Pretreatment with saturated and unsaturated fatty acids regulates fatty acid oxidation in Madin-Darby bovine kidney cells

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ABSTRACT

Metabolic fates of fatty acids in tissue may be influenced by extracellular concentration and profile of fatty acids. Previous work has demonstrated the 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 synthesis and connects gluconeogenesis from lactate and fatty acid metabolism. Our objective was to determine the effects of co-presence 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 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 21 h 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 3 combinations: C16:0/C18:3n-3 *cis*, 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 3 h to determine the rate of metabolism to CO₂ and ASP collection (per μg DNA⁻¹·h⁻¹). Pretreatment with either C16:0 or C18:0 alone significantly depressed subsequent oxidation of [1-¹⁴C]C16:0 to ASP by 62.7 and 41.2%, respectively, compared with C18:3n-3 *cis* pretreatment. Similar patterns were observed for [1-¹⁴C]C16:0 oxidation to CO₂. Expression of *PC* mRNA was significantly decreased with exposure to either C16:0 or C18:0 compared with expression after exposure to either C18:3n-3 *cis* or control 1% BSA in Dulbecco's modified Eagle's medium. Expression of cytosolic phosphoenolpyruvate carboxykinase (*PCK1*) mRNA followed a similar pat-

tern. Fatty acid treatments containing C18:1n-9 *cis* did not alter *PC* or *PCK1* expression from control or C18:3n-3 *cis* results. Pearson 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 was positively correlated ($r = 0.63$) with *PC* expression, whereas ASP production from [1-¹⁴C]C16:0 only tended to positively correlate ($r = 0.51$) with *PC* mRNA expression. Production of CO₂ or ASP from [1-¹⁴C]C16:0 were both positively correlated ($r = 0.80$ and $r = 0.69$, respectively) with *PCK1* expression. Results show a regulation of ketone production by Madin-Darby bovine kidney cells in response to saturated and unsaturated fatty acid pretreatments. **Key words:** 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 cataplerotically to drain carbon intermediates from TCA cycle toward gluconeogenesis. In cattle, the latter is represented by the combined activities of the cytosolic (**PCK1**) and mitochondrial (**PCK2**) forms of the enzyme, yet to date only PCK1 has been shown to be metabolically responsive (Zhang et al., 2015, 2016). 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 depends on 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 that PCK activity does 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

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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, in contrast to concentrations near 0.2 mM during the prepartum period (Rukkwamsuk et al., 2000). Likewise, fatty acid concentration are transiently elevated in response to stress in calves (Sartorelli et al., 1992). Circulating NEFA are taken up by liver in proportion to their presence in blood (Emery et al., 1992) and are metabolized either through complete or partial oxidation or through triglyceride synthesis and subsequent deposition or loading onto very low density lipoprotein (VLDL) particles for export (reviewed by Drackley et al., 2001; Bobe et al., 2004; van Knegsel 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), an increase in activity of genes associated with liver triacylglycerol synthesis (Loor et al., 2005), 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 in lactating cows. A similar metabolic flexibility may provide opportunities to alleviate metabolic stress in calves.

Increases in fatty acid concentration in plasma at calving and in response to feed restriction during lactation are primarily due to increased palmitic (C16:0), stearic (C18:0), and oleic (C18:1n-9 *cis*) acids with minor contributions from linoleic (C18:2n-6 *cis*) and linolenic (C18:3n-3) acids (Rukkwamsuk et al., 2000; Contreras et al., 2010). Fatty acids are known activators of transcription factors, including peroxisome proliferator-activated receptor- α (PPAR- α ; Jump, 2008) that exert control on gluconeogenesis through changes in gene expression. We have previously determined the direct effects of fatty acids on the bovine PC promoter 1 expression (White et al., 2012). We observed a repression of PC promoter 1 activity with stearic acid and palmitic acid, activation by PPAR- α agonists, and an increase in PC with a cocktail of fatty acids that resemble the plasma pattern observed at calving (White et al., 2012). The data suggest that overall PC expression

is determined by the profile and overall concentration of NEFA. However, 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 co-presence of SFA and UFA 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 SFA to UFA 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- μ m 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 μ g/mL streptomycin, and 250 ng/mL amphotericin B (antibiotic-antimycotic solution; Sigma-Aldrich Corp.) at 37°C in 5% CO₂ and 95% air. Medium was refreshed every 2 to 3 d. Cells were subcultured to near confluence following removal of medium with trypsin-EDTA (Sigma-Aldrich Corp.). Trypsin activity was inhibited by addition of 10 mL of fresh DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 μ g/mL streptomycin, and 250 ng/mL amphotericin B. 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. Experiments were conducted using 3 separate cell preparations and triplicate wells or plates within each replicate. Samples from triplicates were pooled within cell preparation and statistical analysis performed for the 3 independent cell preparations within each experiment.

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% con-

fluence. Medium was changed after 24 h and cells were incubated for 24 h at 37°C and exposed to the same treatment medium 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.

Fatty Acid Pretreatments

Fatty acids C16:0, C18:0, C18:1n-9 *cis*, and 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), 1.0 mM individual fatty acid (bound to BSA), or combinations of fatty acids in 10:90, 25:75, 50:50, or 75:25 molar ratios from combinations of C16:0 and C18:3n-3 *cis*, and in 25:75, 50:50, 75:25, or 90:10 molar ratios from combinations of C18:0 and C18:3n-3 *cis* or C18:1n-9 *cis* and 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 3 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 [$1\text{-}^{14}\text{C}$]C16:0 for 3 h before CO_2 and ASP collection, as previously described (Donkin and Armentano, 1994). Briefly, pretreatment medium was removed and each 35-mm dish (base) was placed into a straight-sided, 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). The MDBK cells were then incubated in DMEM containing 1% BSA and 1.0 mM BSA-bound C16:0 containing $1\text{-}^{14}\text{C}$ labeled C16:0 (Perkin Elmer, Waltham, MA) at approximately 1,000,000 decays per minute (DPM) per flask. Cells were gassed with 95% O_2 :5% CO_2 , immediately sealed, and incubated at 37°C for 3 h. Incubations were terminated by the addition of 0.2 mL of 5 N HClO_4 injected through the stopper of the flask into the medium. A volume of 0.2 mL of phenethylamine was added to each hanging center well containing a filter paper wick to trap $^{14}\text{CO}_2$. 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 was determined by liquid scintillation counting. The rate of fatty acid

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

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 described above. After exposure to treatments for 24 h, medium was removed and cells were disrupted and lysed by adding 350 μL of Buffer RLT (RNeasy Mini Kit, Qiagen Inc., Germantown, MD). Plates were shaken on an orbital shaker (Barnstead International, Dubuque, IA) for 10 min and cell lysates collected in 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.). Purified RNA was quantified for each sample by absorbance at 260 nm using a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE); sample integrity was determined by the ratio of absorbance at 260 to 280 nm. Only samples with a ratio of 260:280 ≥ 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.). 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*, CCAC-GAGTTCTCCAACACCT (forward), TTCTCCTC-CAGCTCCTCGTA (reverse); bovine *PCK1*, AGGGAAATAGCAGGCTCCAGGAAA (forward), CACACGCATGTGCACACACACATA (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 measured via threshold cycle values was not affected by treatment ($P = 0.77$).

Statistical Analysis

Data were analyzed for normality using the Univariate procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC). Analyses of variance was performed using PROC MIXED 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 $[1\text{-}^{14}\text{C}]\text{C16:0}$ substrate conversion to either CO_2 or ASP (nmol per $\mu\text{g DNA}^{-1}\cdot\text{h}^{-1}$) were obtained using PROC CORR (SAS Institute Inc.).

RESULTS AND DISCUSSION

Use of the 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 increased 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.

Analysis of cellular integrity after fatty acid treatments on MDBK cells by the TACS MTT Cell Proliferation Assay indicated significant ($P < 0.05$) reductions in cell viability of 18.3, 24.4, 19.5, and 15.9% with the addition of 1.00 mM C16:0, C18:0, C18:1n-9 *cis*, and C18:3n-3 *cis*, respectively, compared with viability levels of 10% FBS-treated controls in MDBK cells. Despite the effects of single fatty acids, any evaluated combination of 2 fatty acids did not differ ($P > 0.10$) for cell viability measurement from the control 1% BSA DMEM (Figure 1). This indicates that a more optimal environment for cellular growth and proliferation occurs when any 2 of the fatty acids evaluated here were present in the treatment medium 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 medium, 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). Although exposure of hepatocytes to 0.25 mM C18:0 significantly reduced MTT-measured cell viability, co-treatment of 0.25 mM C18:0 with either 0.15 or 0.25 mM C18:3n-3 *cis* restored viability 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 acid 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 expression of *PC* or other transcripts. Although decreases were seen in cell viability in the current study under single fatty acid treatments compared with treatment combinations of fatty acids, these decreases did not occur from treatments containing multiple fatty acids. Furthermore, when observed in the presence of a single fatty acid, the effect on cell viability was considerably less than changes in gene expression and fatty acid metabolism. Therefore, gene expression data, discussed below with

Figure 1. Effect of 24-h fatty acid treatments on cellular viability in Madin-Darby bovine kidney (MDBK) cells. Cells were plated on 96-well cell culture plates and grown to 80% confluence. Medium was changed after 24 h and cells were incubated for 24 h at 37°C and exposed to treatment medium. 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 Viability 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. Means without a common letter (a-c) differ ($P < 0.05$).

combinations of fatty acids, are not likely to be the result of differences in viability but are the result of fatty acid treatment.

Expression of *PC* mRNA was significantly decreased ($P < 0.05$) after exposure to 1.0 mM C16:0 or C18:0, compared with mRNA expression after exposure to C18:3n-3 *cis* or to control 1% BSA DMEM (Table 1). To ameliorate this SFA-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 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 responses 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 with mRNA expression after exposure to C18:3n-3 *cis* or to control 1% BSA DMEM (Table 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 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 increased 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 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 [1-¹⁴C]C16:0 to ASP by 62.7 and 41.2%, respectively, compared with C18:3n-3 *cis* pretreatments in MDBK cells (Figure 2, 3). Similar patterns were seen for [1-¹⁴C]C16:0 oxidation to CO₂. It is well established in rodent models that PUFA 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 CO₂ or ASP using bovine liver slices noted decreased oxidation to CO₂ in liver samples from fasted, compared with control, animals, whereas fasting has no effect on C16:0 metabolism to ASP (Jesse et al., 1986). Dairy cows fed a high-fat diet during the dry period exhibited lower total liver lipid accumulation and lower liver triglyceride deposition during the postpartum period compared with cows receiving an isocaloric high-grain dry period diet or to the control (Grum et al., 1996). The same study illustrated increased prepartum plasma NEFA concentrations but decreased postpartum plasma NEFA

Table 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) cells¹

Transcript	Fatty acid treatment ² ratio: C16:0/C18:3n-3 <i>cis</i>								SEM
	0.00/0.00	1.00/0.00	0.90/0.10	0.75/0.25	0.50/0.50	0.25/0.75	0.10/0.90	0.00/1.00	
<i>PC</i>	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
<i>PCK1</i>	2.13 ^{ab}	0.25 ^c	ND	1.02 ^d	1.65 ^{bcd}	2.17 ^{abc}	2.31 ^{ab}	2.53 ^a	0.27
	Fatty acid treatment ratio: C18:0/C18:3n-3 <i>cis</i>								
	0.00/0.00	1.00/0.00	0.90/0.10	0.75/0.25	0.50/0.50	0.25/0.75	0.10/0.90	0.00/1.00	
<i>PC</i>	2.42 ^a	0.13 ^b	0.46 ^b	2.01 ^a	2.13 ^a	2.26 ^a	ND	2.29 ^a	0.32
<i>PCK1</i>	2.13 ^{bc}	0.06 ^c	0.59 ^{de}	1.28 ^d	1.60 ^{cd}	3.19 ^a	ND	2.53 ^{ab}	0.30
	Fatty acid treatment ratio: C18:1n-9 <i>cis</i> /C18:3n-3 <i>cis</i>								
	0.00/0.00	1.00/0.00	0.90/0.10	0.75/0.25	0.50/0.50	0.25/0.75	0.10/0.90	0.00/1.00	
<i>PC</i>	2.42	2.34	2.13	2.27	2.09	2.51	ND	2.29	0.39
<i>PCK1</i>	2.13	2.02	1.98	1.87	1.92	2.28	ND	2.53	0.44

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

¹Data are LSM for 3 separate cell preparations, with associated SEM. Data are presented as ratio of PC:18S mRNA expression or ratio of PCK1:18S mRNA expression (arbitrary units).

²Fatty 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), 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.

³Not determined.

concentrations in the high-fat group compared with the high-grain or control groups (Grum et al., 1996). 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.

Treatment fatty acid levels in the current study totaled 1.0 mM and were consistent with circulating

Figure 2. 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 for 3 separate cell preparations with associated standard errors. Within substrate fraction, CO₂ or ASP, means without a common letter (a-c) differ ($P < 0.05$).

Figure 3. Oxidation of $[1-^{14}\text{C}]\text{C16:0}$ to CO_2 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 for 3 separate cell preparations with associated standard errors. Within substrate fraction, CO_2 or ASP, means without a common letter (a-c) differ ($P < 0.05$).

NEFA levels in transition dairy cows (Rukkamsuk et al., 1999). In contrast with a normal physiological setting, treatments of single fatty acids alone were 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 whereas C18:1 levels are similar pre- and postpartum (Rukkamsuk et al., 1999; Douglas et al., 2007). These data merited selection of the fatty acids evaluated 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 (Lomax et al., 1983; Armentano et al., 1991; Drackley et al., 1991), which raises the question of relationship to PC activity and links to the current research. Addition of pyruvate to hepatocytes isolated from fasted and fed sheep stimulated C16:0 complete oxidation to CO_2 (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 me-

tabolism 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 with isolated hepatocytes from control animals (Malewiak et al., 1988). Although data from the current study demonstrated significantly ($P < 0.05$) greater production of ASP from $[1-^{14}\text{C}]\text{C16:0}$ compared with CO_2 for all fatty acid pretreatments examined (Figures 2, 3, and 4), production of CO_2 from $[1-^{14}\text{C}]\text{C16:0}$ was greater after fatty acid pretreatment with C18:1n-9 *cis* alone compared with pretreatment combinations containing C18:3n-3 *cis* (Figure 4). 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 with 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 coefficient correlations were used to associate PC mRNA expression and rate of $[1-^{14}\text{C}]\text{C16:0}$ me-

Figure 4. Oxidation of $[1-^{14}\text{C}]\text{C16:0}$ to CO_2 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 for 3 separate cell preparations with associated standard errors. Within substrate fraction, CO_2 or ASP, means without a common letter (a–c) differ ($P < 0.05$).

tabolism to CO_2 or ASP, including ketones. Production of CO_2 from $[1-^{14}\text{C}]\text{C16:0}$ was positively correlated ($r = 0.63$, $P < 0.05$) with *PC* expression, whereas ASP production from $[1-^{14}\text{C}]\text{C16:0}$ only tended to be positively correlated ($r = 0.51$, $0.05 < P < 0.01$) with *PC* mRNA expression (Figure 5). 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 UFA on subsequent mitochondrial and peroxisomal oxidation (Mashek et al., 2002). Results demonstrate increased oxidation of fatty acids to CO_2 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 3 h compared with 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 PUFA, 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. Although a very similar experiment was completed after 48-h treatments in hepatocyte monolayer cultures, the system did not concurrently measure complete oxidation to CO_2 , and only data for ASP production are available (Mashek and Grummer, 2003). Treatment of bovine hepatocytes with C18:0 included in the treatment medium resulted in the highest rates of ASP production from C16:0 (Mashek and Grummer, 2003). Taken together, these data indicate a potential role for SFA in the promotion of substrate metabolism to ASP, perhaps linked to UFA as a poor substrate for triglyceride esterification and promotion of substrate metabolism to CO_2 .

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 coefficient correlations were also used to associate *PCK1* mRNA expression and rate of $[1-^{14}\text{C}]\text{C16:0}$ metabolism to CO_2 or ASP. Production of CO_2 or ASP from $[1-^{14}\text{C}]\text{C16:0}$ were both positively cor-

Figure 5. Relationship between pyruvate carboxylase (*PC*) mRNA expression and rate of C16:0 metabolism to CO₂ or acid-soluble products (ASP), including ketones. Madin-Darby bovine kidney (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 0.10:0.90, 0.25:0.75, 0.50:0.50, or 0.75:0.25 molar ratios from combinations of C16:0/C18:3n-3 *cis*, and in 0.25:0.75, 0.50:0.50, 0.75:0.25, or 0.90:0.10 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 for 3 separate cell preparations. Pearson correlation coefficients for PC mRNA expression and nanomoles of ¹⁴C substrate conversion to either ¹⁴CO₂ or ASP (per μg DNA⁻¹·h⁻¹) are shown.

related ($r = 0.80$ and $r = 0.69$, respectively; $P < 0.05$) with *PCK1* expression (Figure 6). Understanding the balance of PC and PCK1 activities is key to determining 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. Although additional research is needed to establish the mechanisms by which fatty acids act to modulate *PC* mRNA expression, evidence is accumulating for a role of 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 with 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 both occur 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).

The transition to lactation, transient feed restriction, the interval around the weaning transition in calves, and the response to elevated glucocorticoids with stress, or exogenously administered glucocorticoids all represent physiological conditions in dairy cattle that are accompanied by elevated plasma NEFA. Our goal was to determine effects of the elevated co-presence of SFA and UFA pretreatments on cellular partitioning of fatty acid metabolism in a bovine model of cellular energy metabolism. The proportions of fatty acids tested were intended to represent the extremes of SFA to UFA ratios. Although these single and dual mixtures

Figure 6. Relationship between cytosolic phosphoenolpyruvate carboxykinase (*PCK1*) mRNA expression and rate of C16:0 metabolism to CO₂ or acid-soluble products (ASP), including ketones. Madin-Darby bovine kidney (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 0.10:0.90, 0.25:0.75, 0.50:0.50, or 0.75:0.25 molar ratios from combinations of C16:0/C18:3n-3 *cis*, and in 0.25:0.75, 0.50:0.50, 0.75:0.25, or 0.90:0.10 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 for 3 separate cell preparations. Pearson correlation coefficients for *PCK1* mRNA expression and nanomoles of ¹⁴C substrate conversion to either ¹⁴CO₂ or ASP (per μg DNA⁻¹·h⁻¹) are shown.

of fatty acids represent extremes and are not likely to occur in vivo, there are examples where C18:3n-3 is elevated. Concentrations of C18:3n-3 represented 10% of total fatty acids in lactating cows fed extruded flaxseed (Gonthier et al., 2004), 7% C18:3n-3 has been observed in plasma from cows fed flaxseed oil from prepartum through 100 DIM (Moallem et al., 2013), and plasma concentration of 17% C18:3n-3 has been observed in preweaned calves fed flaxseed oil in milk replacer (Schossow, 2019). In this study, we indicated the potential of C18:3n-3 *cis* to affect PC expression and fatty acid concentration. Further effort is needed to determine the potential role of elevated C18:3n-3 on modulating the capacity for fatty acid oxidation in vivo in both cows and calves. Likewise, information on the responses to elevated C18:3n-3 when present in complex mixtures of fatty acids is needed to provide a more complete understanding of the role of C18:3n-3 in regulating PC and PCK1 expression and lipid metabolism in dairy cattle. A working hypothesis for the 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 whether a similar relationship holds for bovine liver as for the MDBK cell model examined here.

CONCLUSIONS

We demonstrated regulation of ketone production by MDBK cells in response to saturated and unsaturated fatty acid pretreatments. Although oxidation of [1-¹⁴C] C16:0 to either CO₂ or ASP was depressed by treatments with the SFA C16:0 or C18:0 alone, correlation of CO₂ values to *PC* mRNA expression indicated the potential for increased anaplerotic flux of substrates through PC. We detected a tendency for correlation of ASP oxidation values with *PC* mRNA expression, implying either inadvertent activation of enzymes involved in ketogenesis or activation of other enzymes, including PCK1, involved in gluconeogenesis.

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