Effects of propionate concentration on short-term metabolism in liver explants from dairy cows in the postpartum period

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ABSTRACT

Our objective was to determine the temporal effects of increasing supply of propionate on propionate metabolism in liver tissue of dairy cows in the postpartum (PP) period. A total of 6 dairy cows [primiparous: $n = 3$, 9.00 ± 1.00 d PP (mean $\pm$ SD) and multiparous: $n = 3$; 4.67 ± 1.15 d PP] were biopsied for liver explants in a block-design experiment. Explants were treated with 3 concentrations of [$^{13}\text{C}_3$]sodium propionate of 1, 2, or 4 mM. Explants were incubated in 2 mL of Medium 199 supplemented with 1% BSA, 0.6 mM oleic acid, 2 mM sodium L-lactate, 0.2 mM sodium pyruvate, and 0.5 mM L-glutamine at 38°C and sampled at 0.5, 15, and 60 min. Increasing the concentration of [$^{13}\text{C}_3$]propionate increased total $^{13}\text{C}\%$ enrichment of propionyl coenzyme A (CoA), succinate, fumarate, malate, and citrate with time. Concentration of propionate did not affect total $^{13}\text{C}\%$ enrichment of hepatic glucose or acetyl CoA, but total $^{13}\text{C}\%$ enrichment increased with time for hepatic glucose. The ^{13}C labeling from propionate was incorporated into acetyl CoA, but increased concentrations of propionate did not result in greater labeling of acetyl CoA. However, increases in $^{13}\text{C}\%$ enrichment of [M+4] citrate and [M+5]citrate concentrations of [$^{13}\text{C}_3$]propionate indicate propionate conversion to acetyl CoA and subsequent entry of acetyl CoA into the tricarboxylic acid cycle in dairy cows in the PP period. This research presents evidence that despite an increase in hepatic acetyl CoA concentration and general consensus on the upregulation of gluconeogenesis of dairy cows during the PP period, carbon derived from propionate contributes to the pool of acetyl CoA, which increases as concentration of propionate increases, in addition to

stimulating oxidation of acetyl CoA from other sources. Because of the hypophagic effects of propionate, but importance of propionate as a glucose precursor, a balance of propionate supply to dairy cows could lead to improvements in dry matter intake, and subsequently, health and production in dairy cows.

Key words: propionate, hepatic metabolism, dairy cow

INTRODUCTION

Dairy cows in the immediate postpartum (PP) period experience negative energy balance from increased demands for milk production (Bauman and Currie, 1980). These animals exhibit a suppression in appetite (Allen and Piantoni, 2013), exacerbating the negative energy balance. To increase energy intake, cereal grains containing starch are frequently substituted for fiber sources in the diet of early-lactation cows. Propionic acid produced from starch fermentation is a primary glucose precursor in dairy cows. However, propionate has hypophagic effects on dairy cows in the PP period (Allen, 2000) and has been shown to decrease DMI to a greater extent than the glucose precursors lactate (Gualdrón-Duarte and Allen, 2018) and glycerol (Gualdrón-Duarte and Allen, 2017).

The metabolic pathway of propionate in the liver of ruminants is well-established. However, the majority of work examining propionate metabolism in ruminants was conducted in cell or explant cultures from calves (Aiello et al., 1989; Donkin and Armentano, 1995), goats (Aiello and Armentano, 1987; Demigné et al., 1991), or cows past the PP period (Drackley et al., 1991; Knapp et al., 1992; Zhang et al., 2015). Dairy cows in the PP period are in a lipolytic state with higher concentrations of hepatic acetyl CoA compared with late-lactation dairy cows (Piantoni et al., 2015), and the majority of this pool is derived from hepatic β -oxidation of circulating plasma free fatty acids. Additionally, gluconeogenesis and pyruvate carboxylase, the enzyme responsible for converting pyruvate to oxaloacetate, are upregulated during this stage of lacta-

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tion (Greenfield et al., 2000; Aschenbach et al., 2010). Therefore, the majority of propionate is used for glucose synthesis. However, propionate might contribute to the pool of acetyl CoA as well. As part of gluconeogenesis, carbon derived from propionate exits the tricarboxylic acid (TCA) cycle as malate and converts to phosphoenolpyruvate through oxaloacetate. Phosphoenolpyruvate can either continue through gluconeogenesis to synthesize glucose or convert to pyruvate. Pyruvate, in turn, can be decarboxylated to acetyl CoA through the pyruvate dehydrogenase complex. Although elevated acetyl CoA and ATP concentrations inhibit the pyruvate dehydrogenase complex, a recent study using liver slices from early lactation dairy cows (~12 DIM) incubated for 2 h with labeled pyruvate and unlabeled propionate reported an increase in flux of pyruvate to acetyl CoA (Garcia et al., 2015). Likewise, the short-term presence of unlabeled propionate reduced the flux of radiolabeled lactate to glucose in bovine hepatocytes (Donkin and Armentano, 1994), indicating offsetting changes in pyruvate metabolism. In a previous study in our laboratory, potential metabolic bottlenecks of propionate metabolism, such as succinate dehydrogenase and enzymes sensitive to NADH concentrations, were identified within 20 min of a pulse dose of propionic acid, including an increase in pyruvate concentration (Kennedy and Allen, 2019a). Understanding the short-term hepatic metabolism of propionate in dairy cows in the PP period may help to develop improved feeding and management strategies during this period.

Our objective was to determine the direct, short-term temporal effects of increasing concentrations of propionate on propionate metabolism in liver tissue of dairy cows in the PP period. We hypothesized that the majority of propionate is used for synthesis of glucose, but that increasing the concentration of propionate will increase carbon derived from propionate converted to acetyl CoA for oxidation in the TCA cycle.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at Michigan State University approved all animal experimental procedures for this experiment.

Experimental Design, Treatments, and Animal Management

Six dairy cows (3 primiparous and 3 multiparous) were used in a block-design experiment. Cows were housed in tiestalls at the Michigan State University Dairy Cattle Teaching and Research Center (East Lansing, Michigan). Animals were blocked by parity and fed

a common ration formulated to contain 27% NDF, 23% forage NDF, 25% starch, and 18.4% CP that consisted of corn silage, ground corn, soybean meal, alfalfa silage, alfalfa hay, soybean hulls, and a premix of minerals and vitamins. Primiparous animals were 9.00 ± 1.00 (mean $\pm$ SD) d PP at the time of biopsy and had a BCS of 3.08 ± 0.30 and BW of 618 ± 74.9 kg. The multiparous animals (≥ 3 parity) were 4.67 ± 1.15 d PP at the time of biopsy and had a BCS of 3.50 ± 0.65 and BW of 755 ± 73.9 kg. Treatments consisted of 3 concentrations of [$^{13}\text{C}_3$]sodium propionate (99 atom % ^{13}C , Sigma Aldrich, St. Louis, MO) at 1, 2, or 4 mM and sampled at 0.5, 15, and 60 min. The propionate concentrations (PrC) were chosen to bracket the 2.5 mM propionate concentration used in previous experiments (Donkin and Armentano, 1995; Zhang et al., 2015).

Data Collection and Analysis

Prior to the liver biopsy, animals were moved to a surgery room at the Michigan State Dairy Cattle Research and Teaching Center. Liver was biopsied before the morning feeding at approximately 0800 h. Cows were blocked from feed for approximately 30 min before biopsy and 1 cow was biopsied per day. Liver tissue (~300–400 mg) for the experiment was biopsied as described by Kennedy and Allen (2019a). Tissue was rinsed in sterile ice-cold saline to remove excess blood and then placed in ice-cold supplemented Medium 199 (Sigma Aldrich) for immediate transport to the laboratory. Explants were prepared from biopsied tissue as described by Zhang et al. (2015) and were approximately 4 mm thick and 9 mm in diameter. Explants were incubated in 2 mL of Medium 199 supplemented with 1% BSA (GenDEPOT, Katy, TX), 0.6 mM oleic acid (Sigma Aldrich), 2 mM sodium L-lactate (Sigma Aldrich), 0.2 mM sodium pyruvate (Sigma Aldrich), 0.5 mM L-glutamine (Sigma Aldrich), and either 1, 2, or 4 mM [$^{13}\text{C}_3$]propionate in 20-mL glass scintillation vials (Research Products International, Mt. Prospect, IL). Prior to addition of tissue, the medium was equilibrated to 38°C. Vials were flushed with 95% O_2 :5% CO_2 , capped, and then gently agitated at 40 strokes per minute at 38°C in a Precision Reciprocal Shaking Bath (Thermo Fisher Scientific, Waltham, MA) until the designated sampling time. Samples were then removed from the medium with sterile tweezers, rapidly blotted (~10 s) on Kimwipes (Kimtech Science, Kimberly-Clark Professional, Roswell, GA), placed in 2-mL screw-cap tubes with O-ring caps (Thomas Scientific; Swedesboro, NJ), and dropped in liquid nitrogen. Samples were stored at -80°C until analysis. Tissue not used for explants was frozen in liquid nitrogen as

described and used as a baseline (0 min) and correction factor for the natural abundance of isotopes.

Explants were analyzed for citrate, isocitrate, succinate, fumarate, malate, pyruvate, lactate, glutamate, oxaloacetate (**OAA**), α -ketoglutarate, and glucose by GC-MS and for acetyl CoA and propionyl CoA by liquid chromatography–tandem MS (**LC-MS/MS**). Samples were prepared for mass spectrometry analysis and analyzed on GC-MS and LC-MS/MS using the methods described by Kennedy and Allen (2019b), except additional m/z values were monitored to account for complete ^{13}C tracer incorporation into the metabolites ($[M + n]$). Samples analyzed on GC-MS were derivatized by methoximation and silylation as described by Kennedy and Allen (2019a). Machine run was defined as the group of samples analyzed on a mass spectrometer within a single session (GC-MS). Area quantification of peaks were integrated using MassLynx Mass Spectrometry Software (version 4.1, Waters Corporation, Milford, MA). Naturally low abundance of isocitrate caused data to be undetectable or unreliable with higher isotopologues, thus the data were not included. Additionally, an unknown mass at $[M+2]$ for OAA and α -ketoglutarate interfered with those isotopologues by masking their abundance, including unlabeled samples, and thus the data were not included. Quantified area was corrected using the recorded weight of each explant (grams of wet weight). Limit of detection was calculated (Armbruster and Pry, 2008) and undetectable values were assigned a value of limit of detection/2 (Hornung and Reed, 1990). Total percent of ^{13}C enrichment of each metabolite was calculated as modified from Wang and Jones (2014) using the following equation:

$$^{13}\text{C}\% \text{ enrichment} = \frac{\left(\frac{\bar{M}_L - \bar{M}_U}{^{13}\text{C}_{0.00335} - ^{12}\text{C}} \right) \times 100}{n \text{ carbons in metabolite}},$$

where $\bar{M}_L$ is the weighted average isotopologue mass of the labeled metabolite, $\bar{M}_U$ is the weighted average isotopologue mass of the unlabeled metabolite, and the denominator is the difference between ^{13}C and ^{12}C atomic masses. Additionally, this equation adjusts for the number of carbons within each metabolite, not including carbons within CoA. This equation is a simple representation of stable isotope labeling and calculates the total percent increase of ^{13}C over its natural abundance that is present within a metabolite (i.e., it does not distinguish between the distribution of isotopologues within the metabolite).

Additionally, the isotope enrichment, expressed as molar percent, was calculated for the isotopologues $[M+3]$ propionyl CoA, $[M+2]$ pyruvate, $[M+1]$ acetyl

CoA, $[M+2]$ acetyl CoA, $[M+3]$ citrate, $[M+4]$ citrate, $[M+5]$ citrate, $[M+2]$ glutamate, $[M+3]$ glutamate, $[M+2]$ glucose, and $[M+3]$ glucose as follows: (1) integrated areas per gram were normalized to $[M+0]$ as a percentage, (2) they were multiplied by a correction matrix based on the stable isotope distribution of each compound in unlabeled samples (Fernandez et al., 1996), and (3) each isotopologue was divided by the total isotopologue abundance (Wilson et al., 2017). The enrichment of each isotopologue is relative to the mass distribution of all the isotopologues in the metabolite and is on a scale of 0 to 100%. The isotopologues $[M+2]$ pyruvate, $[M+1]$ acetyl CoA, $[M+2]$ acetyl CoA, $[M+3]$ citrate, $[M+4]$ citrate, and $[M+5]$ citrate were examined because they are key points in determining if acetyl CoA was synthesized from propionate and entered the TCA cycle for oxidation. Because of known labeling patterns of labeled propionate (Supplemental Figure S1, <https://doi.org/10.3168/jds.2020-18914>; Kelleher, 1986; Previs and Kelley, 2015), an increase in $[M+2]$ pyruvate or $[M+3]$ pyruvate is expected, which would result in $[M+1]$ acetyl CoA or $[M+2]$ acetyl CoA through the pyruvate dehydrogenase complex. The labeled acetyl CoA will then condense with $[M+2]$ - or $[M+3]$ oxaloacetate to produce $[M+4]$ - or $[M+5]$ citrate. Because the carbons derived from acetyl CoA are not oxidized during the initial turn of the TCA cycle, unlabeled acetyl CoA will dilute labeled citrate; as such, an enrichment greater than $[M+3]$ citrate would be unexpected from the anaplerosis of $[^{13}\text{C}_3]$ propionate in ruminants. Therefore, enrichment of $[M+4]$ citrate or $[M+5]$ citrate indicates labeled pyruvate entering the TCA cycle via labeled acetyl CoA. All $[M + n]$ of glutamate from propionate results from flux of the TCA cycle as the labeling must have derived from α -ketoglutarate through glutamate dehydrogenase (Garcia et al., 2015; Zhang et al., 2015).

Statistical Analysis

All data were analyzed with mixed models (PROC MIXED) and repeated measures using SAS software (version 9.4, SAS Institute Inc., Cary, NC). Total $^{13}\text{C}\%$ enrichment of each metabolite and isotope enrichment of select isotopologues except for CoA compounds (LC-MS/MS analysis) were analyzed with the following model:

$$Y_{ijklm} = \mu + C_i(P_j, R_k) + P_j + R_k + T_l + S_m + T_l S_m + T_l P_j + T_l R_k + P_j S_m + T_l S_m P_j + e_{ijklm},$$

where Y_{ijklm} = the response variable; μ = overall mean; $C_i(P_j, R_k)$ = random effect of cow i nested within parity

j and machine run k ; P_j = fixed effect of parity j ; R_k = fixed effect of machine run k ; T_l = fixed effect of PrC l ; S_m = fixed effect of sampling time m ; $T_l S_m$ = interaction of PrC and sampling time; $T_l P_j$ = interaction of PrC and parity; $T_l R_k$ = interaction of PrC and machine run; $P_j S_m$ = interaction of parity and sampling time; $T_l S_m P_j$ = interaction of PrC, sampling time, and parity; and e_{ijklm} = residual. The CoA compounds were analyzed with the same model as above except the effect of machine run was not included in the model as all samples were analyzed within the same machine run.

Time was used in the repeated statement to account for repeated measures and the subject was defined as PrC by either cow nested within parity by machine-run interaction (GC-MS analyses) or cow nested within parity (LC-MS/MS analysis). The covariance structure was compound symmetry and denominator degrees of freedom were estimated by using the Kenward-Roger option in the MODEL statement. Paired differences were determined using the diff option with a Tukey adjustment in PROC MIXED.

Main effects and interactions were declared significant at $P \leq 0.05$ and tendencies for significant main effects and interactions were declared at $0.05 < P \leq 0.10$. Box-Cox transformation analyses were applied to response variables that violated the assumptions of normality, homogeneity of residuals, or both as a guide for determining the necessary transformations on the data using JMP Pro (version 13.2, SAS Institute Inc.). Data transformed are noted in the tables and figures along with the transformation used for statistical analysis. All transformed data has been back-transformed for interpretation.

RESULTS

Total $^{13}\text{C}\%$ Enrichment

All metabolites had highly significant increases in total $^{13}\text{C}\%$ enrichment over time (Figure 1; $P < 0.0001$) except for acetyl CoA ($P = 0.19$). An increase in total $^{13}\text{C}\%$ enrichment between baseline and 0.5 min was observed for propionyl CoA, succinate, fumarate, and glucose (Table 1; pairwise tests, $P \leq 0.01$). No significant PrC by machine run interactions were detected ($P > 0.15$).

Increased concentrations of [$^{13}\text{C}_3$]propionate increased total $^{13}\text{C}\%$ enrichment of succinate, fumarate, malate, citrate, glutamate, pyruvate, and lactate over time (Table 2; Figure 1; interaction, $P \leq 0.01$). Propionyl CoA tended to differ for a 3-way interaction of PrC by parity by time (Figure 1A; $P = 0.08$). However, parity groups did not differ among PrC at each time point for pro-

pionyl CoA. Propionyl CoA also significantly increased over time with increased concentrations of [$^{13}\text{C}_3$]propionate ($P < 0.01$). Total $^{13}\text{C}\%$ enrichment of propionyl CoA was increased for the 4 mM PrC compared with the 1 mM and 2 mM PrC at 0.5 min (1 mM: 17.4%, 2 mM: 23.2%, 4 mM: 33.8% enrichment; pairwise tests, $P \leq 0.05$), but we detected no difference between the 1 and 2 mM PrC at 0.5 min (pairwise tests, $P = 0.74$). Additionally, total $^{13}\text{C}\%$ enrichment of propionyl CoA was increased for 2 and 4 mM PrC compared with 1 mM PrC at 15 (1 mM: 35.2%, 2 mM: 47.8%, 4 mM: 50.7% enrichment) and 60 min (1 mM: 51.3%, 2 mM: 62.5%, 4 mM: 65.0% enrichment; pairwise tests, $P \leq 0.03$), but we detected no difference between the 2 and 4 mM PrC at 15 and 60 min (pairwise tests, $P = 1.00$).

The PrC tended to interact with parity for total $^{13}\text{C}\%$ enrichment of glucose ($P = 0.08$) and citrate ($P = 0.09$); however, no differences were detected between parities at each PrC (pairwise tests, $P > 0.10$). Multiparous cows had increased total $^{13}\text{C}\%$ enrichment of citrate with the 2 mM and 4 mM PrC compared with 1 mM PrC (1 mM: 3.97% vs. 2 mM: 5.41% or 4 mM: 6.00% enrichment; pairwise tests, $P \leq 0.01$). Primiparous cows also had increased total $^{13}\text{C}\%$ enrichment of citrate with the 2 mM and 4 mM PrC compared with 1 mM PrC (1 mM: 3.62% vs. 2 mM: 4.41% or 4 mM: 4.86% enrichment; pairwise tests, $P < 0.05$). The PrC concentrations did not differ within multiparous or primiparous cows for total $^{13}\text{C}\%$ enrichment of glucose ($P > 0.35$).

Concentration of [$^{13}\text{C}_3$]propionate had no effect on total $^{13}\text{C}\%$ enrichment of glutamate, glucose or acetyl CoA (Table 2; $P > 0.15$). We detected a main effect ($P \leq 0.01$) for [$^{13}\text{C}_3$]propionate concentration on total $^{13}\text{C}\%$ enrichment of propionyl CoA, succinate, fumarate, malate, citrate, pyruvate, and lactate.

$^{13}\text{C}\%$ Enrichment of Isotopologues

All isotopologues increased in $^{13}\text{C}\%$ enrichment over time (Table 3; Figure 2; $P < 0.0001$) except for [M+1]- and [M+2]acetyl CoA ($P > 0.50$). A tendency for a PrC by machine run interaction was detected for [M+2]pyruvate ($P = 0.09$); however, no differences were detected between machine runs within PrC (pairwise tests, $P > 0.35$).

Effect of different concentrations of [$^{13}\text{C}_3$]propionate on $^{13}\text{C}\%$ enrichment of isotopologues are shown in Table 3. The $^{13}\text{C}\%$ enrichment of [M+2]pyruvate, [M+1]acetyl CoA, [M+3]citrate, [M+4]citrate, [M+5]citrate, and [M+3]glutamate had significant PrC by time interactions (Figure 2; $P \leq 0.05$), and [M+2]glutamate had a tendency for a PrC by time interaction ($P =$

Figure 1. Effects of concentration of $[\text{C}_3]$ propionate on total $^{13}\text{C}\%$ enrichment above natural abundance of hepatic propionyl CoA (A), succinate (B), fumarate (C), malate (D), citrate (E), glutamate (F), pyruvate (G), lactate (H), glucose (I), and acetyl CoA (J) over 60 min. Total $^{13}\text{C}\%$ enrichment is the percent increase of ^{13}C within each metabolite above the natural abundance and adjusted for the number of carbons in each metabolite. Time points are baseline (0 min), 0.5, 15, and 60 min following the initiation of incubation. Graphs are presented as highest level of significance or tendency for significant interaction, and associated P -values can be found in Table 2. Time effects are presented at the bottom of each graph with letters in order of baseline/0.5/15/60 min; different letters a–d differ from each other ($P \leq 0.05$). Concentration of $[\text{C}_3]$ propionate by time interactions at each time point were examined for differences among concentrations of $[\text{C}_3]$ propionate. Letters a–c are presented in the order of 1/2/4 mM $[\text{C}_3]$ propionate concentrations; different letters differ from each other ($P \leq 0.05$), and * between letters signifies a tendency for difference ($0.05 < P \leq 0.10$). Significant results from pairwise tests for the 3-way interactions are described in the text. The total $^{13}\text{C}\%$ enrichment of fumarate and lactate were transformed using $\log(n+1)$ for statistical analysis and back-transformed for interpretation. The total $^{13}\text{C}\%$ enrichment of citrate was transformed using square root ($n+1$) for statistical analysis and back-transformed for interpretation. The total $^{13}\text{C}\%$ enrichment of glutamate and glucose was transformed using inverse ($n+1$) for statistical analysis and back-transformed for interpretation.

Table 1. Difference of total $^{13}\text{C}\%$ enrichment above natural abundance of select hepatic metabolites with significant sampling time effect (Table 2; $P < 0.0001$), assuming 0% total ^{13}C enrichment (baseline, 0 min) and after incubation of explant for approximately 0.5 min in [$^{13}\text{C}_3$]propionate

Metabolite ¹	0 min	0.5 min	<i>P</i> -value ²
Propionyl CoA	0.00 ^(-2.50, 2.50)	24.8 ^(22.3, 27.3)	<0.0001
Succinate	0.00 ^(-0.62, 0.62)	1.77 ^(1.15, 2.39)	<0.01
Fumarate ³	0.00 ^(-0.05, 0.06)	0.45 ^(0.37, 0.53)	<0.0001
Malate	0.00 ^(-0.79, 0.79)	0.38 ^(-0.42, 1.17)	0.76
Citrate ⁴	0.00 ^(-0.17, 0.18)	0.09 ^(-0.08, 0.28)	0.76
Glutamate ⁵	0.00 ^(-0.02, 0.02)	0.00 ^(-0.01, 0.02)	0.99
Pyruvate	0.00 ^(-0.26, 0.26)	0.02 ^(-0.24, 0.28)	1.00
Lactate ³	0.00 ^(-0.06, 0.07)	-0.06 ^(-0.12, 0.01)	0.29
Glucose ⁵	0.00 ^(-0.06, 0.07)	-0.08 ^(-0.13, -0.02)	0.01

¹Total percent of ^{13}C enrichment above natural abundance adjusted for number of carbons in each metabolite. Values reported as LSM^(95% CI).

²Pairwise comparisons were adjusted with Tukey's method.

³Data transformed using log ($n + 1$) for statistical analysis and back-transformed for interpretation.

⁴Data transformed using square root ($n + 1$) for statistical analysis and back-transformed for interpretation.

⁵Data transformed using inverse ($n + 1$) for statistical analysis and back-transformed for interpretation.

0.08). The $^{13}\text{C}\%$ enrichment of [M+3]citrate tended to be greater with the 4 mM than 2 mM PrC at 60 min (pairwise test, $P = 0.06$). Additionally, $^{13}\text{C}\%$ enrichment of [M+2]glutamate tended to be greater with the 2 mM than 1 mM PrC at 60 min (pairwise test, $P = 0.10$), and $^{13}\text{C}\%$ enrichment of [M+3]glutamate tended to be greater with the 4 mM than 1 mM PrC at 60 min (pairwise test, $P = 0.06$). The PrC, time, and parity tended to interact for $^{13}\text{C}\%$ enrichment of [M+3]

propionyl CoA (Figure 2A; $P = 0.10$); however, parity did not differ among PrC at each time point for [M+3]propionyl CoA.

The PrC interacted with parity for $^{13}\text{C}\%$ enrichment of [M+2]pyruvate, [M+2]glucose, and [M+3]glucose ($P < 0.05$), and PrC tended to interact with parity for $^{13}\text{C}\%$ enrichment of [M+3]citrate ($P = 0.10$; Figure 3). Differences among PrC within parity are shown in Figure 3. The $^{13}\text{C}\%$ enrichment of [M+2]pyruvate and [M+3]glucose was greater for multiparous cows than primiparous cows with the 2 mM PrC (2.54% vs. 1.75% and 0.16% vs. 0.07%, respectively; pairwise tests, $P \leq 0.05$). Additionally, [M+3]glucose was greater for multiparous cows than primiparous cows with the 4 mM PrC (0.16% vs. 0.08%; pairwise test, $P = 0.05$), and [M+3]citrate tended to be greater for multiparous cows than primiparous cows with the 4 mM PrC (12.6% vs. 10.0%; pairwise test, $P = 0.07$). The $^{13}\text{C}\%$ enrichment of [M+2]glucose did not differ between parity within each PrC (pairwise tests, $P > 0.10$).

DISCUSSION

Total $^{13}\text{C}\%$ enrichment of acetyl CoA did not increase with increased concentrations of [$^{13}\text{C}_3$]propionate, nor did $^{13}\text{C}\%$ enrichment of acetyl CoA increase over time compared with the baseline. Although no significant PrC or time effects were detected for [M+2]acetyl CoA enrichment, [M+4]citrate and [M+5]citrate enrichment significantly increased over time and with increased PrC. Significant [M+3]citrate enrichment would be ex-

Table 2. Effects of 3 different concentrations of [$^{13}\text{C}_3$]propionate (1, 2, or 4 mM) on total $^{13}\text{C}\%$ enrichment of select hepatic metabolites over 60 min

Metabolite ¹	Concentration of [$^{13}\text{C}_3$]propionate			<i>P</i> -value				
	1 mM	2 mM	4 mM	PrC ²	Time ³	PrC × time	PrC × parity	PrC × time × parity
Propionyl CoA	26.0 ^{(23.8, 28.1)a}	33.4 ^{(31.2, 35.5)b}	37.4 ^{(35.2, 39.5)c}	<0.0001	<0.0001	<0.01	0.71	0.08
Succinate	2.21 ^{(1.62, 2.79)a}	3.52 ^{(2.94, 4.11)b}	4.47 ^{(3.88, 5.05)c}	<0.01	<0.0001	<0.0001	0.41	0.60
Fumarate ⁴	2.58 ^{(2.41, 2.77)a}	3.38 ^{(3.16, 3.61)b}	3.96 ^{(3.72, 4.22)c}	<0.0001	<0.0001	<0.0001	0.17	0.35
Malate	5.61 ^{(4.82, 6.40)a}	8.47 ^{(7.68, 9.26)b}	10.2 ^{(9.42, 11.0)c}	<0.0001	<0.0001	<0.0001	0.36	0.35
Citrate ⁵	3.79 ^{(3.43, 4.16)a}	4.90 ^{(4.50, 5.31)b}	5.42 ^{(5.00, 5.85)c}	<0.0001	<0.0001	<0.0001	0.09	0.74
Glutamate ⁶	0.35 ^(0.32, 0.38)	0.39 ^(0.36, 0.43)	0.38 ^(0.35, 0.41)	0.15	<0.0001	0.01	0.62	0.27
Pyruvate	0.90 ^{(0.64, 1.16)a}	1.45 ^{(1.19, 1.71)b}	1.78 ^{(1.53, 2.04)c}	<0.0001	<0.0001	<0.0001	0.12	0.71
Lactate ⁴	0.29 ^{(0.20, 0.37)a}	0.49 ^{(0.39, 0.59)b}	0.59 ^{(0.49, 0.70)b}	<0.01	<0.0001	<0.0001	0.17	0.48
Glucose ⁶	0.16 ^(0.08, 0.25)	0.16 ^(0.08, 0.25)	0.18 ^(0.10, 0.28)	0.59	<0.0001	0.84	0.08	0.48
Acetyl CoA	0.20 ^(-0.81, 1.20)	0.32 ^(-0.69, 1.32)	0.77 ^(-0.24, 1.77)	0.54	0.19	0.64	0.46	0.18

^{a-c}Means within a row with different superscripts differ ($P \leq 0.05$).

¹Total percent of ^{13}C enrichment above natural abundance adjusted for number of carbons in each metabolite. Values reported as LSM^(95% CI).

²PrC = concentration of [$^{13}\text{C}_3$]propionate.

³Time = sampling time; sampling time points are baseline, 0.5, 15, and 60 min.

⁴Data transformed using log ($n + 1$) for statistical analysis and back-transformed for interpretation.

⁵Data transformed using square root ($n + 1$) for statistical analysis and back-transformed for interpretation.

⁶Data transformed using inverse ($n + 1$) for statistical analysis and back-transformed for interpretation.

Table 3. Effects of 3 different concentrations of [$^{13}\text{C}_3$]propionate (1, 2, or 4 mM) on $^{13}\text{C}\%$ enrichment of select hepatic metabolite isotopologues over 60 min

Metabolite ¹	Concentration of [$^{13}\text{C}_3$]propionate			P-value				
	1 mM	2 mM	4 mM	PrC ²	Time ³	PrC × time	PrC × parity	PrC × time × parity
Propionyl CoA[M+3]	46.4 ^{(42.2, 50.6)a}	57.8 ^{(53.6, 62.1)b}	63.1 ^{(58.9, 67.3)b}	<0.01	<0.0001	0.56	0.70	0.10
Pyruvate[M+2]	1.39 ^{(1.14, 1.64)a}	2.15 ^{(1.90, 2.40)b}	2.54 ^{(2.29, 2.79)c}	<0.0001	<0.0001	<0.0001	0.02	0.72
Acetyl CoA[M+1]	3.36 ^(0.71, 6.01)	3.03 ^(0.38, 5.68)	2.87 ^(0.22, 5.52)	0.93	0.54	0.05	0.24	0.54
Acetyl CoA[M+2]	1.65 ^(0.44, 2.85)	2.09 ^(0.89, 3.30)	2.92 ^(1.72, 4.13)	0.23	0.91	0.15	0.11	0.44
Citrate[M+3]	8.43 ^{(7.59, 9.27)a}	10.5 ^{(9.65, 11.3)b}	11.3 ^{(10.5, 12.1)b}	<0.01	<0.0001	<0.0001	0.10	0.48
Citrate[M+4]	2.36 ^{(1.90, 2.81)a}	3.30 ^{(2.84, 3.75)b}	4.00 ^{(3.55, 4.45)c}	<0.01	<0.0001	<0.0001	0.43	0.65
Citrate[M+5]	1.28 ^{(1.07, 1.49)a}	1.81 ^{(1.59, 2.02)b}	2.30 ^{(2.09, 2.51)c}	<0.01	<0.0001	<0.0001	0.32	0.44
Glutamate[M+2] ⁴	0.81 ^(0.72, 0.91)	0.95 ^(0.85, 1.06)	0.93 ^(0.83, 1.04)	0.11	<0.0001	0.08	0.35	0.51
Glutamate[M+3] ⁵	0.45 ^(0.41, 0.51)	0.52 ^(0.47, 0.58)	0.50 ^(0.44, 0.55)	0.17	<0.0001	0.05	0.53	0.57
Glucose[M+2]	0.19 ^(0.11, 0.27)	0.23 ^(0.15, 0.31)	0.25 ^(0.16, 0.33)	0.16	<0.0001	0.17	0.01	0.40
Glucose[M+3] ⁵	0.09 ^{(0.07, 0.11)a}	0.11 ^{(0.09, 0.13)a}	0.12 ^{(0.09, 0.14)b}	0.05	<0.0001	0.28	0.04	0.11

^{a-c}Means within a row with different superscripts differ ($P \leq 0.05$).

¹Enrichment of each isotopologue is relative to the mass distribution of all the isotopologues in the metabolite (molar percent) and is on a scale of 0 to 100%. Values reported as LSM^(95% CI).

²PrC = concentration of [$^{13}\text{C}_3$]propionate.

³Time = sampling time; sampling time points are 0.5, 15, and 60 min.

⁴Data transformed using inverse square root ($n + 1$) for statistical analysis and back-transformed for interpretation.

⁵Data transformed using inverse ($n + 1$) for statistical analysis and back-transformed for interpretation.

pected as [$^{13}\text{C}_3$]propionate feeds into the TCA cycle, but labeling beyond [M+3]citrate requires labeled acetyl CoA. Enrichment of pyruvate and [M+2]pyruvate significantly increased with PrC over time, indicating that propionate was converted to pyruvate. Because ATP citrate lyase and malic enzyme are negligible in the liver of ruminants (Hanson and Ballard, 1967; Young et al., 1969), the labeled acetyl CoA is likely derived from labeled pyruvate. However, the pyruvate dehydrogenase complex is tightly regulated (Williamson and Cooper, 1980) and the rate of synthesis of acetyl CoA was not likely affected by the concentration of pyruvate because pyruvate carboxylase is upregulated during this stage of lactation (Greenfield et al., 2000). Therefore, the tight regulation of the pyruvate dehydrogenase complex could partially explain the lack of PrC effect detected for enrichment of acetyl CoA when incubated with a concentration of propionate from 1 to 4 mM. However, dilution from naturally high concentrations of hepatic acetyl CoA in dairy cows in the PP period (Piantoni et al., 2015) due to lipolysis likely contributed to detecting minimal enrichment of ^{13}C -labeled acetyl CoA as well. Contrary to Greenfield et al. (2000), Garcia et al. (2015) reported an increase in the relative activity of the pyruvate dehydrogenase complex versus pyruvate carboxylase when liver slices from early-lactation cows were incubated with a pyruvate and propionate treatment compared with a pyruvate only treatment for 2 h. Notably, Garcia et al. (2015) did not measure enrichment of acetyl CoA directly, but estimated [M+2]acetyl

CoA enrichment from glutamate enrichment. Although pyruvate carboxylase may incorporate some $^{13}\text{CO}_2$ for the conversion of pyruvate to OAA, this contribution is likely negligible. Additionally, this incorporation would not result in [M+5]citrate, of which we detected differences among PrC.

In addition to its anaplerotic capabilities, propionate is a primary glucose precursor in dairy cows. Estimates of propionate contribution to glucose synthesis range from 61 to 95% (Wiltrout and Satter, 1972; Armentano, 1992). However, enrichment of intracellular hepatic glucose was approximately 0.5% when adjusted to a per carbon basis at its greatest enrichment, and no PrC nor PrC by time effects were detected. Similar to total $^{13}\text{C}\%$ enrichment of glucose, [M+3]glucose enrichment increased over time, but enrichment did not exceed 1%. However, [M+3]glucose enrichment was higher in multiparous than primiparous animals at higher [$^{13}\text{C}_3$]propionate concentrations. Notably, we measured the ^{13}C enrichment of the accumulated, free glucose located within the hepatic tissue and not the extracellular labeling of glucose, but free glucose is released from hepatic tissue for use by other tissues. Any glucose that is retained within cells is stored as glycogen, approximately 8% on a DM basis for dairy cows 7 to 14 DIM (Vazquez-Añon et al., 1994), but we did not measure glycogen in this experiment. Furthermore, the enzymes glucokinase and hexokinase (EC 2.7.1.2 and EC 2.7.1.1, respectively) are minimal in the liver of dairy cows (Ballard, 1965), and as such, recycling and

Figure 2. Effects of concentration of $^{13}\text{C}_3$ propionate on $^{13}\text{C}\%$ enrichment of hepatic [M+3]propionyl CoA (A), [M+1]acetyl CoA (B), [M+2]pyruvate (C), [M+3]citrate (D), [M+4]citrate (E), [M+5]citrate (F), [M+2]glutamate (G), [M+3]glutamate (H), [M+2]glucose (I), and [M+3]glucose (J) over 60 min. The enrichment of each isotopologue is relative to the mass distribution of all the isotopologues in the metabolite (molar percent) and is on a scale of 0 to 100%. Time points are 0.5, 15, and 60 min. Graphs are presented as highest level of significant or tendency for significant interaction, and associated P -values can be found in Table 3. All graphs had significant time effects that differed among each time point ($P < 0.01$) except $^{13}\text{C}\%$ enrichment of [M+1]- and [M+2]acetyl CoA, which had no time effects ($P > 0.50$). Concentration of $^{13}\text{C}_3$ propionate by time interactions at each time point were examined for differences among concentrations of $^{13}\text{C}_3$ propionate. Letters a and b are presented in the order of 1/2/4 mM $^{13}\text{C}_3$ propionate concentrations, and different letters differ from each other ($P \leq 0.05$). Significant results from pairwise tests for the 3-way interaction are described in the text. The [M+2]glutamate was transformed using inverse square root ($n + 1$) for statistical analysis and back-transformed for interpretation. The [M+3]glutamate and [M+3]glucose were transformed using inverse ($n + 1$) for statistical analysis and back-transformed for interpretation.

Figure 3. The effect of parity and concentration of [$^{13}\text{C}_3$]propionate on $^{13}\text{C}\%$ enrichment of hepatic [M+2]pyruvate (A), [M+3]citrate (B), [M+2]glucose (C), and [M+3]glucose (D). The enrichment of each isotopologue is relative to the mass distribution of all the isotopologues in the metabolite (molar percent) and is on a scale of 0 to 100%. Letters a–c are presented in the order of 1/2/4 mM [$^{13}\text{C}_3$]propionate concentrations; different letters differ from each other ($P \leq 0.05$), and * between letters signifies a tendency for difference ($0.05 < P \leq 0.10$). The [M+3]glucose was transformed using inverse $(n + 1)$ for statistical analysis and back-transformed for interpretation.

absorption of extracellular glucose is likely negligible as well. Consequently, we suspect that because extracellular glucose represents a sink for [^{13}C]propionate, we were unable to observe changes in glucose enrichment. Additionally, the detected enrichment of [^{13}C]glucose is likely an artifact of the accumulated residual glucose in the medium, which is likely very diluted due to the [^{12}C]glucose initially present in the medium. Therefore, the appearance of the isotope in the intracellular pool does not likely reflect the labeling of glucose, nor can this be determined from analysis of the medium due to the initial presence of [^{12}C]glucose in the medium that would assuredly dilute isotope enrichment.

Although propionate is the primary glucose precursor in dairy cows, its contribution slightly decreases

in the immediate PP period (approximately 60%), despite increased propionate uptake and glucose output. Lactate, also a gluconeogenic precursor, contributes approximately 25% to glucose synthesis during the PP period (Aschenbach et al., 2010). Because lactate was present in our medium, glucose synthesis from lactate may have also diluted the observed enrichment of free glucose from propionate. Additionally, extracellular [^{12}C]lactate from the medium also likely diluted the detected accumulation of ^{13}C enriched lactate to some extent.

Our PrC were chosen to bracket the 2.5 mM propionate concentration used in previous experiments, which suggest that a concentration of 2.5 mM of propionate for a 2-h incubation supplies enough propionate for a

steady state to be achieved (Donkin and Armentano, 1995; Zhang et al., 2015). Although our lowest PrC was 1 mM [$^{13}\text{C}_3$]propionate, Reynolds et al. (2003) stated that 795 mmol/h of propionate was measured from net portal-drained viscera, and that plasma flow through the portal vein was 1,287 L/h for dairy cows 11-d PP on a 24% starch diet. Therefore, a mean of approximately 0.62 mM plasma propionate was present in the portal vein for those dairy cows. A similar concentration (0.44 mM) was reported by Bergman et al. (1966) for sheep consuming a full ration and infused with ~25 mmol/h of propionic acid into the rumen vein. Whereas both of these circulating concentrations are below our lowest PrC concentration (1 mM), they are mean concentrations over hours and ruminal production and portal-drained viscera flux of propionate is highly variable over time relative to meals (Benson et al., 2002). Also, hepatocytes might have greater capacity for propionate metabolism beyond physiological levels as suggested by Armentano (1992).

Enrichment of metabolites increased as concentration of [$^{13}\text{C}_3$]propionate increased for the majority of measured metabolites from 1 to 4 mM, which suggests an increase in anaplerosis at higher concentrations of [$^{13}\text{C}_3$]propionate in the range of 1 to 4 mM. The observed increase in enrichment of glutamate over time supports increased anaplerosis by propionate and suggests that α -ketoglutarate was enriched as well because glutamate can be synthesized from α -ketoglutarate via the glutamate dehydrogenase reaction (EC 1.4.1.2). This reaction oxidizes NADH to NAD^+ , which in turn replenishes the supply of NAD^+ that is used as a co-factor in many reactions within and associated with the TCA cycle. Additionally, increased enrichment of glutamate over time supports that the explants were alive and continuing to properly function metabolically throughout our experiment.

Enrichment of metabolites associated with propionate metabolism increased with time as expected, and significant detection of enrichment was present for all metabolites except acetyl CoA by 15 min. In our experiment, we were interested in the short-term effects of propionate metabolism and effect of different concentrations of propionate on its metabolism. Furthermore, we chose to use explants for this experiment, which enabled us to provide a similar physiological situation seen in an adult dairy cow in the PP period. However, explants have a shorter life span compared with cell cultures or other in vitro techniques, which informed our choice of time points. Interestingly, ^{13}C enrichment of some metabolites appeared to occur soon after introduction of explants to the incubation medium, as propionyl CoA, succinate, fumarate, and glucose had

significant increases in total $^{13}\text{C}\%$ enrichment between baseline and exposure to [$^{13}\text{C}_3$]propionate-enriched medium for ~0.5 min, independent of [$^{13}\text{C}_3$]propionate concentration. The 0.5-min samples were originally proposed as baseline samples. They were taken by adding an explant to medium treated with either 1, 2, or 4 mM [$^{13}\text{C}_3$]propionate and then immediately removing the tissue from the medium, blotting it, and then freezing it in liquid nitrogen as previously described. However, rapid incorporation of the stable tracer was observed for some of the measured metabolites; therefore, these samples were unsuitable as baseline values. This rapid incorporation of labeling indicated a near instantaneously labeled pool (within 5 min). Additionally, a quasi-steady state was reached for some metabolites by 15 min, such as fumarate.

Propionate is a major volatile fatty acid produced by the rumen of dairy cows and a major glucose precursor, yet research suggests it can decrease DMI, especially during the transition period (Allen and Piantoni, 2013). Dry matter intake is a function of the size and frequency of meals, and adequate DMI is important for transition cow success. Metabolic reactions proceed quickly (within minutes) and dynamic changes occur within a system when studied on a minute time scale that can affect meal size. Therefore, by understanding the short-term metabolism of nutrients, we can begin to imagine new ideas and strategies to improve and manage nutrition in dairy cows to minimize the metabolic stress associated with transition to lactation. Further research is needed to understand the extent of and scenarios in which propionate contributes to oxidation through acetyl CoA production both in in vitro and in vivo models.

CONCLUSIONS

Despite high concentrations of hepatic acetyl CoA in dairy cows in the PP period, our results indicated that propionate converts to acetyl CoA and enters the TCA cycle for oxidation; however, propionate does not appreciably accumulate as acetyl CoA, likely due to a rapid turnover rate. Furthermore, the contribution of propionate to oxidation via acetyl CoA increases as concentrations of propionate increase within a 1 to 4 mM range as evidenced by an increase in citrate.

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