of coactivator proteins. The predominant form in liver is PPAR α , which had been implicated in a central role of lipid metabolism and fatty acid oxidation (Lee et al., 1995). The PPAR α subtype is also expressed in brown adipose tissue, as well as cardiac and skeletal muscle (Georgiadi and Kersten, 2010). Subtype

PPAR γ is highly expressed in adipocytes and macrophages where it promotes differentiation of preadipocytes and lipid storage, while subtype PPAR δ exhibits a more ubiquitous expression (Sugden et al., 2010).

Long chain fatty acids, and in particular polyunsaturated fatty acids (PUFA), preferentially bind to all PPAR subtypes (Krey et al., 1997). The role for PPAR α in mediating hepatic gene expression through unsaturated fatty acids was confirmed from gene expression profiles in PPAR α null mice provided with synthetic dietary triglycerides comprised of only one fatty acid (Sanderson et al., 2008). Additionally, PPAR α null mice in the fed state exhibit reduced lactate production and gluconeogenesis, while fasted PPAR α null mice are unable to switch to fatty acid oxidation (Xu et al., 2002). Although PC mRNA expression or enzyme activity was not measured in the PPAR α null mice, the role for PPAR α in gluconeogenesis from lactate is clear and may directly relate to PC activity and potential regulation by fatty acids.

Interestingly, differences are found between the ability of circulating fatty acids, arising from mobilization of adipose tissue, compared to fatty acids of dietary origin, to activate PPAR α in liver (Chakravarthy et al., 2005; Sanderson et al., 2009). This inability of circulating fatty acids to activate PPAR α may be due to differences in intracellular pools of fatty acids as only fatty acids of dietary origin acted through PPAR α to influence glucose or fatty acid metabolism in liver-specific fatty acid synthase knockout mice (Chakravarthy et al., 2005). Differences are also seen in PPAR α activation in response to physiological cues during fasting compared to response to pharmacological agonists where PPAR δ is responsive to circulating fatty acids, but the same is not true for PPAR α in mouse liver (Sanderson et al., 2009). This difference in response to fatty acids of

dietary or adipose origin may be of particular interest in the periparturient dairy cow when feed intake electively decreases and adipose tissue mobilization increases. However, implications from these studies must carefully be considered because of the lack of lipoprotein lipase or hepatic triglyceride lipase in ruminant liver (Cordle et al., 1983; Liesman et al., 1984) which limits utilization, by liver, of fatty acids from chylomicrons of dietary origin. Studies evaluating the response of different PPAR isoforms to fatty acids of either dietary or adipose origin in ruminants are lacking, but lipoprotein lipase is shown to be increased by PPAR α ligands in human macrophages (Li et al., 2002).

Direct regulation of genes involved in fatty acid metabolism through PPAR α has been examined in both rodent and bovine. Research in rodent models had indicated that the proximal PC promoter is responsive to PPAR α activation (Jitrapakdee et al., 2008). Increased PC expression is seen in isolations of pancreatic islets from rats after treatment with either long chain fatty acids or synthetic PPAR α agonist, fibrates, concurrent with an increase in PPAR α expression (Yoshikawa et al., 2001). In bovine, responsiveness of carnitine palmitoyltransferase (CPT) I activity is seen in Madin-Darby bovine kidney (MDBK) cells after exposure to the PPAR α agonist Wy-14643 (Bionaz et al., 2008a), while direct activation of PPAR α by long chain fatty acid is seen in the same cell model (Thering et al., 2009). Specific regulation of bovine PC promoter 1 is seen with activation by PPAR α and a decrease in promoter activity after exposure to saturated fatty acids (White et al., 2011c). Finally, both PPAR α and PPAR δ have been shown to increase lipid oxidation in heart, intestine, kidney, and liver (Sugden et al., 2010; Jump et al., 2013). In neonatal bovine hepatocyte isolations, exposure to a PPAR α agonist increased oxidation

of C16:0 to CO₂, compared to control (Litherland et al., 2010). The importance of fatty acid oxidation in relation to the role of PC is discussed below. Together, these data lend support to the hypothesis that fatty acid influence on PC mRNA expression is mediated through PPAR α .

Regulation by SREBP

Sterol regulatory element binding proteins are members of the basic helix-loop-helix leucine zipper family of transcription factors and exhibit three isoforms, SREBP-1a, SREBP-1c and SREBP-2, from two separate genes. The isoforms are expressed in a tissue-specific manner and elicit their effects on different target genes with SREBP-1c activating genes for *de novo* lipogenesis and SREBP-2 activating genes involved in the synthesis and uptake of cholesterol (Georgiadi and Kersten, 2010). Synthesized as membrane-bound precursors in the endoplasmic reticulum, activation of SREBP requires cleavage by cholesterol-sensing proteins, known as SREBP cleavage activating proteins (SCAP). Cleavage and release of SREBP allows for nuclear translocation and binding to response elements (Hua et al., 1995). Serving, in effect, as cholesterol-sensing proteins, SCAP promote cleavage and SREBP nuclear translocation when cholesterol levels are low. Increased cellular cholesterol concentrations leave the SREBP-SCAP complex sequestered by endoplasmic reticulum (ER) membrane proteins (Hua et al., 1995).

All SREBP isoforms are inhibited by PUFA, as these fatty acids act to both decrease SREBP mRNA levels, and prevent the cleavage and processing required for SREBP maturation (Xu et al., 1999). Inhibition of SREBP through PUFA has recently been attributed to the inhibition of Ubx8, a membrane-bound protein responsible for

degrading the proteins anchoring the SREBP-SCAP complex to the ER (Lee et al., 2010). Inhibition of Ubx8 and the related anchoring protein leaves the SREBP-SCAP inactivated and stranded in the ER.

The predominant isoform in liver, SREBP-1c, plays a direct role in the regulation of lipogenesis and is responsible for the activation of fatty acid and triglyceride synthesis-related gene transcription (Shimano, 2009). Expression of SREBP-1c is increased during periods of elevated glucose concentrations and during over-nutrition, and is upregulated by insulin and agonists of LXR. Long-term elevation of SREBP-1c can induce insulin resistance and fatty liver (Horton et al., 1998; Nakamura et al., 2004). In opposition, SREBP-1c is downregulated by PUFA (Shimano, 2009). Recent research has established a relationship between elevated C18:0 levels in Type II diabetes patients with hyperlipidemia from increased fatty acid synthesis due to signaling through SREBP-1c (Chu et al., 2013). Any potential relationship between SREBP signaling and PC expression has not been examined in ruminants or nonruminants.

Regulation by LXR and Relationship to SREBP

Liver X receptors belong to the nuclear receptor superfamily, are responsive to sterols, and are relevant to several metabolic systems, including growth and development, reproduction, overall metabolism, and, specifically, Type II diabetes (Ding et al., 2014). The LXR are activated by oxysterols, derivatives of cholesterol, and activate genes for cholesterol and bile excretion (Nakamura et al., 2014). Liver X receptor null mice exhibit aberrant cholesterol accumulation and liver metabolism (Peet et al., 1998). Effects of LXR on other metabolic aspects occur through interactions with either SREBP-1c or

PPAR α . Two functional LXR response elements are found in the promoter region of the SREBP-1c gene (Repa et al., 2000). Through activation of SREBP-1c, LXR also induces genes for *de novo* lipogenesis (Nakamura et al., 2014). Evaluation of cross-talk between PPAR α and LXR demonstrated that overexpression of PPAR α in human embryonic kidney cells inhibited LXR-induced SREBP-1c activity (Yoshikawa et al., 2003).

Confirmed through gel shift assays and incubation with PPAR α agonists, the activation of PPAR α is likely competitively reducing formation of a LXR-RXR heterodimer to reduce signaling through the LXR-SREBP-1c pathway (Yoshikawa et al., 2003).

Regulation by TLR4

Toll-like receptors are highly conserved proteins intricately involved in the innate immune response which recognize pathogen-associated molecular patterns and mediate cytokine responses (Galli and Calder, 2009). Effects of fatty acids on inflammatory response have been extensively studied and data largely support a proinflammatory role of saturated fatty acids and an anti-inflammatory role of n-3 PUFA (Galli and Calder, 2009). While much of the influence from fatty acid metabolism is likely from metabolites, including prostaglandins, leukotrienes, and diacylglycerols, a direct role also exists for fatty acids in both pro- and anti-inflammatory pathways. The TLR4 subtype is of particular interest because of the activation of the receptor by both lipopolysaccharides and fatty acids. In both adipocytes and monocytes, saturated C16:0 and C18:0 serve as direct ligands to stimulate TLR4 signaling and elicit downstream NF κ B activation and proinflammatory cytokine release (Kopp et al., 2009; Schaeffler et al., 2009).

Taken together, previous research strongly suggests a role of both unsaturated and saturated fatty acids, through their influence on PPAR α and SREBP-1c, respectively, in regulating hepatic gene expression. Presently, the role of these lipid classes as regulators of hepatic gene expression in bovine, and transition dairy cows in particular, is unknown. Data for PC regulation in nonruminants and emerging data for ruminants suggests dominant regulation by unsaturated fatty acid on potential PPAR α response elements of PC. A role of LXR as a cotranscription factor is also plausible when considering the established role of cross-talk between PPAR α , LXR, and SREBP-1c. Research is lacking that links these pathways to regulation of metabolically controlled genes, such as PC, in cattle.

FATTY ACID METABOLISM AND β -OXIDATION

Long-chain fatty acids are mobilized from adipose tissue during increased energy requirements, which exceed energy supply, in the form of NEFA that bind to serum albumin and are transported in blood (Dole, 1956; Gordon and Cherkas, 1956). In dairy cows, circulating NEFA taken up by liver in proportion to their concentration in blood (Emery et al., 1992) and are subject to one of several metabolic fates, including complete oxidation to CO₂ or partial oxidation to yield ketone bodies (reviewed in full below). Because PC is allosterically regulated by acetyl-CoA, and because the anaplerotic potential of PC depends on acetyl-CoA condensing with OAA to enter the TCA cycle, supply of acetyl-CoA from fatty acid β -oxidation is of particular interest.

Fatty acid β -oxidation pathway

The catabolic breakdown of fatty acids in mitochondria yields acetyl-CoA, which can enter the TCA cycle, and NADH and FADH₂, which are used in the electron transport chain (Berg et al., 2002). In healthy individuals, decreases in circulating glucose and insulin levels during fasting permit the activation of adipose tissue hormone-sensitive lipase (HSL) by opposing endocrine signals to increase NEFA mobilization (Bennett, 2010). Activation of HSL in adipose tissue occurs through the protein kinase A cascade promoting HSL to translocate and hydrolyze triglycerides from lipid droplets. In contrast to nonruminants, adipose triglyceride lipase is decreased during the periparturient period in dairy cows (Koltes and Spurlock, 2011). Fatty acids are oxidized in mitochondria but must first be activated, at the outer mitochondrial membrane, by acyl-CoA synthetase / fatty acid thiokinase to long chain fatty acyl-CoA. Activated long chain fatty acyl-CoA are then conjugated to carnitine for transport across the mitochondrial membrane, facilitated by the outer-membrane-bound CPT I (Berg et al., 2002). Four enzyme-catalyzed reactions are then required for fatty acid β -oxidation: dehydrogenation by acyl-CoA dehydrogenase, hydration by enoyl-CoA hydratase, dehydrogenation by 3-L-hydroxyacyl-CoA dehydrogenase, and cleavage by thiolase. The resulting fatty acyl-CoA is shortened by two carbons while acetyl-CoA, NADH and FADH₂ are generated (Berg et al., 2002). Resulting acetyl-CoA can then continue to complete oxidation through the TCA cycle (pathway discussed above) only if fat and carbohydrate metabolism are balanced. If OAA is unavailable for condensation with acetyl-CoA, acetyl-CoA is then diverted to ketone body formation (Berg et al., 2002).

Figure 1.2. Fatty acid β -oxidation; substrates, products, and associated enzymes.

Activated fatty acyl-CoA are conjugated to carnitine for transport across the mitochondrial membrane, facilitated by carnitine palmitoyltransferase (CPT) I, prior to β -oxidation. Acetyl-CoA resulting from the final cleavage step can be oxidized through the tricarboxylic acid (TCA) cycle to yield ATP and CO₂.

Role of fatty acid β -oxidation in liver and kidney tissue

Complete oxidation of fatty acids to CO_2 necessitates a balance between mitochondrial β -oxidation and peroxisomal β -oxidation. Peroxisomal β -oxidation is utilized for breakdown of fatty acids longer than 20 carbons before further oxidation in the mitochondria proceeds (Osmundsen et al., 1991). While mitochondrial β -oxidation is, in large part, dependent upon CPT I activity (McGarry and Brown, 1997), limitations in CPT I activity have not been linked to propensity for metabolic disorders in periparturient dairy cows (Dann and Drackley, 2005).

Kidney and liver are the only tissues in which both mitochondrial β -oxidation and peroxisomal β -oxidation of fatty acids can occur (Osmundsen et al., 1991; Zaar, 1992). Complete oxidation of long chain fatty acids, specifically C16:0, to CO_2 by extrahepatic tissues was first demonstrated in vitro in murine kidney slices (Weinhouse et al., 1950). Oxidation of C16:0 to CO_2 in kidney slices or homogenates was equal to or greater than oxidation of C16:0 to CO_2 in liver slices or homogenates (Weinhouse et al., 1950). Early liver explant incubations in bovine illustrated that oxidation of C16:0 is stimulated by carnitine and inhibited by propionate (Jesse et al., 1986). In liver of dairy cows, peroxisomal β -oxidation contributes 50% of first cycle total C16:0 β -oxidation (Grum et al., 1994). Importance of the capacity for oxidation in dairy cows is emphasized around parturition and was previously shown to increase in the oxidation of C16:0 in liver tissue slices (Grum et al., 1996). In a rodent model, regulation of fatty acid oxidation is demonstrated after dietary fat containing high amounts of C18:3n-3 *cis* increase both mitochondrial and peroxisomal fatty acid β -oxidation in rat liver (Ide et al., 1996). Experiments performed in bovine hepatocytes examined the influence of fatty acids after

48 h exposure and found a decrease in fatty acid esterification to triglycerides when C18:3n-3 *cis* was included in treatment media (Mashek and Grummer, 2003). An increase in gluconeogenesis is also seen after exposure to C18:3n-3 *cis* in hepatocytes (Mashek and Grummer, 2003). The negative effects of overfeeding during the dry period on enzymes related to NEFA oxidation in liver have also been established (Murondoti et al., 2004), while lower rates of C16:0 oxidation to CO₂ are observed after overfeeding during the far-off dry period with a concurrent increase in the esterification of triglycerides (Litherland et al., 2011). These studies effectively established a method for analysis of fatty acid substrate metabolism, and illustrated influences of diet and, specifically, fatty acids, but did not measure PC mRNA expression or enzyme activity, and the exact role of PC remains uncertain.

Fatty acid β -oxidation also occurs in proximal kidney tubules of the renal cortex and is increased during diabetes (Guder et al., 1986). Potentially strengthening the link between fatty acid β -oxidation and PC activity, fatty acids that are increased in circulating concentrations during diabetes are found to induce expression of several fatty acid β -oxidation enzymes and promote the utilization of fatty acids (Ouali et al., 1998). Furthermore, all PPAR isoforms are detected in kidney cortex (Wang, 2010). As bovine PC promoter 1 activated by PPAR α agonist (White et al., 2011c), the relevance of examining kidney metabolism in the current research holds true.

ROLE OF PYRUVATE CARBOXYLASE IN ABERRANT METABOLISM

Evolutionary conservation and biological importance

The PC gene has been highly conserved through evolution and is found in both bacteria and eukaryotic cells. Well characterized in *Saccharomyces cerevisiae*, this yeast contains two functional PC gene copies; *PYC1* and *PYC2*, with both isozymes being located in the cytoplasm (Walker et al., 1991). This is in contrast to cellular localization of PC in the mitochondrial matrix, near the mitochondrial inner membrane, in higher eukaryotes (Rhode et al., 1991). A single PC gene is found in rats, humans, and cows with high similarity; containing 19 coding exon regions and 18 introns (Jitrapakdee and Wallace, 1999; Hazelton et al., 2008b). Mammalian control of PC expression is intricately regulated by distinct promoters, discussed in full detail in previous sections. The pivotal role of PC in several physiological states will be the focus of the following section. The biological importance of the enzymes is perhaps most easily illustrated by a brief review of studies of compromised PC function.

Generation of PC-deficient mutants in *S. cerevisiae* result in altered phenotypes, while defects in either the *PC1* or *PC2* genes led to growth failure in yeast on glucose media (Walker et al., 1991; Brewster et al., 1994). Deficiency of PC in humans is an autosomal, recessive trait, which manifests as one of three, nearly distinct, phenotypes (Gray et al., 2014). Type A PC deficiency, found in North American populations, is characterized by decreased, though not abolished, PC activity, episodic lactic acidosis, elevated alanine and proline levels, hypoglycemia, and developmental delays (Robinson, 2006). Decreases in both gluconeogenesis and activity of the TCA cycle are responsible

for decreased OAA and glucose, and increased alanine, lactate, and pyruvate in these PC-deficient individuals (Jitrapakdee and Wallace, 1999). Life expectancy of Type A-deficiency patients is usually several years (Gray et al., 2014). In contrast, Type B PC deficiency, historically reported in French populations, is characterized by near absence of PC protein or enzyme activity. Severe lactic acidosis and neurological deficits occur, and lifespan in these patients is usually only several months (Robinson, 2006). Type C PC deficiency is, in comparison, a very mild form of the disease, lacking neurological complications and exhibiting limited and infrequent lactic acidosis (Gray et al., 2014). Point mutations leading to altered protein stability or lower enzyme activity have been identified in patients with Type A PC deficiency, and PC activity among these patients is around 50% (Wexler et al., 1998). The genotype for Type B deficiency has not yet been identified. The severe implications of PC deficiency emphasize the central role for the enzyme in numerous metabolic pathways.

Obesity and Type II Diabetes Mellitus

Type II diabetes mellitus, or noninsulin-dependent diabetes mellitus, is a metabolic disorder arising from both genetic and lifestyle factors characterized by hyperglycemia, insulin resistance, and insulin deficiency. Increases in all gluconeogenic genes; PC, PCK, fructose-1,6-bisphosphatase, and glucose-6-phosphatase have been reported during diabetes mellitus (Filsell et al., 1969; Wimbhurst and Manchester, 1970; Weinberg and Utter, 1980). Insulin has been previously shown to decrease this elevated PC expression, by two-fold, in diabetic rats (Weinberg and Utter, 1980). Genetically obese rats also exhibit dramatic increases in PC expression, which has been specifically

attributed to a response by the distal PC promoter in pancreatic β -cells (Jitrapakdee et al., 1998). Increased gluconeogenesis in diabetes was later specifically attributed to an increase in flux through PC in its cataplerotic role in both mice and rats (Salto et al., 1996).

In a genetically inherited Type II diabetic rat model, elevated glucose levels due to insulin resistance results in a “glucotoxicity loop” and concomitant decrease in PC activity (MacDonald et al., 1996a,b; Poitout et al., 2002). The ensuing “lipotoxicity loop” only serves to increase insulin resistance, decrease PC activity, and decrease pancreatic β -cell function (Poitout et al., 2002). Finally, identification of a single nucleotide polymorphism in the PC gene has been identified and linked to the acute insulin response phenotype, an independent predictor of Type II diabetes mellitus (Palmer et al., 2006).

Ketosis and Fatty liver

Hepatic steatosis, or the accumulation of excessive lipid in liver, has been linked to the development of nonalcoholic fatty liver disease (Malhi and Gores, 2008).

Accumulation of these liver lipids occurs from an imbalance between the rates of synthesis and disposal. How liver responds to the excess lipid load during the increased influx determines potential susceptibility to development of hepatic steatosis.

The 6 wk period surrounding parturition in dairy cows is aptly referred to as the transition period. As this moniker implies, drastic metabolic changes are occurring as a homeorhetic response to increased nutrient needs of the fetus, and the onset of lactogenesis. During this period of extreme negative energy balance, adipose tissue reserves are mobilized, releasing NEFA into the bloodstream which are transported to

liver for mitochondrial metabolism (Baird et al., 1968; LeBlanc, 2010). Concurrent with increased circulating NEFA from adipose tissue triglyceride lipolysis, the rate of blood flow increases during the periparturient period in dairy cows (Lomax and Baird, 1983). Circulating NEFA in dairy cows are taken up by liver proportional to their concentration in blood (Emery et al., 1992) and are subject to one of four possible fates: complete oxidation through the tricarboxylic acid (TCA) cycle yielding ATP and CO₂, partial oxidation yielding ketone bodies, esterification for storage in liver as triglycerides, or transport from the liver as very low density lipoprotein (VLDL) for use by extrahepatic tissues (reviewed by Grummer, 1993; Drackley et al., 2001; Bobe et al., 2004; van Knegsel et al., 2005). With the cost of hepatic steatosis to the United States dairy industry estimated at US\$60 million annually and the prevalence of hepatic steatosis in early lactation dairy cows at over 50% (Bobe et al., 2004), potential strategies to mitigate the severity of the disease and the cost to dairy producers are necessary.

Dairy cows exhibit an inherently low capacity for export of VLDL from liver, and display an upper limit of extrahepatic tissues to utilize ketone bodies (Kleppe et al., 1988; Armentano et al., 1991; Emmison et al., 1991). This is, in part, due to the fact that ruminants express limited concentrations of the proteins necessary for VLDL packaging, including apolipoprotein B and apolipoprotein A-I (Grummer, 1993, Bobe et al., 2004). This puts a dairy cow at an even greater risk for developing ketosis or fatty liver. Decreases in feed intake during the transition period may result in decreased carbon supply from propionate and decreased availability of OAA to serve as a substrate (van Knegsel et al., 2005). Increased production of acetyl-CoA, resulting from fatty acid β -oxidation, during a period of increased energy demand could surpass the capacity of the

TCA cycle for complete oxidation. If OAA and acetyl-CoA are not present in an equal ratio to serve as TCA cycle substrates, other metabolic fates of acetyl-CoA will predominate. Increased rates of ketogenesis to acetate and acetoacetate from acetyl-CoA, along with increased esterification to storage triglycerides (Grummer 1993, Drackley et al., 2001, van Knegsel et al., 2005) would simply exacerbate the delicate metabolic situation of the transition cow. While the decrease in feed intake is usually greater in obese cows compared to healthy individuals (Stockdale, 2001), NEFA mobilization in obese dairy cows is also more severe (McNamara, 2000). Because liver lipids can accumulate very rapidly and fatty liver can develop in as little as 96 h in dairy cows (Grummer et al., 1990), the risk to development of metabolic disease is clear. Additionally, postpartum concentration of C16:0, C18:0, and C18:1n-9 *cis* were significantly increased in lactating dairy cows with fatty liver, compared to levels in control cows (Rukkwamsuk et al., 2000), indicating a potential detrimental effect of these particular fatty acids in whole body metabolism.

Recent research has extensively examined the response of PC promoter to fatty acids and PPAR α agonist (White et al., 2011b,c), but it remains unclear the dominance of control of specific fatty acids on PC mRNA expression or promoter activity, and if changes in PC will indeed correlate to changes in oxidative pathway flux or the capacity of the TCA cycle.

CONCLUSIONS AND FUTURE DIRECTIONS

The central role that PC plays in several biosynthetic pathways is clear. Pyruvate carboxylase is responsible for the conversion of pyruvate to OAA, with the cellular fate of OAA determined by the energy status of the cell. Pyruvate carboxylase expression is tissue-specific due to regulation by, in bovine, three separate promoters that give rise to six unique 5' UTR variants. The response of PC to physiological stressors, including and elevated circulating fatty acid profile, is of utmost importance to dairy cows during the period of negative energy balance surrounding the transition period.

The guiding hypothesis for this dissertation is that the circulating fatty acid profile, and specifically the ratio of saturated to unsaturated fatty acids, influences the capacity for cellular oxidation and the production of OAA, through specific influences on PC and PC promoter 1. These data are needed to provide valuable insight into the control of PC and fatty acid metabolism. If the intricate balance present between the anaplerotic and cataplerotic roles of PC and OAA may be manipulated through plasma fatty acid profiles to either prevent, or better respond to, metabolic stressors inherent to the periparturient period in dairy cows.

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**CHAPTER 2. FATTY ACID CHAIN LENGTH AND DEGREE OF
SATURATION REGULATE EXPRESSION OF PYRUVATE
CARBOXYLASE MESSENGER RNA IN MADIN-DARBY BOVINE KIDNEY
CELLS AND BOVINE PRIMARY HEPATOCYTES**

ABSTRACT

Pyruvate carboxylase (PC) catalyzes oxaloacetate (OAA) synthesis and links gluconeogenesis and the anaplerotic generation of OAA. Previous work links bovine PC mRNA expression to nonesterified fatty acids (NEFA). Our objective was to determine effects of chain length and degree of saturation through copresence of saturated and unsaturated fatty acids on PC mRNA. For Experiment 1, Madin-Darby bovine kidney (MDBK) cells were grown to 80% confluence and exposed for 24 h to either individual fatty acids bound to BSA (C16:0, C18:0, C18:1n-9 *cis* or C18:3n-3 *cis*) or their combinations at increasing molar ratios for C16:0: C18:3n-3 *cis* or C18:0: C18:3n-3 *cis* or C18:1n-9 *cis*: C18:3n-3 *cis*. For Experiment 2, 20 h after seeding, isolated bovine hepatocytes were similarly exposed for 24 h to individual fatty acids (C16:0, C18:0, or C18:3n-3 *cis*) or their combinations at increasing molar ratios for C16:0: C18:3n-3 *cis* or C18:0: C18:3n-3 *cis*. Total fatty acid concentration was 1.00 mM for all incubations. Both C16:0 and C18:0 independently decreased ($P < 0.05$) PC mRNA relative to control (0.48, 0.16, and 2.33 ± 0.48 for C16:0, C18:0, and control, respectively) in MDBK cells. The ratio of 75:25 C18:0: C18:3n-3 *cis* increased PC expression compared to C18:0

treatment alone (1.97 vs. 0.16 ± 0.34 , for 75:25 C18:0: C18:3n-3 *cis*, and C18:0, respectively; $P < 0.05$) in MDBK cells. The ratio of 75:25 C16:0: C18:3n-3 *cis* significantly increased PC mRNA expression compared with C16:0 alone, but only 50:50 C16:0: C18:3n-3 *cis* could recover PC expression to levels similar to control (1.41 , 0.48 , 2.08 , and 2.33 ± 0.48 for 75:25 C16:0: C18:3n-3 *cis*, C16:0, 50:50 C16:0: C18:3n-3 *cis*, and control, respectively; $P < 0.05$) in MDBK cells. For primary bovine hepatocyte, exposure to C18:3n-3 *cis* significantly elevated ($P < 0.05$) PC mRNA expression relative to control, C16:0 or C18:0. There was no effect of C16:0, C18:0 or combinations of C16:0 or C18:0 with C18:3n-3 *cis*. Results indicate that C18:3n-3 *cis* is a more potent alleviator of PC depression in C18:0 treated MDBK cells compared to C16:0: C18:3n-3 *cis* treatments. C18:1n-9 *cis* treatments did not depress PC ($P > 0.10$) in MDBK cells, revealing fatty acid saturation is key in PC expression. PC activation by unsaturated fatty acids may play a critical role in setting the capacity for OAA synthesis.

Keywords: bovine, fatty acid, pyruvate carboxylase

INTRODUCTION

Accumulation of liver lipids, known as hepatic steatosis, is a predisposing condition to nonalcoholic fatty liver disease and occurs from an imbalance in lipid synthesis and disposal (Malhi and Gores, 2008). The capacity of liver to metabolize fatty acids released from adipose tissue determines, at least in part, susceptibility to hepatic steatosis. Likewise, both the type and amount of dietary fatty acids have been shown to influence whole-body metabolism, as well as hepatic gene expression (Ooi et al., 2013). In rodents, diets high in saturated fat increase serum triglyceride levels and promote the development of obesity and insulin resistance (Kraegen et al., 1991). Previous studies show that regulation of gene expression by circulating fatty acids occurs either through indirect mechanisms to alter cellular metabolism or directly by acting to regulate transcription factor activity (Jump et al., 2013). Therefore, circulating fatty acids may influence susceptibility to metabolic diseases by altering responsiveness to endocrine and metabolic signals.

During the transition to lactation, the 6 wk period surrounding parturition, a dairy cow experiences a period of negative energy balance when adipose tissues are mobilized and fatty acids are released into blood and transported to liver for metabolism (LeBlanc, 2010). In dairy cows, circulating fatty acids are taken up by liver in proportion to their concentration in blood (Emery et al., 1992). In liver, fatty acids are metabolized and have one of four possible fates: complete oxidation through the tricarboxylic acid (TCA) cycle yielding ATP and CO₂, incomplete oxidation yielding ketone bodies, transport from the liver as very low density lipoprotein (VLDL), or esterification for storage in liver as

triglycerides (reviewed by Grummer, 1993; Drackley et al., 2001; Bobe et al., 2004; van Knegsel et al., 2005). Because the prevalence of hepatic steatosis is over 50% in dairy cows with a cost to the United States dairy industry of approximately US\$60 million annually (Bobe et al., 2004), strategies are sought to reduce the incidence and severity of the disease. With the inherently low capacity of ruminant liver to export VLDL (Kleppe et al., 1988; Armentano et al., 1991; Emmison et al., 1991,), as well as a maximum level for use of ketone bodies by extrahepatic tissues, a promising avenue to reduce the incidence of hepatic steatosis in dairy cows may be to promote alternative metabolism of fatty acids through oxidation to CO₂.

Pyruvate carboxylase (PC; EC 6.4.1.1) catalyzes oxaloacetate (OAA) synthesis and ostensibly links the cataplerotic pull of carbons away from the tricarboxylic acid cycle (TCA), specifically through gluconeogenesis, and the anaplerotic generation of OAA. Expansion of the OAA pool can increase the potential capacity of the TCA cycle and promote an increase in complete oxidation of acetyl-CoA as long as the activities of cytosolic phosphoenolpyruvate carboxykinase (PCK1) and mitochondrial phosphoenolpyruvate carboxykinase (PCK2) are not increased (Jitrapakdee et al., 2006). A reduction in PC activity has long been associated with a decrease in the pool of OAA in mitochondria of glucogenic tissues during increased gluconeogenesis in ketotic cows (Baird et al., 1968).

Pyruvate carboxylase mRNA expression is significantly increased around the time of calving and during feed restriction in dairy cows while other enzymes related to glucose production are not affected (Greenfield et al., 2000; Velez and Donkin, 2005). Changes in the profile of circulating fatty acids around parturition may influence

gluconeogenic enzymes, including PC. Fatty acids have previously been shown to regulate PC gene expression in several species (Jitrapakdee et al., 2008). Previous work also demonstrates activation of bovine PC mRNA in response to a peroxisome proliferator-activated receptor- α (PPAR α) agonist but a decrease with exposure to saturated fatty acids (White et al., 2011b).

Diets fed to dairy cows during the dry period have been shown to alter circulating fatty acid profiles and concentrations at calving (Rukkwamsuk et al., 2000). Dairy cows with induced fatty liver postpartum had significantly greater concentrations of serum C16:0, C18:0, and C18:1n-9 *cis* relative to fatty acid serum concentrations from control cows (Rukkwamsuk et al., 2000). Furthermore, feeding diets rich in flaxseed, a source of C18:3n-3 *cis*, starting from 6 wk before calving significantly decreased the accumulation of liver triglycerides after calving compared to control cows or those receiving saturated fat supplements during the dry period (Petit et al., 2007), suggesting that inclusion of dietary C18:3n-3 *cis* alters hepatic fatty acid metabolism. Long chain fatty acids have also been shown to regulate fatty acid oxidation in rodents (Ide et al., 1996) and dietary fat containing high amounts of C18:3n-3 *cis* increased both mitochondrial and peroxisomal oxidation rates, as well as increased activities of enzymes related to β -oxidation in rat liver.

We hypothesized that bovine PC mRNA expression is differentially regulated by saturated and unsaturated fatty acids and the balance of fatty acids determines PC mRNA abundance. Our objective was to determine the effects of fatty acid chain length, degree of saturation, and copresence of saturated and unsaturated fatty acids on PC mRNA expression. We first utilized Madin-Darby bovine kidney (MDBK) cells as a model

system for these experiments as enzymes associated with glucogenic pathways have previously been studied in MDBK cells (Bionaz et al., 2008a,b; Thering et al., 2009; White et al., 2012). Additionally, this cell line permits prolonged exposure to fatty acids and other factors. A parallel experiment was completed in isolated bovine hepatocytes to examine the effects of fatty acids on PC mRNA expression in bovine liver.

MATERIALS AND METHODS

Experiment 1

Cell Culture. Madin-Darby bovine kidney epithelial cells (NBL-1 ATCC CCL-22) were obtained at passage 110 from the American Type Culture Collection (ATCC, Manassas, VA). Cells were propagated in sterile polystyrene 150-cm² canted-neck, 0.2 µm vent cap flasks (Corning, Corning, NY) in Dulbecco's Modified Eagle's Medium (DMEM) containing 1 g/L D-glucose, L-glutamine, and 110 mg/L sodium pyruvate (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Corp., St. Louis, MO), 100 units/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B (Antibiotic-Antimycotic Solution; Sigma-Aldrich Corp., St. Louis, MO) at 37°C in 5% CO₂ and 95% air. Media was refreshed every two to three d. Cells were subcultured to near confluence following media removal with trypsin-EDTA (Sigma-Aldrich Corp., St. Louis, MO). Trypsin activity was inhibited by addition of 10 mL of fresh DMEM supplemented with 10% FBS (Sigma-Aldrich Corp.), 100 units/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B

(Antibiotic-Antimycotic Solution; Sigma-Aldrich Corp.). For each experimental replicate, cells were plated on 6-well plates (Corning, Glendale, AZ) at a density of 5×10^5 cells per 35-mm diameter well and incubated in 10% FBS DMEM with antibiotics, as indicated above, to achieve approximately 80% confluence before exposure to treatments.

Fatty acid treatments. Fatty acids C16:0, C18:0, C18:1n-9 *cis*, C18:3n-3 *cis* (Sigma-Aldrich Corp.) were bound to BSA (Probumin; Millipore, Billerica, MA) in a 4:1 molar ratio as described previously (Berry et al., 1991). MDBK cells at 80% confluence were cultured in DMEM and exposed for 24 h to either 1% BSA (no addition control), or to 1.0 mM individual fatty acid (bound to BSA), or to combinations of fatty acids in 10:90, 25:75, 50:50, or 75:25 molar ratios from combinations of C16:0: C18:3n-3 *cis*, and in 25:75, 50:50, 75:25, or 90:10 molar ratios from combinations of C18:0: C18:3n-3 *cis*, or C18:1n-9 *cis*: C18:3n-3 *cis*. Total fatty acid concentration in each combination was 1.0 mM and total BSA concentration was 1%. Treatments were applied in triplicate to three separate MDBK cell replicates.

Experiment 2

Care and Use of Hepatocyte Donor Animals. All experimental procedures involving animals were approved by the Purdue University Animal Care and Use Committee. Intact male dairy calves were fed colostrum at birth followed by milk diet at 10% of birthweight and delivered as two equal portions twice daily. Animals were selected as hepatocyte donors within 7 d of age, were given heparin (1,000 USP, Sagent Pharmaceuticals, Schaumburg, IL) intravenously and anesthetized with 1% propofol (6 mg/kg calf body weight, Hospira Inc. Lake Forest, IL) via intravenous injection. The

caudate process was excised and the animals were euthanized immediately by intravenous injection of saturated potassium chloride (150 mg/kg calf body weight; Sigma-Aldrich Corp., St. Louis, MO).

Hepatocyte Monolayer Preparation and Culture. Liver cells were dispersed via collagenase perfusion as described previously (Donkin and Armentano, 1993). The isolated hepatocytes were plated onto 35-mm Falcon Primaria Easy Grip tissue culture dish (Becton Dickinson, Lincoln Park, NJ) at an approximate density of 5.3×10^4 cells/cm² in DMEM containing 20% FBS and 100 units/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B (Antibiotic-Antimycotic Solution; Sigma-Aldrich Corp., St. Louis, MO). After 4 h, the plating media and unattached cells were removed by aspiration and media was replaced with DMEM containing 10% FBS and 1% antibiotic, antimycotic solution. Twenty hours after seeding plates, media was removed by aspiration and replaced with DMEM containing 1% BSA and the fatty acid treatments described below.

Fatty acid treatments. Fatty acids C16:0, C18:0, C18:3n-3 *cis* (Sigma-Aldrich Corp.) were bound to BSA (Probumin; Millipore, Billerica, MA) in a 4:1 molar ratio as described previously (Berry et al., 1991). Twenty hours after seeding, hepatocytes were cultured in DMEM and exposed for 24 h to either 1% BSA (no addition control), or to 1.0 mM individual fatty acid (bound to BSA), or to combinations of fatty acids in 10:90, 25:75, 50:50, or 75:25 molar ratios from combinations of C16:0: C18:3n-3 *cis*, and in 25:75, 50:50, 75:25, or 90:10 molar ratios from combinations of C18:0: C18:3n-3 *cis*. Total fatty acid concentration in each combination was 1.0 mM and total BSA

concentration was 1%. Treatments were applied in triplicate to five separate hepatocyte isolations.

Cell Sample Collection, RNA Isolation, and Transcript Quantification

Samples from both Experiment 1 and Experiment 2 were processed identically. After exposure to treatments for 24 h, media was removed and cells were disrupted and lysed by adding 350 μ L Buffer RLT (RNeasy Mini Kit, Qiagen Inc., Germantown, MD). Plates were shaken on an orbital shaker (Barnstead International, Dubuque, IA) for 10 minutes and cell lysates collected to a 1.5 mL microcentrifuge tube. Lysates were stored at -80°C until subsequent RNA isolation.

Cell lysates were homogenized by vortexing and processed using the RNeasy Mini Kit according to manufacturer directions (Qiagen Inc., Germantown, MD). Purified RNA was quantified for each sample by absorbance at 260 nm using a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and sample integrity was determined by the ratio of absorbance at 260 nm to 280 nm. Only samples with a ratio of 260:280 greater than or equal to 1.8 were used for further analysis. An aliquot of 1 μ g of purified RNA from each sample was reverse transcribed to cDNA using an Omniscript reverse transcriptase kit (Qiagen, Inc., Thousand Oaks, CA), random decamers (Ambion, Foster City, CA) and oligo-dT (Qiagen, Inc., Thousand Oaks, CA). Abundance of cDNA for each sample was quantified using real-time PCR, Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Inc., Santa Clara, CA) and primers described below. A no-reverse-transcriptase template control was formed by combining equal quantities of purified RNA from each sample. A cDNA pool

was formed from equal quantities of cDNA from each sample and diluted with DNase-free water in a 1:4 dilution series to generate a standard curve. DNase-free water served as the no-template control. The abundance of PC (EC 6.4.1.1), PCK1 (EC 4.1.1.32), and 18S (NCBI Gene number 493779) mRNA was determined using quantitative real-time PCR. Primers were as follows: bovine PC, CCACGAGTTCTCCAACACCT (forward), TTCTCCTCCAGCTCCTCGTA (reverse); and bovine 18S, ACCCATTCGAACGTCTGCCCTATT (forward), TCCTTGGATTGTGGTAGCCGTTTCT (reverse). Reactions were as follows: 1 cycle at 95°C for 3 min; 40 cycles of 95°C for 10 s, 60°C for 20 s; and 1 cycle of 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. Reaction efficiencies were between 90% and 110% based on standard curve analysis. All samples, standards, and controls were analyzed in triplicate and mean values normalized to 18S mRNA abundance within each sample. Data are expressed as arbitrary units of mRNA relative to 18S abundance within sample. Abundance of 18S mRNA (Ct values) was not affected by treatment ($P = 0.76$).

Assessment of Cellular Integrity

To assess the effects of cell manipulation and fatty acid treatments on viability, MDBK cells were plated on 96-well cell culture plates and grown to 80% confluence. Media was changed after 24 h and cells were incubated for 24 h at 37° and exposed to the same treatment media used for assessment of RNA expression. The TACS MTT Cell Proliferation Assay (Trevigen, Gaithersburg, MD) was used according to the manufacturer's directions to determine the effect of fatty acid treatment on cell viability.

Statistical Analysis

Data were analyzed for normality using the Univariate procedure of SAS 9.3 (SAS Institute Inc., Cary, NC). Analyses of variance was performed using the Proc Mixed procedure of SAS. The model accounted for the fixed effects of treatment and the random effects of cell replicate. Means were considered different when $P < 0.05$ and tended to differ if $0.05 \leq P \leq 0.10$. Tukey-Kramer studentized adjustments were used for multiple comparisons to separate treatment means. Data are reported as least squares means with associated standard errors. Data from Experiment 1 and Experiment 2 were analyzed separately.

RESULTS AND DISCUSSION

Madin-Darby bovine kidney (MDBK) cells are used here to examine regulation of bovine PC mRNA expression by fatty acids and their combinations. The MDBK cell model has been used previously to study control of enzymes for gluconeogenesis and fatty acid metabolism, including PC (Bionaz et al., 2008a,b; Thering et al., 2009; White et al., 2012). Expression patterns of periparturient cow liver tissue and MDBK transcriptomes showed strong similarities (Bionaz et al., 2008b). Furthermore, response to long-chain fatty acids and to peroxisome proliferator-activated receptor agonist (PPAR) agonist have been evaluated in MDBK cells (Bionaz et al., 2008a). This cell culture system was most recently used to analyze transcript abundance of glucogenic genes after treatment with fatty acid profiles that mimic circulating NEFA profiles in

dairy cows during the periparturient period, or under a model of induced fatty liver (White et al., 2012).

Analysis of cellular integrity after fatty acid treatments on MDBK cells by TACS MTT Cell Proliferation Assay indicated a significant ($P < 0.05$) reduction in cell viability of 13.12%, 15.78%, 12.26%, and 8.7% with the addition of 1.00 mM C16:0, C18:0, C18:1n-9 *cis*, or C18:3n-3 *cis*, respectively, compared to viability levels of 10% FBS-treated controls in MDBK cells. Treatments with each fatty acid alone, at all evaluated concentrations (0.25 mM, 0.50 mM, 0.75 mM, or 1.00 mM) did not differ ($P > 0.10$) in viability compared to the 10% FBS or 1% BSA treatments (Figure 2.1). Treatments with each fatty acid alone, at all evaluated concentrations (0.25 mM, 0.50 mM, 0.75 mM, or 1.00 mM) showed a slight, but significant decrease ($P < 0.05$) in viability compared to the combination of C18:3n-3 *cis* at any evaluated ratio (Figure 2.1). Despite the effects of single fatty acids, any evaluated combination of two fatty acids did not differ ($P > 0.10$) for cell viability measurement from the control 1% BSA DMEM or 10% FBS DMEM media (Figure 2.1). This indicates a more optimal environment for cellular growth and proliferation occurs when any two of the fatty acids evaluated here are present in the treatment media for MDBK cells.

Previous work in the human hepatoblastoma HepG2 cell line demonstrated a high percentage of apoptosis, measured with DAPI (4',6-diamidino-2-phenylindole dihydrochloride), after 24 h treatment with 0.66 mM C16:0 (Ricchi et al., 2009). Percent apoptosis was significantly reduced when C18:1n-9 *cis* was added to treatment media, even when total treatment concentrations equaled 1.0 or 2.0 mM (Ricchi et al., 2009). Similarly, when evaluated in a model of hepatic steatosis, human hepatocytes exhibited

decreased cell viability after treatment with C16:0 alone (Chen et al., 2014). In the same study, treatments containing combinations of C16:0 with C18:1n-9 *cis* were able to reduce the C16:0-induced inhibition of cell growth (Chen et al., 2014). Treatment combinations of C16:0 with C18:1n-9 *cis* were not evaluated in the current study. Fatty acid treatments combining C18:0 with C18:3n-3 *cis* have been studied in isolated rat primary hepatocytes (Zhang et al., 2011). While exposure of hepatocytes to 0.25 mM C18:0 significantly reduced MTT-measured cellular viability, co-treatment of 0.25 mM C18:0 with either 0.15 mM or 0.25 mM C18:3n-3 *cis* restored viability measurement to levels similar to the control (Zhang et al., 2011). This knowledge base, combined with data from the current study, indicates that co-incubation with more than one fatty acids allows greater cellular viability, potentially from more closely mimicking in vivo circulating fatty acid concentrations. Consequently, these data support the value of any effects of fatty acid combinations on PC expression or other transcripts. While decreases are seen in cellular viability in the current study under single fatty acid treatments, compared to treatment combinations of fatty acids, these decreases did not occur from treatments containing multiple fatty acids. Therefore, gene expression data, discussed below, are not likely attributed to differences in viability and are the result of fatty acid treatment.

Treatment with C16:0 or C18:0 independently decreased ($P < 0.05$) PC mRNA expression compared to either C18:3n-3 *cis* treatment alone or the control of no additional fatty acid in MDBK cells (Figure 2.2). In contrast, exposure to C18:1n-9 *cis* or C18:3n-3 *cis* treatment alone did not differ ($P > 0.10$) from the control (Figure 2.2) in MDBK cells. Previous research in the MIN6 mouse pancreatic β -cell line demonstrated a

significant decrease in PC transcript abundance after 48 h treatment with either 0.4 mM C16:0 or 0.4 mM C18:1n-9*cis* (Busch et al., 2002). In the current study, a differential response is clearly seen in PC mRNA expression after treatment with saturated, compared to mono- or polyunsaturated fatty acids alone. The bovine PC gene contains three promoters (Agca et al., 2004) and expresses six 5' untranslated region variants that are transcribed from these promoters (Hazelton et al., 2008). PC expression regulation by fatty acids occurs through activation of promoter 1 (White et al., 2011a). Specifically, responsiveness of PC promoter 1 to C18:0 was established where depression of promoter 1 occurred with increasing concentrations, up to 1.0 mM, of C18:0 (White et al., 2011b). While the current study examines PC mRNA expression, it is clear that some regulation by fatty acids occurs. Whether this regulation occurs through changes in transcription factor binding or through metabolic influences remains to be elucidated.

To determine the dominance of control of PC mRNA expression by saturated or unsaturated fatty acids in MDBK cells, combinations of fatty acids in 10:90, 25:75, 50:50, or 75:25 molar ratios from combinations of C16:0: C18:3n-3 *cis*, and in 25:75, 50:50, 75:25, or 90:10 molar ratios from combinations of C18:0: C18:3n-3 *cis*, or C18:1n-9 *cis*: C18:3n-3 *cis* were examined for their potential influence on PC mRNA expression. The ratio of 75:25 C16:0: C18:3n-3 *cis* significantly increased ($P < 0.05$) PC expression compared to C16:0 alone, though values were still significantly lower ($P < 0.05$) than either C18:3n-3 *cis* treatment alone or the control of no additional fatty acid. Ratios of 50:50, 25:75, or 10:90 for C16:0: C18:3n-3 *cis* were able to significantly ($P < 0.05$) recover PC mRNA expression in MDBK cells to levels similar to control or C18:3n-3 *cis* treatment. Regulation through C18:3n-3 *cis* is therefore stronger than

regulation through C16:0 as when these two fatty acids were present in equal ratios in the treatment media, PC mRNA expression was similar to expression after 1.0 mM C18:3n-3 *cis* treatment, and greater than expression after 1.0 mM C16:0 treatment. Fatty acid combinations of 75:25, 50:50, or 25:75 C18:0: C18:3n-3 were able to significantly ($P < 0.05$) recover PC mRNA expression to levels similar to control or C18:3n-3 *cis* treatment in MDBK cells. Again, the dominance of control on PC gene expression can be seen in response to lesser ratios of C18:3n-3 *cis*. Combinations of C18:1n-9 *cis* with C18:3n-3 *cis* in any ratio did not significantly ($P > 0.05$) alter PC mRNA expression in MDBK cells (Figure 2.2). Interestingly, a recent study examining the responsiveness in MDBK cells of several genes involved in gluconeogenesis and fatty acid oxidation, including PC, after 6 h exposure to several different long-chain fatty acids showed no change in PC mRNA expression after treatment with either C16:0, C18:0, C18:1n-9 *cis*, or C18:3n-3 *cis* compared to control (Bionaz et al., 2012.) However, only single fatty acid treatments at 0.15 mM were interrogated (Bionaz et al., 2012.). Additionally, an in vivo experiment examined effects of supplemental long-chain fatty acids from either saturated fat sources (Energy Booster) or fish oil supplements from around 25 d before calving to 10 d postpartum (Akbar et al., 2013). Pyruvate carboxylase mRNA expression was increased in liver biopsy samples from the control group (no supplemental long-chain fatty acids) postpartum compared to prepartum, while PC mRNA expression was unchanged pre- to postpartum with either peripartal fat supplement, but was significantly lower than control (Akbar et al., 2013). Concurrently, lower concentrations of C18:3n-3 *cis* in liver phospholipids at 10 d prepartum were recorded in both fat supplemented groups

compared to control (Akbar et al., 2013), which further supports the relationship between liver C18:3n-3 *cis* concentration and PC mRNA expression.

Fatty acid treatments, in any examined combination, in bovine hepatocytes did not elicit a significant response in PC mRNA expression ($P > 0.10$; Figure 2.3). When PC mRNA expression in response to only single fatty acid treatments and control 1% BSA is examined, treatment with C18:3n-3 *cis* alone did significantly increase ($P < 0.05$) PC mRNA expression compared to the control or saturated fatty acid treatment (1.86, 1.00, 1.04, and 0.66 ± 0.17 for C18:3n-3 *cis*, 1% BSA DMEM control, C16:0, and C18:0, respectively). These data differ from the response seen in MDBK cells in that C18:3n-3 *cis* numerically increases PC mRNA expression to levels greater than 1% BSA DMEM control in primary hepatocytes. As the duration of treatment was identical, this difference may be due, in part, to the sensitivity of hepatocytes to unsaturated fatty acids, compared to MDBK cells. Previous work examined response of bovine hepatocytes to long-term fatty acid treatments over a 48 h period and found influences of fatty acids on glucose metabolism (Mashek and Grummer, 2003). Treatments that included C18:3n-3 *cis* increased gluconeogenesis and decreased triglyceride deposition, though PC was not evaluated (Mashek and Grummer, 2003). Longer incubation periods may be necessary to elicit a greater response to fatty acids in primary hepatocytes. The MDBK cell model may be more sensitive to fatty acid uptake as the 24 h treatment period was adequate in this model. Accordingly, we must also consider that primary hepatocytes are exposed to circulating fatty acid concentrations in vivo, before harvest, and were weaned off of that profile after isolation, while the MDBK cells are cultured in a background media containing very low fatty acid levels.

Carboxylation of pyruvate to OAA by PC maintains the pool of OAA available for use in the TCA cycle. Maintaining or increasing this pool is necessary to promote oxidative capacity in the cell. Any increase in the OAA pool that is generated by PC may be opposed by enzymes that pull carbons out of the OAA pool, specifically in gluconeogenesis by PCK1 and PCK2. Pyruvate carboxylase enzyme activity in bovine has been previously correlated with PC mRNA expression (Greenfield et al., 2000). Therefore, if activity of PC increases, and this increase is not matched by PCK activity, then an increase in potential cycling and oxidative capacity of the TCA may occur. Elevated PC expression and activity at calving in the absence of changes in PCK1 (Greenfield et al., 2000) would favor an increased oxidative capacity and may be part of the adaptations that enable transition cows to metabolize greater quantities of fatty acids in liver.

Mobilization of adipose tissue stores during negative energy balance around parturition is necessary to meet increased energy demand. Inability to cope with this increased NEFA load can lead to development of ketosis or fatty liver (Vernon, 2005). The circulating NEFA profile as well as concentration at calving are influenced by diets fed to dry cows (Rukkwamsuk et al., 2000). Increases in C16:0 and C18:1, presumably from adipose tissue lipolysis, were elevated in liver of cows with fatty liver compared to control animals (Rukkwamsuk et al., 2000). Changes in circulating NEFA and the activation of glucogenic enzymes have also been previously linked in nonruminants (Pawar and Jump, 2003). Long chain fatty acids are known activators of transcription factors, including PPAR α , that regulate genes for gluconeogenesis (Pawar and Jump, 2003). While exposure to Wy14643 (a PPAR α agonist) at 0.10 mM in rat hepatoma cells

has previously been shown to increase the activity of a transfected bovine PC promoter 1 (White et al., 2011b), no change in PC mRNA expression was seen in MDBK cells exposed for 6 h to 0.15 mM Wy14643 (Bionaz et al., 2012). Effects of other PPAR α agonists, or the direct effects of unsaturated fatty acids on PC mRNA expression remain uncertain.

The fatty acid concentrations of 1.0 mM tested in the present study are relevant with regards to total circulating concentrations of NEFA in dairy cows around the time of parturition (Rukkwamsuk et al., 1999), but are not directly applicable to most physiological settings because adipose triglycerides and dietary lipids are rarely comprised of a single fatty acid. However, during the negative energy balance associated with transition to lactation, plasma NEFA profiles consist predominantly of C16:0, C18:0, and C18:1 (Rukkwamsuk et al., 1999). Shifts that occur in the profile of plasma fatty acids may influence expression of genes responsible for gluconeogenesis or fatty acid metabolism. An increase in the concentration of plasma C18:0 has been reported in periparturient dairy cows (Rukkwamsuk et al., 1999). While C18:1 levels are similar during the pre- or post-partum periods, C18:0 levels show a peak at calving (Rukkwamsuk et al., 1999; Douglas et al., 2007). Increases in plasma NEFA also occur after supplementation with calcium soaps of long-chain fatty acids (Megalac-R) compared to controls (Moallem et al., 2007). Dairy cows receiving 230 g/d Megalac-R during the dry period and 215 g/d Megalac-R postpartum until 100 DIM had significantly higher plasma NEFA levels postpartum compared to cows receiving a control dry cow diet without supplemental fatty acids (Moallem et al., 2007). Similarly, supplementation of dry cow diets with calcium soaps of fatty acids from 30 d before calving through 100 d

postpartum increased plasma NEFA and BHBA levels compared to cows receiving hydrogenated palm oil triglyceride at equal net energy supply during the same period (Karcagi et al., 2009). Although neither of these studies were specifically designed to examine the influences of dietary fat on plasma fatty acid profile, data is available indicating the effect of feeding whole flaxseed, a source of polyunsaturated fatty acids, on plasma NEFA profiles (Petit, 2002). Postpartum diets containing flaxseed increased plasma C18:1n-9 *cis* and C18:3n-3 *cis* levels compared to plasma levels in cows receiving postpartum diets supplemented with micronized soybeans (Petit, 2002). Data from the current study indicate that stearic acid directly depresses bovine PC mRNA expression and suggest that the ratio of saturated to unsaturated plasma fatty acids regulate changes in PC mRNA around calving. When combined with observations of changes in fatty acid profiles at calving with diet and predisposing physiological states, a working hypothesis emerges that dietary fatty acid profile can determine PC expression to potentially alter the cellular OAA pool. Additional work is needed to better characterize the interplay between these fatty acids and potential effects on bovine liver.

Results presented in the current study indicate that C18:3n-3 *cis* is a more potent alleviator of PC mRNA depression in C18:0: C18:3n-3 *cis* treated MDBK cells compared to C16:0: C18:3n-3 *cis* treatments. C18:1n-9 *cis* treatments did not depress PC mRNA expression, revealing that fatty acid saturation is key in fatty acid regulation of PC expression. These data indicate a potential differential response of unsaturated or saturated fatty acids to signals induced by fatty acid treatments.

CONCLUSIONS

Results indicate a significant depression of PC mRNA expression in MDBK cells after 24 h treatment with either C16:0 or C18:0. Inclusion of C18:3n-3 *cis* in treatment media, in the higher evaluated ratios, proved to successfully ameliorate this depression and recover PC mRNA expression to levels similar to control. Response of bovine hepatocytes to fatty acid exposure was muted, possibly due to duration of treatment, but PC mRNA expression did increase in bovine hepatocytes after C18:3n-3 *cis*, compared to single saturated fatty acid treatment or to control. PC activation by unsaturated fatty acids may play a critical role in setting the capacity for OAA synthesis. Both fatty acid chain length and degree of saturation appear to be key in regulation of PC mRNA expression by fatty acids.

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Figure 2.1. Effect of 24 h fatty acid treatments on cellular viability in Madin-Darby bovine kidney (MDBK) cells. To assess the effects of cell manipulation and fatty acid treatments on viability, cells were plated on 96-well cell culture plates and grown to 80% confluence. Media was changed after 24 h and cells were incubated for 24 h at 37° and exposed to treatment media. Fatty acid treatments were either single fatty acids (C16:0, C18:0, C18:1n-9 *cis*, or C18:3n-3 *cis*) or combinations of C16:0 with C18:3n-3 *cis*, or C18:0 with C18:3n-3 *cis*, or C18:1n-9 *cis* with C18:3n-3 *cis*, in the molar ratios indicated. All treatments totaled 1.0 mM in Dulbecco's Modified Eagle's Medium (DMEM) with 1% BSA. Control cells were incubated in DMEM with 1% BSA and no additional fatty acid. The TACS MTT Cell Proliferation Assay (Trevigen, Gaithersburg, MD) was used according to the manufacturer's directions to determine the effect of fatty acid treatment on cell viability. Data are reported as least squares means with associated standard errors. Within panel, means without a common superscript differ ($P < 0.05$).

Figure 2.2. Effect of 24 h exposure to fatty acids on expression of pyruvate carboxylase (PC) mRNA in Madin-Darby bovine kidney (MDBK) cells. MDBK cells at 80% confluence were cultured with BSA-bound fatty acids (FA) for 24 h. Fatty acid treatments were either single fatty acids (C16:0, C18:0, C18:1n-9 *cis*, or C18:3n-3 *cis*) or combinations of C16:0 with C18:3n-3 *cis* (top), or C18:0 with C18:3n-3 *cis* (middle), or C18:1n-9 *cis* with C18:3n-3 *cis* (bottom), in the molar ratios listed. All treatments totaled 1.0 mM in Dulbecco's Modified Eagle's Medium (DMEM) containing 1% BSA. Control treatment is DMEM with 1% BSA and no added fatty acid. Data are presented as ratio of PC:18S mRNA expression in arbitrary units (AU) and reported as least squares means with associated standard errors. Within panel, means without a common superscript differ ($P < 0.05$).

Figure 2.3. Effect of 24 h exposure to fatty acids on expression of pyruvate carboxylase (PC) mRNA in bovine primary hepatocytes. Twenty hours after seeding plates, hepatocytes were exposed to BSA-bound fatty acids (FA) for 24 h and then harvested for mRNA analysis. Fatty acid treatments were either single fatty acids (C16:0, C18:0, or C18:3n-3 *cis*) or combinations of C16:0 with C18:3n-3 *cis* (top), or C18:0 with C18:3n-3 *cis* (bottom), in the molar ratios listed. All treatments totaled 1.0 mM in Dulbecco's Modified Eagle's Medium (DMEM) containing 1% BSA. Control treatment is DMEM with 1% BSA and no added fatty acid. Data are presented as ratio of PC:18S mRNA expression, relative to 1% BSA DMEM control, in arbitrary units (AU) and reported as least squares means with associated standard errors. Within panel, means without a common superscript differ ($P < 0.05$). Analysis of PC mRNA expression in response to only single fatty acid treatments and control 1% BSA determined treatment with C18:3n-3 *cis* alone significantly increased ($P < 0.05$) PC mRNA expression compared to the 1% BSA DMEM control or saturated fatty acid treatment (1.86, 1.00, 1.04, and 0.66 ± 0.17 for C18:3n-3 *cis*, 1% BSA DMEM control, C16:0, and C18:0, respectively).

CHAPTER 3. PRETREATMENT WITH SATURATED AND UNSATURATED FATTY ACIDS REGULATES SUBSEQUENT PALMITATE METABOLISM IN MADIN-DARBY BOVINE KIDNEY CELLS

ABSTRACT

Metabolic fates of fatty acids in tissue may be influenced by extracellular concentration and profile of fatty acids. Previous work has demonstrated an ability of C18:3n-3 *cis* to ameliorate the effects of C16:0- or C18:0-induced depression of pyruvate carboxylase (PC) mRNA expression. Pyruvate carboxylase catalyzes oxaloacetate (OAA) synthesis and connects gluconeogenesis from lactate and fatty acid metabolism. Our objective was to determine the effects of a copresence of saturated and unsaturated fatty acids on cellular partitioning of [1-¹⁴C]C16:0 metabolism to CO₂ or acid-soluble products (ASP) in Madin-Darby bovine kidney (MDBK) cells and the role of PC in this relationship. We hypothesized that the ratio of saturated to unsaturated fatty acid pretreatments regulates [1-¹⁴C]C16:0 partitioning to CO₂ or ASP. Cells were exposed for 21h to either individual fatty acids, C16:0, C18:0, C18:1n-9 *cis* or C18:3n-3 *cis*, or to fatty acid combinations in 10:90, 25:75, 50:50, 75:25 or 90:10 ratios for combinations of C16:0: C18:3n-3 *cis* or C18:0: C18:3n-3 *cis* or C18:1n-9 *cis*: C18:3n-3 *cis*. Total fatty acid concentration was 1.0 mM during the 21 h pretreatment phase. Following the 21 h incubation phase with fatty acid combinations, cells were incubated in the presence of 1.0

mM [1-¹⁴C]C16:0 for 3h to determine the rate of metabolism to CO₂ and ASP collection per h · μg DNA⁻¹. Pretreatments with either C16:0 or C18:0 alone significantly ($P < 0.01$) depressed subsequent oxidation of [1-¹⁴C]C16:0 to ASP by 62.7% and 41.2%, respectively, compared to C18:3n-3 *cis* pretreatments. Similar patterns were observed for [1-¹⁴C]C16:0 oxidation to CO₂. Expression of PC mRNA was significantly decreased ($P < 0.05$) with exposure to either C16:0 or C18:0, compared to expression after exposure to either C18:3n-3 *cis* or control 1% BSA DMEM. Expression of cytosolic phosphoenolpyruvate carboxykinase (PCK1) mRNA followed a similar pattern. Fatty acid treatments containing C18:1n-9 *cis* did not alter PC or PCK1 expression from control or C18:3n-3 *cis* results. Pearson's coefficient correlations were determined for PC mRNA expression and rate of [1-¹⁴C]C16:0 metabolism to CO₂ or ASP, including ketones, and for PCK1 mRNA expression and rate of [1-¹⁴C]C16:0 metabolism to CO₂ or ASP. Production of CO₂ from [1-¹⁴C]C16:0 positively correlated ($r = 0.63$; $P < 0.05$) with PC expression, while ASP production from [1-¹⁴C]C16:0 only tended to positively correlate ($r = 0.51$; $0.05 < P < 0.10$) with PC mRNA expression. Production of CO₂ or ASP from [1-¹⁴C]C16:0 both positively correlated ($r = 0.80$; $r = 0.69$, respectively; $P < 0.05$) with PCK1 expression. Results show a regulation of ketone production by MDBK cells in response to saturated and unsaturated fatty acid pretreatments.

Keywords: bovine, fatty acid oxidation, ketogenesis

INTRODUCTION

Pyruvate carboxylase (PC; EC 6.4.1.1) is a critical enzyme, responsible for the anaplerotic regeneration of tricarboxylic acid cycle (TCA) intermediates. Conversely, phosphoenolpyruvate carboxykinase (PCK; EC 4.1.1.32) acts to cataplerotically drain carbon intermediates from TCA cycle towards gluconeogenesis. Pyruvate carboxylase is responsible for the carboxylation of pyruvate to oxaloacetate (OAA), which subsequently condenses with acetyl-CoA in the first step of the TCA cycle, therefore, the capacity for fatty acid oxidation as well as gluconeogenesis is dependent upon the OAA status within the cell. As a result, the maintenance of the OAA pool through the action of PC flux could promote complete oxidation of acetyl-CoA in the TCA cycle, providing PCK1 and PCK2 activities do not exceed PC flux (Jitrapakdee et al., 2006).

Nutritional control of PC is well established in rats (Bizeau et al., 2001), mice (Hagopian et al., 2004), and lactating dairy cows, specifically during a period of imposed feed restriction (Velez and Donkin 2005), and transition to lactation (Greenfield et al., 2000). In periparturient dairy cows, plasma nonesterified fatty acid (NEFA) concentrations increase in response to the negative energy balance experienced around calving. After calving, plasma NEFA concentrations in excess of 1.5 mM are observed and contrast to concentrations near 0.2 mM during the prepartum period (Rukkwamsuk et al., 2000). Circulating NEFA are taken up by liver in proportion to their presence in blood (Emery et al., 1992) and are either metabolized through either complete or partial oxidation, or triglyceride synthesis and either deposition or loading onto very low density lipoprotein (VLDL) particles for export (reviewed by Drackley et al., 2001; Bobe et al.,

2004; van Kneysel et al., 2005). Ruminants and nonruminants differ with respect to hepatic capacity for VLDL export, with the rate of export by nonruminants being 25 times the capacity of VLDL export for ruminants (Kleppe et al., 1988). Increases in triglyceride synthesis thus put dairy cows at increased risk for developing ketosis and fatty liver. Furthermore, overfeeding during the prepartum interval is linked to a decrease in the activity of hepatic enzymes involved in NEFA oxidation (Murondoti et al., 2004), and a decrease in the rate of C16:0 oxidation to CO₂ (Litherland et al., 2011). Strategies to alleviate these pathways of fatty acid metabolism and promote alternative fatty acid oxidative pathways could potentially decrease susceptibility to these metabolic disorders.

The role of PC mRNA expression in relation to the potential for cellular complete or partial fatty acid oxidation remains unclear. Our objective was therefore to determine effects of copresence of saturated and unsaturated fatty acid pretreatments on cellular partitioning of [1-¹⁴C]C16:0 metabolism to CO₂ or acid-soluble products (ASP) in Madin-Darby bovine kidney (MDBK) cells, a bovine model cell line for the study of cellular energy metabolism. We hypothesized that the ratio of saturated to unsaturated fatty acid pretreatments regulates [1-¹⁴C]C16:0 partitioning to CO₂ and ASP.

MATERIALS AND METHODS

Cell Culture

Madin-Darby bovine kidney epithelial cells (NBL-1 ATCC CCL-22) were obtained at passage 110 from the American Type Culture Collection (ATCC, Manassas,

VA). Cells were propagated in sterile polystyrene 150-cm² canted-neck, 0.2 µm vent cap flasks (Corning, Corning, NY) in Dulbecco's Modified Eagle's Medium (DMEM) containing 1 g/L D-glucose, L-glutamine, and 110 mg/L sodium pyruvate (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Corp., St. Louis, MO), 100 units/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B (Antibiotic-Antimycotic Solution; Sigma-Aldrich Corp., St. Louis, MO) at 37°C in 5% CO₂ and 95% air. Media was refreshed every two to three d. Cells were subcultured to near confluence following media removal with trypsin-EDTA (Sigma-Aldrich Corp., St. Louis, MO). Trypsin activity was inhibited by addition of 10 mL of fresh DMEM supplemented with 10% FBS (Sigma-Aldrich Corp.), 100 units/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B (Antibiotic-Antimycotic Solution; Sigma-Aldrich Corp.). For each experimental replicate, cells were plated onto 35-mm Falcon Primaria Easy Grip tissue culture dishes (Becton Dickinson, Lincoln Park, NJ) at a density of 5×10^5 cells per well and incubated in 10% FBS DMEM with antibiotics, as indicated above, to achieve approximately 80% confluence before exposure to treatments.

Fatty acid pretreatments

Fatty acids C16:0, C18:0, C18:1n-9 *cis*, C18:3n-3 *cis* (Sigma-Aldrich Corp.) were bound to BSA (Probumin; Millipore, Billerica, MA) in a 4:1 molar ratio as described previously (Berry et al., 1991). Cells at 80% confluence were cultured in DMEM and exposed for 21 h to either 1% BSA (no addition control), or to 1.0 mM individual fatty acid (bound to BSA), or to combinations of fatty acids in 10:90, 25:75, 50:50, or 75:25

molar ratios from combinations of C16:0: C18:3n-3 *cis*, and in 25:75, 50:50, 75:25, or 90:10 molar ratios from combinations of C18:0: C18:3n-3 *cis*, or C18:1n-9 *cis*: C18:3n-3 *cis*. Total fatty acid concentration in each combination was 1.0 mM and BSA concentration was 1%. Pretreatments were applied in triplicate to three separate cell replicates.

Measurement of metabolic flux

Following exposure to pretreatments for 21 h, cells were incubated in the presence of DMEM containing 1% BSA and 1.0 mM [1-¹⁴C]C16:0 for 3h before CO₂ and ASP collection, as previously described (Donkin and Armentano, 1994). Briefly, pretreatment media was removed and each 35-mm dish (base) was placed into a straight-side wide-mouth 60-mL jar (Nalgene, Thermo Scientific, Waltham, MA) with lids modified to hold rubber plug-type stoppers (Kimble Chase, Vineland, NJ) and fitted with a hanging plastic center well (Kimble Chase, Vineland, NJ). MDBK cells were then incubated in DMEM containing 1% BSA and 1.0 mM BSA-bound C16:0 containing 1-¹⁴C labelled C16:0 (Perkin Elmer, Waltham, MA) at approximately 1,000,000 DPM per flask. Cells were gassed with 95% O₂ : 5% CO₂, immediately sealed and incubated at 37°C for 3 h. Incubations were terminated by the addition of 0.2 mL of 5N HClO₄ injected through the stopper of the flask into the media. A volume of 0.2 mL phenethylamine was added to each hanging center well containing a filter paper wick to trap ¹⁴CO₂. Samples were incubated for 1 h at room temperature after which center wells were removed, placed in scintillation vials with scintillation cocktail (Ecolite; MP Biomedical LLC, Solon, OH), and radioactivity determined by liquid scintillation

counting. The rate of fatty acid incorporation into ASP was determined from acidified media. Media was removed from plates, collected in 15 mL conical tubes and plates were rinsed 2× with ice cold saline and the saline rinse combined with acidified media. The collected media and saline mixture was centrifuged for 10 min at $700 \times g$, neutralized with KOH, and radioactivity determined by liquid scintillation counting. DNA content of each culture plate was determined as previously described (Labarca and Paigen, 1980). The rate of oxidation was determined and expressed as $\text{nmol } ^{14}\text{C substrate metabolized to } ^{14}\text{CO}_2 \cdot \mu\text{g DNA}^{-1} \cdot \text{h}^{-1}$. The rate of synthesis of ASP, including ketones, is expressed as $\text{nmol } ^{14}\text{C substrate incorporated in ASP} \cdot \mu\text{g DNA}^{-1} \cdot \text{h}^{-1}$.

Cell Sample Collection, RNA Isolation, and Transcript Quantification

A parallel incubation was completed utilizing the same passage of cells interrogated for the oxidation experiment. Cells were exposed to C16:0: C18:3n-3 *cis* or C18:0: C18:3n-3 *cis* fatty acid treatment combinations in the molar ratios describe above. After exposure to treatments for 24 h, media was removed and cells were disrupted and lysed by adding 350 μL Buffer RLT (RNeasy Mini Kit, Qiagen Inc., Germantown, MD). Plates were shaken on an orbital shaker (Barnstead International, Dubuque, IA) for 10 minutes and cell lysates collected to a 1.5 mL microcentrifuge tube. Lysates were stored at -80°C until subsequent RNA isolation.

Cell lysates were homogenized by vortexing and processed using the RNeasy Mini Kit according to manufacturer directions (Qiagen Inc., Germantown, MD). Purified RNA was quantified for each sample by absorbance at 260 nm using a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and sample integrity

was determined by the ratio of absorbance at 260 nm to 280 nm. Only samples with a ratio of 260:280 greater than or equal to 1.8 were used for further analysis. An aliquot of 1 µg of purified RNA from each sample was reverse transcribed to cDNA using an Omniscript reverse transcriptase kit (Qiagen, Inc., Thousand Oaks, CA), random decamers (Ambion, Foster City, CA) and oligo-dT (Qiagen, Inc., Thousand Oaks, CA). Abundance of cDNA for each sample was quantified using real-time PCR, Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Inc., Santa Clara, CA) and primers described below. The no-reverse-transcriptase template control was formed by combining equal quantities of purified RNA from each sample. A cDNA pool was formed from equal quantities of cDNA from each sample and diluted with DNase-free water in a 1:4 dilution series to generate a standard curve. DNase-free water served as the no-template control. The abundance of PC (EC 6.4.1.1), PCK1 (EC 4.1.1.32), and 18S (NCBI Gene number 493779) mRNA was determined using quantitative real-time PCR. Primers were as follows: bovine PC, CCACGAGTTCTCCAACACCT (forward), TTCTCCTCCAGCTCCTCGTA (reverse); bovine PCK1, AGGGAAATAGCAGGCTCCAGGAAA (forward), CACACGCATGTGCACACACATA (reverse); and bovine 18S, ACCCATTCGAACGTCTGCCCTATT (forward), TCCTTGGATTGTGGTAGCCGTTTCT (reverse). Reactions were as follows: 1 cycle at 95°C for 3 min; 40 cycles of 95°C for 10 s, 60°C for 20 s; and 1 cycle of 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. Reaction efficiencies were between 90% and 110% based on standard curve analysis. All samples, standards, and controls were analyzed in triplicate and mean values normalized to 18S mRNA abundance within each sample.

Data are expressed as arbitrary units of mRNA relative to 18S abundance within sample.

Abundance of 18S mRNA (Ct values) was not affected by treatment ($P = 0.77$).

Statistical Analysis

Data were analyzed for normality using the Univariate procedure of SAS 9.3 (SAS Institute Inc., Cary, NC). Analyses of variance was performed using the Proc Mixed procedure of SAS. The model accounted for the fixed effects of fatty acid pretreatment and the random effects of cell replicate. Means were considered different when $P < 0.05$ and tended to differ if $0.05 \leq P \leq 0.10$. Tukey-Kramer studentized adjustments were used for multiple comparisons to separate treatment means. Data are reported as least squares means with associated standard errors. Pearson correlation coefficients for PC mRNA expression and nmol [$1\text{-}^{14}\text{C}$]C16:0 substrate conversion to either CO_2 or ASP (per $\mu\text{g DNA}^{-1} \cdot \text{h}^{-1}$) were obtained using the PROC CORR procedure of SAS (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Use of the Madin-Darby bovine kidney (MDBK) cell line for the current study is supported by previous studies examining metabolism and activities of enzymes for gluconeogenesis and fatty acid oxidation (Bionaz et al., 2008a,b; Thering et al., 2009; White et al., 2012). Notable similarities have been illustrated between mRNA expression patterns in MDBK cells and liver from periparturient dairy cows (Bionaz et al., 2008b).

The metabolic role of kidney to oxidize fatty acids, especially during times of increase circulating NEFA load (Salto et al., 1996; Huang et al., 2006) further justifies the use of a kidney cell line for the current substrate oxidation experiments.

Similar to results presented in CHAPTER 2, effects of fatty acids on expression of PC mRNA were evaluated. Expression of PC mRNA was significantly decreased ($P < 0.05$) after exposure to 1.0 mM C16:0 or C18:0, compared to mRNA expression after exposure to C18:3n-3 *cis* or to control 1% BSA DMEM (Table 3.1). To ameliorate this saturated fatty acid-induced depression, treatment ratios greater than or equal to 0.25:0.75 C18:0: C18:3n-3 *cis* or 0.50:0.50 C16:0: C18:3n-3 *cis* were required to restore PC mRNA expression to levels similar ($P > 0.10$) to those after 1.0 mM C18:3n-3 *cis* treatment (Table 3.1). Expression of PC mRNA did not differ ($P > 0.10$) after exposure, at any ratio, to C18:1n-9 *cis*. To better determine the balance of cataplerotic and anaplerotic response occurring after exposure to fatty acid treatments, PCK1 mRNA expression was also determined. Expression of PCK1 mRNA was significantly decreased ($P < 0.05$) after exposure to 1.0 mM C16:0 or C18:0, compared to mRNA expression after exposure to C18:3n-3 *cis* or to control 1% BSA DMEM (Table 3.1). Treatment ratios greater than or equal to 0.75:0.25 C16:0: C18:3n-3 *cis* or C18:0: C18:3n-3 *cis* were required to restore PCK1 mRNA expression to levels similar ($P > 0.10$) to those after 1.0 mM C18:3n-3 *cis* treatment (Table 3.1). Similar to results from PC mRNA expression, treatments including any ratio of C18:1n-9 *cis* did not alter PCK1 mRNA expression. Maintenance of activity or increase activity of either enzyme, using a proxy measure of mRNA expression (Greenfield et al., 2000), can be used to infer the pool size available for the cataplerotic or anaplerotic fate of OAA.

Fatty acid β -oxidation occurs in both the mitochondria and peroxisome and effectively shortens fatty acids by two carbons with each cycle. Fatty acid pretreatments in the current substrate oxidation study with either 1.0 mM C16:0 or C18:0 alone significantly ($P < 0.01$) depressed subsequent oxidation of [1- ^{14}C]C16:0 to ASP by 62.7% and 41.2%, respectively, compared to C18:3n-3 *cis* pretreatments in MDBK cells (Figure 3.1, 3.2). Similar patterns were seen for [1- ^{14}C]C16:0 oxidation to CO_2 . It is well established in rodent models that polyunsaturated acids are capable of causing increases in both mitochondrial and peroxisomal oxidation, while leading to a decrease in esterification to triglycerides (Ikeda et al., 1998; Ide et al., 2000). Early studies in ruminants examining metabolism of C16:0 to CO_2 or ASP using bovine liver slices noted decreased oxidation to CO_2 in liver samples from fasted, compared to control, animals, while fasting has no effect on C16:0 metabolism to ASP (Jesse et al., 1986). Dairy cows receiving a high fat diet during the dry period exhibited lower total liver lipid accumulation and lower liver triglyceride deposition during the postpartum period compared to cows receiving an isocaloric high grain dry period diet, or to the control (Grum et al., 1996). The fat source was provided from 50 d prepartum and was comprised mainly of 42% C18:1n-9 *cis*, 24% C16:0, 14% C18:0, 8% C18:2n-6 *cis*, less than 1% C18:3n-3 *cis*, and other fatty acid sources (Grum et al., 1996). The same study illustrated increased prepartum plasma NEFA concentrations, but decreased postpartum plasma NEFA concentrations in the high fat group, compared to the high grain or control groups (Grum et al., 1996). While total β -oxidation of C16:0 did not differ in liver tissue samples taken from the high grain, high fat, or control fed cows, the rate of peroxisomal β -oxidation of C16:0 was significantly higher in samples from cows receiving the high fat

diet (Grum et al., 1996). Mitochondrial and peroxisomal β -oxidation were not separately measured in the current study, but peroxisomal β -oxidation has been shown to be elevated during diabetes and excess NEFA mobilization (Osmundsen et al., 1991). This suggests that fatty acid β -oxidation is adjustable at transition as a potential mechanism for transition cows to cope with the excess NEFA load experienced during this period. Therefore, ways to increase fatty acid β -oxidation through the fatty acid profile to which cells are exposed is of utmost interest.

Treatment fatty acid levels in the current study totaled 1.0 mM and are consistent with circulating NEFA levels in transition dairy cows (Rukkwamsuk et al., 1999). In contrast with a normal physiological setting, treatments of single fatty acids alone are included in the current study to evaluate the effect of individual fatty acids on subsequent partitioning and metabolism. Plasma NEFA profiles during the periparturient period are primarily comprised of C16:0, C18:0, and C18:1 fatty acids, and C18:0 levels have been shown to peak at calving while C18:1 levels are similar pre- and post-partum (Rukkwamsuk et al., 1999; Douglas et al., 2007). These data merited selection of the fatty acids interrogated in the current study.

The ability to increase the cellular capacity for NEFA β -oxidation has been extensively discussed (Drackley and Andersen, 2006). Potential influences to NEFA oxidation have been examined through effects of pyruvate (Armentano et al., 1991; Drackley et al., 1991; Lomax et al., 1983), which raises the question of relationship to PC activity and links to the current research. Addition of pyruvate to isolated hepatocytes from fasted and fed sheep stimulated C16:0 complete oxidation to CO₂ (Lomax et al., 1983). Pyruvate was shown to decrease triglyceride accumulation from C18:1n-9 *cis* in

isolated hepatocytes from lactating goats (Armentano et al., 1991). A comprehensive study of biopsy samples from multiparous dairy cows also demonstrated the ability of pyruvate to increase C16:0 oxidation (Drackley et al., 1991). Any alterations to PC mRNA expression or enzyme activity concurrent with changes in NEFA metabolism were not examined in previous studies, so the direct influence of NEFA on PC activity or flux toward anaplerosis or cataplerosis was not determined.

Previous research in rodents examined the effects of dietary sources of long chain fatty acids and their influence on β -oxidation (Malewiak et al., 1988; Kumamoto and Ide, 1998). Metabolism of C18:1n-9 *cis* in isolated hepatocytes from fat-fed rats resulted in an increase in ASP formation and an increase in esterification to triglycerides compared to isolated hepatocytes from control animals (Malewiak et al., 1988). While data from the current study demonstrates significantly ($P < 0.05$) greater production of ASP from [1- ^{14}C]C16:0 compared to CO_2 for all fatty acid pretreatments examined (Figure 3.1, 3.2, 3.3), production of CO_2 from [1- ^{14}C]C16:0 was greater after fatty acid pretreatment with C18:1n-9 *cis* alone, compared to pretreatment combinations containing C18:3n-3 *cis* (Figure 3.3). Interestingly, increased rates of both mitochondrial and peroxisomal C16:0 oxidation are seen in isolated hepatocytes from rats fed a fat mixture high in 18:3n-3 *cis*, compared to C16:0 oxidation from rats fed sources of C16:0 or C18:2n-6 *cis* (Kumamoto and Ide, 1998), indicating the potential for differences in responses to fatty acids between ruminants and nonruminants, or differences between in vitro experiments and those examining dietary fat sources.

Pearson's coefficient correlations were used to associate PC mRNA expression and rate of [1- ^{14}C]C16:0 metabolism to CO_2 or ASP, including ketones. Production of

CO₂ from [1-¹⁴C]C16:0 positively correlated ($r = 0.63$, $P < 0.05$) with PC expression, while ASP production from [1-¹⁴C]C16:0 only tended to positively correlate ($r = 0.51$, $0.05 < P < 0.01$) with PC mRNA expression (Figure 3.4). Previous research using bovine primary hepatocytes examined the effects of 3 h exposure to C16:0 alone, or concurrent exposure to C16:0 combined with several different unsaturated fatty acids, on subsequent mitochondrial and peroxisomal oxidation (Mashek et al., 2002). Results demonstrate increased oxidation of fatty acids to CO₂ when treatment combinations of C16:0 with either C20:5n-3 *cis* (eicosapentaenoic acid; EPA) or C22:6n-3 *cis* (docosahexaenoic acid; DHA) were introduced to hepatocytes for 3h, compared to fatty acid treatments of C16:0 alone or C16:0 with C18:1 (Mashek et al., 2002). Similarly, 3 h treatment of bovine hepatocytes with C16:0 with either C18:3, C20:5n-3 *cis*, or C22:6n-3 *cis* reduced total cellular lipid deposition from C16:0 metabolism (Mashek et al., 2002). Results also indicated that polyunsaturated fatty acids, including C18:3n-3 *cis*, were poor substrates for esterification to triglycerides (Mashek et al., 2002). Although esterification of substrates to triglyceride formation was not measured in the current study, limitation of triglyceride formation by C18:3n-3 *cis* in a primary hepatocyte model illustrates the potential for promotion through other fates, namely oxidation, of fatty acids after C18:3n-3 *cis* exposure in bovine. While a very similar experiment was completed after 48h treatments in hepatocyte monolayer cultures, the system did not concurrently measure complete oxidation to CO₂, and only data for ASP production is available (Mashek and Grummer, 2003). Treatment of bovine hepatocytes with C18:0 included in the treatment media resulted in the highest rates of ASP production from C16:0 (Mashek and Grummer, 2003). Taken together, these data indicate a potential role for saturated fatty

acid in the promotion of substrate metabolism to ASP, perhaps linked to unsaturated fatty acid as a poor substrate for triglyceride esterification and promotion of substrate metabolism to CO₂.

Studies examining the influence of C18:3n-3 *cis* on hepatic fatty acid metabolism in rodents indicate that rats fed linseed oil had increased activity of enzymes involved in mitochondrial and peroxisomal β -oxidation when fed this source of C18:3n-3 *cis* (Kabir and Ide, 1996). Enzymes for fatty acid oxidation were also increased in rats fed C18:3n-3 *cis* enzyme activity from rats receiving dietary sources of either C18:2n-6 *cis* or C16:0 (Kumamoto and Ide, 1998). These data further support the role for C18:3n-3 *cis* in promoting fatty acid oxidation pathways.

Finally, Pearson's coefficient correlations were also used to associate PCK1 mRNA expression and rate of [1-¹⁴C]C16:0 metabolism to CO₂ or ASP. Production of CO₂ or ASP from [1-¹⁴C]C16:0 both positively correlated ($r = 0.80$; $r = 0.69$, respectively; $P < 0.05$) with PCK1 expression (Figure 3.5). Understanding the balance of PC and PCK1 activities is key to determine the net anaplerotic or cataplerotic pull of TCA cycle carbons. To maximize the energy production potential of both systems, there is a need to identify agents or combinations of agents that independently activate PC to promote fatty acid β -oxidation and TCA cycle capacity. Such an agent would, simultaneously, maintain, but not increase, PCK1 activity to fulfill the need for carbon pull to gluconeogenesis.

The present data establish a relationship between regulation of PC mRNA expression by fatty acids and cellular oxidative capacity. While additional research is needed to establish the mechanisms through which fatty acids act to modulated PC

mRNA expression, evidence is accumulating for a role of peroxisome proliferator-activated receptor α (PPAR α). Research on hepatocyte isolations from 7-wk-old calves after 5 d administration of either fish oil or clofibrate, a PPAR α agonist, found an increased rate of C16:0 conversion to CO₂ in 8 h incubations from clofibrate-treated calves, compared to fish oil- or control-treated hepatocyte isolations (Litherland et al., 2010), indicating an increase in capacity for fatty acid β -oxidation. An even greater increase in substrate oxidation to CO₂ is seen in lactating goats treated with Wy-14643, a known PPAR α agonist (Cappon et al., 2002). Furthermore, previous research from our laboratory indicates a response of bovine PC proximal promoter to PPAR α agonist (White et al., 2011). Increased mRNA expression of acyl-CoA dehydrogenase and PPAR α are both during early lactation (Loor et al., 2005) and may link increases in PC at calving to elevated fatty acid β -oxidation in transition cows. In further support of this aspect, increases in mRNA expression for genes involved in fatty acid β -oxidation, including acyl-CoA synthetase long chain, carnitine palmitoyl-transferase 1A, and acyl-CoA dehydrogenase very long chain, are seen after parturition in liver samples from transition dairy cows (Loor, 2010; Weber et al., 2013).

A working hypothesis for role of PC in determining cellular partitioning of fatty acid metabolism to either complete or partial oxidation is supported by the correlation of PC mRNA expression to substrate oxidation to CO₂. Additional experiments are needed to determine if a similar relationship holds for bovine liver as for the MDBK cell model examined here.

CONCLUSIONS

Results show a regulation of ketone production by MDBK cells in response to saturated and unsaturated fatty acid pretreatments. While oxidation of [1-¹⁴C]C16:0 to either CO₂ or ASP was depressed by treatments with saturated fatty acids C16:0 or C18:0 alone, correlation of CO₂ values to PC mRNA expression does indicate potential for increased anaplerotic flux of substrates through PC, but the tendency for correlation of ASP oxidation values to PC mRNA expression infers either inadvertent activation of enzymes involved in ketogenesis, or activation of other enzymes, including PCK1, involved in gluconeogenesis.

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Table 3.1. Pyruvate carboxylase (PC) and cytosolic phosphoenolpyruvate carboxykinase (PCK1) mRNA expression after 24 h exposure to fatty acid treatments in Madin-Darby bovine kidney (MDBK) cells.

Transcript ²	Fatty acid treatment ratio ¹									SEM
	C16:0	0.00	1.00	0.90	0.75	0.50	0.25	0.10	0.00	
	C18:3n-3 <i>cis</i>	0.00	0.00	0.10	0.25	0.50	0.75	0.90	1.00	
PC		2.42 ^a	0.36 ^c	ND ³	1.33 ^b	1.97 ^{ab}	2.41 ^a	2.36 ^a	2.29 ^a	0.36
PCK1		2.13 ^{a,b}	0.25 ^e	ND	1.02 ^d	1.65 ^{bcd}	2.17 ^{abc}	2.31 ^{ab}	2.53 ^a	0.27

Transcript ²	Fatty acid treatment ratio									SEM
	C18:0	0.00	1.00	0.90	0.75	0.50	0.25	0.10	0.00	
	C18:3n-3 <i>cis</i>	0.00	0.00	0.10	0.25	0.50	0.75	0.90	1.00	
PC		2.42 ^a	0.13 ^b	0.46 ^b	2.01 ^a	2.13 ^a	2.26 ^a	ND	2.29 ^a	0.32
PCK1		2.13 ^{b,c}	0.06 ^e	0.59 ^{de}	1.28 ^d	1.60 ^{cd}	3.19 ^a	ND	2.53 ^{ab}	0.30

Transcript ²	Fatty acid treatment ratio									SEM
	C18:1n-9 <i>cis</i>	0.00	1.00	0.90	0.75	0.50	0.25	0.10	0.00	
	C18:3n-3 <i>cis</i>	0.00	0.00	0.10	0.25	0.50	0.75	0.90	1.00	
PC		2.42	2.34	2.13	2.27	2.09	2.51	ND	2.29	0.39
PCK1		2.13	2.02	1.98	1.87	1.92	2.28	ND	2.53	0.44

^{a-e}Within fatty acid treatment ratio panel, means with different superscripts within transcript are significantly different ($P < 0.05$).

¹Fatty acid treatments were either single fatty acids (C16:0, C18:0, C18:1n-9 *cis*, or C18:3n-3 *cis*) or combinations of C16:0 with C18:3n-3 *cis* (top), or C18:0 with C18:3n-3 *cis* (middle), or C18:1n-9 *cis* with C18:3n-3 *cis* (bottom) in the molar ratios listed. All treatments totaled 1.0 mM in Dulbecco's Modified Eagle's Medium (DMEM) with 1% BSA. Control treatment is DMEM with 1% BSA and no additional fatty acid.

²Data are presented as ratio of PC:18S mRNA expression or ratio of PCK1:18S mRNA expression (Arbitrary Units). Data are reported as least squares means with associated standard errors.

³ND : Not determined.

Figure 3.1. Oxidation of [1-¹⁴C]C16:0 to CO₂ or acid-soluble products (ASP) after pretreatment with C16:0 and C18:3n-3 *cis*. Oxidation by Madin-Darby bovine kidney (MDBK) cells was measured over a 3 h period after 21 h pretreatments with BSA-bound fatty acids (FA). Fatty acid pretreatments were either single fatty acids (C16:0 or C18:3n-3 *cis*) or combinations of C16:0 with C18:3n-3 *cis* in the molar ratios listed. All treatments totaled 1.0 mM in Dulbecco's Modified Eagle's Medium (DMEM) with 1% BSA. Control is DMEM with 1% BSA and no additional FA. Data are reported as least squares means with associated standard errors. Within substrate fraction, CO₂ or ASP, means without a common superscript differ ($P < 0.05$).

Figure 3.2. Oxidation of [1-¹⁴C]C16:0 to CO₂ or acid-soluble products (ASP) after pretreatment with C18:0 and C18:3n-3 *cis*. Oxidation by Madin-Darby bovine kidney (MDBK) cells was measured over a 3 h period after 21 h pretreatments with BSA-bound fatty acids (FA). Fatty acid pretreatments were either single fatty acids (C18:0 or C18:3n-3 *cis*) or combinations of C18:0 with C18:3n-3 *cis* in the molar ratios listed. All treatments totaled 1.0 mM in Dulbecco's Modified Eagle's Medium (DMEM) with 1% BSA. Control is DMEM with 1% BSA and no additional FA. Data are reported as least squares means with associated standard errors. Within substrate fraction, CO₂ or ASP, means without a common superscript differ ($P < 0.05$).

Figure 3.3. Oxidation of [1-¹⁴C]C16:0 to CO₂ or acid-soluble products (ASP) after pretreatment with C18:1n-9 *cis* and C18:3n-3 *cis*. Oxidation by Madin-Darby bovine kidney (MDBK) cells was measured over a 3 h period after 21 h pretreatments with BSA-bound fatty acids (FA). Fatty acid pretreatments were either single fatty acids (C18:1n-9 *cis* or C18:3n-3 *cis*) or combinations of C18:1n-9 *cis* with C18:3n-3 *cis* in the molar ratios listed. All treatments totaled 1.0 mM in Dulbecco's Modified Eagle's Medium (DMEM) with 1% BSA. Control is DMEM with 1% BSA and no additional FA. Data are reported as least squares means with associated standard errors. Within substrate fraction, CO₂ or ASP, means without a common superscript differ ($P < 0.05$).

Figure 3.4. Relationship between pyruvate carboxylase (PC) mRNA expression and rate of C16:0 metabolism to CO₂ or acid-soluble products (ASP), including ketones. MDBK cells at 80% confluence were cultured with BSA-bound fatty acids (FA) for 24 h (mRNA expression) or 21 h (substrate oxidation). Fatty acid treatments were combinations of fatty acids in 10:90, 25:75, 50:50, or 75:25 molar ratios from combinations of C16:0: C18:3n-3 *cis*, and in 25:75, 50:50, 75:25, or 90:10 molar ratios from combinations of C18:0: C18:3n-3 *cis*, or C18:1n-9 *cis*: C18:3n-3 *cis*. All treatments totaled 1.0 mM in Dulbecco's Modified Eagle's Medium (DMEM) with 1% BSA. Data are reported as least squares means with associated standard errors. Means without a common superscript differ ($P < 0.05$). Pearson correlation coefficients for PC mRNA expression and nmol ¹⁴C substrate conversion to either ¹⁴CO₂ or ASP (per μg DNA⁻¹ · h⁻¹) are shown.

Figure 3.5. Relationship between cytosolic phosphoenolpyruvate carboxykinase (PCK1) mRNA expression and rate of C16:0 metabolism to CO₂ or acid-soluble products (ASP), including ketones. MDBK cells at 80% confluence were cultured with BSA-bound fatty acids (FA) for 24 h (mRNA expression) or 21 h (substrate oxidation). Fatty acid treatments were combinations of fatty acids in 10:90, 25:75, 50:50, or 75:25 molar ratios from combinations of C16:0: C18:3n-3 *cis*, and in 25:75, 50:50, 75:25, or 90:10 molar ratios from combinations of C18:0: C18:3n-3 *cis*, or C18:1n-9 *cis*: C18:3n-3 *cis*. All treatments totaled 1.0 mM in Dulbecco's Modified Eagle's Medium (DMEM) with 1% BSA. Data are reported as least squares means with associated standard errors. Means without a common superscript differ ($P < 0.05$). Pearson correlation coefficients for PCK1 mRNA expression and nmol ¹⁴C substrate conversion to either ¹⁴CO₂ or ASP (per μ g DNA⁻¹ $\cdot$ h⁻¹) are shown.

CHAPTER 4. PYRUVATE CARBOXYLASE PROXIMAL PROMOTER ACTIVITY IS REGULATED BY SATURATED AND UNSATURATED FATTY ACIDS IN MADIN-DARBY BOVINE KIDNEY CELLS

ABSTRACT

An increase in bovine pyruvate carboxylase (PC; EC 6.4.1.1) at calving and during feed restriction corresponds with increased circulating nonesterified fatty acids (NEFA) as a consequence of negative energy balance. Regulation of PC mRNA and impact of saturated and unsaturated fatty acid profile has not yet to be explored. Our objective was to determine effects of chain length, degree of saturation and copresence of saturated and unsaturated fatty acids on activity of bovine PC promoter 1 (PCP1). For these experiments MDBK cells were transfected with a full length bovine PCP1 construct from -1002 to +3 bp relative to the bovine PC gene transcription start site (bovine PCP1_(-1002_+3)) ligated to a Firefly luciferase reporter, or with one of a series of nested 5' serial truncations (bovine PCP1_(-773_+3), bovine PCP1_(-494_+3), or bovine PCP1_(-222_+3)). Cells were exposed for 23 h to either individual fatty acids bound to BSA (C16:0, C18:0, or C18:3n-3 *cis*) or to fatty acid mixtures in ratios of 75:25, 50:50, or 25:75 corresponding to combinations of C16:0: C18:3n-3 *cis* or C18:0: C18:3n-3 *cis*. Total fatty acid concentration was 1.00 mM. Exposure to either C16:0 or C18:3n-3 *cis* alone elicited a significant ($P < 0.05$) increase in bovine PCP1_(-1002_+3) activity compared to 1% BSA

DMEM control treatment (33.97, 50.05, and 19.64 ± 6.15 Fold of promoter induction (AU) for C16:0, C18:3n-3 *cis*, and control, respectively). Treatment with C18:3n-3 *cis* alone caused a greater increase ($P < 0.05$) in promoter activity compared to C16:0 alone, indicating a lesser response to C16:0 alone. Interestingly, inclusion of C18:3n-3 *cis*, at any level in fatty acid ratios examined, increased promoter activity of bovine PCP1_(-773_+3) or bovine PCP1_(-222_+3) compared to activity of bovine PCP1_(-1002_+3). Data from the bovine PCP1 truncation and fatty acid copresence experiments reveal the potential for response elements of unsaturated fatty acids or fatty acid ligands in several bovine PCP1 promoter regions. In silico analysis of bovine PCP1 identified putative peroxisome proliferator-activated receptor (PPAR) α and sterol regulatory element binding protein (SREBP) binding sites which may be implicated in fatty acid signaling to alter bovine PCP1 activity. Pyruvate carboxylase promoter 1 activity that is mediated by unsaturated fatty acids acting through elements within -1002 and -222 of bovine PCP1, may determine PC response during periods of negative energy balance in dairy cows.

Keywords: bovine, fatty acid, pyruvate carboxylase proximal promoter

INTRODUCTION

Pyruvate carboxylase (PC; EC 6.4.1.1) plays a central role in the anaplerotic supply of oxaloacetate (OAA) for the tricarboxylic acid (TCA) cycle and the cataplerotic supply of carbons for gluconeogenesis. Expression of PC mRNA is significantly elevated in dairy cows around the time of calving and during feed restriction, and has been linked to increased fatty acid concentrations (Greenfield et al., 2000; Velez and Donkin, 2005; White et al., 2011a,b). Diets fed during the prepartum interval alter both the concentration and profile of plasma fatty acids in dairy cows at calving (Rukkwamsuk et al., 2000; Douglas et al., 2007; Petit et al., 2007). Likewise, changes in PC mRNA during feed restriction are linked to changes in serum fatty acid concentrations and profiles (White et al., 2011b) and these effects are mediated through promoter 1 and located proximal to the transcription start site (PCP1) of the bovine PC gene. However, there appears to be opposing effects of saturated fatty acids on PC promoter activity (White et al., 2011b,c).

Regulation of PC gene expression by fatty acids has been characterized in several species and appears to be linked through peroxisome proliferator-activated receptors (PPAR; Jitrapakdee et al., 2008). The contrasting effects of unsaturated fatty acids and saturated fatty acids that serve as PPAR agonists and antagonists for bovine PC may be a consequence of differences in modes of signaling for these fatty acids and cross talk that involve sterol regulatory element binding protein (SREBP)-1c and liver X receptor (LXR; Yoshikawa et al., 2003). Direct effects of saturated and unsaturated fatty acids and the interplay between these fatty acids on bovine PCP1 have not been fully elucidated.

Based on previous data indicating opposing effects of C18:0 and C18:3n-3 *cis* on bovine PC mRNA, we hypothesized that fatty acid profile, and specifically the ratio of saturated to unsaturated fatty acids, regulates the activity of bovine PCP1. Our objective was to determine the effects of fatty acid chain length and degree of saturation and the copresence of saturated and unsaturated fatty acids on the activity of bovine PCP1.

Madin-Darby bovine kidney (MDBK) cells were utilized in the current study. Previous research on enzymes associated with gluconeogenic pathways in MDBK cells (Bionaz et al., 2008a,b; Thering et al., 2009; White et al., 2012), and examination of the transcriptomes of MDBK cells compared to bovine liver (Bionaz et al., 2008b) support the use of this cell model system.

MATERIALS AND METHODS

Cell Culture

Madin-Darby bovine kidney epithelial cells (NBL-1 ATCC CCL-22) were obtained at passage 110 from the American Type Culture Collection (ATCC, Manassas, VA). Cells were propagated in sterile polystyrene 150-cm² canted-neck, 0.2 µm vent cap flasks (Corning, Corning, NY) in Dulbecco's Modified Eagle medium (DMEM) containing 1 g/L D-glucose, L-glutamine, and 110 mg/L sodium pyruvate (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Corp., St. Louis, MO), 10 mg/L piperacillin (Sigma-Aldrich Corp.), and 10 mg/L ciprofloxacin (Sigma-Aldrich Corp.), at 37°C in 5% CO₂ and 95% air. Media

was refreshed every two to three d. Cells were subcultured to near confluence following media removal with trypsin-EDTA (Sigma-Aldrich Corp., St. Louis, MO). Trypsin activity was inhibited by addition of 10 mL of fresh DMEM supplemented with 10% FBS (Sigma-Aldrich Corp.), 10mg/L piperacillin and 10mg/L ciprofloxacin. For each experimental replicate, cells were plated on 24-well plates (Corning, Glendale, AZ) at a density of 1.5×10^5 cells per 15.6-mm diameter well and incubated in 10% FBS DMEM with antibiotics, as indicated above, to achieve approximately 80% confluence before exposure to treatments.

Promoter Constructs and DNA Transfections

To test the effects of fatty acids on bovine PCP1 activity, cells were transfected with one of four possible promoter-luciferase constructs containing segments of bovine PCP1 truncated from the 5' end of the bovine PC gene and linked to a reporter gene for firefly luciferase. The full length construct for bovine PCP1 contained bases 613,522 through 612,518 of bovine chromosome 29 (GenBank accession NW_00149451.1), which corresponds to the region from -1,001 through +3 bp relative to the transcription start site of bovine PCP1 (Hazelton et al., 2008). The ligated pGL3 constructs were as previously described (White et al., 2011c), including the full length construct of bovine PCP1 (PCP1_(-1002_+3)), and truncations consisting of -773 through +3 bp (bovine PCP1_(-773_+3)), -494 through +3 bp (bovine PCP1_(-494_+3)), and -222 through +3 bp (bovine PCP1_(-222_+3)) relative to the transcription start site, respectively.

The pGL3 family of plasmids (Promega, Madison, WI) was utilized for the current experiments. The pGL3-Basic plasmid, lacking a promoter sequence, was used as

a negative control for transfection experiments. A luciferase reporter plasmid containing the SV40 promoter, pGL3-SV40, served as a positive control. Co-transfection of cells with a *Renilla* luciferase plasmid, pRL-CMV (Promega), provided a normalization control for transfection efficiency.

Transient transfections in MDBK cells were performed using Lipofectamine 2000 (Invitrogen Inc., Waltham, MA) according to the manufacturer's instructions. For transfection, cells were incubated in media containing 1% BSA (Probumin; Millipore, Billerica, MA) without antibiotics. A volume of 100 μ L of the Lipofectamine-plasmid mixture containing 98 μ L of Opti-MEM I Reduced Serum Medium (Life Technologies Inc., Grand Island, NY), 2 μ L of Lipofectamine, 0.8 μ g of each tested plasmid and 0.008 μ g of a *Renilla* pRL-CMV luciferase plasmid was added to each well. Five hours after transfection, media was removed by aspiration, and replaced with the treatment additions to DMEM containing 1% BSA and without antibiotics. Basal promoter activity and responsiveness to presence of single fatty acids or their combinations were tested during a 23 h treatment interval.

Treatments

Fatty acids C16:0, C18:0, C18:3n-3 *cis* (Sigma-Aldrich Corp.) were bound to BSA (Probumin; Millipore, Billerica, MA) in a 4:1 molar ratio as described previously (Berry et al., 1991). Cells at 80% confluence were cultured in DMEM and exposed for 23 h to either 1% BSA (no addition control), or to 1.0 mM individual fatty acid (bound to BSA), or to combinations of fatty acids in 25:75, 50:50, or 75:25 molar ratios from combinations of C16:0: C18:3n-3 *cis* or C18:0: C18:3n-3 *cis*. Total fatty acid

concentration in each combination was 1.0 mM and total BSA concentration was 1%.

Treatments were applied in triplicate to three cell replicates.

Luciferase Activity

Following 23 h exposure to treatments, media was removed by aspiration and cells were rinsed with ice-cold 1x PBS and harvested in 80 μ L 1 $\times$ passive lysis buffer (Promega, Madison, WI). Cells were removed to a 0.65 mL microcentrifuge tube and stored at -20°C until further processing. Luciferase activity in 20 μ L of cell lysates was initiated by the addition of 100 μ L of firefly luciferase detection reagent followed by addition of 100 μ L of *Renilla* luciferase detection agent (Promega, Madison, WI). Luminescence was quantified using a Tecan GENios Pro spectrofluorometer with Magellan 5.0 software (Tecan, Research Triangle Park, NC). Data were normalized for transfection efficiency by dividing the relative light units detected for firefly luciferase by the corresponding *Renilla* luciferase relative light units (firefly: *Renilla*) for each well. Activity of bovine PCP1 or bovine PCP1 truncations for each treatment was normalized to expression of PGL3-basic. All experiments were repeated in triplicate to three separate cell preparations.

Statistical Analysis

Data were analyzed for normality or error variances using the Univariate procedure of SAS 9.3 (SAS Institute Inc., Cary, NC). Analyses of variance was performed using the Proc Mixed procedure of SAS. The model accounted for the fixed effects of treatment and the random effects of cell replicate. Means were considered

different when $P < 0.05$ and tended to differ if $0.05 \leq P \leq 0.10$. Tukey-Kramer studentized adjustments were used to separate treatment means. Data are reported as least squares means with associated standard errors.

In Silico Promoter Sequence Analysis

Bovine PCP1 sequence (-1002/+3) was analyzed using the PROMO virtual laboratory of ALGGEN, which utilizes TRANSFAC version 8.3 (Messeguer et al., 2002; Farré et al., 2003). The search parameters were limited to liver specific factors, high-quality matrices, and set to minimize false positives.

RESULTS AND DISCUSSION

The MDBK cell line is utilized in the current study to examine the effects of saturated and unsaturated fatty acids on the activity of bovine PCP1. Previous research supports the use of the MDBK cell model through similarities between transcriptomes of the cell line and liver samples from periparturient dairy cows and responsiveness to fatty acids (Bionaz et al., 2008a,b; Thering et al., 2009; White et al., 2012).

Bovine PC contains three promoters which give rise to six 5' UTR variants (Agca et al., 2004; Hazelton et al., 2008). Development of a novel quantitative real-time RT-PCR multiplex assay confirmed the correlation between transcript abundance and transcription rate for bovine 5' UTR variants (White et al., 2011a). Fatty acid treatments created to mimic circulating nonesterified fatty acid (NEFA) profiles around calving or

during induced fatty liver increased expression of PC 5' UTR variant products of bovine PCP1 in MDBK cells (White et al., 2012). This response was not seen after treatment with fatty acid profiles representing the pre- or postpartum period, nor was a response seen in 5'UTR variants of PCP2 or PCP3 (White et al., 2012).

The current study utilized the pGL3-luciferase family of promoter constructs. The negative control was the promoterless parent vector, pGL3-Basic and the pGL3-SV40 Promoter served as the positive control. The positive control pGL3-SV40 Promoter drove luciferase expression by almost 4 fold relative to pGL3-Basic ($P < 0.05$; Figure 4.1). Bovine PCP1_(-1002_+3), as well as any of the examined 5' bovine PCP1 truncations, were able to drive luciferase expression at least 1.5 fold compared to the pGL3-Basic promoterless negative control ($P < 0.05$; Figure 4.1). Bovine PCP1_(-773_+3) and bovine PCP1_(-222_+3)-coupled expression did not differ ($P > 0.10$) from pGL3-SV40 Promoter-driven luciferase expression (Figure 4.1). Bovine PCP1_(-494_+3) drove luciferase expression and did not differ ($P > 0.10$) from bovine PCP1_(-1002_+3)-coupled luciferase expression.

To examine the effects of single fatty acids on bovine PCP1_(-1002_+3) activity, MDBK cells transfected with bovine PCP1_(-1002_+3) were exposed for 23 h to either 1% BSA DMEM, or to 1.0 mM C16:0, C18:0, or C18:3n-3 *cis*. Response in promoter activity was normalized to activity of pGL3-Basic promoterless vector (Figure 4.2). Cells exposed to either C16:0 or C18:3n-3 *cis* alone elicited a significant ($P < 0.05$) increase in bovine PCP1_(-1002_+3) activity (Figure 4.2). Treatment with C18:3n-3 *cis* alone caused a significantly greater increase ($P < 0.05$) in promoter activity compared to C16:0 alone, indicating a greater magnitude of response to C18:3n-3 *cis* alone (Figure 4.2).

Bovine PCP1 was further evaluated to determine the effects of fatty acid treatments on activity of bovine PCP1 5' serial truncations. Compared to bovine PCP1_(-1002_+3), bovine PCP1_(-773_+3) exhibited significantly greater ($P < 0.05$) activity relative to control incubations in 1% BSA DMEM, or after exposure to 1.0 mM C18:3n-3 *cis* (Figure 4.2). Bovine PCP1_(-494_+3) showed a decreased ($P < 0.05$) response to C18:3n-3 *cis* compared to bovine PCP1_(-1002_+3), and response of bovine PCP1_(-494_+3) to other single fatty acids or control did not differ from bovine PCP1_(-1002_+3) response. In contrast, bovine PCP1_(-222_+3) exhibited increased ($P < 0.05$) activity, compared to bovine PCP1_(-1002_+3), after exposure to control 1% BSA DMEM, C16:0, C18:0, or C18:3n-3 *cis* (Figure 4.2). Results presented here indicate potential for C18:3n-3 *cis*-induced response in bovine PCP1_(-1002_+3), bovine PCP1_(-773_+3), and bovine PCP1_(-222_+3) truncation regions, and the potential for C16:0 or C18:0-induced response in bovine PCP1_(-222_+3).

Previously, expression of bovine PCP1 showed a linear decrease in response to increasing concentrations (from 0.25 to 1.0 mM) of C18:0 when bovine PCP1 truncations were transfected into H4IIE rat hepatoma cells (White et al., 2011c). Results from previous studies demonstrate that the activity of bovine PCP1 is repressed after exposure to 1.0 mM C18:0, regardless of the bovine PCP1 truncation, indicating a C18:0-responsive region in within the bovine PCP1_(-222_+3) (White et al., 2011c), which is in agreement with current findings. When compared to PC mRNA expression data in response to these same fatty acid treatment ratios (CHAPTER 2), the bovine PCP1 activity data presented in the current study do not parallel the repression caused by saturated fatty acids compared to control treatments. Because such dramatic changes are seen in mRNA expression, without similar patterns in promoter activity, this suggests that

other factors, such as mRNA degradation or stability, are influenced by the presence of saturated fatty acids and independent experiments are necessary to confirm these effects.

Activity of bovine PCP1 was significantly increased with exposure to serum from feed-restricted, compared to control, cows when bovine PCP1_(-1002_+3) was transfected into the H4IIE rat hepatoma cell line (White et al., 2011b). Taken together with previous data showing an increase in PC mRNA during feed restriction in dairy cows (Velez and Donkin, 2005), and that bovine PC enzyme activity is directly correlated with PC mRNA expression (Greenfield et al., 2000), the potential influence of circulating factors in the serum from feed-restricted cows on PC activity emerges. Modifying the serum from control cows to contain increased concentrations of fatty acids, in proportion to their presence in serum from feed-restricted cows, elicited an increase in bovine PCP1 activity compared with exposure to serum from feed-restricted cows (White et al., 2011b). Data reported in the current study supports the regulation of bovine PCP1 by fatty acids and extends these observations to the direct influence of C16:0, C18:0, and C18:3n-3 on bovine PCP1 activity.

During the transition period, or the 6-wk period surrounding calving in dairy cows, the predominate plasma NEFA profile consists mainly of C16:0, C18:0, and C18:1 fatty acids (Rukkwamsuk et al., 1999). During this time, C18:0 plasma levels increase while C18:1 levels are similar pre- or postpartum (Rukkwamsuk et al., 1999; Douglas et al., 2007). Levels of C16:0 and C18:1 increase in liver of dairy cows that have been induced with fatty liver, compared to control animals, mainly due to release of these fatty acids from adipose tissue lipolysis (Rukkwamsuk et al., 2000). The fatty concentrations in the current experiment (1.0 mM) are similar in magnitude to total circulating NEFA

levels at parturition in dairy cows (Rukkwamsuk et al., 1999). Although treatments with a single fatty acid are not realistic in a physiological setting, previous research that has examined the effect of serum from control and feed restricted cows on bovine PCP1 activity (White et al., 2011b) indicates induction of PC, but the role of specific fatty acids in this regard was unclear. Data from the current study indicates that the increase in PC mRNA is associated with location-specific promoter activation of bovine PCP1 by C18:3n-3 *cis*.

To further examine the interplay for saturated or unsaturated fatty acids on PC gene expression, and to more closely mimic physiological fatty acid profiles, the effects of treatment combinations of saturated and unsaturated fatty acids were interrogated using nested 5' deletions of bovine PCP1. Treatment with 0.75:0.25, 0.50:0.50, or 0.25:0.75 C16:0: C18:3n-3 *cis* increased ($P < 0.05$) activity of bovine PCP1_(-773_+3), compared to activity of bovine PCP1_(-1002_+3) (Figure 4.3). Similarly, treatment with 0.75:0.25, 0.50:0.50, or 0.25:0.75 C16:0: C18:3n-3 *cis* increased ($P < 0.05$) activity of bovine PCP1_(-222_+3), compared to activity of bovine PCP1_(-1002_+3) (Figure 4.3). Similar to treatment with C18:3n-3 *cis* alone, treatments of 0.50:0.50 or 0.25:0.75 C16:0: C18:3n-3 *cis* elicited a significant ($P < 0.05$) decrease in truncated promoter activity compared to activity of bovine PCP1_(-1002_+3) within each treatment (Figure 4.3). Activity of bovine PCP1_(-494_+3) decreased ($P < 0.05$), compared to bovine PCP1_(-1002_+3) activity after exposure to 0.50:0.50, or 0.25:0.75 C16:0: C18:3n-3 *cis* (Figure 4.3). Similar response patterns were seen over all bovine PCP1 truncations with treatment combinations of C18:0: C18:3n-3 *cis* (Figure 4.4). These data again point to both activating and suppressive influences of fatty acids on different bovine PCP1 regions. Because response

to inclusion, at any level, of C18:3n-3 *cis* increased promoter activity of bovine PCP1_(-773_+3) or bovine PCP1_(-222_+3) compared to activity of bovine PCP1_(-1002_+3), these data support the potential for response elements of unsaturated fatty acids or fatty acid ligands in this region.

Transcriptional regulation of genes by long chain fatty acids occurs through a complement of transcription factors, including PPAR α , hepatocyte nuclear factor (HNF)4, and LXR (Pawar and Jump, 2003; Jump, 2013). Because primary regulators of these transcription factors include C18- and C20-carbon fatty acids (Jump, 2013), changes in the circulating NEFA profile concurrent with feed restriction or during parturition in dairy cows are of particular interest.

Ligands capable of binding to PPAR include fatty acids, peroxisome proliferators, antidiabetic, and hypolipidemic drugs (Schoonjans et al., 1996). Activated, ligand-bound PPAR forms a heterodimer with retinoid X receptor before binding to response elements. In liver, the predominant form is PPAR α , which is linked to both fatty acid β -oxidation and lipid metabolism (Lee et al., 1995). Bovine hepatocyte isolations from 7-wk-old calves given treatment of a PPAR α agonist for 5 d showed increased conversion of C16:0 to CO₂ compared to control (Litherland et al., 2010). The activity of bovine PCP1 is upregulated through PPAR α agonists, and suppressed by C18:0 in rat hepatoma cells transfected with bovine PCP1 (White et al., 2011c). Furthermore, responsiveness of bovine PCP1 is regulated by serum fatty acid profiles that are altered during feed restriction and negative energy balance (White et al., 2011b), but the dominance of control of saturated and unsaturated fatty acids, and the concentration required to elicit a response have not yet been defined.

Sterol regulatory element-binding proteins are a family of transcription factors intricately involved in both lipogenesis and cholesterol synthesis. The predominant isoform in liver, SREBP-1c, is increased during over nutrition or with increased circulating glucose concentrations in rodents (Horton et al., 1998), and is downregulated by polyunsaturated fatty acids (Shimano, 2009). Recently, SREBP-1c mediated signaling has been implicated in postprandial increase in C18:0 associated with insulin resistance in diabetic humans and rats (Chu et al., 2013). Liver X receptors are activated by oxysterols and also activate the expression of SREBP-1c (Yoshikawa et al., 2001). This link between cholesterol metabolism and fatty acid metabolism has been further solidified as PPAR α and PPAR α agonists have been shown to reduce the binding LXR to LXR response elements in the SREBP-1c promoter by reducing LXR dimerization to retinoid X receptor (Yoshikawa et al., 2003). This crosstalk between PPAR α and LXR to limit SREBP-1c supports the potential for interplay between saturated and unsaturated fatty acids to influence gene expression and subsequent cellular lipid metabolism.

In silico analysis of the bovine PCP1 using the PROMO virtual laboratory of ALGGEN, which utilizes TRANSFAC version 8.3 (Messeguer et al., 2002; Farré et al., 2003), revealed several putative binding sites for both PPAR α and SREBP (Figure 4.5). Putative PPAR α binding sites are identified at -989 to -933, -401 to -395, and from -73 to -67 bp relative to bovine PCP1_(-1002_+3) transcription start site (TSS; Figure 4.5). Putative SREBP sites are identified at -411 to -397 bp relative to bovine PCP1_(-1002_+3) TSS for SREBP-1 and at -665 to -654 and -408 to -398 bp relative to bovine PCP1_(-1002_+3) TSS for SREBP-2 (Figure 4.5). Finally, a putative LXR site is identified at -74 to -68 bp relative to bovine PCP1_(-1002_+3) TSS (Figure 4.5). Abbreviations for additional putative

transcription factors identified in Figure 4.5 are listed in Table 4.1. Promoter truncation activity in response to fatty acids treatments supports the potential for activation by the presence of C18:3n-3 *cis* of bovine PCP1_(-1002_+3), and bovine PCP1_(-222_+3); regions which contain a putative PPAR α binding site. The lack of response to C18:3n-3 *cis* inclusion for bovine PCP1_(-494_+3) may be attributed to the presence of putative SREBP-1 and SREBP-2 sites, or the potential for crosstalk between these transcription factors and their binding sites. This hypothesis is further supported by the upregulation of bovine PCP1 through PPAR α agonists, and suppression by C18:0 in transfected rat hepatoma cells (White et al., 2011c), and by the potential for crosstalk between PPAR α and LXR to reduce SREBP-1c-induced signaling (Yoshikawa et al., 2003). Taken together, the data reported in the current study support the involvement of both PPAR α and SREBP as the underlying signaling pathways mediating the expression of bovine PCP1.

CONCLUSIONS

Data presented here indicates several potential fatty acid-responsive regions of bovine PCP1. Specifically, that increased activity of bovine PCP1_(-1002_+3), and bovine PCP1_(-222_+3) in response to inclusion of C18:3n-3 *cis* may be due to potential signaling through putative PPAR α binding sites identified in these regions. Furthermore, repression of bovine PC may be linked to putative SREBP binding sites within region -494 through +3 of bovine PCP1. Additional research is necessary to confirm involvement of these transcription factors in regulating bovine PC expression and to determine the precise mechanisms by which fatty acids interact to regulate bovine PCP1 activity.

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Figure 4.1. Basal promoter activity of bovine pyruvate carboxylase promoter 1 (PCP1) and bovine PCP1 regions serially truncated from the 5' end. The longest construct, bovine PCP1_(-1002_+3), and serial 5' truncations, bovine PCP1_(-773_+3), bovine PCP1_(-494_+3), and bovine PCP1_(-222_+3), were individually ligated to Firefly luciferase reporter and transfected in Madin-Darby bovine kidney (MDBK) cells. Cotransfection of *Renilla* pRL-CMV luciferase plasmid was used to normalize transfection efficiency. Five hours after transfection, cells were incubated in DMEM containing 1% BSA without antibiotics for 23 h and luciferase activities were then determined in cell lysates. The pGL3-Basic is promoterless and used as a negative control, and the pGL3-SV40 Promoter served as positive control. Promoter activity is expressed as ratio of Firefly luciferase to *Renilla*

luciferase. Data are reported as least squares means with associated standard errors.

Means without a common superscript differ ($P < 0.05$).

Figure 4.2. Effect of 23 h single fatty acid treatments, C16:0, C18:0, or C18:3n-3 *cis*, on induction of bovine pyruvate carboxylase promoter 1 (PCP1) and bovine PCP1 regions serially truncated from the 5' end in Madin-Darby bovine kidney (MDBK) cells. The longest construct, bovine PCP1_(-1002_+3), and serial 5' truncations, bovine PCP1_(-773_+3), bovine PCP1_(-494_+3), and bovine PCP1_(-222_+3), were individually ligated to Firefly luciferase reporter and transfected into MDBK cells. Cotransfection of *Renilla* pRL-

CMV luciferase plasmid was used to normalize transfection efficiency. Five hours after transfection, cells were exposed to single fatty acid treatments (C16:0, C18:0, or C18:3n-3 *cis*). All treatments totaled 1.0 mM in Dulbecco's Modified Eagle's Medium (DMEM) with 1% BSA. Control treatment is DMEM with 1% BSA and no additional fatty acid. Promoter activity is expressed as ratio of Firefly luciferase to *Renilla* luciferase, normalized to pGL3-Basic promoterless negative control basal expression. Data are reported as least squares means with associated standard errors. Within treatment, asterisk indicates a significant ($P < 0.05$) difference from bovine PCP1_(-1002_+3). Within bovine PCP1 truncation, means without a common superscript differ ($P < 0.05$).

Figure 4.3. Effect of 23 h C16:0: C18:3n-3 *cis* fatty acid treatments on induction of bovine pyruvate carboxylase promoter 1 (PCP1) and bovine PCP1 regions serially truncated from the 5' end in Madin-Darby bovine kidney (MDBK) cells. The longest construct, bovine PCP1_(-1002_+3), and serial 5' truncations, bovine PCP1_(-773_+3), bovine PCP1_(-494_+3), and bovine PCP1_(-222_+3), were individually ligated to Firefly luciferase reporter and transfected into MDBK cells. Cotransfection of *Renilla* pRL-CMV

luciferase plasmid was used to normalize transfection efficiency. Five hours after transfection, cells were exposed to fatty acid combinations in 25:75, 50:50, or 75:25 ratios for the fatty acid combinations of C16:0: C18:3n-3 *cis*. All treatments totaled 1.0 mM in Dulbecco's Modified Eagle's Medium (DMEM) with 1% BSA. Control treatment is DMEM with 1% BSA and no additional fatty acid. Promoter activity is expressed as ratio of Firefly luciferase to *Renilla* luciferase, normalized to pGL3-Basic promoterless negative control basal expression. Data are reported as least squares means with associated standard errors. Within treatment, asterisk indicates a significant ($P < 0.05$) difference from bovine PCP1_(-1002_+3). Within bovine PCP1 truncation, means without a common superscript differ ($P < 0.05$).

Figure 4.4. Effect of 23 h C18:0: C18:3n-3 *cis* fatty acid treatments on induction of bovine pyruvate carboxylase promoter 1 (PCP1) and bovine PCP1 regions serially truncated from the 5' end in Madin-Darby bovine kidney (MDBK) cells. The longest construct, bovine PCP1_(-1002_+3), and serial 5' truncations, bovine PCP1_(-773_+3), bovine PCP1_(-494_+3), and bovine PCP1_(-222_+3), were individually ligated to Firefly luciferase reporter and transfected into MDBK cells. Cotransfection of *Renilla* pRL-CMV

luciferase plasmid was used to normalize transfection efficiency. Five hours after transfection, cells were exposed to fatty acid combinations in 25:75, 50:50, or 75:25 ratios for the fatty acid combinations of C18:0: C18:3n-3 *cis*. All treatments totaled 1.0 mM in Dulbecco's Modified Eagle's Medium (DMEM) with 1% BSA. Control treatment is DMEM with 1% BSA and no additional fatty acid. Promoter activity is expressed as ratio of Firefly luciferase to *Renilla* luciferase, normalized to pGL3-Basic promoterless negative control basal expression. Data are reported as least squares means with associated standard errors. Within treatment, asterisk indicates a significant ($P < 0.05$) difference from bovine PCP1_(-1002_+3). Within bovine PCP1 truncation, means without a common superscript differ ($P < 0.05$).

Figure 4.5. A diagram of bovine pyruvate carboxylase promoter 1 (PCP1; -1002/+3 bp relative to transcription start site) and putative transcription factor binding sites. Putative transcription factor binding sites were identified using the PROMO virtual laboratory of ALGGEN, which utilizes TRANSFAC version 8.3 (Messeguer et al., 2002; Farré et al., 2003). The search parameters were limited to liver specific factors, high-quality matrices, and set to minimize false positives. Abbreviations for common transcription factors are listed in Table 4.1.

Table 4.1. Abbreviations for common transcription factors whose putative binding sites are identified in bovine pyruvate carboxylase promoter 1. Putative transcription factors were identified using the PROMO virtual laboratory of ALGGEN, which utilizes TRANSFAC version 8.3 (Messeguer et al., 2002; Farré et al., 2003).

Abbreviation	Common name
ATF	Activating transcription factor
ATF-4	Activating transcription factor 4
CAR	Constitutive androstane receptor
C-Jun	Jun proto-oncogene
COUP	Chicken ovalbumin upstream transcription factor
CREB	cAMP responsive element binding protein
Ebox	Viral oncogene homolog
FXR	Farnesoid X receptor
GR	Glucocorticoid receptor
HNF1 α	Hepatocyte nuclear factor-1 α
HNF1 β	Hepatocyte nuclear factor-1 β
LRH1	Liver receptor homologous protein
LXR	Liver X receptor
p50	p50; nuclear factor κ B light chain gene enhancer
p65	p65; nuclear factor κ B light chain gene enhancer
PPAR α	Peroxisome proliferator-activated receptor α
PXR	Pregnane X receptor
RAR	Retinoic acid receptor
RXR	Retinoid X receptor
SREBP1	Sterol regulatory element binding protein 1
SREBP2	Sterol regulatory element binding protein 2
USF	Upstream specificity factor
USF1	Upstream specificity factor 1
USF2	Upstream specificity factor 2

CHAPTER 5. CONCLUSIONS AND IMPLICATIONS

Pyruvate carboxylase (PC) is responsible for the synthesis of oxaloacetate (OAA) from pyruvate, a reaction critical to both the anaplerotic push and cataplerotic pull of carbons through the tricarboxylic acid (TCA) cycle. The pool of available OAA appears to be critical in determining the oxidative capacity of the cell and the maintenance of this pool is a function of the balance of anaplerotic and cataplerotic processes in the cell. If the pull of carbons to gluconeogenesis, namely through actions of the enzyme phosphoenolpyruvate carboxykinase (PCK), is less than the supply of carbons to the TCA cycle by PC, then the oxidative capacity of the cell should be increased, yielding increased oxidation of acetyl-CoA. The study of factors, including fatty acids, which influence PC activity and subsequent OAA supply is necessary for a better understanding of the relationship between carbohydrate and lipid metabolism. The central hypothesis of this dissertation is that the ratio of the most prevalent circulating nonesterified saturated and unsaturated fatty acids in dairy cows regulates PC flux and subsequent fatty acid metabolism. The objectives of this dissertation were to determine: 1) the response of PC mRNA to the copresence of the most abundant saturated and unsaturated fatty acids present in circulation in periparturient dairy cows; 2) the direct effects of these saturated and unsaturated fatty acids, and their combinations, on the cellular oxidation of fatty

acids, and the potential relationship to PC; 3) the effects of the copresence of these saturated and unsaturated fatty acids on the transcriptional activity of bovine PC proximal promoter (PCP1).

Characterization of PC response to the copresence of saturated and unsaturated fatty acids was tested in a bovine kidney cell line and in bovine primary hepatocytes. The fatty acids tested included C16:0, C18:0, C18:1n-9 *cis*, and C18:3n-3 *cis*, as these are the predominant circulating fatty acids in periparturient dairy cows. There is a significant depression of PC mRNA expression after exposure to either C16:0 or C18:0 alone in bovine kidney cells; this depression is ameliorated by inclusion of C18:3n-3 *cis* in the kidney cell model. Monounsaturated C18:1n-9 *cis* did not elicit a change in PC mRNA expression, indicating that degree of fatty acid saturation is key for influence on PC. Bovine primary hepatocytes displayed a similar, though not significantly different, numerical pattern in response to fatty acid treatments. In contrast to data from bovine kidney cells, PC expression increased nearly two-fold with exposure to C18:3n-3 *cis* alone in primary hepatocytes. This discrepancy may reflect differences in sensitivity to fatty acid exposure between cell types, cell or in vivo conditions prior to initiation of the experiments, or both.

To determine if changes in PC mRNA expression were related to oxidative capacity of cell, substrate flux was determined following exposure to the copresence of saturated and unsaturated fatty acids. Fatty acid flux to CO₂ was correlated to PC mRNA expression, while fatty acid substrate flux to ketone body production, representing incomplete oxidation, was less evident but still tended to correlate with PC mRNA expression. Substrate flux representing either complete oxidation to CO₂ or partial

oxidation to ketone bodies was depressed by saturated fatty acids and these fluxes both correlate with PCK1 mRNA expression. Activation of enzymes involved in ketogenesis or gluconeogenesis, including PCK, may be tempered by inclusion of factors that specifically activate PC, while only maintaining PCK to promote cellular oxidative capacity.

Activity of bovine PCP1 was directly examined in response to saturated and unsaturated fatty acid treatments using a promoter reporter construct and nested 5' serial promoter truncations. Bovine PCP1 activity increased after exposure to either unsaturated C18:3n-3 *cis* or saturated 16:0 treatments. Promoter truncation experiments evaluating specific regions of bovine PCP1 reveal a region between -773 to -494 bp (bovine PCP1_(-773_+3)) relative to transcription start site (TSS) that is highly responsive to fatty acids. In silico analysis of bovine PCP1 showed several transcription factor binding site consensus sequences within this region. Bovine PCP1 response to the presence of C18:3n-3 *cis* may be due to potential signaling through peroxisome proliferator activated receptor (PPAR) α at potential binding sites within bovine PCP1_(-1002_+3) or within bovine PCP1_(-222_+3). A lack of response to C18:3n-3 *cis* of bovine PCP1_(-494_+3) may be linked to sterol regulatory element binding proteins (SREBP) binding sites, which are responsive to saturated fatty acids. A more rigorous evaluation of these putative transcription factor binding sites is necessary to definitively assign these elements to control of the bovine PC gene.

Taken together, these data indicate that bovine PC mRNA expression is responsive to fatty acids through changes in the activity of the PC proximal promoter.

Furthermore, PC mRNA expression is correlated with complete substrate oxidation to CO₂, supporting the potential role for PC flux to promote cellular oxidative capacity.

This new research adds to our knowledge of the balance between anaplerosis and cataplerosis and the response to metabolic conditions by PC. This information provides new insight that may be applied to better understand the physiological response of dairy cows to the onset of lactation and potential changes in hepatic oxidative capacity during this interval. Nonesterified fatty acids (NEFA) that are mobilized during periods of negative energy balance, including the periparturient period in dairy cattle, are taken up by liver and subsequently are either completely or partially oxidized. The current work confirms that the fates of NEFA are determined, in part, by PC activity thus promoting the pull of acetyl-CoA through the TCA cycle for complete oxidation (Figure 5.1). Because it is also well established that acetyl-CoA serves as an allosteric regulator of PC, and the current data effectively demonstrate the influence that fatty acids have in determining both PC mRNA expression and substrate pull towards oxidation. If the availability of acetyl-CoA is greater than the supply of OAA, excess acetyl-CoA will be incompletely oxidized to ketone bodies. This puts a periparturient dairy cow at increased risk for developing ketosis if PC activity cannot maintain the OAA pool. Novel findings from the current research illustrate the pivotal role for PC in the anaplerotic generation of TCA cycle intermediates, and emphasize the importance of identifying potential factors that specifically promote PC flux while only maintaining but not expanding the pull, by PCK, of carbons for gluconeogenesis. If the ratio of PCK : PC increases, carbon flux will be promoted to gluconeogenesis rather than into the TCA cycle. Bovine PCP1 is responsive to the same physiological factors, primarily circulating fatty acids, which

dramatically increase during the negative energy balance experienced by dairy cattle during the periparturient period.

Data in this dissertation demonstrates for the first time that the response of PC mRNA expression, through bovine PCP1 promoter activity, is dependent on the ratio of saturated to unsaturated fatty acids, with polyunsaturated n-3 fatty acid having strong stimulatory influence. Additional work is necessary to translate this fundamental discovery to application but the potential exists, based on the present data, to program hepatic metabolism in transition cows towards enhanced capacity to metabolize NEFA in favor of complete and partial oxidation and alleviate the potential for hepatic lipid accumulation that is prevalent in the industry. Manipulation of the ratio of fatty acids in plasma through feeding management strategies during critical periods of metabolic stress, if successful, may favor a shift in hepatic metabolism that would aid in the prevention of metabolic disorders in dairy cows. While the present work has focused on lipid metabolism in gluconeogenic tissues, it will be critical going forward to determine the impact of manipulating fatty acid profiles more holistically in the dairy cow and the overall impact on animal health and productivity.

Figure 5.1. A diagram of fatty acid metabolism in dairy cows and impact of pyruvate carboxylase (PC). Nonesterified fatty acids (NEFA) are mobilized from adipose tissue reserves during periods of negative energy balance, including the periparturient period in dairy cows. Metabolized NEFA can have several fates, including esterification and deposition as liver triglycerides (TG), partial oxidation, yielding ketones, or complete oxidation of acetyl-CoA to ATP and CO₂. The capacity of the tricarboxylic acid (TCA) cycle to oxidize acetyl-CoA is dependent upon the available pool of oxaloacetate (OAA). The anaplerotic regeneration of TCA cycle intermediates is supported by PC, which catalyzes the conversion of pyruvate to OAA. If PC activity increases, supply of OAA to the TCA cycle can be increased, only if the activity of phosphoenolpyruvate

carboxykinase (PCK) is maintained, but not concurrently increased. Expression of PC mRNA is, in part, regulated by the profile of fatty acids present, with specific effects of saturated fatty acids (C16:0 or C18:0) to depress PC mRNA expression, which can be recovered with exposure to polyunsaturated C18:3n-3 *cis*. This change in PC mRNA expression directly correlates to the partitioning of C16:0 to CO₂, or a laboratory measurement of ketones. Fatty acids may act to further regulate cellular metabolism by activating transcription factors with response elements in the bovine PC proximal promoter, further potentiating the response of PC to fatty acids. Cytosolic phosphoenolpyruvate carboxykinase, PCK1; mitochondrial phosphoenolpyruvate carboxykinase, PCK2; phosphoenolpyruvate, PEP.

VITA

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Katie Boesche was born in October, 1986 in DeKalb, Illinois. She completed her Bachelor of Science degree at the University of Illinois in 2009 where she was active in the Illini Dairy Club and on the Dairy Judging and Dairy Challenge teams. Katie began her graduate studies at Ohio State University and, in 2011, earned her Master of Science degree in mammary growth and development under the direction of Dr. Kristy Daniels. Katie then continued on to a PhD program at Purdue University where her work with major professor Dr. Shawn Donkin in ruminant nutrition and physiology focuses on bovine pyruvate carboxylase expression in response to saturated and unsaturated fatty acids. Katie is a member of the American Dairy Science Association, the American Society for Nutrition, and on the American Registry of Professional Animal Scientists.