Propionate induces mRNA expression of gluconeogenic genes in bovine calf hepatocytes

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ABSTRACT

Hepatocytes monolayers from neonatal calves were used to determine the responses of the cytosolic phosphoenolpyruvate carboxykinase (*PCK1*) mRNA expression to propionate and direct hormonal cues including cyclic AMP (cAMP), dexamethasone, and insulin. The responses of other key gluconeogenic genes, including mitochondrial phosphoenolpyruvate carboxykinase (*PCK2*), pyruvate carboxylase (*PC*), and glucose-6-phosphatase (*G6PC*), were also measured. Expression of *PCK1* was linearly induced with increasing propionate concentrations in media and 2.5 mM propionate increased *PCK1* mRNA at 3 and 6 h of incubation; however, the induction disappeared at 12 and 24 h. The induction of *PCK1* mRNA by propionate was mimicked by 1 mM cAMP, or in combination with 5 μ M dexamethasone, but not by dexamethasone alone. The induction of *PCK1* mRNA by propionate or cAMP was eliminated by addition of 100 nM insulin. Additionally, expression of *PCK2* and *PC* mRNA was also induced by propionate in a concentration-dependent manner. Consistent with *PCK1*, propionate-stimulated *PCK2* and *PC* mRNA expression was inhibited by insulin. Expression of *G6PC* mRNA was neither affected by propionate nor cAMP, dexamethasone, insulin, or their combinations. These findings demonstrate that propionate can directly regulate its own metabolism in bovine calf hepatocytes through upregulation of *PCK1*, *PCK2*, and *PC* mRNA expression.

Key words: *PCK1*, hormonal regulation, gluconeogenesis

INTRODUCTION

The importance of gluconeogenesis in ruminants is highlighted by the extensive fermentation of simple sugars and starch in the rumen, despite extensive meta-

bolic needs for glucose by tissues and as a precursor for milk lactose synthesis (Aschenbach et al., 2010). Hypoglycemia, ketosis, and related metabolic disorders are often observed when gluconeogenic capacity in the liver fails to adapt to the increased tissue demands for glucose in dairy cattle (Aschenbach et al., 2010). The prevalence of this insufficiency is highlighted by reports that the incidence of ketosis in commercial dairy herds may be as high as 17% (Dohoo and Martin, 1984). Because propionate contributes approximately 50% of the carbon for gluconeogenesis (Huntington, 1990), strategies to increase propionate supply, including monensin feeding, have successfully reduced the incidence of ketosis in transition dairy cows (Duffield et al., 1998). In addition to the documented effect of monensin on feed efficiency and increased ruminal propionate production (Sauer et al., 1989), a portion of the effect of monensin in transition cows may be linked to increased capacity for gluconeogenesis through enhanced expression of cytosolic phosphoenolpyruvate carboxykinase (*PCK1*), a key gene for glucose synthesis (Karcher et al., 2007).

Control of *PCK1* enzyme activity has been studied extensively in nonruminants and is primarily exerted through transcription of the gene via activation of basal, tissue-specific, and hormone-dependent promoter elements within the 5'-flanking region of the *PCK1* gene (Hanson and Reshef, 1997). Expression of *PCK1* mRNA in nonruminants is activated by glucagon and glucocorticoids and insulin counteracts these effects (O'Brien and Granner, 1990). A lack of response for *PCK1* to feed restriction in dairy cows (Velez and Donkin, 2005) and sheep (Filsell et al., 1969; Taylor et al., 1971; Smith et al., 1982) is in direct contrast with data from nonruminants demonstrating that feed restriction induces the *PCK1* gene (Hanson and Reshef, 1997). Although in vivo administration of glucagon in dairy cattle suggests *PCK1* induction in dairy cattle (Bobe et al., 2009), these data are equivocal perhaps due to counter-regulatory release of insulin and other hormones (Bobe et al., 2009) in the experimental models used.

Regulation of *PCK1* in response to glucagon and other hormones has been extensively studied in rat hepatocytes and hepatoma cells. The mRNA for *PCK1*

Received August 25, 2015.

Accepted December 4, 2015.

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is maximally induced by cyclic AMP (cAMP) in presence of glucocorticoids within 3 h (Christ et al., 1988). Likewise, short-chain fatty acids, including propionate, induce *PCK1* mRNA in rat hepatocytes (Massillon et al., 2003), indicating the potential for propionate to control its own metabolism. Effects of glucagon on propionate and lactate metabolism in hepatocytes from neonatal calves indicate increased gluconeogenesis from propionate and lactate, but not glycerol (Donkin and Armentano, 1994), suggesting a regulation point of *PCK1* by glucagon. However, the direct effects of propionate and hormones on *PCK1* mRNA in bovine hepatocytes have not been determined. We hypothesized that propionate and cAMP act to induce *PCK1* mRNA expression and insulin opposes these effects on *PCK1* mRNA in liver hepatocytes.

The primary objective of the current research was to determine the direct effects of propionate, cAMP, dexamethasone, and insulin on mRNA expression of *PCK1* in the neonatal calf hepatocytes. The secondary objective was to evaluate the relative effects of propionate and these hormonal cues on mRNA expression of other key gluconeogenic genes, including mitochondrial phosphoenolpyruvate carboxykinase (*PCK2*), pyruvate carboxylase (*PC*), and glucose-6-phosphatase (*G6PC*).

MATERIALS AND METHODS

Care and Use of Hepatocyte Donor Animals

All experimental procedures involving animals were approved by the Purdue University Animal Care and Use Committee. Three intact male dairy calves were used as hepatocyte donor animals. All calves received 3.8 L of superior colostrum for the first feeding postpartum and 1.9 L of superior colostrum during the first 48 h of life, for a total of 5.7 L. Calves subsequently received milk at 10% of BW in 2 equal feedings per day. Calves were housed in a drying pen for the first 24 h, then moved to individual calf hutches. At less than 7 d of age, the calves (47 ± 3 kg of BW) were given heparin (1,000 USP, Sagent Pharmaceuticals, Schaumburg, IL) intravenously and anesthetized with 1% propofol (6 mg/kg of calf BW, 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 of calf BW, Sigma-Aldrich, 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 on 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 Dulbecco's Modified Eagles Medium (DMEM) containing 20% fetal bovine serum and 1% antibiotic, antimycotic solution (Sigma-Aldrich). After 4 h, the plating medium and unattached cells were removed by aspiration and media was replaced by DMEM containing 10% fetal bovine serum and 1% antibiotic, antimycotic solution.

Twenty hours after seeding, the media was removed by aspiration and replaced with DMEM supplemented with 1% BSA (Merck Millipore, Billerica, MA) and 1% antibiotic, antimycotic solution, and the respective treatment additions. Time-dependent effect of propionate exposure was determined in the presence of either 0 or 2.5 mM propionate for 3, 6, 12, and 24 h. Concentration dependence to propionate was determined in the presence of 0, 0.625, 1.25, 2.5, and 5.0 mM propionate for 6 h. The effects of hormone additions and their relationship with propionate were tested using 1 mM 8-Br-cAMP, 5 μ M dexamethasone, 100 nM insulin, 2.5 mM propionate, or their designated combinations over a period of 6 h.

RNA Extraction and PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Inc., Thousand Oaks, CA) and quantified by absorbance at 260 nm using a ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE). A total of 2 μ g of purified sample was reverse transcribed to cDNA using an Omniscript reverse transcriptase kit (Qiagen, Inc.), random decamers (Ambion, Foster City, CA), and oligo-dT (Qiagen, Inc.). Abundance of cDNA product for each RNA sample was quantified using real-time PCR, Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Inc., Santa Clara, CA) with primers described below. A cDNA pool was generated from an equivalent quantity of cDNA from each sample. A 1:4 dilution series of the cDNA pool was used to generate the standard curve. No-template control (using water as template) and no-reverse transcription control (using RNA pool as template) were included in the real-time PCR analysis. The forward and reverse primers, respectively, for *PCK1*, *PCK2*, *PC*, *G6PC*, *GAPDH*, and *18S* were: bovine *PCK1* (GenBank accession: NM_174737.2), AGGGAAATAG-CAGGCTCCAGGAAA, CACACGCATGTGCA-CACACACATA; bovine *PCK2* (GenBank accession: NM_001205594.1), TGACTGGGCAAGGGGAGCCG, GGGGCCACCCCAAAGAAGCC; bovine *PC* (GenBank accession: NM_177946.4), CCACGAGT TCTC-CAACACCT, TTCTCCTCCAGCTCCTCGTA; bovine *G6PC* (GenBank accession: NM_001076124.2),

TGATGGACCAAGAAAGATCCAGGC, TATGATTGACCTCACTGGCCCTCTT; bovine *GAPDH* (GenBank accession: NM_001034034.2), CATGTTGTGATGGGCGTGAACCA, TGATGGCGTG-GACAGTGGTCATAA; and bovine *18S* (GenBank accession: NR_036642.1), ACCCATTCGAACGTCTGCCCTATT, TCCTTGGATGTGGTAGCCGTTTCT. Reactions were 1 cycle at 95°C for 3 min; 40 cycles at 95°C for 10 s, 60°C for 20 s; and 1 cycle at 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. Samples were analyzed in triplicate and PCR reaction efficiencies were between 90 and 110%. The cycle threshold values for treatments were evaluated independently for *GAPDH* and *18S* as potential housekeeping genes. There was no effect of treatment on either transcript. The copy number for *18S* showed less variation among samples compared with *GAPDH*, and mean values for transcripts evaluated were normalized to mRNA abundance of *18S* within each sample. Data are expressed as arbitrary units of mRNA adjusted for *18S* abundance.

Statistical Analysis

All experiments were conducted in 3 separate cell preparations from 3 calves using 3 replicates per treatment. Data were analyzed for normality using the Univariate procedure of SAS 9.2 (SAS Inst. Inc., Cary, NC), and any heteroscedastic data were adjusted to achieve normality using a natural logarithm transformation. Analyses of variance were performed using the Proc Mixed procedure of SAS. For analyzing data associated with time, the model accounted for the random effect of cell preparation, the fixed effect of time, treatment, and time by treatment interaction. For data that had a significant time by treatment interaction, comparisons between treatments at the same time point were made using pdiff option. For data not associated with an effect of time, the model accounted for the random effect of cell preparation and fixed effects of treatment. Dunnett's test was used for comparisons between each hormonal treatment and the no-addition control. Data are reported as least squares means and standard errors. Means were considered different when $P \leq 0.05$ and a tendency for difference was declared when $0.05 < P \leq 0.10$.

RESULTS

Abundance of *PCK1*, *PCK2*, and *PC* mRNA was induced ($P \leq 0.05$) by 2.5 mM propionate, but no effect of propionate on *G6PC* was noted (Figure 1). A treatment by time interaction ($P \leq 0.05$) was detected for *PCK1* mRNA in response to incubation with 2.5 mM propionate. Propionate induced *PCK1* mRNA to 188%

of control at 3 h (2.65 vs. 1.41) and 163% of control at 6 h (2.43 vs. 1.49), but the response to propionate dissipated by 12 h so that a lack of effect ($P > 0.10$) was observed for both 12 and 24 h. Expression of *PCK2* mRNA was induced ($P \leq 0.05$) to approximately 350% of control at 3 (0.74 vs. 0.21), 6 (0.67 vs. 0.19), 12 (0.54 vs. 0.15), and 24 h (0.35 vs. 0.10). Propionate induced ($P \leq 0.05$) *PC* mRNA to approximately 130 to 140% of control at 3 (0.96 vs. 0.69), 6 (1.00 vs. 0.72), 12 (1.07 vs. 0.79), and 24 h (1.21 vs. 0.94). The expression of *G6PC* mRNA was not affected ($P > 0.10$) by propionate at any time point tested.

The effect of increasing propionate concentration on expression of *PCK1*, *PCK2*, *PC*, and *G6PC* mRNA in bovine hepatocytes was investigated by exposing to initial concentrations of propionate at 0, 0.625, 1.25, and 2.5 mM for 6 h (Figure 2). Expression of *PCK1*, *PCK2*, and *PC* showed a linear trend ($P \leq 0.05$) with increasing propionate concentrations. In contrast, the expression of *G6PC* mRNA was not affected ($P > 0.10$) by any of the concentrations tested.

In addition to the significant induction ($P \leq 0.05$) of *PCK1* mRNA expression by propionate, expression of *PCK1* mRNA also tended to increase ($0.05 < P \leq 0.10$) in response to incubation with 1.0 mM cAMP and the combination of 1.0 mM cAMP and 5.0 μ M dexamethasone, but was not altered ($P > 0.10$) by 5.0 μ M dexamethasone alone (Figure 3). In presence of 100 nM insulin, the expression of *PCK1* mRNA by propionate, cAMP, or the combination of cAMP and dexamethasone was repressed to the basal level. Similarly, propionate increased ($P \leq 0.05$) mRNA expression of *PCK2* and *PC*, which was inhibited back to the basal level by insulin, whereas cAMP and dexamethasone had no effects on these 2 genes (Figure 3). The *G6PC* mRNA was not affected ($P > 0.10$) by any of the hormonal combinations tested (Figure 3).

DISCUSSION

Cytosolic phosphoenolpyruvate carboxykinase catalyzes formation of phosphoenolpyruvate from oxaloacetate (OAA), a critical reaction for gluconeogenesis in liver and kidney. The induction of *PCK1* mRNA by cAMP in cultured calf hepatocytes is consistent with the effect observed for this second messenger of glucagon action in rat hepatocytes (Salavert and Iynedjian, 1982; Christ et al., 1988) and rat hepatoma cells (Sasaki et al., 1984; Granner et al., 1986). Likewise, the lack of induction of *PCK1* mRNA by dexamethasone alone is in agreement with the observations in rat hepatocytes showing dexamethasone as a permissive factor for induction of *PCK1* mRNA by glucagon rather than a stimulatory factor when present alone (Salavert and

Iynedjian, 1982; Christ et al., 1988). The dominant inhibitory effect of insulin on cAMP-induced *PCK1* mRNA is again consistent with studies in using rat liver cells (Granner et al., 1983). The data support that the overall responses to these hormone signals on *PCK1* mRNA are similar for rat and bovine.

An increase in *PCK1* mRNA has been associated with increased feed intake during early lactation in dairy cows (Greenfield et al., 2000), a circumstance also linked to increased ruminal propionate production. We determined the direct role of propionate on regulation of *PCK1* mRNA expression in bovine by using

Figure 1. (A) Effect of propionate on expression of cytosolic phosphoenolpyruvate carboxykinase (*PCK1*) mRNA, (B) mitochondrial phosphoenolpyruvate carboxykinase (*PCK2*) mRNA, (C) pyruvate carboxylase (*PC*) mRNA, and (D) glucose-6-phosphatase (*G6PC*) mRNA in bovine calf hepatocytes. After 24 h, hepatocytes were exposed to either 0 (dotted line, open circle) or 2.5 mM propionate (solid line, solid square) for the interval indicated and harvested for mRNA analysis. Data are expressed as arbitrary units of mRNA adjusted for 18S abundance. Values are LSM $\pm$ SE ($n = 3$ cell preparations). The symbol (*) indicates means within the same time point differ ($P \leq 0.05$).

a primary hepatocyte culture system and, therefore, avoided any compensatory responses to nutrient supply or hormonal status often observed *in vivo* in response to shift in nutrient status. The induction of *PCK1* mRNA by propionate observed here expands the previous observation that propionate induces *PCK1* mRNA (Massillon et al., 2003) to include both rat and bovine. The opposing effect of insulin to repress propionate induction of *PCK1* mRNA confirms previous speculation that insulin blunts the maximal effect of propionate on *PCK1* mRNA *in vivo* during the postprandial infusion of propionate in lactating dairy cows (Zhang et al., 2015). This is the first study, to our knowledge, that has investigated the relationship of propionate

and insulin in regulating *PCK1* mRNA in any species. Whether the regulation of *PCK1* mRNA by propionate is transcriptionally controlled in bovine, as is the case for *PCK1* in rat liver cells (Hanson and Patel, 1994; Hanson and Reshef, 1997), remains to be determined.

In contrast to *PCK1*, expression of *PCK2* mRNA is generally considered constitutive and lacks acute hormonal and nutritional regulation (Beale et al., 2007). Our data confirmed the lack of regulation of *PCK2* by hormones, including cAMP and dexamethasone; however, propionate induces *PCK2* mRNA expression in a concentration-dependent manner in bovine hepatocytes. The significant role of *PCK2* in gluconeogenesis, lipid metabolism, and trichloroacetic acid cycle activity

Figure 2. (A) Effects of propionate concentration on expression of cytosolic phosphoenolpyruvate carboxykinase (*PCK1*) mRNA, (B) mitochondrial phosphoenolpyruvate carboxykinase (*PCK2*) mRNA, (C) pyruvate carboxylase (*PC*) mRNA, and (D) glucose-6-phosphatase (*G6PC*) mRNA in bovine calf hepatocytes. After 24 h, hepatocytes were exposed to 0, 0.625, 1.25, or 2.5 mM propionate for 6 h and then harvested for mRNA analysis. Data are expressed as arbitrary units of mRNA adjusted for 18S abundance. Values are LSM $\pm$ SE ($n = 3$ cell preparations). Expression of *PCK1* and *PCK2* shows a linear effect ($P \leq 0.05$) with increasing propionate concentrations.

Figure 3. Effects of propionate, insulin, cyclic AMP (cAMP), dexamethasone, and combinations on mRNA expression of (A) cytosolic phosphoenolpyruvate carboxykinase (*PCK1*), (B) mitochondrial phosphoenolpyruvate carboxykinase (*PCK2*), (C) pyruvate carboxylase (*PC*), and (D) glucose-6-phosphatase (*G6PC*) in bovine calf hepatocytes. After 24 h, hepatocytes were exposed to treatments for 6 h and then harvested for mRNA analysis. Data are expressed as fold induction of *PCK1* mRNA for each treatment relative to the no-addition control. Values are LSM $\pm$ SE ($n = 3$ cell preparations). Means with the symbol (*) differ ($P \leq 0.05$) from the no-addition control, and means with the symbol (#) tend to differ ($0.05 < P \leq 0.10$) from the no-addition control.

has been recently emphasized by overexpression models of *PCK2*, in *PCK1* knockout mice and in hepatocytes (Méndez-Lucas et al., 2013), and gene silencing models of *PCK2* (Stark et al., 2014). In contrast to the rat and mouse liver, in which *PCK2* only accounts for about 5 and 1% of the total phosphoenolpyruvate carboxykinase activity, respectively (Nordlie and Lardy, 1963; Wiese et al., 1991), ruminants present approximately equal activities of *PCK1* and *PCK2* (Agca et al., 2002). Approximately 60% of propionate flux to glucose depends

on the enzyme *PCK2* in goat hepatocytes (Aiello and Armentano, 1987). The inductive effect of propionate on *PCK2* gene expression and counteractive effect of insulin on propionate-stimulated *PCK2* gene expression has brought new insights regarding the nutritional and hormonal regulation of *PCK2* expression. However, limited available supporting data makes it difficult to predict whether the changes in *PCK2* mRNA would result in changes of in the enzyme expression and activity and relevant physiological consequences.

Pyruvate carboxylase catalyzes the formation OAA from pyruvate and is an important intermediate for both gluconeogenesis and fatty acid oxidation (Jitrapakdee et al., 2008). The expression of *PC* mRNA is increased during feed restriction (Velez and Donkin, 2005) and at parturition in transition dairy cows (Greenfield et al., 2000), and serves to support increased gluconeogenesis from lactate and gluconeogenic AA for lactation (Aschenbach et al., 2010). Our data demonstrate that increased propionate can also induce *PC* mRNA expression, even though gluconeogenesis from propionate does not directly rely on *PC*. Our results are in accordance with a previous report that shows linear induction of *PC* mRNA expression by propionate in neonatal bovine hepatocytes (Xu and Wang, 2006). An increase in *PC* mRNA expression directly reflects an increased enzyme activity in dairy cattle (Greenfield et al., 2000). Because OAA pool size is determined by the activities of *PCK* and *PC*, a matched increase in both enzymes may be important for maintaining the OAA pool and preventing the drainage of OAA from the trichloroacetic acid cycle. Reduction of OAA pool has been considered as a major cause of ketosis in dairy cows (Baird et al., 1968). The propionate-induced *PC* mRNA expression is not mimicked by the hormonal cues in the present study, which is consistent with previous reports that the transcriptional activities of bovine *PC* promoters are not regulated by hormones such as dexamethasone (White et al., 2011). The regulation of *PC* expression by fatty acids through activation of bovine *PC* promoter 1 indicates nutritional control of bovine *PC* gene transcription (White et al., 2011), which also appears to be the case for propionate. The inhibition of insulin on propionate-stimulated *PC* mRNA expression is consistent with previous studies in which gluconeogenesis from propionate is decreased by insulin in isolated bovine primary hepatocytes (Donkin and Armentano, 1994, 1995). Insulin was reported to inhibit rat *PC* gene transcription through its proximal promoter (Jitrapakdee et al., 1997). Nevertheless, the underlying molecular mechanism by which propionate and insulin regulate *PC* mRNA expression in bovine remains to be elucidated.

Expression of *G6PC* mRNA is not regulated by propionate in the present study, despite the increases in other key gluconeogenic genes. The metabolism of glucose-6-phosphate (**G6P**) by *G6PC* is necessary to release glucose from the hepatocyte. Intracellular G6P may also be metabolized to glycogen by glycogen kinase. Propionate has been shown to stimulate glycogen synthesis in hepatocytes isolated from fed sheep in a concentration-dependent manner, and the induction is mediated through increased intracellular concentration of metabolic intermediaries, including OAA, phospho-

enolpyruvate, and G6P (Morand et al., 1990). Lack of stimulation of *G6PC* mRNA to match changes in *PCK1*, *PCK2*, and *PC* mRNA may indicate either increased glycogen storage or adequate reserve capacity of *G6PC* to accommodate additional G6P, potentially resulting from increased *PCK1*, *PCK2*, and *PC* mRNA in response to propionate, or a combination of both processes.

Glucose production from propionate and lactate in neonatal calf hepatocytes have been shown to be regulated by substrates concentrations (Donkin and Armentano, 1994). However, the rate of gluconeogenesis from lactate is lower relative to propionate (Donkin and Armentano, 1994) and attributed to unknown factor(s) that limit conversion of lactate, but not propionate to glucose (Armentano, 1992; Donkin and Armentano, 1994). The present study demonstrates that propionate can directly induce expression of mRNA expression of *PCK1*, *PCK2*, and *PC* in bovine hepatocytes, which may be the underlying mechanism for the stimulation of glucose production from propionate.

CONCLUSIONS

Overall, these data indicate a role of propionate to regulate its own metabolism through induction of *PCK1*, *PCK2*, and *PC* mRNA expression in isolated bovine hepatocytes. The effect of propionate on *PCK1* mRNA expression is mimicked by cAMP. Propionate and cAMP induction of *PCK1* mRNA expression is eliminated by the addition of insulin. These data provide evidence of a direct action of propionate to control expression of mRNA for key enzymes for gluconeogenesis in ruminants.

ACKNOWLEDGMENTS

This project was supported in part by National Research Initiative Competitive Grant no. 2006-35206-16646 from the USDA National Institute of Food and Agriculture (Washington, DC).

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