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## Long-term elevation of $\beta$ -hydroxybutyrate in dairy cows through infusion: Effects on feed intake, milk production, and metabolism

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### ABSTRACT

Elevation of ketone bodies in dairy cows frequently occurs in early lactation, usually concomitantly with a lack of energy and glucose. The objective of this study was to induce an elevated plasma  $\beta$ -hydroxybutyrate (BHBA) concentration over 48 h in mid-lactating dairy cows (i.e., during a period of positive energy balance and normal glucose plasma concentrations). Effects of BHBA infusion on feed intake, metabolism, and performance were investigated. Thirteen cows were randomly assigned to 1 of 2 infusion groups, including an intravenous infusion with Na-DL- $\beta$ -OH-butyrates (1.7 mol/L) to achieve a plasma concentration of 1.5 to 2.0 mmol/L of BHBA (HyperB;  $n = 5$ ), or an infusion of 0.9% saline solution (control;  $n = 8$ ). Blood was sampled before and hourly during the 48 h of infusion. In the liver, mRNA transcripts related to gluconeogenesis (pyruvate carboxylase, glucose 6-phosphatase, mitochondrial phosphoenolpyruvate carboxykinase), phosphofructokinase, pyruvate dehydrogenase complex, and fatty acid synthesis (acetyl-coenzyme A carboxylase, fatty acid synthase) were measured by real-time PCR. Glyceraldehyde-3-phosphate dehydrogenase and ubiquitin were used as housekeeping genes. Changes (difference between before and after 48-h infusion) during the infusion period were evaluated by ANOVA with treatment as fixed effect, and area under the curve of variables was calculated on the second day of experiment. The plasma BHBA concentration in HyperB cows was  $1.74 \pm 0.02$  mmol/L (mean  $\pm$  SE) compared with  $0.59 \pm 0.02$  mmol/L for control cows. The change in feed intake, milk yield, and energy corrected milk did not differ between the 2 experimental groups. Infusion of BHBA reduced the plasma glucose concentration ( $3.47 \pm 0.11$  mmol/L) in HyperB compared with

control cows ( $4.11 \pm 0.08$  mmol/L). Plasma glucagon concentration in HyperB was lower than the control group. All other variables measured in plasma were not affected by treatment. In the liver, changes in mRNA abundance for the selected genes were similar between 2 groups. Results demonstrate that intravenous infusion of BHBA decreased plasma glucose concentration in dairy cows, but this decrease could not be explained by alterations in insulin concentrations or key enzymes related to gluconeogenesis. Declined glucose concentration is likely functionally related to decreased plasma glucagon concentration.

**Key words:** ketone bodies, glucose,  $\beta$ -hydroxybutyrate, metabolism

### INTRODUCTION

High-yielding dairy cows need a lot of energy and nutrients for maintenance and milk synthesis during peak lactation, in particular. Despite increased feed intake in early lactation (Agenäs et al., 2003), after the periparturient nadir this adaptation cannot cover the requirements during this period. Typical metabolic changes during this period of negative energy balance are low plasma concentrations of glucose and high concentrations of plasma NEFA, as well as a subsequent elevation of ketone bodies (van Dorland et al., 2009; Gross et al., 2011). Although ketone bodies can be used as an alternative fuel for some tissues, such as brain and heart (Laffel, 1999; Veech, 2004), kidney (Weidemann and Krebs, 1969), skeletal muscles (Ruderman and Goodman, 1973) and lactating mammary glands (Shaw, 1943), the utilization of ketone bodies is limited. Unused ketone bodies concentration increase tremendously in blood (Duffield et al., 2009). This elevation of ketone bodies in blood causes reduced feed intake and increases the risk of clinical ketosis, displaced abomasums, metritis, and subsequent decrease of milk production (Duffield et al., 2009). The most used method to detect subclinical ketosis is the measurement of BHBA in serum or plasma (Duffield, 2000; Herdt, 2000); in ruminants, BHBA is the major ketone body found in

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circulation (Bergman, 1971). Ospina et al. (2010) suggested a threshold value of 1,200  $\mu\text{mol/L}$  to distinguish between normal cows and subclinical ketotic cows.

Subcutaneous BHBA infusion in rats (Moore et al., 1976; Langhans et al., 1983), intraperitoneal BHBA infusion in goats (Rossi et al., 2000), and intracerebroventricular BHBA infusion in dairy cows (Kuhla et al., 2011) led to inhibited feed intake in these animals. Intravenous BHBA infusion led to decreased plasma concentration of glucose in dogs (Madison et al., 1964), pigs (Müller et al., 1984), and ewes (Schlumbohm and Harmeyer, 2003, 2004). The reason for declined glucose during the infusion of BHBA remains unclear, but those authors suggested that infusion of BHBA induced hypoglycemia through direct or indirect inhibition of gluconeogenesis, or by a change in the insulin concentration in pig and sheep (Müller et al., 1984; Schlumbohm and Harmeyer, 2004). This mechanism has not yet been investigated in dairy cows. Our objective was to induce an elevated plasma BHBA concentration over 48 h and to investigate its effects on feed intake, performance, and metabolism in the dairy cow. More specifically, this study was carried out to confirm that in the dairy cows, as in sheep and pigs, elevated plasma BHBA concentration affects plasma glucose concentration, and to investigate the underlying mechanisms.

## MATERIALS AND METHODS

### *Animals and Management*

The current trial followed the guidelines of Swiss Law on Animal Protection permitted by the Committee of Animal Experiments of the Canton Fribourg, Switzerland. The study was carried out with 13 diestrus Holstein dairy cows with a parity of  $3.5 \pm 0.10$  at  $28 \pm 0.3$  (mean  $\pm$  SD) wk in milk. Cows in later lactation were selected to allow the investigation of the specific effect of BHBA infusion without influences of the characteristic endocrine and metabolic changes during the transition period. The cows were healthy at the start of the experiment, which was confirmed by a routine blood glutaraldehyde coagulation test (Sandholm, 1976) and by measuring milk SCC (DeLaval cell counter DCC, DeLaval International AB, Tumba, Sweden), which had to be less than 150,000 cells/mL in all 4 quarters.

Two weeks before the start of the experiment, animals were allowed to adapt to tiestall housing and to the experimental feeding conditions. Animals were fed ad libitum with hay. In addition, cows were fed a protein- and energy-rich concentrate (barley 23.5%, oats 14.0%, wheat bran 20.0%, expeller soybean 17.0%, linseed meal 15.0%, salt livestock 0.6%, carbonate of lime 2.2%, kaf premix 0.4%, molasses 4.0%, and alikon

3.0%) twice daily according to each cow's individual extra energy requirement for milk production, and 50 g of minerals once daily. Water was available ad libitum. Milking was performed twice daily at 0530 and 1600 h.

### *Experimental Design and Treatments*

The 13 cows were randomly assigned to one of 2 infusion treatments. The treatments included (1) an infusion with BHBA (**HyperB**;  $n = 5$ ) to obtain a plasma BHBA concentration between 1.5 to 2.0 mmol/L and (2) an infusion with a 0.9% saline solution (NaCl, 20 mL/h; control;  $n = 8$ ). One day before the infusions started, cows were fitted with indwelling intravenous catheters (Cavafix Certo Splittocan, B. Braun Melsungen AG, Melsungen, Germany) with a length of 32 cm and a diameter of 16 gauge in both jugular veins. The infusions through one of the catheters started at 0900 h and continued until 0900 h two days later. The initial infusion rate ( $8.5 \pm 0.6 \mu\text{mol/kg}$  per min) was determined on the basis of BW of each animal and the molarity of the solution was followed by instant continued adjustments throughout the 48 h to maintain a plasma BHBA concentration between 1.5 to 2.0 mmol/L by a peristaltic pump (Perpex Jubile pump, H. J. Guldener, Zürich, Switzerland). During the first 2 h of BHBA infusion, the blood samples were taken every 15 min to measure the plasma BHBA concentration and adjust the BHBA infusion rate to reach the plasma BHBA concentration between 1.5 and 2.0 mmol/L. The control cows were treated with 0.9% saline solution at an infusion rate of 20 mL/h administered using an automatic pump (Perfuser, B. Braun Melsungen AG).

### *Preparation of the BHBA Solution*

To achieve a 1.7 M BHBA solution, 279.053 g of DL-BHBA acid sodium salt (H6501, Sigma-Aldrich, Basel, Switzerland) was dissolved in bi-distilled water to reach an amount of 1.0 L, followed by homogenization using a rotor mixer for 10 to 20 min. The pH of the solution was adjusted with HCl to 7.4 and sterilized at 100 kPa and 131°C for 50 min. The solution was filtered through a 0.2- $\mu\text{m}$  filter (Millipore Express Plus Membrane 45 mm, Millipore, Billerica, MA) and stored at 4°C until infusion.

### *Data Collection and Sampling*

**Feed and Milk Samples.** The individual intake of hay was recorded daily for each cow by weighing the amount given and subtracting it by the remainder of hay found the next morning. The milk yield was record-

ed for each cow individually at every milking during the adaptation and experimental period. Proportional milk samples were taken during milking in the afternoon and in the morning before the start of the experimental infusions. The milk sampling was repeated in the afternoon of d 2 (32 h after the start of infusion), and at the morning milking at the end of experiment.

**Blood Samples.** One week before the experiment, and immediately before the start of the experimental infusions on d 1, reference blood samples of each cow were taken from a jugular vein via vacuum tubes containing tri-potassium-EDTA at 0730 h, after milking and before feeding. The tubes were kept on wet ice until they were centrifuged for 20 min at  $3,000 \times g$  and the plasma was obtained. Until the samples were analyzed for concentrations and activities of plasma variables, the plasma was frozen at  $-20^{\circ}\text{C}$ . After analysis, the mean value of these samples was calculated and used as the reference concentration in the statistical evaluation. During the experimental infusions, blood samples were obtained from the contralateral jugular catheter, which was not used for the infusion, by using tubes containing tri-potassium-EDTA. The collected blood samples were partly analyzed immediately for BHBA concentration (described under Laboratory Procedures) as a basis for adjustments of the infusion rate, or were handled similar to the reference samples.

**Liver Biopsies.** One week before the infusions and 48 h after the start of the infusions, following the blood sampling at around 0800 h, a liver biopsy was taken. Liver tissue (60–100 mg) was obtained under local anesthesia with 10 mL of Lidocain 2% (Streuli Pharma AG, Uznach, Switzerland) by blind liver puncture with a 14-gauge and 150 mm or 200 mm long biopsy needle (Tru-Cut, Provet AG, Lyssach, Switzerland). The samples were placed directly into RNA stabilization reagent (RNAlater, Ambion, Applied Biosystems, Austin, TX), kept at  $5^{\circ}\text{C}$  for 24 h, and then stored at  $-80^{\circ}\text{C}$  until RNA extraction.

### Laboratory Procedures

**Feed and Milk.** The nutritional composition of hay (DM content, 890g/kg of fresh matter; on DM basis, consisting of 153 g of CP/kg, 235.0 g of crude fiber/kg, and 5.7 MJ of  $\text{NE}_L$ /kg) and concentrate (DM content, 881 g/kg of fresh matter; on DM basis, consisting of 217 g of CP/kg, 73.9 g of crude fiber/kg, and 7.6 MJ of  $\text{NE}_L$ /kg) were determined by using routine analyses as described by van Dorland et al. (2009). The nutritive energy balance was calculated as the difference between the energy intake, and the energy output [ $\text{NE}_L$ , requirements for maintenance and milk production,

determined from tabulated values and recommended equations according as to van Dorland et al. (2009)].

Milk samples were analyzed at the laboratory of the Holstein Association of Switzerland (Grangeneuve, Switzerland) for content of fat, protein, lactose, and urea (CombiFoss 6000, Gerber Instrument AG, Effretikon, Switzerland). The ECM was calculated based on Sjaunja et al. (1991), using the formula  $\text{ECM (kg/d)} = \{[0.038 \times \text{Fat (g/kg)} + 0.024 \times \text{Protein (g/kg)} + 0.017 \times \text{Lactose (g/kg)}] \times \text{Milk Yield [kg/d]}\} / 3.14$ .

**Blood Plasma.** Plasma metabolites were measured enzymatically with an automated analyzer (Cobas Mira 2, Hoffmann-La Roche, Basel, Switzerland) by the use of commercial kits as described by van Dorland et al. (2009) and Kreipe et al. (2011). Plasma insulin and IGF-1 were measured by RIA as described by Vicari et al. (2008), and total cortisol was measured by RIA as described by Blum et al. (1985). Plasma glucagon concentration was measured by using a RIA kit (cat. #GL-32K, Millipore, Zug, Switzerland).

**Liver Tissue.** Total mRNA was extracted from liver samples using peqGOLD TriFast (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's protocol, and afterward quantity and purity were determined by absorbance at 260 and 280 nm using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Subsequently, 1  $\mu\text{g}$  of total mRNA was reverse transcribed with 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase RNAase H Minus, Point Mutant (Promega Corp., Madison, WI) using 100 pmol of random hexamer primers (Invitrogen, Leek, the Netherlands). A master mix was prepared containing the following components: 0.8  $\mu\text{L}$  of PCR water, 1.0  $\mu\text{L}$  of forward primer (5 pmol), 1.0  $\mu\text{L}$  of reverse primer (5 pmol), 5.2  $\mu\text{L}$  of  $2 \times$  SensiMix plus SYBR-Green (1 mM  $\text{MgCl}_2$ ). The selected and measured genes encoding for hepatic enzymes involved in gluconeogenesis, glycolysis, FA oxidation, citrate synthase, FA and triglyceride synthesis, cholesterol synthesis, pyruvate dehydrogenase complex, and ketogenesis are shown in Table 1. The primer sequences for pyruvate carboxylase (PC) and mitochondrial phosphoenolpyruvate carboxykinase were according to Hammon et al. (2003); FA synthase, acyl-CoA synthetase long-chain, carnitine palmitoyltransferase 1A, and citrate synthase were according to van Dorland et al. (2009); acetyl-CoA carboxylase (ACoAC), carnitine palmitoyltransferase 2, glucose-6-phosphatase, and 3-hydroxyl-3-methylglutaryl-CoA synthase 1 (HMGCS1) were according to Graber et al. (2010). Lactate dehydrogenase A, lactate dehydrogenase B, phosphofructokinase-liver, dihydrolipoamide branched chain transacylase E2, dihydrolipoamide

dehydrogenase, pyruvate dehydrogenase (lipomide)  $\alpha$  1, glucagon receptor, glycogen synthase 2, and protein kinase AMP-activated  $\alpha$  1 (**PRKAA1**) primers were designed to amplify cDNA.

The PCR quantification was performed by using a Rotor-Gene 6000 (Corbett Research, Sydney, Australia), using the software version 1.7.40. Fluorescence take-off was calculated with the threshold program op-

**Table 1.** Polymerase chain reaction primer information (for = forward, rev = reverse), annealing temperature, and the PCR product length

Gene <sup>1</sup>		Sequence 5'-3' <sup>2</sup>	Gene Bank accession no.	Annealing temperature (°C)	Length (bp)
Gluconeogenesis-related variables					
<i>G6PC</i>	for	GCCAACCTACAGATTTTCGGTG	NM-001076124	58	140
	rev	CAATGCCTGACAAGACTCCAG			
<i>PC</i>	for	ATCTCCTACACGGGTGACGT	NM_177946	60	214
	rev	TGTCGTGGGTGTGGATGTGCA			
<i>PEPCKm</i>	for	TACGAGGCCCTTCAACTGGCGT	XM_583200	60	365
	rev	AGATCCAAGGCGCCTTCCTTA			
Phosphofructokinase, liver and lactate dehydrogenase					
<i>LDHA</i>	for	CGGTTCCACAAACAAGTGGTTG	NM-174099	60	166
	rev	GAGACCCTTAATCATGGTGG			
<i>LDHB</i>	for	CAGCATTCTGGGAAAGTCTC	NM-174100	60	141
	rev	GAGTAATCTTTGTCTGCCAC			
<i>PFKL</i>	for	TTGACTGCAGGACCAACGTC	NM-001080244	60	120
	rev	GCAGCTTCTCCGACATCCAG			
Glucagon receptor, glycogen synthase, protein kinase AMP-activated $\alpha$ 1					
<i>GCGR</i>	for	TCCCAGTGTGATGGATGA	NM-001192414	60	207
	rev	CTTGAGCATGAAGGACACGA			
<i>GYS2</i>	for	AAACTTCCTGCTGAGGATGC	NM-001192905	58	138
	rev	GAATGTGCAGTGTCCCATAG			
<i>PRKAA1</i>	for	AAACTGTACCAGGTCATCAG	NM-001109802	58	326
	rev	GAAATTACTTCTGGTGCAGC			
Pyruvate dehydrogenase complex variables					
<i>DBT</i>	for	GTGCGCTTGCAATGAAAAAC	NM-173905	60	248
	rev	GTGAAAGCCTTTCACGGGTTTC			
<i>DLD</i>	for	TGGTCAAGAGAGGCCATTTC	NM-001206170	60	16
	rev	AACTGGGCAGCTTTAATAGC			
<i>PDHA1</i>	for	ACACAGCATGAGTGATCCTG	NM-001101046	60	127
	rev	CTTCAACACTGGCGGAGATTG			
FA and cholesterol synthesis-related variables					
<i>ACoAC</i>	for	CTCTTCCGACAGGTTCAAGC	AJ-132890	61	248
	rev	ACCATCCTGGCAAGTTTCAC			
<i>FASN</i>	for	CTGAGTCCGAGAACCCTGGAG	NM_001012669	63	232
	rev	ACAAATGGCCTCGTAGGTGAC			
<i>HMGCS1</i>	for	TGTACGGCTCCCTGGCTTCTG	BC-102850	60	313
	rev	CATGTTCTTCGAAGAGGGAATC			
FA oxidation-related variables					
<i>ACSL1</i>	for	TGACTGTTGCTGGAGACTGG	NM-001076085	61	199
	rev	CAGCCGTCTTTATCCAGAGC			
<i>CPT1A</i>	for	CAAAACCATGTTGTACAGCTTCCA	BF039285	54	111
	rev	GCTTCCTTCATCAGAGGCTTCA			
<i>CPT2</i>	for	CAGCCTGCCAGGCTGCCTAT	BC105423	60	163
	rev	AGTGACCAGCTGCTCATGCA			
Citrate synthase and ketogenesis-related variables					
<i>CS</i>	for	TGGACATGATGTATGGTGG	BC-114138	60	217
	rev	AGCCAAGATACCTGTTCCCTC			
<i>HMGCS2</i>	for	TCTGGCCCATCACTCTGCC	NM-001045883	60	126
	rev	AGTGGGGAGCCTGGAGAAGC			

<sup>1</sup>G6PC = glucose-6-phosphatase; PC = pyruvate carboxylase; PEPCKm = mitochondrial phosphoenolpyruvate carboxylase; LDHA = lactate dehydrogenase A; LDHB = lactate dehydrogenase B; PFKL = phosphofructokinase liver; GCGR = glucagon receptor; GYS2 = glycogen synthase 2; PPKA1 = protein kinase AMP-activated  $\alpha$  1; DBT = dihydrolipoamide branched chain transacylase E2; DLD = dihydrolipoamide dehydrogenase; PDHA1 = pyruvate dehydrogenase (lipomide)  $\alpha$  1; ACoAC = acetyl-CoA carboxylase; FASN = fatty acid synthase; HMGCS1 = 3-hydroxyl-3-methylglutaryl-CoA synthase 1; ACSL1 = acyl-CoA synthetase long-chain; CPT1A = carnitine palmitoyltransferase 1A; CPT2 = carnitine palmitoyltransferase 2; CS = citrate synthase; HMGCS2 = 3-hydroxyl-3-methylglutaryl-CoA synthase 2.

<sup>2</sup>for = forward; rev = reverse.

tion. Eight microliters of master mix and 2  $\mu$ L of sample volume, containing 20 ng of cDNA, were used. A 3-step PCR program was performed with (1) denaturation for 10 min at 95°C, (2) 40 cycles of amplification (each consisting of 15 s at 95°C, the primer-specific annealing temperature for 30 s; Pfaffl et al., 2002; Graber et al., 2010) and extension at 72°C for 20 s and quantification of fluorescence, and (3) a melting curve program (60–95°C). The mRNA levels were calculated relative to the mean expression level of the housekeeping genes GAPDH and ubiquitin, which were stable across time points as described by Kreipe et al. (2011).

### Statistical Analysis

Prior to data analysis, the change ( $\Delta$ ) for each variable (gene expression, plasma metabolites, feed intake, milk yield, and ECM) was calculated by subtracting the value measured after the infusion treatments from the value measured before the infusion treatments. The  $\Delta$  for each variable (difference between before and after 48-h infusion) was statistically evaluated using the general linear models procedure of SAS (SAS Institute Inc., Cary, NC, 2002–2008, Release 9.2), including treatment (BHBA or NaCl) as fixed effect. Differences between means were determined by the Tukey test. In addition, means of  $\Delta$  values obtained within each treatment were tested for their difference from zero. Furthermore, blood plasma variables and the area under the curve was calculated for d 2 of the treatments and subsequently assessed statistically with the model as described above. Data obtained before the treatment were included as covariates in the model to compensate for initial differences between individuals. Data are presented as means  $\pm$  SEM, and differences were considered significant if  $P < 0.05$  and as a trend if  $P < 0.10$ .

## RESULTS

### Infusion Rates and Plasma Concentrations of BHBA

The mean infusion rate of BHBA over 48 h was  $8.5 \pm 0.6$   $\mu$ mol/kg per min (Figure 1). The mean plasma BHBA concentration reached and maintained was  $1.7 \pm 0.1$  mmol/L for 48 h. The infusion of BHBA resulted in a marked increase in plasma BHBA concentration in HyperB cows ( $P < 0.001$ ). Plasma BHBA concentration in control cows remained unchanged. Consequently, larger area under the curve was observed (Table 2) for plasma BHBA concentration in HyperB ( $1.74 \pm 0.02$  mmol/L) cows compared with the control cows ( $0.59 \pm 0.02$  mmol/L) on d 2 ( $P < 0.001$ ).

### Feed Intake, Energy Balance, and ECM

Dry matter intake of hay and concentrate before the infusions (d 0) is shown in Table 3. On d 1 of the infusions, DMI of hay and concentrate was  $19.1 \pm 0.4$  and  $16.9 \pm 1.9$  kg/d for HyperB and control cows, respectively. On d 2 of the infusions, DMI of hay and concentrate was  $19.0 \pm 0.9$  and  $17.9 \pm 1.0$  kg/d for HyperB and control cows, respectively. The DMI was not affected by BHBA infusion.

The nutritive energy balance was not affected by BHBA infusion and was similar in both groups across the study period. The  $\Delta$  from d 0 to d 2 was  $-1.04 \pm 8.67$  and  $1.72 \pm 4.9$  MJ/d for HyperB and control cows, respectively. Milk yield and ECM were not affected by BHBA infusion.

### Blood Metabolites and Enzymes

Plasma glucose concentration in HyperB cows decreased ( $P = 0.03$ ; Table 4) and was lower compared with control group on d 2 ( $P = 0.0012$ ; Table 2 and Figure 2).  $\beta$ -Hydroxybutyrate infusion decreased the plasma glucagon concentration on d 2 ( $P = 0.03$ ; Figure 3). Plasma concentrations of NEFA, IGF-1, urea, triglyceride, cortisol, and insulin (Figure 4) remained unchanged in both HyperB and control groups during the course of infusion. The plasma urea, IGF-1, and triglycerides decreased within control group, and the plasma glucose and urea decreased within HyperB group.

### Liver Gene Expression

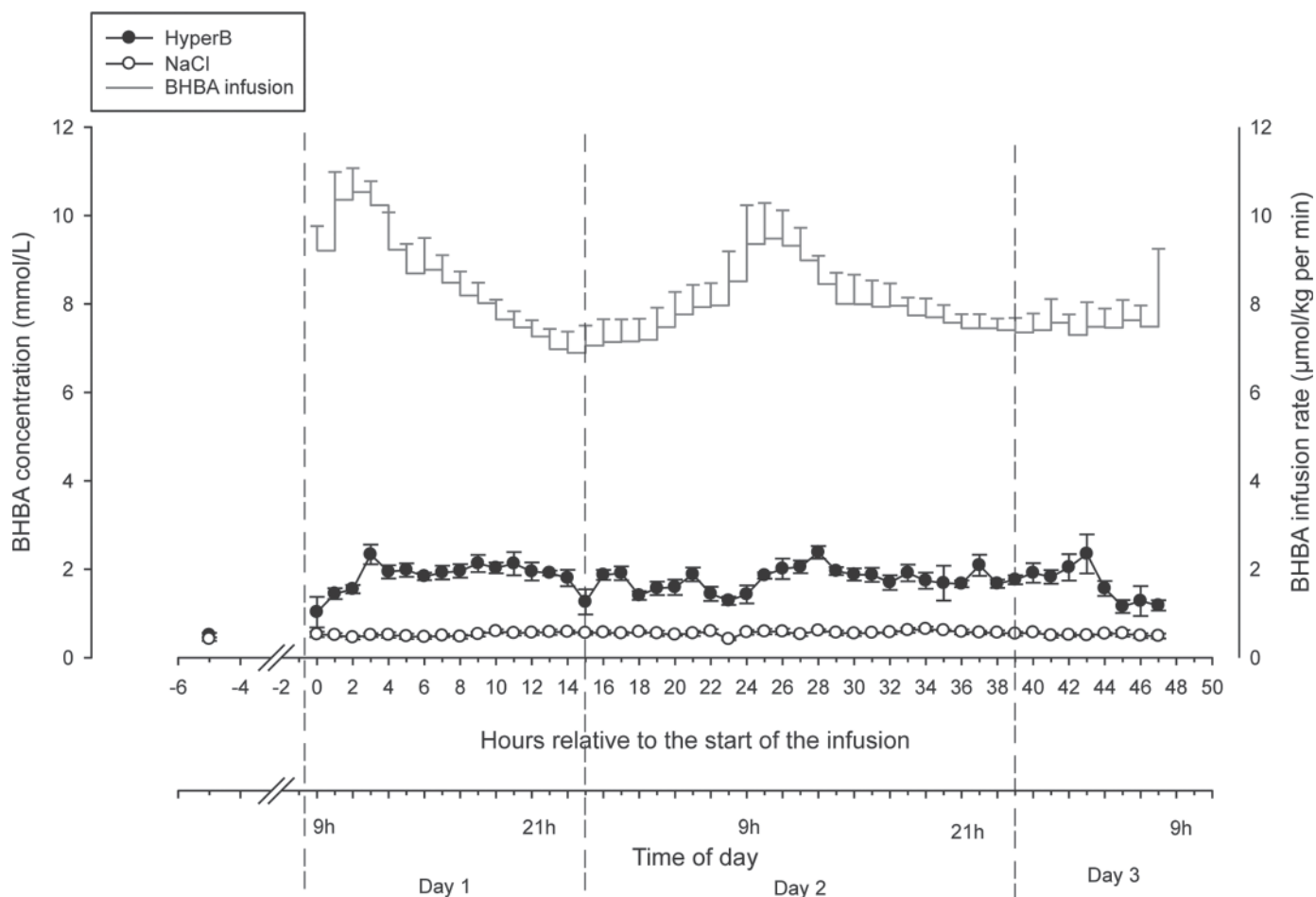
The mRNA abundance in liver of the studied variables did not differ between treatments (Table 5).  $\beta$ -Hydroxybutyrate infusion affected abundance of PRKAA1on mRNA and tended to be lower in the HyperB group than the control group ( $P = 0.08$ ). The mRNA abundance of PC increase and that of pyruvate dehydrogenase (lipomide)  $\alpha$  1, and HMGCS1 decreased ( $P < 0.05$ ) over time within the control cows. In the HyperB group, the mRNA abundance of HMGCS1 decreased significantly over time.

## DISCUSSION

The objective was to induce an elevated plasma concentration of BHBA, without the metabolic situation as observed during the early lactation, to solely investigate the effect of BHBA on feed intake, milk yield, and metabolism. To the best of our knowledge, this study is the first that has induced an elevated plasma

**Table 2.** Least squares means  $\pm$  SEM of area under the curve for plasma variables concentration in dairy cows infused with BHBA (HyperB) or saline (NaCl) on d 2

Variable	Group	LSM $\pm$ SEM d 2	ANOVA ( <i>P</i> -value, group)
Glucose (mmol/L)	HyperB	3.47 $\pm$ 0.11	<0.01
	NaCl	4.11 $\pm$ 0.08	
Insulin (mU/L)	HyperB	12.7 $\pm$ 1.4	0.54
	NaCl	13.9 $\pm$ 1.1	
BHBA (mmol/L)	HyperB	1.74 $\pm$ 0.02	<0.001
	NaCl	0.59 $\pm$ 0.02	
NEFA (mmol/L)	HyperB	0.06 $\pm$ 0.03	0.51
	NaCl	0.09 $\pm$ 0.02	
Urea (mmol/L)	HyperB	3.77 $\pm$ 0.31	0.63
	NaCl	3.97 $\pm$ 0.24	
Glucagon (pg/mL)	HyperB	97.4 $\pm$ 3.3	<0.05
	NaCl	107.7 $\pm$ 2.6	
IGF-1 (ng/mL)	HyperB	90 $\pm$ 4.5	0.11
	NaCl	80 $\pm$ 3.6	
Triglycerides (mmol/L)	HyperB	0.15 $\pm$ 0.01	0.58
	NaCl	0.14 $\pm$ 0.01	
Cortisol (ng/mL)	HyperB	2.41 $\pm$ 0.55	0.78
	NaCl	2.62 $\pm$ 0.43	



**Figure 1.**  $\beta$ -Hydroxybutyrate infusion rate during 48-h infusion in BHBA cows (HyperB). Plasma BHBA concentration in control cows and cows infused with BHBA before the start of the infusion (d 0) and during 48-h infusion. Values represent mean  $\pm$  SEM.

**Table 3.** Milk yield, ECM, DMI, and energy balance (EB) of dairy cows infused with BHBA (HyperB) or saline (NaCl) before the start of the infusion (d 0) and difference ( $\Delta$  d 2-d 0) between before and after 48-h infusion (d 2); values represent mean  $\pm$  SEM

Variable	Group	d 0	$\Delta$ d 2-d 0	ANOVA ( <i>P</i> -value, group)
Milk yield (kg/d)	HyperB	23.31 $\pm$ 0.78	-3.30 $\pm$ 1.43*	0.3
	NaCl	21.78 $\pm$ 2.53	-1.76 $\pm$ 0.68	
ECM (kg/d)	HyperB	21.00 $\pm$ 0.28	-1.58 $\pm$ 0.76	0.79
	NaCl	21.95 $\pm$ 2.58	-1.21 $\pm$ 0.85	
DMI (kg/day)	HyperB	19.82 $\pm$ 0.66	-0.80 $\pm$ 1.22	0.76
	NaCl	18.33 $\pm$ 1.28	-0.38 $\pm$ 0.76	
EB (MJ/d)	HyperB	15.04 $\pm$ 2.18	-1.04 $\pm$ 8.67	0.77
	NaCl	1.67 $\pm$ 4.90	1.72 $\pm$ 4.90	

\*Delta is different from 0 ( $P < 0.05$ ).

BHBA concentration for 48 h in dairy cows by infusion of BHBA. The plasma BHBA concentration reached was  $1.7 \pm 0.1$  mmol/L, which was clearly above the considered concentration of 1.2 to 1.4 mmol/L referring to subclinical ketosis (Duffield et al., 2009; Ospina et al., 2010).

The expected reduction of DMI in HyperB cows, as shown in cows with spontaneously elevated ketone body concentrations (Bareille et al., 2003; González et al., 2008), was not observed in our study. Subcutaneous injection of BHBA (10 mmol/kg of BW) in rats (Moor et al., 1976; Langhans et al., 1983), intraperitoneal BHBA infusion (15 mmol/kg<sup>0.75</sup>) in pigmy goats (Rossi et al., 2000), and intracerebroventricular BHBA infusion (18mM/d) in dairy cows (Kuhla et al., 2011) decreased feed intake.  $\beta$ -Hydroxybutyrate can play a role in the regulation of feed intake (Langhans et al., 1983). In an in vitro experiment, elevation of the

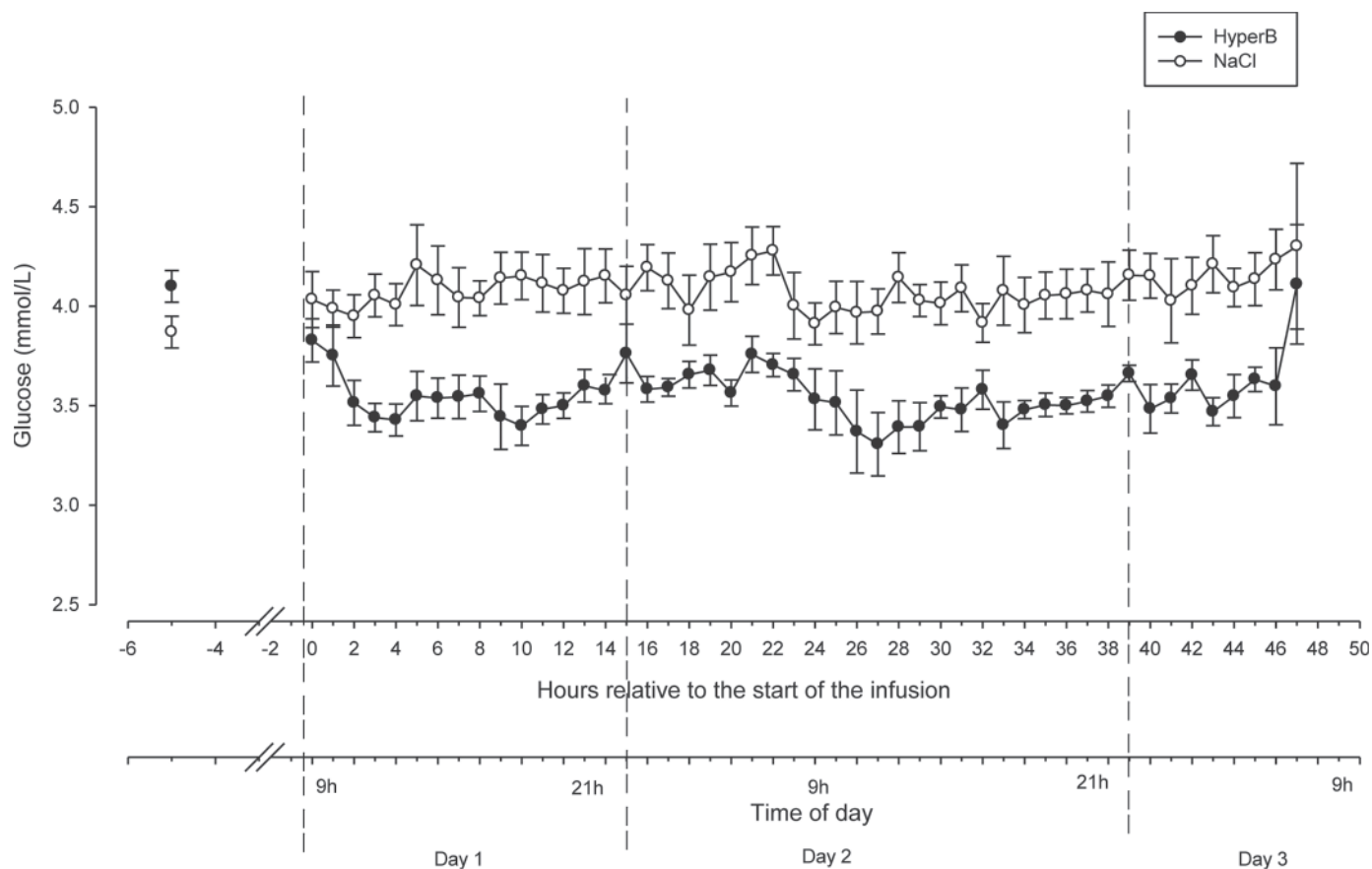
BHBA to glucose ratio to 1:1 in early lactation, compared to nonketotic cows with a 1:4 ratio, can decrease feed intake via stimulation of dephosphorylation of 5' adenosine monophosphate-activated protein kinase (AMPK) after parturition (Laeger et al., 2012). In the current study, BHBA infusion did not affect feed intake throughout the experiment, which may relate to a low ratio of BHBA to glucose.

During the experiment plasma glucose concentration decreased dramatically due to the infusion of BHBA. This finding is consistent with other reports of decreased plasma glucose concentration associated with elevated BHBA in pregnant sheep (Schlumbohm and Harmeyer 2003, 2004), dogs (Felts et al., 1964; Madison et al., 1964), and pigs (Müller et al., 1984). However, our findings are in contrast to other reports that described a hyperglycemia in rabbits (Mirsky and Broh-Kahn, 1937) induced by ketone bodies infusion.

**Table 4.** Plasma variables concentrations in dairy cows infused with BHBA (HyperB) or saline (NaCl) before the start of the infusion (d 0) and difference ( $\Delta$  d 2-d 0) between before and after 48-h infusion (d 2); values represent mean  $\pm$  SEM

Variable	Group	d 0	$\Delta$ d 2-d 0	ANOVA ( <i>P</i> -value, group)
Glucose (mmol/L)	HyperB	4.10 $\pm$ 0.08	-0.46 $\pm$ 0.18*	<0.05
	NaCl	3.87 $\pm$ 0.08	0.10 $\pm$ 0.14	
Insulin (mU/L)	HyperB	8.74 $\pm$ 0.78	-1.56 $\pm$ 0.92	0.30
	NaCl	6.78 $\pm$ 1.62	0.14 $\pm$ 1.08	
BHBA (mmol/L)	HyperB	0.51 $\pm$ 0.05	0.74 $\pm$ 0.15*	<0.001
	NaCl	0.42 $\pm$ 0.04	0.09 $\pm$ 0.05	
NEFA (mmol/L)	HyperB	0.11 $\pm$ 0.02	-0.02 $\pm$ 0.02	0.7
	NaCl	0.16 $\pm$ 0.03	-0.04 $\pm$ 0.04	
Urea (mmol/L)	HyperB	4.73 $\pm$ 0.34	-1.25 $\pm$ 0.3*	0.73
	NaCl	5.0 $\pm$ 0.26	-1.05 $\pm$ 0.4*	
IGF-1 (ng/mL)	HyperB	91 $\pm$ 6.1	-6.65 $\pm$ 2.5	0.55
	NaCl	100 $\pm$ 10.0	-9.87 $\pm$ 3.7*	
Glucagon (pg/mL)	HyperB	115.2 $\pm$ 19.1	-17.3 $\pm$ 1.3*	0.06
	NaCl	109.6 $\pm$ 5.4	-1.0 $\pm$ 6.1	
Triglycerides (mmol/L)	HyperB	0.18 $\pm$ 0.01	-0.02 $\pm$ 0.01	0.91
	NaCl	0.16 $\pm$ 0.01	-0.02 $\pm$ 0.01*	
Cortisol (ng/mL)	HyperB	5.74 $\pm$ 1.4	-2.37 $\pm$ 1.9	0.67
	NaCl	2.92 $\pm$ 0.8	-1.56 $\pm$ 0.9	

\*Delta is different from 0 ( $P < 0.05$ ).

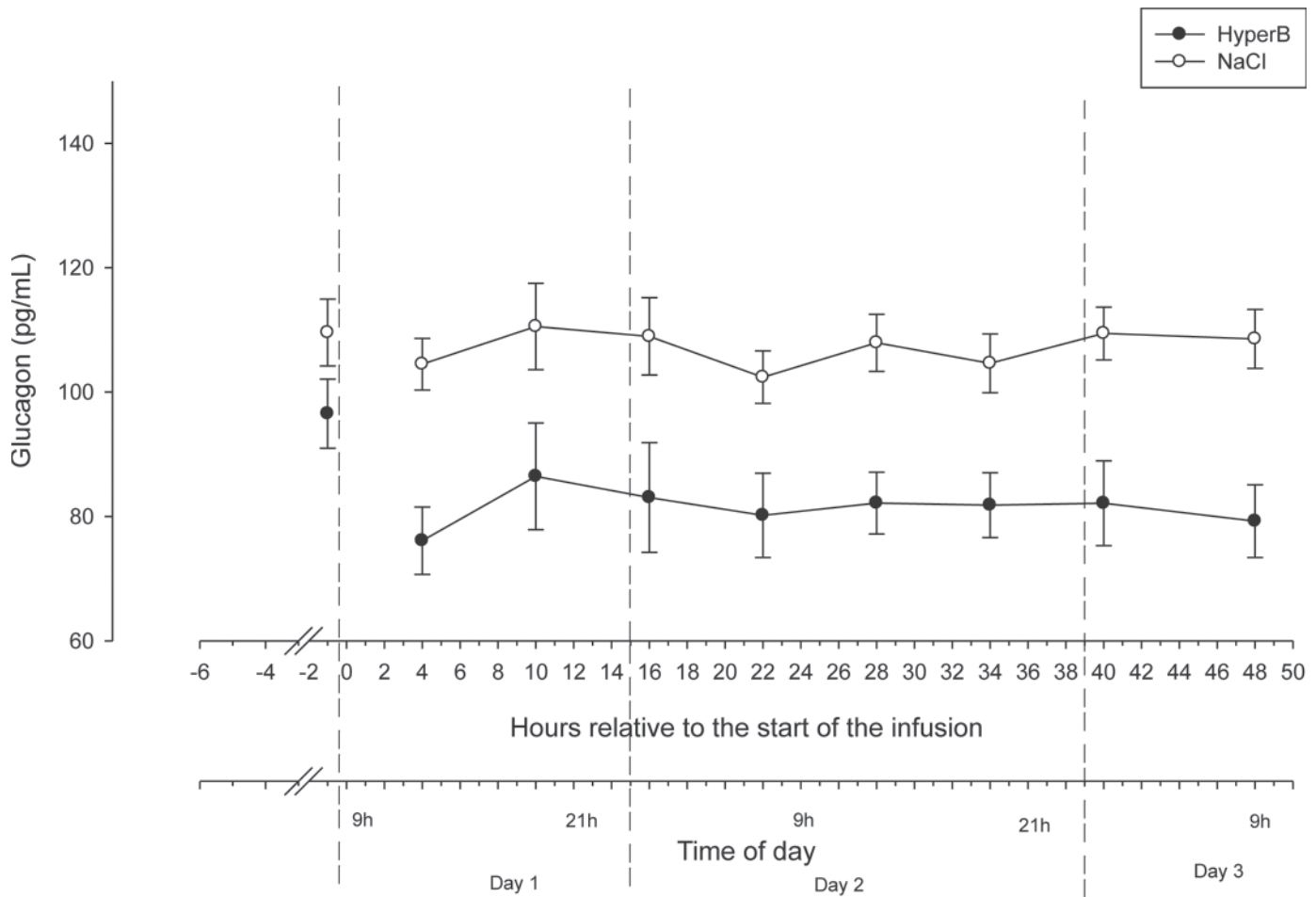


**Figure 2.** Plasma glucose concentration in control cows and cows infused with BHBA (HyperB) before the start of the infusion (d 0) and during 48-h infusion. Values represent mean  $\pm$  SEM.

Information about the reasons for decreased plasma glucose concentration due to the elevated BHBA concentration is limited; hypothetically, insulin plays a role in this effect. Infusion of BHBA increases insulin secretion in pigs (Müller et al., 1984). Madison et al. (1964) suggested that pancreatic beta cells respond to the high plasma BHBA concentration with increased insulin secretion in dogs. It appears that insulin suppresses the glucose production throughout an inhibitory effect on the regulatory enzymes of gluconeogenesis (Brockman and Larrveld, 1986; Hayirli, 2006). This release of endogenous insulin can lead to a decline in plasma glucose concentration and reduce hepatic glucose production. The unchanged insulin concentration in response to the BHBA infusion in the present study may be explained by the fact that ketone bodies represent only a modest stimulus on insulin secretion in ruminants (Jordan and Philips, 1978; Heitmann and Fernandez, 1986). Insulin obviously did not play a significant role in mediating the lowering effect of BHBA on plasma glucose concentration in sheep (Schlumbohm and Harmeyer, 2003). Decreased glucose concentration and hepatic glucose

output due to the infusion of BHBA can reduce the availability of glucose for the peripheral tissues because exhausted glucose stores must be replaced by a catabolism of proteins during emergency conditions for the vital tissues, such as central nervous system (Madison et al., 1964). The molarity of ketone bodies in plasma is the same or higher than that of glucose. Thus, enhanced plasma BHBA concentration can increase the efficiency of BHBA as a competitor to glucose used by peripheral tissues, and has been shown to inhibit peripheral glucose utilization (Madison et al., 1964). Glucose is required as a unique source for mammary lactose synthesis or energy for red blood cells. However, during limited glucose availability in the body, extra hepatic tissues (e.g., brain) can use ketone bodies as an alternative fuel, and ketone bodies have a glucose-sparing effect in the tissues where they can be used as energy source. Mebane and Madison (1962) demonstrated that ketone body infusion leads to a 30% reduction in glucose utilization by peripheral tissues. Previous studies also reported that the oxidation of glucose was inhibited by ketone body infusion (Neptune



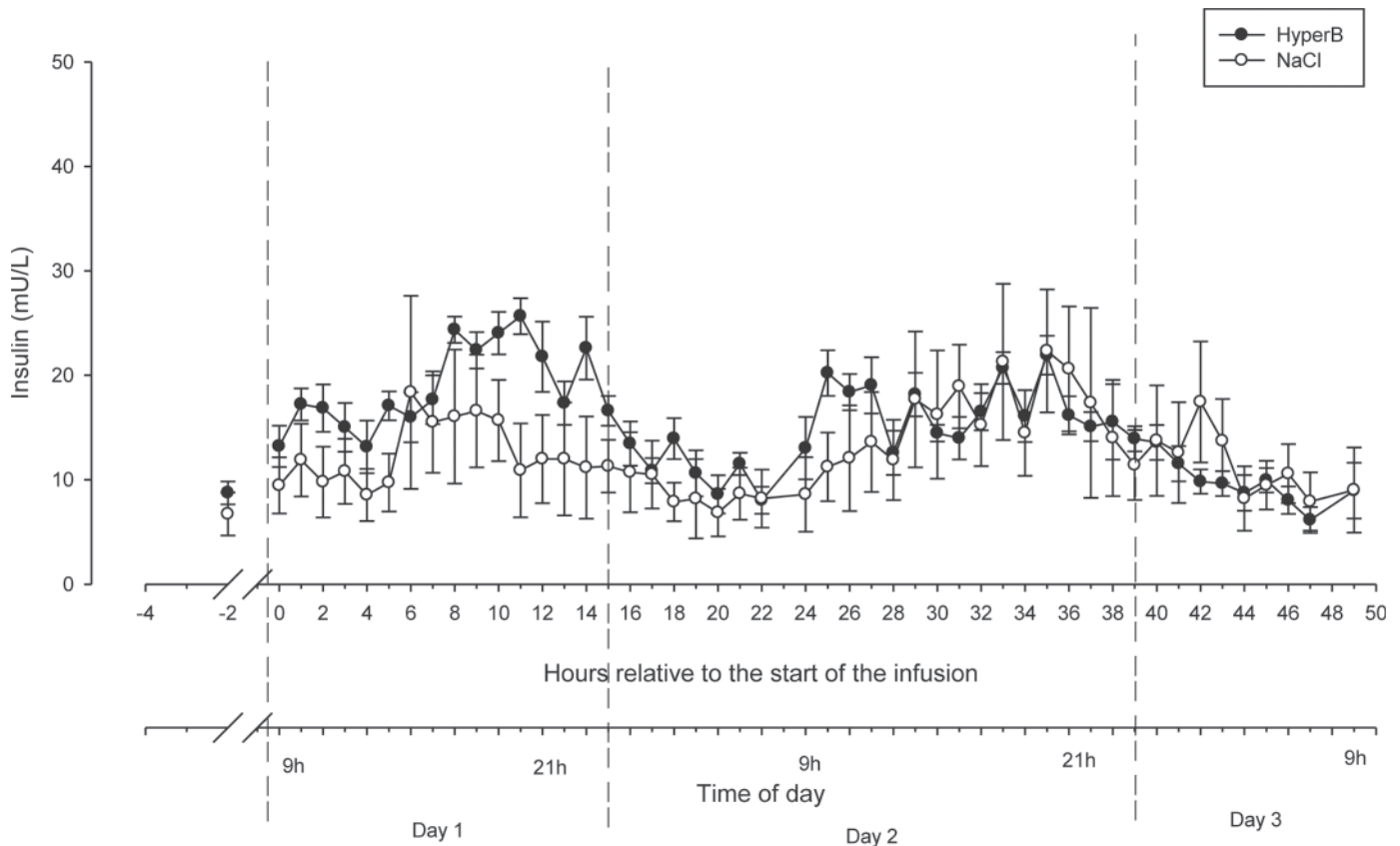


**Figure 3.** Plasma glucagon concentration in control cows and cows infused with BHBA (HyperB) before the start of the infusion (d 0) and during 48-h infusion. Values represent mean  $\pm$  SEM.

et al., 1961; Williamson and Krebs, 1961). In contrast, as mentioned previously, Schlumbohm and Harmeyer (2004) suggested that BHBA infusion decreased endogenous glucose production but did not affect glucose utilization in pregnant sheep. Furthermore, they suggested that the elevated concentration of ketone bodies in blood initially decreases glucose production via the depressed signal on hepatic gluconeogenesis in liver.

In contrast to a previous study that found increased glucagon concentrations after BHBA infusion in pigs (Müller et al., 1984), in the current study BHBA infusion decreased glucagon concentration in HyperB compared with the control group. Plasma glucose is derived from the feed, glycogen, and by gluconeogenesis, and it is regulated by several hormones, mainly glucagon and insulin. Glucagon increases plasma glucose concentration through stimulation of gluconeogenesis and glycogenolysis (Aronoff et al., 2004; Taborsky, 2010). Changes in glucagon secretion from the physiological range results in changes of plasma glucose concentra-

tion (Taborsky, 2010); some substrates, such as NEFA and ketone bodies, suppress glucagon secretion (Gerich et al., 1974;1976; Goberna et al., 1974). Evidence exists that glucagon secretion is inhibited by the neighboring beta cell through insulin (Weir et al., 1976) and  $\gamma$ -aminobutyric acid (**GABA**; Adeghate et al., 2000; Wendt et al., 2004).  $\gamma$ -Aminobutyric acid is an inhibitory neurotransmitter in the brain. In an in vitro study it was demonstrated that BHBA increases GABA concentration in the epileptic brain (Suzuki et al., 2009). Therefore, it can be hypothesized that the decrease in glucagon concentration in the current study is related to elevated BHBA concentration—as BHBA is used as an alternative fuel and energy source for the tissues—or glucagon secretion was inhibited by GABA, whose concentration likely increased during elevated BHBA. Subsequently, a decline of glucagon secretion decreased glucose production through the effect on glycogenolysis or gluconeogenesis during the experiment. A decrease of plasma NEFA concentration was expected in response



**Figure 4.** Plasma insulin concentration in control cows and cows infused with BHBA (HyperB) before the start of the infusion (d 0) and during 48-h infusion. Values represent mean  $\pm$  SEM.

to the BHBA infusion and a subsequent increase of insulin (Madison et al., 1964); however, both variables did not change during the experimental period.

$\beta$ -Hydroxybutyrate can replace glucose to be utilized in peripheral tissues (Madison et al., 1964), which can also potentially contribute to reduced FA mobilization from adipose tissues. Conversely, after a decline in glucose during negative energy balance, NEFA plasma concentration increased in early lactation (van Dorland et al., 2009). The main difference of the current study from the previous one is the absence of negative energy balance despite decreased glucose, with the respective differences of insulin secretion. However, as the main site of ketone body synthesis from fatty acid oxidation, the liver cannot utilize ketone bodies for the synthesis of acetoacetyl CoA, and subsequently FA, due to a lack of succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial (Goodridge and Sul, 2000).

#### **Effects of BHBA Infusion on Hepatic Gene Expression**

The present results of mRNA abundance of key enzymes related to hepatic gluconeogenesis (PC, glucose-

6-phosphatase, mitochondrial phosphoenolpyruvate carboxykinase) are in contrast with suggestions from previous studies that indicated an inhibition of gluconeogenesis by elevated plasma BHBA concentration (Müller et al., 1984; Schlumbohm and Harmeyer, 2003). Müller et al. (1984) suggested that ketone bodies probably have a direct inhibitory action on gluconeogenesis. They mentioned that the effect of ketone bodies on endogenous glucose production is probably mediated by insulin. However, in ruminants, the effect of ketone bodies on insulin secretion is not strong (Jordan and Philips, 1978; Heitmann and Fernandez, 1986), and insulin concentration did not change in the current study. Soling and Kleineke (1976) suggested that ketone bodies have a glucose-sparing effect in tissues through the mitochondrial formation of citrate, an allosteric inhibitor, phosphofructo-1,6-kinase, acetyl-CoA inhibition of pyruvate dehydrogenase and activation of PC.  $\beta$ -Hydroxybutyrate infusion did not affect key enzymes related to gluconeogenesis, glycolysis, pyruvate dehydrogenase complex, and citrate synthase mRNA expression. According to the mRNA abundance of some enzymes related to FA oxidation, BHBA infusion did

**Table 5.** Liver mRNA expression in dairy cows infused with BHBA (HyperB) or saline (NaCl) before the start of the infusion (d 0) and difference ( $\Delta$  d 2–d 0) between before and after 48-h infusion (d 2); values represent mean  $\pm$  SEM

Variable <sup>1</sup>	Group	d 0	$\Delta$ d 2–d 0	ANOVA ( <i>P</i> -value, group)
Gluconeogenesis-related variables				
<i>G6PC</i>	HyperB	22.89 $\pm$ 0.46	–0.57 $\pm$ 0.50	0.44
	NaCl	21.05 $\pm$ 0.49	–0.18 $\pm$ 0.24	
<i>PC</i>	HyperB	17.42 $\pm$ 0.22	0.43 $\pm$ 0.08	0.87
	NaCl	17.57 $\pm$ 0.13	0.48 $\pm$ 0.23*	
<i>PEPCKm</i>	HyperB	14.60 $\pm$ 0.43	0.46 $\pm$ 0.29	0.18
	NaCl	14.85 $\pm$ 0.20	–0.21 $\pm$ 0.32	
Phosphofructokinase, liver and lactate dehydrogenase				
<i>LDHA</i>	HyperB	16.58 $\pm$ 0.22	–0.58 $\pm$ 0.32	0.80
	NaCl	16.50 $\pm$ 0.30	–0.45 $\pm$ 0.34	
<i>LDHB</i>	HyperB	19.47 $\pm$ 0.32	0.01 $\pm$ 0.18	0.52
	NaCl	19.68 $\pm$ 0.38	–0.36 $\pm$ 0.45	
<i>PFKL</i>	HyperB	15.00 $\pm$ 0.25	0.35 $\pm$ 0.37	0.35
	NaCl	15.38 $\pm$ 0.21	–0.22 $\pm$ 0.40	
Glucagon receptor, glycogen synthase, protein kinase AMP-activated $\alpha$ 1				
<i>GCGR</i>	HyperB	17.92 $\pm$ 0.19	–0.33 $\pm$ 0.24	0.91
	NaCl	16.98 $\pm$ 0.56	–0.24 $\pm$ 0.28	
<i>GYS2</i>	HyperB	19.03 $\pm$ 0.28	–0.90 $\pm$ 0.35	0.83
	NaCl	17.56 $\pm$ 0.49	–0.57 $\pm$ 0.29	
<i>PRKAA1</i>	HyperB	15.19 $\pm$ 0.22	–0.57 $\pm$ 0.30	0.08
	NaCl	14.11 $\pm$ 0.36	0.11 $\pm$ 0.21	
Pyruvate dehydrogenase complex variables				
<i>DBT</i>	HyperB	18.19 $\pm$ 0.18	–0.09 $\pm$ 0.23	0.90
	NaCl	17.64 $\pm$ 0.27	–0.21 $\pm$ 0.68	
<i>DLD</i>	HyperB	18.14 $\pm$ 0.17	–0.52 $\pm$ 0.31	0.84
	NaCl	17.90 $\pm$ 0.14	–0.38 $\pm$ 0.48	
<i>PDHA1</i>	HyperB	17.94 $\pm$ 0.14	–0.58 $\pm$ 0.46	0.53
	NaCl	18.09 $\pm$ 0.17	–1.03 $\pm$ 0.46*	
FA and cholesterol synthesis-related variables				
<i>ACoAC</i>	HyperB	10.41 $\pm$ 0.18	–0.44 $\pm$ 0.34	0.64
	NaCl	8.66 $\pm$ 0.51	–0.74 $\pm$ 0.43	
<i>FASN</i>	HyperB	14.0 $\pm$ 0.4	–0.8 $\pm$ 0.3	0.82
	NaCl	14.0 $\pm$ 0.51	–0.9 $\pm$ 0.5	
<i>HMGCS1</i>	HyperB	20.95 $\pm$ 0.24	–1.61 $\pm$ 0.51*	0.69
	NaCl	18.84 $\pm$ 0.46	–1.24 $\pm$ 0.60*	
FA oxidation-related variables				
<i>ACSL</i>	HyperB	19.76 $\pm$ 0.17	0.05 $\pm$ 0.19	0.65
	NaCl	18.87 $\pm$ 0.49	–0.07 $\pm$ 0.16	
<i>CPT1</i>	HyperB	14.82 $\pm$ 0.22	–0.28 $\pm$ 0.29	0.55
	NaCl	13.16 $\pm$ 0.36	–0.09 $\pm$ 0.16	
<i>CPT2</i>	HyperB	18.07 $\pm$ 0.12	–0.33 $\pm$ 0.10	0.54
	NaCl	17.19 $\pm$ 0.29	–0.46 $\pm$ 0.14	
Citrate synthase, and ketogenesis-related variables				
<i>CS</i>	HyperB	14.48 $\pm$ 0.06	–0.26 $\pm$ 0.15	0.91
	NaCl	12.77 $\pm$ 0.40	–0.23 $\pm$ 0.20	
<i>HMGCS2</i>	HyperB	21.62 $\pm$ 0.33	–0.23 $\pm$ 0.33	0.77
	NaCl	21.14 $\pm$ 0.32	–0.10 $\pm$ 0.29	

<sup>1</sup>G6PC = glucose-6-phosphatase; PC = pyruvate carboxylase; PEPCKm = mitochondrial phosphoenolpyruvate carboxykinase; LDHA = lactate dehydrogenase A; LDHB = lactate dehydrogenase B; PFKL = phosphofructokinase liver; GCGR = glucagon receptor; GYS2 = glycogen synthase 2; PPKA1 = protein kinase AMP-activated  $\alpha$  1; DBT = dihydrolipoamide branched chain transacylase E2; DLD = dihydrolipoamide dehydrogenase; PDHA1 = pyruvate dehydrogenase (lipomide)  $\alpha$  1; ACoAC = acetyl-CoA carboxylase; FASN = fatty acid synthase; HMGCS1 = 3-hydroxy-3-methylglutaryl-CoA synthase 1; ACSL1 = acyl-CoA synthetase long-chain; CPT1A = carnitine palmitoyltransferase 1A; CPT2 = carnitine palmitoyltransferase 2; CS = citrate synthase; HMGCS2 = 3-hydroxy-3-methylglutaryl-CoA synthase 2.

\*Delta is different from 0 ( $P < 0.05$ ).

not affect FA oxidation. These results are not in agreement with previous reports (Soling and Kleineke, 1976; Morio and Wolfe, 2005) that mentioned ketone bodies are an inhibitor for FA oxidation through increase acetyl CoA production, inhibit carnitine palmitoyltransferase activity, and stimulate malonyl CoA synthesis.

As mentioned previously, ketone bodies suppressed the glucagon secretion, and this inhibition could have been regulated via cyclic AMP. The mechanism of this inhibition is related to the generation of ATP or ADP by substrate, such as ketone bodies, then decrease of cAMP, and finally prevention of glucagon secretion

(Toyota et al., 1975; Gerich et al., 1976; Mitrakou et al., 1991; Adeghate et al., 2000). In the present study, the PRKAA1 expression tended to decrease in HyperB compared with control group ( $P < 0.1$ ). Conversely, BHBA infusion decreased the PRKAA1 expression, and subsequently glucagon excretion was decreased.

## CONCLUSIONS

Results from this study demonstrate that intravenous BHBA infusion successfully induced elevated plasma BHBA concentration over 48 h in dairy cows. Feed intake was not affected by elevated BHBA concentration during a period when energy balance is positive. Decreased plasma glucose concentration during BHBA administration cannot be related to changes of insulin concentration or to mRNA abundance of genes related to hepatic gluconeogenesis, FA synthesis, citrate synthase, pyruvate dehydrogenase complex, and glycolysis. Although mRNA expression indicated high activity of enzyme production, the effects of BHBA infusion most likely occurred at a posttranscriptional level. Reduced glucose concentration may be related to a decrease in plasma glucagon concentration, which can decrease glucose production from gluconeogenesis or glycogen storage. Thus, BHBA seems to have a glucose-sparing effect. However, glucose entry rate was probably reduced by the treatment, and additional studies will be necessary to clarify the underlying mechanisms.

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