Induced hypoglycemia for 48 hours indicates differential glucose and insulin effects on liver metabolism in dairy cows

L. Kreipe, M. C. M. B. Vernay, A. Oppliger, O. Wellnitz, R. M. Bruckmaier, and H. A. van Dorland¹

Veterinary Physiology, Vetsuisse Faculty, University of Bern, Bremgartenstrasse 109a, 3001 Bern, Switzerland

ABSTRACT

Hypoglycemia is a characteristic condition of early lactation dairy cows and is subsequently dependent on, and may affect, metabolism in the liver. The objective of the present study was to investigate the effects of induced hypoglycemia, maintained for 48 h, on metabolic parameters in plasma and liver of mid-lactation dairy cows. The experiment involved 3 treatments, including a hyperinsulinemic hypoglycemic clamp (HypoG, $n = 6$) to obtain a glucose concentration of 2.5 mmol/L, a hyperinsulinemic euglycemic clamp (EuG, $n = 6$) in which the effect of insulin was studied, and a control treatment with a 0.9% saline solution (NaCl, $n = 6$). Blood samples for measurements of insulin, metabolites, and enzymes were taken at least once per hour. Milk yield was recorded and milk samples were collected before and after treatment. Liver biopsies were obtained before and after treatment to measure mRNA abundance by real-time, quantitative reverse transcription-PCR of 12 candidate genes involved in the main metabolic pathways. Milk yield decreased in HypoG and NaCl cows, whereas it remained unaffected in EuG cows. Energy-corrected milk yield (kg/d) was only decreased in HypoG cows. In plasma, concentration of β -hydroxybutyrate decreased in response to treatment in EuG cows and was lower (0.41 ± 0.04 mmol/L) on d 2 of the treatment compared with that in HypoG and NaCl cows (on average 0.61 ± 0.03 mmol/L, respectively). Nonesterified fatty acids remained unaffected in all treatments. In the liver, differences between treatments for their effects were only observed in case of mitochondrial phosphoenolpyruvate carboxykinase (PEPCKm) and glucose-6-phosphatase (G6PC). In HypoG, mRNA abundance of PEPCKm was upregulated, whereas in EuG and NaCl cows, it was downregulated. The EuG treatment downregulated mRNA expression of G6PC, a marked effect compared with the unchanged transcript expression in NaCl. The mRNA abundance of the in-

sulin receptor remained unaffected in all treatments, and no significant treatment differences were observed for genes related to lipid metabolism. In conclusion, low glucose concentrations in dairy cows affect liver metabolism at a molecular level through upregulation of PEPCKm mRNA abundance. Metabolic regulatory events in the liver are directed, apart from hormones, by the level of metabolites, either in excess (e.g., free fatty acids) or in shortage (e.g., glucose).

Key words: liver, hypoglycemia, hyperinsulinemia, gene expression

INTRODUCTION

The most challenging time for the metabolism of the dairy cow, even when kept under optimal management conditions, is the time around parturition. During this period, dairy cows undergo remarkable endocrine and metabolic changes that are necessary to cover the sudden increased nutrient and energy demand at the onset of milk production. However, the demands for lactation exceed the intake capacity of the dairy cow, leading to a status of negative energy balance. Characteristic of a cow in negative energy balance compared with a cow in positive energy balance is a low plasma concentration of glucose, which is normally accompanied by elevated plasma concentrations of NEFA and ketone bodies (van Dorland et al., 2009). Each of these metabolites is dependent on and may affect metabolic regulation in the liver. For instance, a cow's requirement for glucose increases 2- to 3-fold during the transition period, which coincides with an increase in hepatic gluconeogenesis postpartum (Drackley et al., 2001). In addition, during a situation of decreased plasma glucose concentration, glycogenolysis in the liver and kidney is stimulated (Overton et al., 1998). Undoubtedly, the liver plays a major role in metabolic regulation during this period, and failure of hepatic functioning may lead to metabolic-related disorders such as ketosis or fatty liver (Drackley et al., 2005). Identification of the specific adjustments or failure of hepatic metabolism during the physiological condition described above may increase our understanding of the pathogenesis of metabolic disorders. The investigation of exclusive effects

Received January 25, 2011.

Accepted July 24, 2011.

¹Corresponding author: anette.vandorland@physio.unibe.ch

of individual parameters of the complex physiological condition of early lactation may aid in obtaining this understanding. In the present study, the effects of a long-term induced hypoglycemia (for 48 h) on key enzymes of metabolic regulation in the liver of mid-lactation dairy cows were investigated. An exclusive state of hypoglycemia, without elevated plasma concentrations of NEFA or BHBA, was achieved through long-term infusion of insulin in mid-lactation cows. The hypothesis tested was that a condition of hypoglycemia for 48 h affects hepatic metabolism. This was studied through quantification of mRNA abundance for candidate genes involved in metabolic pathways in the liver and through measurements of concentrations and activities of plasma parameters.

MATERIALS AND METHODS

Animals and Management

The experimental procedures followed the Swiss Law on Animal Protection and were approved by the Committee of Animal Experiments of the Canton Fribourg, Switzerland. The experiment was carried out in 18 anestrus dairy cows with a parity of 2.9 ± 1.3 , at 28 ± 6 (mean $\pm$ SD) wk postpartum, of the Holstein ($n = 16$) and Swiss Fleckvieh (Red Holstein $\times$ Simmental, $n = 2$) breeds. To study the exclusive effect of hypoglycemia on hepatic metabolism, the complex endocrine and physiological changes related to the period around parturition were avoided by using dairy cows in mid lactation. The cows were confirmed as being in good health at the start of the experiment through evaluation of a routine blood analysis, a glutaraldehyde coagulation test (Sandholm, 1976), and a milk SCC (DeLaval cell counter DCC, Sursee, Switzerland), which had to be $<150,000$ cells/mL in all 4 quarters. The animals were kept in a tie-stall barn, where they were milked twice daily at approximately 0530 and 1600 h. Cows had free access to hay and water, and they were provided with salt at 50 g/d per cow. In addition, cows were fed a concentrate according to each cow's individual extra energy requirement for milk production. Prior to the experiment, an adaptation period of at least 3 wk was applied to adapt the cows to the feeding and housing conditions.

Experimental Design and Treatments

The present study involved 3 experimental groups with 6 cows per group, which were assigned to 1 of the 3 groups at random. The experimental period included 48 h of infusion across 3 d from 0900 h on d 1 to 0900 h on d 3.

The first group of cows was treated with a hyperinsulinemic hypoglycemic clamp (**HypoG**) for 2 consecutive days. The second group of cows was treated with a hyperinsulinemic euglycemic clamp (**EuG**) for 2 consecutive days. The EuG treatment was performed to differentiate the effect of insulin on liver metabolism from the effect of hypoglycemia. The EuG cows were therefore infused with a mean insulin infusion rate as used in the HypoG cows. The third group received physiological saline solution, thereby acting as a control group (NaCl).

One day before the infusions started, cows were fitted with 2 sterile intravenous catheters (16 gauge, 32 cm long; Cavafix Certo Splittocan, B. Braun Melsungen AG, Melsungen, Germany) in both jugular veins. The treatment started at 0900 h the following day (d 1) and was continued for 48 h until 0900 h on d 3. In the HypoG cows, the glucose concentration was decreased by infusion of 4 mU of insulin/kg per minute using a peristaltic pump (Perpex Jubile pump, H. J. Guldener, Zürich, Switzerland) to a glucose target concentration of 2.5 mmol/L (approximately during the first 30 min). After reaching the target glucose concentration, the insulin solution was infused at a rate of 1 mU of insulin/kg per minute, using an automatic pump (Perfuser, B. Braun Melsung AG) to maintain the intended level of plasma glucose for 48 h until the end of the experiment. Throughout the experimental infusion, the rate of insulin infusion was adjusted in response to plasma glucose measurements to maintain a blood glucose level close to the target concentration.

For the EuG cows, the blood glucose level was first decreased to 3.0 mmol/L by giving 5-mL boli of 4 mU/kg insulin solution, applied with a syringe once every 5 min. After approximately 30 min, a continuous insulin infusion was applied with a concentration of 1 mU/kg through an automatic pump as described above. In addition, a glucose infusion was applied (either 40 or 20% glucose depending on the required infusion rate) by using a peristaltic pump as described above. The infusion rate of the insulin solution was kept stable at 0.62 mU/kg per minute for 48 h. The glucose infusion rate was adjusted in response to glucose concentration measurements in the blood to maintain the plasma glucose level measured for each cow individually before the experiment.

The NaCl cows were treated with 0.9% saline solution with an infusion rate of 20 mL/h administered using an automatic pump or a peristaltic pump as described above.

Preparation of the Insulin Solution

For preparation of the insulin solution for the HypoG and EuG cows, a stock solution was prepared on

the day before the infusion. For 500 mL of stock solution, 11.85 g of potassium chloride (Merck Sharp and Dohme-Chibret AG, Luzern, Switzerland), 4.688 g of sodium chloride (Merck Sharp & Dohme-Chibret AG), and 0.521 g of BSA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were dissolved in 500 mL of double-distilled water. Thereafter, the stock solution was sterile filtered (Millipore Express PLUS Membrane 0.22 μ m, Millipore, Billerica, MA) and kept at 5°C. The final insulin solution was prepared at various times during the treatment, whereby bovine insulin (27 U/mg, I4011, Sigma-Aldrich Chemie GmbH) and 4.17% freshly obtained heparin plasma were added to the stock solution. The heparinized plasma, from the respective experimental cow, was obtained through centrifugation of blood at $3,000 \times g$ for 20 min. The insulin solution was prepared at 2 concentrations, containing 4 or 1 mU of insulin/kg of BW.

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 from the remainder of hay found the next morning. Milk yield was recorded for each cow individually at every milking during the adaptation and experimental periods. Milk samples were taken in the afternoon and in the morning before the start of the experimental infusions. Milk sampling was repeated in the afternoon of the second day and the morning of the third day of the experiment.

Blood Sampling. One week before the experiment and directly before the start of the experiment, on d 1, reference blood samples of each cow were taken at 0730 h, after milking and before feeding. Blood was obtained from the jugular vein by using tubes containing tri-potassium-EDTA. The tubes were kept on wet ice until they were centrifuged for 20 min at $3,000 \times g$, and the plasma was obtained. Plasma was frozen at -20°C until the samples were analyzed for concentrations and activities of plasma parameters.

During experimental infusions, minimally once per hour, blood samples were obtained from the contralateral catheter (i.e., that not used for the infusion) by using tubes containing tri-potassium-EDTA. Part of the collected blood samples were analyzed immediately for glucose concentrations using a selective analyzer (Cobas Mira 2, Hoffmann-La Roche, Basle, Switzerland) and a commercial kit (described under laboratory procedures), or were handled as the reference samples described above. The automated analyzer was located in a room organized as small laboratory near the experimental cow.

Liver Biopsies. One week before the infusions and on d 3 of the infusions, following the blood sampling, a biopsy of the liver was performed at around 0800 h. Liver tissue (60 to 100 mg) was obtained under local anesthesia with 10 mL of lidocaine 2% (Streuli Pharma AG, Uznach, Switzerland) by blind percutaneous puncture with a 14-gauge and 150- 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°C for 24 h, and thereafter stored at -80°C until RNA extraction.

Laboratory Procedures

Feed and Milk. The chemical composition of hay (DM content, 880 g/kg of fresh matter (FM); on DM basis, consisting of 153 g of CP/kg and 5.6 MJ of NE_L /kg) and concentrate (DM content, 881 g/kg of FM; on DM basis, consisting of 217 g of CP/kg and 7.6 MJ of NE_L /kg) were determined using routine analyses as described by van Dorland et al. (2009). The energy balance was calculated as the difference between energy intake and energy output (NE_L , requirements for maintenance and milk production, determined from tabulated values and recommended equations according as described in van Dorland et al., 2009).

Milk samples were analyzed at the laboratory of the Holstein Association of Switzerland (Grangeneuve, Switzerland) for contents 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 $\text{ECM (kg/d)} = [0.038 \times \text{fat (g/kg)} + 0.024 \times \text{protein (g/kg)} + 0.017 \times \text{lactose (g/kg)}] \times \text{milk yield (kg/d)}$.

Blood Plasma. Plasma metabolites and enzymes were measured enzymatically with a selective analyzer (Cobas Mira 2, Hoffmann-La Roche) and by the use of commercial kits as described by van Dorland et al. (2009). Concentrations of glucose, urea, triacylglycerides, and activity of aspartate aminotransferase (**ASAT**) were measured with kits (no. 61269, 61974, 61236 and 63212, respectively; bioMerieux, Marcy l'Etoile, France). Concentrations of NEFA and BHBA were measured with kits FA115 and RB1007 (Randox Laboratories Ltd., Ibach, Switzerland). Activity of glutamate dehydrogenase (**GLDH**) in plasma was measured with kit no. 1192992216 from Hoffmann-La Roche. Plasma insulin was measured by RIA as described by van Dorland et al. (2009).

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 µg of extracted 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 containing the following components were prepared: 0.8 µL of nuclease-free water, 1.0 µL of forward primer (5 pmol), 1.0 µL of reverse primer (5 pmol), 5.2 µL of 2× SensiMix Plus SYBR-Green (1 mM MgCl₂). The mRNA abundance in the liver was measured of 12 candidate genes encoding enzymes involved in gluconeogenesis [glucose-6-phosphatase (**G6PC**), pyruvate carboxylase (**PC**), cytosolic phosphoenolpyruvate carboxykinase (**PEPCKc**), mitochondrial phosphoenolpyruvate carboxykinase (**PEPCKm**); fatty acid oxidation [acyl-coenzyme A (**CoA**) dehydrogenase very long chain (**ACADVL**), acyl-CoA synthetase long-chain 1 (**ACSL1**), carnitine palmitoyltransferase 1A (**CPT1A**), and carnitine palmitoyltransferase 2 (**CPT2**); fatty acid synthesis [fatty acid synthase (**FASN**) and mitochondrial glycerol-3-phosphate acyltransferase (**GPAM**); ketogenesis (3-hydroxy-3-methylglutaryl-coenzyme A synthase 2, **HMGCS2**); and the insulin receptor (**INSR**). The primer sequences for G6PC, PC, PEPCKc, PEPCKm, ACADVL, ACSL1, CPT1A, CPT2, FASN, GPAM, and HMGCS2 were according to Graber et al. (2010), and the primer sequence for INSR was according to Pfaffl et al. (2002).

The PCR quantification was performed with the Rotor-Gene 6000 (Corbett Research, Sydney, Australia), using software version 1.7.40. Fluorescence take off was calculated with the "threshold" program option. Eight microliters of master mix and 2 µL of sample volume, containing 20 ng of cDNA, were used. The following 3-step PCR program was performed: denaturation for 10 min at 95°C, 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), extension at 72°C for 20 s, and quantification of fluorescence], and finally a melting curve program (60 to 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 (Pfaffl et al., 2002; Graber et al., 2010).

Statistical Analysis

For each parameter, the change (delta) was calculated by subtracting the value measured after the

infusion treatment from the value measured before the infusion treatment. The delta for each parameter was statistically evaluated using the PROC GLM procedure of SAS (SAS Institute, 2001), including treatment (HypoG, EuG, or NaCl) as fixed effect. Differences between means were determined by the Tukey test. In addition, deltas obtained within each treatment were tested with the null hypothesis. Furthermore, in case of the blood plasma parameters, the area under the curve (**AUC**) was calculated for d 2 of the treatments and subsequently assessed statistically with the model as described above. Data obtained before the treatment (d 0) were included as covariate in the model to compensate for initial differences between the groups.

Data are reported as means ± SEM and were considered significant if $P < 0.05$ and a trend if $P < 0.10$.

RESULTS

Infusion Rates and Plasma Concentrations of Insulin and Glucose

The infusion treatments (Figure 1) resulted in a marked increase in plasma insulin concentration in HypoG and EuG cows (Table 1; $P < 0.05$). Plasma insulin concentration in NaCl cows remained unchanged (Table 1). Consequently, larger AUC were observed (Figure 2) for plasma insulin concentration in HypoG (41.9 ± 8.1 mU/L, mean ± SEM) and EuG (57.8 ± 7.8 mU/L) cows compared with the NaCl cows (12.2 ± 2.8 mU/L) on d 2 ($P < 0.002$; Figure 2).

In EuG cows, insulin and glucose were simultaneously infused, the latter at a rate of 2.47 ± 0.05 mmol/kg per minute (mean ± SEM on d 2, Figure 1). Plasma glucose decreased in response to the treatment in HypoG cows ($P < 0.05$; Table 1) and in EuG cows ($P < 0.05$). The lowest AUC were observed ($P < 0.001$, Figure 3) for plasma glucose concentration in HypoG (2.25 ± 0.05 mmol/L, mean ± SEM) compared with EuG (3.80 ± 0.16 mmol/L) and NaCl cows (4.17 ± 0.10 mmol/L) on d 2.

Feed Intake and Energy Balance

The concentrate fed during the experiment was 2.6 ± 0.2 kg of DM/d per cow across treatments. Prior to the infusions (d 0), DMI of hay and concentrate was 19.9 ± 1.1 , 20.3 ± 0.8 , and 20.4 ± 1.1 kg/d for HypoG, EuG, and NaCl cows, respectively. On d 1 of the infusions, DMI of hay and concentrate was 20.2 ± 0.8 , 18.9 ± 0.8 , and 17.1 ± 2.6 kg/d for HypoG, EuG, and NaCl cows, respectively. On d 2 of the infusions, DMI of hay and concentrate was 19.0 ± 1.2 , 16.7 ± 0.9 , and 18.5 ± 1.3 kg/d for HypoG, EuG, and NaCl cows, respectively. For

Figure 1. Insulin and glucose infusion rates (mean $\pm$ SEM) during a 48-h infusion of insulin and glucose in a hypoglycemic (HypoG) and euglycemic (EuG) clamp.

EuG cows, the DMI decreased ($P < 0.05$) from d 0 to 2. For NaCl cows, the DMI decreased ($P < 0.05$) from d 0 to 1. However, the change in DMI among groups on d 1 or 2 relative to d 0 was not different between groups.

The net energy balance remained unaffected within treatment group and was similar between treatment groups on d 2 of the infusions: -11.7 ± 4.5 , 12.4 ± 7.6 , and 4.0 ± 4.5 MJ/d for HypoG, EuG, and NaCl cows, respectively.

Milk Yield and Milk Components

The treatments decreased the milk yield in HypoG and NaCl cows ($P < 0.05$; Table 2), and milk yield remained unaffected for EuG cows. Compared with milk yield, ECM tended to decrease in HypoG cows ($P < 0.1$). With regard to milk components, fat and protein contents increased, and lactose content and total lactose and urea yield decreased in HypoG cows in response to the infusion treatment ($P < 0.05$). Furthermore, total lactose yield was decreased in response to the treatment in NaCl cows ($P < 0.05$). However, total lactose yield decreased more in HypoG cows than in NaCl cows ($P < 0.001$).

Blood Metabolites and Enzymes

Even though plasma NEFA remained unaffected by treatment (Tables 1 and 3), BHBA concentration in EuG cows decreased ($P < 0.05$) and was lower compared with HypoG and NaCl cows on d 2 ($P < 0.01$; Tables 1 and 3). In addition, in EuG cows, activities of ASAT and GLDH increased ($P < 0.05$) in response to treatment and triacylglycerides decreased ($P < 0.05$), although these effects did not result in subsequent differences between treatments for the calculated AUC on d 2. In HypoG and NaCl cows, plasma urea concentration decreased ($P < 0.05$) and a treatment effect ($P < 0.04$) was observed for the calculated AUC on d 2. However, this effect could not be identified through comparison of the means of the treatments by the Tukey t -test.

Liver Gene Expression

With regard to the genes related to gluconeogenesis (Figure 4a), a glucose effect was observed for the mRNA abundance of PEPCKm, which was upregulated in HypoG cows, whereas in EuG and NaCl cows mRNA abundance decreased ($P < 0.05$) in response to treat-

Table 1. Mean $\pm$ SEM of concentrations and activities of plasma parameters at 0730 h before the start of the infusion (0 h) and the change after 48 h of infusion (48 h – 0 h)

Parameter ¹	Group ²	0 h	Change: 48 h – 0 h	ANOVA (<i>P</i> -value, group)
GLC (mmol/L)	HypoG	3.70 $\pm$ 0.12	–1.61 $\pm$ 0.04 ^{a*}	<0.001
	EuG	4.15 $\pm$ 0.18	–0.54 $\pm$ 0.13 ^{b*}	
	NaCl	3.53 $\pm$ 0.10	0.17 $\pm$ 0.17 ^c	
INS (mU/L)	HypoG	3.6 $\pm$ 0.4	18.3 $\pm$ 5.6 ^{a,b*}	<0.01
	EuG	3.4 $\pm$ 0.2	45.2 $\pm$ 10.9 ^{a*}	
	NaCl	4.9 $\pm$ 1.2	–0.6 $\pm$ 1.1 ^b	
BHBA (mmol/L)	HypoG	0.40 $\pm$ 0.04	0.08 $\pm$ 0.05 ^b	<0.001
	EuG	0.61 $\pm$ 0.04	–0.21 $\pm$ 0.05 ^{a*}	
	NaCl	0.42 $\pm$ 0.05	0.12 $\pm$ 0.05 ^b	
NEFA (mmol/L)	HypoG	0.12 $\pm$ 0.02	0.02 $\pm$ 0.05	0.42
	EuG	0.11 $\pm$ 0.03	–0.06 $\pm$ 0.02	
	NaCl	0.16 $\pm$ 0.05	–0.02 $\pm$ 0.04	
ASAT (U/L)	HypoG	70.3 $\pm$ 5.0	–0.33 $\pm$ 1.9	0.07
	EuG	68.3 $\pm$ 2.8	28.08 $\pm$ 14.0*	
	NaCl	77.1 $\pm$ 6.7	3.42 $\pm$ 5.1	
GLDH (U/L)	HypoG	15.4 $\pm$ 1.7	0.9 $\pm$ 2.4	0.09
	EuG	14.0 $\pm$ 1.8	24.3 $\pm$ 11.2*	
	NaCl	32.3 $\pm$ 8.7	3.6 $\pm$ 6.2	
TG (mmol/L)	HypoG	0.19 $\pm$ 0.02	–0.02 $\pm$ 0.02	0.41
	EuG	0.21 $\pm$ 0.02	–0.05 $\pm$ 0.01*	
	NaCl	0.17 $\pm$ 0.01	–0.02 $\pm$ 0.01	
Urea (mmol/L)	HypoG	5.26 $\pm$ 0.40	–1.00 $\pm$ 0.30*	0.58
	EuG	2.48 $\pm$ 0.20	–0.52 $\pm$ 0.16	
	NaCl	5.00 $\pm$ 0.34	–0.97 $\pm$ 0.52*	

^{a–c}Values within a parameter without common superscript letters are different ($P < 0.05$).

¹GLC = glucose; INS = insulin; ASAT = aspartate aminotransferase; GLDH = glutamate dehydrogenase; TG = triacylglyceride.

²HypoG = hypoglycemic hyperinsulinemic clamp group; EuG = euglycemic hyperinsulinemic clamp group; NaCl = physiological saline solution (control) group.

*Different from zero ($P < 0.05$).

ments. Furthermore, the EuG treatment downregulated mRNA expression of G6PC ($P < 0.05$).

With regard to genes related to lipid metabolism in the liver (Figure 4b), a trend for a treatment effect was observed for ACADVL ($P = 0.08$) and CPT2 ($P = 0.053$). Within treatment, for HypoG cows, the mRNA abundance of ASCL1 ($P < 0.05$) and CPT1A ($P < 0.1$) was upregulated, and that for FASN was downregulated ($P < 0.05$). The EuG treatment upregulated mRNA abundance of ACSL1 ($P < 0.1$) and ACADVL ($P < 0.05$). The NaCl treatment downregulated gene expression of CPT2 ($P < 0.05$). Unaffected by treatment were the mRNA abundances of GPAM, HMGCS2 (Figure 4b), and INSR (data not shown).

DISCUSSION

To our knowledge, the present study was the first that applied a continuous intravenous insulin infusion for 48 h in dairy cows. With this treatment, we aimed to evaluate an exclusive condition of hypoglycemia in dairy cows over a long period and its effect on key enzymes involved in hepatic metabolism, without the accompanied changes in NEFA. The hyperinsulinemic

euglycemic clamp was specifically carried out to differentiate the effect of insulin on liver metabolism from the effect of hypoglycemia. The EuG cows were therefore infused with a mean insulin infusion rate as used in the HypoG cows. The hypoglycemia treatment resulted in a successful decrease in blood glucose concentration, which was lower than glucose concentrations observed in other studies with hypoglycemic cows (e.g., Graber et al., 2010). Furthermore, the calculated energy balance for HypoG cows was not affected during the study period.

Effects of Hypoglycemia and Euglycemia on Milk Yield and Milk Components

The observed decreases in milk yield and ECM in response to the hypoglycemia in HypoG cows confirm results from Kronfeld et al. (1963) and Schmidt (1966). The decrease may be explained by the decreased blood glucose concentration and associated reduction in lactose production by the mammary gland. Because milk lactose is the main osmotic factor determining milk yield, the hypogalactia is an explicit result of the hypoglycemia, not an effect from the hyperinsulinemia (Kro-

Figure 2. Insulin concentration (mean $\pm$ SEM) in blood plasma before the start of the infusion (d 0) and during a 48-h infusion. Different letters (a, b) indicate differences ($P < 0.05$) between the means of the 3 treatment groups. HypoG = hypoglycemic hyperinsulinemic clamp group; EuG = euglycemic hyperinsulinemic clamp group; NaCl = physiological saline solution (control) group.

nfeld et al., 1963). Therefore, as expected, milk yield, milk lactose content, and ECM yield in the EuG group remained unchanged (Corl et al., 2006). However, the decrease in milk yield and lactose yield in NaCl cows was unexpected, whereas ECM remained unchanged. We assumed that this resulted from the treatment itself, which included hourly sampling throughout the day and night that may have disturbed the cows (Drackley et al., 2005; Rigalma et al., 2010). As an observed stress response, DMI decreased and the glucose demand of the peripheral tissue increased (McGuire et al., 1989; Drackley et al., 2005), which decreased the supply of glucose for lactose synthesis in the mammary gland.

The decrease in milk yield in HypoG cows resulted in an increased content of milk fat and protein, but not in the yield of the 2 components. These changes were most likely a concentration effect due to the decreased milk yield as also observed by Schmidt (1966), and the elevated plasma insulin concentrations obviously prevented lipolysis as response to the hypoglycemic condition (Brockman, 1979; Corl et al., 2006). The decreased milk urea yield for the HypoG cows likely resulted from the decreased milk yield, because urea content in the milk remained unaffected by treatment. With regard to results for EuG and NaCl cows, McGuire et al. (1995)

also did not find any changes in milk protein and fat content during the first 2 d of a hyperinsulinemic euglycemic clamp. Mackle et al. (1999) described an increase in milk protein of 11%, but this effect appeared in the last 2 d of a 4-d hyperinsulinemic euglycemic clamp treatment. The observed decrease in milk fat percentage and yield by Corl et al. (2006) was not confirmed by our results, the reason being that Corl et al. (2006) used early lactation cows. The use of body fat for milk fat is greatest during early lactation, when insulin activity is low. Infusion of insulin therefore led to a decrease of milk fat percentage and fat yield through inhibition lipolysis, whereas in the present study body fat use for milk fat in mid-lactation cows was low before and during the study, as illustrated by the low NEFA concentrations. Infusion of insulin therefore did not affect milk fat yield in HypoG cows.

Effects of Hypoglycemia and Euglycemia on Blood Plasma Parameters

The decrease in plasma BHBA under influence of a hyperinsulinemic euglycemic clamp, as observed in the EuG cows, was observed previously in mid-lactation cows by Mashek et al. (2001). In addition, Hayirli et al.

Figure 3. Glucose concentration (mean $\pm$ SEM) in blood plasma before the start of the infusion (d 0) and during a 48-h infusion. Different letters (a, b) indicate differences ($P < 0.05$) between the means of the 3 treatment groups. HypoG = hypoglycemic hyperinsulinemic clamp group; EuG = euglycemic hyperinsulinemic clamp group; NaCl = physiological saline solution (control) group.

(2002) concluded that the decreased BHBA is an effect of insulin, because injecting cows with 3 ascending dosages of slow-releasing insulin resulted in a correlated decrease in plasma BHBA concentration. On the one hand, insulin inhibits lipolysis, therefore limiting the availability of NEFA as a substrate for ketogenesis. On the other hand, the effects of insulin on the level of BHBA may be explained by enhanced peripheral tissue ketone oxidation, and altering enzyme activities that are involved in ketogenesis in the liver. Insulin stimulates the activity of acetyl-CoA carboxylase or formation of malonyl-CoA that inhibits activity of CPT I (Zammit, 1983). The generally higher plasma insulin level in EuG compared with HypoG cows may explain why the decrease in BHBA was only observed in the former group.

Plasma NEFA concentrations in the present study did not show significant changes and reflect results from Mashek et al. (2001), who performed a 4-d hyperinsulinemic euglycemic clamp comparing cows in early and mid lactation, and observed that NEFA in plasma is only susceptible to insulin during early lactation, because in mid lactation the NEFA concentration is

already very low. In addition, lipolysis in adipose tissue is inhibited by insulin (Corl et al., 2006).

The observed significant decrease of blood urea in HypoG and NaCl cows but not in EuG cows cannot be explained by hypoglycemia or insulinemia effects. The HypoG and NaCl cows had higher plasma urea concentrations at the start of the treatment compared with the EuG cows that remained higher even after treatments. All values, however, were in the physiological range during the entire study and likely represent physiological fluctuations, which can be as much as 1 mmol/L during an entire day (Blum et al., 2000). Compared with urea concentrations (>5.5 mmol/L) measured by Mashek et al. (2001), the cows in the present study showed a generally low concentration of urea in the blood. Nevertheless, the decrease in plasma urea concentration in HypoG cows explains the concomitant decrease in milk urea yield.

The significant increase in activity of the liver enzymes ASAT and GLDH in plasma observed for the EuG cows may not be attributed to the hyperinsulinemia effect, because the activities remained unchanged in HypoG cows. The high SEM in EuG for ASAT and GLDH compared with HypoG and NaCl is indicative of initial

Table 2. Mean $\pm$ SEM of milk yield and milk constituents before the start of the infusion (0 h) and the change after 48 h of infusion (48 h – 0 h)¹

Parameter	Group ²	0 h	Change: 48 h – 0 h	ANOVA (<i>P</i> -value, group)
Milk yield (kg/d)	HypoG	21.0 $\pm$ 0.40	–4.6 $\pm$ 0.6 ^{a*}	<0.001
	EuG	25.4 $\pm$ 2.1	1.0 $\pm$ 0.7 ^b	
	NaCl	23.1 $\pm$ 3.2	–2.2 $\pm$ 0.8 ^{a*}	
ECM (kg/d)	HypoG	21.7 $\pm$ 1.0	–2.1 $\pm$ 1.4 [†]	0.75
	EuG	24.3 $\pm$ 2.0	–0.9 $\pm$ 1.2	
	NaCl	23.2 $\pm$ 3.2	–1.9 $\pm$ 0.8	
Fat (g/100 g)	HypoG	4.28 $\pm$ 0.26	0.99 $\pm$ 0.59 [*]	0.07
	EuG	3.72 $\pm$ 0.15	–0.49 $\pm$ 0.21	
	NaCl	3.94 $\pm$ 0.18	0.03 $\pm$ 0.35	
Fat yield (kg/d)	HypoG	0.90 $\pm$ 0.07	–0.05 $\pm$ 0.10	0.88
	EuG	0.95 $\pm$ 0.09	–0.10 $\pm$ 0.07	
	NaCl	0.90 $\pm$ 0.12	–0.06 $\pm$ 0.06	
Protein (g/100 g)	HypoG	3.40 $\pm$ 0.08	1.00 $\pm$ 0.10 ^{a*}	<0.001
	EuG	3.15 $\pm$ 0.13	–0.04 $\pm$ 0.13 ^b	
	NaCl	3.48 $\pm$ 0.09	–0.02 $\pm$ 0.03 ^b	
Protein yield (kg/d)	HypoG	0.71 $\pm$ 0.03	0.01 $\pm$ 0.03	0.09
	EuG	0.88 $\pm$ 0.06	–0.01 $\pm$ 0.03	
	NaCl	0.81 $\pm$ 0.12	–0.08 $\pm$ 0.03	
Lactose (g/100 g)	HypoG	4.73 $\pm$ 0.07	–0.46 $\pm$ 0.06 ^{a*}	<0.001
	EuG	4.87 $\pm$ 0.06	–0.01 $\pm$ 0.05 ^b	
	NaCl	4.79 $\pm$ 0.05	0.05 $\pm$ 0.03 ^b	
Lactose yield (kg/d)	HypoG	0.99 $\pm$ 0.02	–0.29 $\pm$ 0.03 ^{a*}	<0.001
	EuG	1.23 $\pm$ 0.10	0.04 $\pm$ 0.04 ^c	
	NaCl	1.11 $\pm$ 0.15	–0.10 $\pm$ 0.04 ^{b*}	
Urea (mg/100 mL)	HypoG	2.7 $\pm$ 0.3	0.1 $\pm$ 0.3	0.16
	EuG	1.4 $\pm$ 0.1	–0.3 $\pm$ 0.3	
	NaCl	2.0 $\pm$ 0.1	0.4 $\pm$ 0.2	
Urea (yield, g/d)	HypoG	5.56 $\pm$ 0.63	–1.74 $\pm$ 0.45 [*]	0.04
	EuG	3.52 $\pm$ 0.56	0.24 $\pm$ 0.81	
	NaCl	4.77 $\pm$ 0.79	0.31 $\pm$ 0.39	

^{a–c}Values within a parameter without common superscript letters are different ($P < 0.05$).

¹0 h = milk yield and constituents from the p.m. and a.m. milking before the start of the infusions; 48 h = milk yield and constituents from the last p.m. and a.m. milking before the end of the infusions.

²HypoG = hypoglycemic hyperinsulinemic clamp group; EuG = euglycemic hyperinsulinemic clamp group; NaCl = physiological saline solution (control) group.

*Different from zero ($P < 0.05$); †different from zero ($P < 0.10$).

variation between individual cows. Nevertheless, the enzyme values remained within physiological range (Kraft and Dürr, 2005), and no significant differences were observed between the groups on d 2. The lack of change in plasma TG is difficult to reconcile with hyperinsulinemia hypoglycemia and hyperinsulinemia euglycemia condition, but may represent adaptive adjustments that were irrelevant with respect to the treatments.

Effects of Hypoglycemia and Euglycemia on Hepatic Gene Expression

Gluconeogenesis and Insulin Receptor. The increase in mRNA abundance of PEPCKm for HypoG cows compared with the decrease observed in the other groups illustrates that low glucose concentration, and not increased insulin, affected PEPCKm expression. Results of O'Brien and Granner (1991) and Greenfield et al. (2000) confirm that the regulation of PEPCKm

mRNA expression is independent of the insulin concentration in the blood. The increase in PEPCKm transcript expression in the present study indicates that hepatic gluconeogenesis during hypoglycemia may be linked to enhanced capacity for mitochondrial rather than cytosolic formation of phosphoenolpyruvate. In addition, changes in PEPCKm in the absence of an increase in PC may suggest that lactate was not an essential precursor for gluconeogenesis because PEPCKm is more suited to gluconeogenesis from lactate (Watford et al., 1981), which is normally observed to be channeled increasingly through pyruvate and oxaloacetate toward gluconeogenesis in early lactation (Greenfield et al., 2000). Nonetheless, the present study demonstrated that low glucose concentration affects gluconeogenesis by increasing PEPCKm transcript expression, thereby playing an important role during the competition for oxaloacetate, which may be used for both the gluconeogenesis and citric acid cycle pathways (Van Knegsel et al., 2005).

Figure 4. Differences in mRNA abundance ($\Delta\Delta$ cycle threshold, CT, $\log_2$) of hepatic candidate genes after 48 h of infusions normalized for the housekeeping genes GAPDH and ubiquitin. G6PC = glucose-6-phosphatase; PC = pyruvate carboxylase; PEPCKc = cytosolic phosphoenolpyruvate carboxykinase; PEPCKm = mitochondrial phosphoenolpyruvate carboxykinase; ACADVL = acyl-CoA dehydrogenase very long chain; ACSL1 = acyl-CoA synthetase long-chain 1; CPT1A = carnitine palmitoyltransferase 1A; CPT2 = carnitine palmitoyltransferase 2; FASN = fatty acid synthase; GPAM = mitochondrial glycerol-3-phosphate acyltransferase; HMGCS2 = 3-hydroxy-3-methylglutaryl CoA synthase 2. Means are different from zero at * $P < 0.05$ and † $P < 0.1$. Different letters (a, b) indicate differences ($P < 0.05$) between the means of the 3 treatment groups. HypoG = hypoglycemic hyperinsulinemic clamp group; EuG = euglycemic hyperinsulinemic clamp group; NaCl = physiological saline solution (control) group. HypoG = hypoglycemic hyperinsulinemic clamp group; EuG = euglycemic hyperinsulinemic clamp group; NaCl = physiological saline solution (control) group.

Apart from the effects of a low glucose concentration on hepatic metabolism, specific insulin effects were observed. Insulin plays a central role in the regulation of hepatic metabolic pathways. It influences enzyme activity by phosphorylation and dephosphorylation of enzymes (O'Brien and Granner, 1991) and can inhibit or stimulate gene expression. It is generally acknowledged that insulin has an inhibitory effect on the enzymes of gluconeogenesis and ketogenesis and a stimulating effect on enzymes of lipogenesis in the liver of dairy cows (Hayirli, 2006). Numerous studies confirmed the inhibitory effect of insulin on G6PC, PEPCKc, and PC (O'Brien et al., 2001; Desvergne et al., 2006) that affect the gluconeogenesis pathway. In the present study, this was confirmed for G6PC mRNA abundance, which decreased in HypoG and EuG cows. A similar change

was not observed for PEPCKc and PC. The absence of changes for PEPCKc and PC mRNA abundance could suggest regulation at a different level; for example, by modification of enzyme activity. However, Greenfield et al. (2000) and Agca et al. (2002) observed a close relationship between mRNA abundance and enzyme activity of both PC and PEPCKc. Therefore, the absence of changes in these 2 enzymes may imply that their contribution to gluconeogenesis was relatively small before the treatment, as up to 60% of propionate flux to glucose is dependent on PEPCKm (Aiello and Armentano, 1987), and therefore effects of insulin on PEPCKc and PC did not become evident.

Even though the role of insulin in metabolism is clear, it does not affect gene expression of its receptor, INSR (Fenwick et al., 2008), as observed in dairy cows

Figure 4 (Continued). Differences in mRNA abundance ($\Delta\Delta$ cycle threshold, CT, $\log_2$) of hepatic candidate genes after 48 h of infusions normalized for the housekeeping genes GAPDH and ubiquitin. G6PC = glucose-6-phosphatase; PC = pyruvate carboxylase; PEPCKc = cytosolic phosphoenolpyruvate carboxykinase; PEPCKm = mitochondrial phosphoenolpyruvate carboxykinase; ACADVL = acyl-CoA dehydrogenase very long chain; ACSL1 = acyl-CoA synthetase long-chain 1; CPT1A = carnitine palmitoyltransferase 1A; CPT2 = carnitine palmitoyltransferase 2; FASN = fatty acid synthase; GPAM = mitochondrial glycerol-3-phosphate acyltransferase; HMGCS2 = 3-hydroxy-3-methylglutaryl CoA synthase 2. Means are different from zero at $*P < 0.05$ and $†P < 0.1$. Different letters (a, b) indicate differences ($P < 0.05$) between the means of the 3 treatment groups. HypoG = hypoglycemic hyperinsulinemic clamp group; EuG = euglycemic hyperinsulinemic clamp group; NaCl = physiological saline solution (control) group. HypoG = hypoglycemic hyperinsulinemic clamp group; EuG = euglycemic hyperinsulinemic clamp group; NaCl = physiological saline solution (control) group.

Table 3. Least squares means $\pm$ SEM of area under the curve of concentrations and activities of plasma parameters during d 2

Parameter ¹	Group ²	LSM $\pm$ SEM	ANOVA (<i>P</i> -value, group)
BHBA (mmol/L)	HypoG	0.59 $\pm$ 0.03 ^a	<0.01
	EuG	0.41 $\pm$ 0.04 ^b	
	NaCl	0.63 $\pm$ 0.03 ^a	
NEFA (mmol/L)	HypoG	0.09 $\pm$ 0.02	0.36
	EuG	0.04 $\pm$ 0.02	
	NaCl	0.08 $\pm$ 0.02	
ASAT (U/L)	HypoG	72.9 $\pm$ 4.5	0.36
	EuG	74.6 $\pm$ 4.5	
	NaCl	80.2 $\pm$ 4.6	
GLDH (U/L)	HypoG	19.7 $\pm$ 4.3	0.36
	EuG	22.6 $\pm$ 4.3	
	NaCl	29.7 $\pm$ 4.8	
TG (mmol/L)	HypoG	0.16 $\pm$ 0.02	0.43
	EuG	0.19 $\pm$ 0.02	
	NaCl	0.16 $\pm$ 0.02	
Urea (mmol/L)	HypoG	3.46 $\pm$ 0.37	0.04
	EuG	2.09 $\pm$ 0.50	
	NaCl	4.00 $\pm$ 0.34	

^{a,b}Values without common superscript letters are different ($P < 0.05$).

¹ASAT = aspartate aminotransferase; GLDH = glutamate dehydrogenase; TG = triacylglyceride.

²HypoG = hypoglycemic hyperinsulinemic clamp group; EuG = euglycemic hyperinsulinemic clamp group; NaCl = physiological saline solution (control) group.

in the present study. Hatada et al. (1989) observed that insulin affects INSR gene transcript through enhanced receptor degradation and not through a decrease in INSR mRNA.

Lipid Metabolism and Ketogenesis. A state of hypoglycemia without concomitant elevated NEFA concentrations, achieved by infusion of insulin, resulted in minimal effects on gene transcripts related to fatty acid β -oxidation, triacylglyceride, and fatty acid biosynthesis. Obviously, low plasma glucose concentrations seem not to be involved in preparing hepatic lipid metabolism for the flux of NEFA in response to hypoglycemia that is commonly observed in early lactation or during feed restriction, even beyond early lactation (Gross et al., 2011). However, in the present study, metabolically related gene transcripts were studied, not the related functional protein quantity, which would allow a more complete image of the physiological events that took place.

Free long-chain fatty acids that are taken up by the liver are converted into fatty acyl-CoA esters by ACSL1, which plays a key role in both lipid biosynthesis and fatty acid oxidation. The increased mRNA abundance of ACSL1 for HypoG and EuG, and the absence of changes in NaCl cows, suggest an effect of insulin. This is in agreement with observations from Coleman et al. (2002) in rat adipocytes treated with insulin. However, the increase in ACSL1 mRNA expression likely did not result in increased triacylglyceride synthesis in the liver because GPAM transcript expression was not

changed in the respective groups. Because regulation of GPAM is not subjected to allosteric regulation and posttranslational modification (Sul and Wang, 1998), these results comply with observations for plasma triacylglyceride concentrations in the present study. The absence of large amounts of fatty acid uptake in the liver, being a plasma concentration-dependent process (Zammit, 1983) most commonly observed in early lactation or during feed restriction (Gross et al., 2011), may explain the absence of effects regarding hepatic triacylglyceride synthesis, as free fatty acids are used for its synthesis. The absence of large amounts of fatty acid uptake in the liver may also explain the observation made for genes involved in hepatic β -oxidation of fatty acids. Loores et al. (2006) observed positive correlations between NEFA and ACADVL and CPT1A mRNA expression in early lactation, whereas in the present study, mRNA abundance of ACADVL and CPT1A was not significantly affected in HypoG cows, which also did not show elevated NEFA levels. Apart from the absence of possible substrate-related effects, insulin may also indirectly affect mRNA expression of CPT1A, but not CPT2 (Declercq et al., 1987), through inhibition of malonyl-CoA, which itself is increasing when insulin increases and vice versa (Drackley et al., 2001). Whether the difference in plasma insulin concentration between HypoG and EuG cows was responsible for the exclusive effects observed in HypoG and EuG in case of ACADVL and CPT1A transcript expression is to be confirmed. Furthermore, the significantly decreased

CPT2 mRNA abundance in NaCl cows is difficult to explain and may illustrate selective inhibition of components of the CPT system (Declercq et al., 1987).

The absence of an increase in fatty acid oxidation resulted in unchanged mRNA abundance of HMGCS2 in HypoG cows. The HMGCS2 gene encodes a key enzyme involved in ketogenesis, and its mRNA expression and protein are greatly increased by starvation (Casals et al., 1992), providing that enough substrate (acetyl-CoA from oxidation of NEFA) is available in the mitochondria (Casals et al., 1992), which explains the absence of effects in the present study. Even though mRNA abundance of HMGCS2 was unaffected by the treatments, the decreased and lowest BHBA plasma concentrations in EuG on d 2 suggest inhibition of ketogenesis by insulin (Brockman, 1979; Nadal et al., 2002). However, HypoG cows also experienced a high insulin plasma concentration, but stimulation of gluconeogenesis through hypoglycemia proceeds in a parallel fashion to ketogenesis (Casals et al., 1992), which may have prevented a decrease of plasma BHBA in HypoG cows.

Supply of substrate, in this case a lack of glucose, may also explain the observations for FASN. In HypoG, the decreased mRNA abundance of FASN may be a response to the low glucose concentration, despite the elevated plasma insulin concentration (O'Brien et al., 2001; Desvergne et al., 2006). The observed glucose effect may be confirmed by the results of Ishii et al. (2004), who discovered a carbohydrate responsive element binding protein in mice hepatocyte cells that promotes higher FASN gene expression at high glucose concentrations.

CONCLUSIONS

Low glucose concentrations in dairy cows affect liver metabolism at the molecular level, through upregulation of PEPCKm mRNA abundance. Although these findings were obtained in mid-lactation cows, which are physiologically different from early lactation cows, the observed adjustment involves the substrate oxaloacetate, for which both the gluconeogenesis and citric acid cycle pathways are competing, to synthesize glucose or to react with acetyl-CoA derived from fatty acid oxidation. Upregulation of the PEPCKm transcript in response to a hypoglycemic condition appears to be a crucial adjustment to avoid impairment of liver metabolism in early lactating dairy cows, which could result in metabolic disorders such as ketosis. Interventions undertaken to assist the dairy cow to adapt to lactation after parturition should specifically involve avoiding hypoglycemia and promoting elevated plasma insulin concentrations, because metabolic regulatory

events that may lead to impairment of hepatic metabolism are directed, apart from hormones, by the level of metabolites, either in excess (e.g., free fatty acids) or in shortage (e.g., glucose).

ACKNOWLEDGMENTS

This study was supported by a grant of the Swiss National Research Foundation (grant no. 320030-120317). The expert laboratory assistance by Yolande Zbinden, Claudine Morel, and Chantal Philipona (Veterinary Physiology, Vetsuisse Faculty, University of Bern, Bern, Switzerland) is gratefully acknowledged. Mohammed Ahmed (Faculty of Veterinary Medicine, South Valley University, Egypt) is thanked for his help around the animals during the experiment.

REFERENCES

- Agca, C., R. B. Greenfield, J. R. Hartwell, and S. S. Donkin. 2002. Cloning and characterization of bovine cytosolic and mitochondrial PEPCK during transition to lactation. *Physiol. Genomics* 11:53–63.
- Aiello, R. J., and L. E. Armentano. 1987. Gluconeogenesis in goat hepatocytes is affected by calcium, ammonia and other key metabolites but not primarily through cytosolic redox state. *Comp. Physiol. B Comp. Biochem.* 88:193–201.
- Blum, J. W., R. M. Bruckmaier, P.-Y. Vacher, A. Munger, and F. Jans. 2000. Twenty four-hour patterns of hormones and metabolites in week 9 and 19 of lactation in high-yielding dairy cows fed triglycerides and free fatty acids. *J. Vet. Med. A* 47:43–60.
- Brockman, R. P. 1979. Roles for insulin and glucagon in the development of ruminant ketosis—A review. *Can. Vet. J.* 20:121–126.
- Casals, N., N. Roca, M. Guerrero, G. Gil-Gomez, J. Ayte, C. J. Ciudad, and F. G. Hegardt. 1992. Regulation of the expression of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene. Its role in the control of ketogenesis. *Biochem. J.* 283:261–264.
- Coleman, R. A., T. M. Lewin, C. G. Van Horn, and M. R. Gonzalez-Baro. 2002. Do long-chain acyl-CoA synthetases regulate fatty acid entry into synthetic versus degradative pathways? *J. Nutr.* 132:2123–2126.
- Corl, B. A., S. T. Butler, W. R. Butler, and D. E. Bauman. 2006. Short communication: Regulation of milk fat yield and fatty acid composition by insulin. *J. Dairy Sci.* 89:4172–4175.
- Declercq, P. E., J. R. Falck, M. Kuwajima, H. Tyminski, D. W. Foster, and J. D. McGarry. 1987. Characterization of the mitochondrial carnitine palmitoyltransferase enzyme system. I. Use of inhibitors. *J. Biol. Chem.* 262:9812–9821.
- Desvergne, B., L. Michalik, and W. Wahli. 2006. Transcriptional regulation of metabolism. *Physiol. Rev.* 86:465–514.
- Drackley, J. K., M. Heather, G. Dann, N. Douglas, N. A. Janovick Guretzky, N. B. Litherland, J. P. Underwood, and J. J. Looor. 2005. Physiological and pathological adaptations in dairy cows that may increase susceptibility to periparturient diseases and disorders. *Ital. J. Anim. Sci.* 4:323–344.
- Drackley, J. K., T. R. Overton, and G. N. Douglas. 2001. Adaptations of glucose and long-chain fatty acid metabolism in liver of dairy cows during the periparturient period. *J. Dairy Sci.* 84(E. Suppl.):E100–E112.
- Fenwick, M. A., R. Fitzpatrick, D. A. Kenny, M. G. Diskin, J. Patton, J. J. Murphy, and D. C. Wathes. 2008. Interrelationships between negative energy balance (NEB) and IGF regulation in liver of lactating dairy cows. *Domest. Anim. Endocrinol.* 34:31–44.
- Graber, M., S. Kohler, T. Kaufmann, M. G. Doherr, R. M. Bruckmaier, and H. A. van Dorland. 2010. A field study on characteristics and diversity of gene expression in the liver of dairy cows during the transition period. *J. Dairy Sci.* 93:5200–5215.

- Greenfield, R. B., M. J. Cecava, and S. S. Donkin. 2000. Changes in mRNA expression for gluconeogenic enzymes in liver of dairy cattle during the transition to lactation. *J. Dairy Sci.* 83:1228–1236.
- Gross, J., H. A. van Dorland, R. M. Bruckmaier, and F. J. Schwarz. 2011. Performance and metabolic profile of dairy cows during a lactational and deliberately induced negative energy balance with subsequent realimentation. *J. Dairy Sci.* 94:1820–1830.
- Hatada, E. N., D. A. McClain, E. Potter, A. Ullrich, and J. M. Olefsky. 1989. Effects of growth and insulin treatment on the levels of insulin receptors and their mRNA in HEP G2 cells. *J. Biol. Chem.* 264:6741–6747.
- Hayirli, A. 2006. The role of exogenous insulin in the complex of hepatic lipidosis and ketosis associated with insulin resistance phenomenon in postpartum dairy cattle. *Vet. Res. Commun.* 30:749–774.
- Hayirli, A., S. J. Bertics, and R. R. Grummer. 2002. Effects of slow-release insulin on production, liver triglyceride, and metabolic profiles of Holsteins in early lactation. *J. Dairy Sci.* 85:2180–2191.
- Ishii, S., K. I. Izuka, B. C. Miller, and K. Uyeda. 2004. Carbohydrate response element binding protein directly promotes lipogenic enzyme gene transcription. *Proc. Natl. Acad. Sci. USA* 101:15597–15602.
- Kraft, W., and U. M. Dürr. 2005. *Klinische Labordiagnostik in der Tiermedizin*. 6. Auflage. Schattauer GmbH, Stuttgart, Germany.
- Kronfeld, D. S., G. P. Mayer, J. McD. Robertson, and F. Raggi. 1963. Depression of milk secretion during insulin administration. *J. Dairy Sci.* 46:559–563.
- Loor, J. J., H. M. Dann, R. E. Everts, R. Oliveira, C. A. Green, N. A. Janovick-Guretzky, N. Litherland, S. L. Rodriguez-Zas, H. A. Lewin, and J. K. Drackley. 2006. Plane of nutrition prepartum alters hepatic gene expression and function in dairy cows as assessed by longitudinal transcript and metabolic profiling. *Physiol. Genomics* 27:29–41.
- Mackie, T. R., D. A. Dwyler, K. L. Ingvarsen, P. Y. Chouinad, J. M. Lynch, D. M. Barbano, and D. E. Bauman. 1999. Effects of insulin and amino acids on milk protein concentration and yield from dairy cows. *J. Dairy Sci.* 82:1512–1524.
- Mashek, D. G., K. L. Ingvarsen, J. B. Andersena, M. Vestergaardb, and T. Larsena. 2001. Effects of a four-day hyperinsulinemic-euglycemic clamp in early and mid-lactation dairy cows on plasma concentrations of metabolites, hormones, and binding proteins. *Domest. Anim. Endocrinol.* 21:169–185.
- McGuire, M. A., D. K. Beede, M. A. DeLorenzo, C. J. Wilcox, G. B. Huntington, C. K. Reynolds, and R. J. Collier. 1989. Effects of thermal stress and level of feed intake on portal plasma flow and net fluxes of metabolites in lactating Holstein cows. *J. Anim. Sci.* 67:1050–1060.
- McGuire, M. A., J. M. Griinari, and D. E. Bauman. 1995. Role of insulin in the regulation of mammary synthesis of fat and protein. *J. Dairy Sci.* 78:816–824.
- Nadal, A., P. F. Marrero, and D. Haro. 2002. Down-regulation of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by insulin: The role of the forkhead transcription factor FKHRL1. *Biochem. J.* 366:289–297.
- O'Brien, R. M., and D. K. Granner. 1991. Regulation of gene expression by insulin. *Biochem. J.* 278:609–619.
- O'Brien, R. M., R. S. Streeper, J. E. Ayala, B. T. Stadelmaier, and L. A. Hornbuckle. 2001. Insulin-regulated gene expression. *Biochem. Soc. Trans.* 29:552–558.
- Overton, T. R., J. K. Drackley, C. J. Ottemann-Abbamonte, A. D. Beaulieu, and J. H. Clark. 1998. Metabolic adaptation to experimentally increased glucose demand in ruminants. *J. Anim. Sci.* 76:2938–2946.
- Pfaffl, M. W., T. Mircheva Georgieva, I. Penchev Georgiev, E. Ontsouka, M. Hageleit, and J. W. Blum. 2002. Real-time RT-PCR quantification of insulin-like growth factor (IGF)-1, IGF-1 receptor, IGF-2, IGF-2 receptor, insulin receptor, growth hormone receptor, IGF-binding proteins 1, 2 and 3 in the bovine species. *Domest. Anim. Endocrinol.* 22:91–102.
- Rigalma, K., C. Duvaux-Ponter, A. Barrier, C. Charles, A. A. Ponter, F. Deschamps, and S. Roussel. 2010. Medium-term effects of repeated exposure to stray voltage on activity, stress physiology, and milk production and composition in dairy cows. *J. Dairy Sci.* 93:3542–3552.
- Sandholm, M. 1976. Coagulation of serum by glutaraldehyde. *Clin. Biochem.* 9:39–41.
- SAS Institute. 2001. *SAS User's Guide: Statistics*. Version 8 ed. SAS Inst. Inc., Cary, NC.
- Schmidt, G. H. 1966. Effect of insulin on yield and composition of milk of dairy cows. *J. Dairy Sci.* 49:381–385.
- Sjaunja, L. O., L. Baevre, L. Junkkarinen, J. Pedersen, and J. Setälä. 1991. A Nordic proposal for an energy corrected milk (ECM) formula. Pages 156–157 in *Performance Recording of Animals State of the Art 1990*. P. Gaillon and Y. Chabert, ed. EAAP Publication 50. Centre for Agricultural Publishing and Documentation, Wageningen, the Netherlands.
- Sul, H. S., and D. Wang. 1998. Nutritional and hormonal regulation of enzymes in fat synthesis: Studies of fatty acid synthase and mitochondrial glycerol-3-phosphate acyltransferase gene transcription. *Annu. Rev. Nutr.* 18:331–351.
- van Dorland, H. A., S. Richter, I. Morel, M. G. Doherr, N. Castro, and R. M. Bruckmaier. 2009. Variation in hepatic regulation of metabolism during the dry period and in early lactation in dairy cows. *J. Dairy Sci.* 92:1924–1940.
- Van Kneegsel, A. T. M., H. van den Brand, J. Dijkstra, S. Tamminga, and B. Kemp. 2005. Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. *Reprod. Nutr. Dev.* 45:665–688.
- Watford, M., Y. Hod, Y. B. Chiao, M. F. Utter, and R. W. Hanson. 1981. The unique role of the kidney in gluconeogenesis in the chicken. The significance of a cytosolic form of phosphoenolpyruvate carboxykinase. *J. Biol. Chem.* 256:10023–10027.
- Zammit, V. A. 1983. Regulation of hepatic fatty acid oxidation and ketogenesis. *Proc. Nutr. Soc.* 42:289–302.