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Feed Restriction Induces Pyruvate Carboxylase but not Phosphoenolpyruvate Carboxykinase in Dairy Cows*

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ABSTRACT

The ability of dairy cattle to adapt to changes in nutrient intake requires appropriately responsive expression of several key genes in liver. Holstein cows were used in 2 experiments to determine the effect of short-term feed restriction on expression of mRNA for gluconeogenic and ureagenic enzymes in liver. In experiment 1, cows were fed a total mixed diet for ad libitum intake for a 5-d period followed by 5 d of 50% of their previous 5-d ad libitum intake followed by 10 d of ad libitum feeding. Liver biopsies and blood samples were obtained on d 5, 10, and 20 of the experiment, the last day of each feeding period. Pyruvate carboxylase (PC) mRNA increased with feed restriction, but phosphoenolpyruvate carboxykinase (PEPCK) was unchanged. Expression of carbamoyl phosphate synthetase (CPS-I), argininosuccinate synthetase, and ornithine transcarbamylase mRNA were not altered by feed restriction; however, CPS-I mRNA expression tended to increase during realimentation. In experiment 2, cows were fed for ad libitum intake for 5 d and then fed 50% of previous intake for 5 d. Liver biopsy samples collected on d 5 and 10 were used for PC mRNA, PEPCK mRNA, and in vitro measure of gluconeogenesis from radiolabelled propionate and lactate. The data indicate expression of genes for key metabolic processes in liver of lactating cows is responsive to feeding level. Expression of PC mRNA is part of the adaptive response to feed intake restriction and is matched by increased capacity for gluconeogenesis from lactate.
(**Key words:** energy, glucose, liver, gene)

Abbreviation key: CPS-I = carbamoyl phosphate synthetase, **FR** = feed restricted, **OTC** = ornithine transcarbamylase, **PC** = pyruvate carboxylase, **PEPCK** = phosphoenolpyruvate carboxykinase, **PEPCK-C** = cytosolic phophoenolpyruvate carboxykinase, **PEPCK-M** = mitochondrial phosphoenolpyruvate carboxykinase, **PUN** = plasma urea nitrogen.

INTRODUCTION

Ruminants rely largely on hepatic gluconeogenesis to support whole body glucose metabolism and to supply glucose for lactose synthesis. The rate of gluconeogenesis is responsive to level of production, substrate availability, and relative concentrations of gluconeogenic precursors (Lomax and Baird, 1983; Huntington, 1990; Donkin and Armentano, 1994). Pyruvate carboxylase (**PC**) and phosphoenolpyruvate carboxykinase (**PEPCK**) are 2 key enzymes for gluconeogenesis in liver that respond to nutritional status in nonruminants (Tilghman et al., 1974; Wallace et al., 1998). Synthesis of cytosolic PEPCK (**PEPCK-C**) in mammalian liver is induced by feed withdrawal (Hanson and Reshef, 1997) and calorie restriction (Dhahbi et al., 1999) and is reduced by a high-carbohydrate diet (Hanson and Reshef, 1997). It is well documented that increased PEPCK activity in nonruminants results from the actions of glucagon (acting through cyclic AMP), glucocorticoids, and thyroid hormone to induce transcription of the PEPCK. Insulin, in contrast, represses PEPCK activity by antagonizing the effects of glucagon on gene transcription (O'Brien and Granner, 1991). Limited data indicate that a similar effect of feed restriction on hepatic PEPCK activity is not evident for ruminants, but PC activity in liver of lactating cows is increased in response to feed withdrawal (Ballard et al., 1968).

Starvation in rats induces both liver and kidney PC activity (Salto et al., 1996). Induction of PC mRNA and activity during the transition to lactation may be part of the physiological adaptations associated with the onset of parturition (Greenfield et al., 2000a), a consequence of the decline in voluntary feed intake that occurs in late gestation, or a combination of both factors. Imposed feed restriction has been used to study the metabolic consequences of nutrient insufficiency on metabolism and milk production (Drackley et al., 1991; Bertics and Grummer, 1999) and, therefore, may provide insight on nutrient-gene interactions in dairy cows.

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Hepatic N disposal and urea synthesis are usually sufficient to accommodate acute increases in amino acid catabolism (Morris, 2002). However, long-term adaptations in the capacity of the urea cycle are observed in response to dietary calorie restriction (Tillman et al., 1996), dietary protein intake level, or catabolism of endogenous protein. Changes in rate of gene transcription represent a major portion of the long-term response in expression of urea cycle enzymes (Morris, 2002) and require several days to be fully manifested (Schmike, 1962). Changes in liver N metabolism, in response to changes in feed intake, occur within 5 d in cattle (Reynolds, 1992). To our knowledge, the molecular basis of these adaptations has not been investigated in cattle.

Despite intense investigation on control genes for key metabolic reactions in liver of nonruminants, there is a paucity of data describing nutritional and hormonal regulation of gene expression in liver of ruminants. The objective of the present study was to determine the effect of imposed feed restriction on expression of PEPCK, PC, carbamoyl phosphate synthetase I (**CPS-I**), ornitine transcarbamylase (**OTC**), and argininosuccinate synthase mRNA in liver of lactating dairy cattle. A second objective was to determine the biological consequences of changes in expression of gluconeogenic enzymes.

MATERIALS AND METHODS

Two experiments were conducted to address the objectives just stated. In experiment 1, the effects of feed restriction on production, energy balance, blood metabolites, hormones, and expression of gene for gluconeogenesis and for ureagenesis were assessed. Experiment 2 focused on the relationship between mRNA expression for PC and PEPCK and the rates of metabolism of lactate and propionate to glucose.

Animals, Management, and Sampling: Experiment 1

Sixteen multiparous Holstein cows selected from the Purdue University Dairy Research and Education Center herd were stratified by previous production and days in milk and were assigned to one of 2 treatment groups. Cows averaged 34.2 ± 0.8 kg milk/d and were 156 ± 6 DIM at the beginning of the trial. Cows were housed in individual tie stalls, had free access to water, were milked twice daily at 0800 and 2000 h, and were fed once daily a diet formulated to meet or exceed requirements for milk production (NRC, 2001) (Table 1). Feed intake was measured daily by difference of feed offered and refused. The Purdue Animal Care and Use Committee approved animal handling and sample collection procedures.

Table 1. Diet ingredients and chemical composition.

Ingredient, % of DM	
Shelled corn, dry rolled	9.50
High moisture shelled corn	8.15
Soy hulls	5.76
Whole cottonseed	3.76
Corn gluten feed	18.60
Alfalfa haylage	16.87
Corn silage	25.04
Aminoplus[1]	2.45
Soybean meal	4.88
Urea	0.06
Tallow	0.27
Vitamin E premix[2]	0.03
Magnesium oxide	0.21
Bentonite	0.11
Sodium bicarbonate	0.63
Dicalcium phosphate	1.40
Limestone	0.78
Dynamate	0.20
Potassium chloride	0.41
Salt	0.50
Selenium	0.15
4-plex[3]	0.02
Trace mineral premix[4]	0.21
Nutrient[5]	
CP	16.8
RUP,[6] % of CP	35.3
ADF	15.0
NDF	25.9
NE_L, Mcal/kg DM	1.65
Ca	0.65
P	0.43

[1]Processed soybean meal (RUP = 58%); Consolidated Nutrition L.C., Decatur, IN.

[2]Contained 44,000 IU/kg of vitamin E.

[3]Zinpro Corporation (Eden Prairie, MN).

[4]Composition: 9180 ppm of Mn, 2511 ppm of Cu, 1,984,000 IU/kg of vitamin A, 500,000 IU/kg of vitamin D, and 400 IU/kg of vitamin E.

[5]Dry matter basis, unless indicated otherwise.

[6]Based on ingredient composition.

Cows were used in a 20-d experiment consisting of 3 phases: 5 d of ad libitum feeding, 5 d of feed restriction, and 10 d of realimentation. During the first 5 d of the experiment, all cows were fed for ad libitum feed intake. On d 6 and continuing through d 10 of the experiment, the feed restricted (**FR**) group (n = 8) was fed 50% of the average daily DMI for the previous 5-d ad libitum feeding period. Feed restriction was imposed for 5 d after which cows were fed for ad libitum intake. Control cows (n = 8) were given free access to feed during the entire experiment. Feed samples were collected during each phase of the trial, dried in a convection oven at 55°C, and used for diet adjustments. Samples of TMR collected during each phase of the trial were used to form a composite sample, which was analyzed by a commercial laboratory (Dairy One, Ithaca, NY) by wet chemistry methods (Table 1).

Individual milk yields were recorded electronically at each milking (HerdMaster Galaxy Management Sys-

tem, Alfa-Laval Agri Inc., Kansas City, MO). Milk samples were obtained for 2 consecutive milkings during each phase of the experiment and analyzed for fat, protein, and SCC by near infrared reflectance and for MUN by the Bentley Chemspec method at the DHIA Laboratory (East Lansing, MI).

Liver biopsy samples were obtained by blind percutaneous needle biopsy (Greenfield et al., 2000a) within 2 to 6 h of feeding; on d 5, 10, and 20 of the trial, which corresponded to the last day of the ad libitum feeding phase; the last day of the feed restriction phase; and after 10 d of realimentation. Liver samples were rinsed in saline, transferred into a 50-mL conical tube containing 10 mL of guanidinium thiocyanate solution [4 *M* guanidinium thiocyanate, 25 m*M* sodium citrate (pH = 7.4), 0.5% sarcosyl, and 0.1 *M* beta-mercaptoethanol], frozen in liquid nitrogen, and stored at –80°C pending RNA extraction.

Blood samples and BW were obtained on the days when liver biopsies were performed. Two blood samples were collected immediately prior to biopsy from a medial coccygeal blood vessel into vacutainers (Becton Dickinson, Franklin Lakes, NJ). One sample collection tube contained potassium oxalate and sodium fluoride, and the other contained heparin. Plasma was separated by centrifugation at 550 × *g* for 15 min and was frozen at –20°C pending analysis.

Animals, Management, and Sampling: Experiment 2

Five cows were selected from the Purdue University Dairy Research and Education Center herd and used to determine the relationship between gluconeogenesis and expression of PC and PEPCK mRNA in liver. Cows averaged 158 ± 6.5 DIM, were producing 40.75 ± 1.6 kg/d of milk/d, and were housed, fed, and managed as described previously for cows in Experiment 1.

Cows were offered feed for ad libitum consumption on d 1 through 6 of the experiment. Feed intake was measured daily by difference of feed offered and feed refused. Approximately 4 h after feeding, on d 6 of the experiment, liver samples were obtained by blind percutaneous needle biopsy (Greenfield et al., 2000b). Samples were rinsed in ice-cold saline, and an aliquot of approximately 250 mg was prepared for mRNA analysis as described previously. An additional 1 g of liver was placed in ice-cold Dulbecco's Modified Eagles medium containing 1% BSA, transported on ice to the laboratory, subdivided into 30- to 50-mg explants, and used to measure the rate of gluconeogenesis from radiolabelled propionate and lactate. On d 7 through 11 of the experiment, all cows were given 50% of their previous ad libitum intake. Liver biopsy samples were obtained again on d 11 of the experiment for RNA analysis and measures of gluconeogenesis from propionate and lactate. The Purdue Animal Care and Use Committee approved animal handling and sample collection procedures.

cDNA Probes

Plasmids bPC1000, bPEPCKC3′, and bPEPCKM3′ were cloned in our laboratory (Agca et al., 2000, 2002) and contained insert DNA for bovine PC, PEPCK-C, and mitochondrial PEPCK (**PEPCK-M**), respectively. Plasmid pAS4, containing a 1.5-kb fragment of the human argininosuccinate synthetase cDNA; plasmid pOTC, containing a 1.2-kb fragment of the human OTC cDNA; and plasmid and plasmid pCPS-I, containing a 5.65-kb fragment of rat CPS-I, were purchased from American Type Culture Collection (Rockville, MD). The plasmid pDF8 containing a 1.06-kb fragment corresponding to the central region of the rat 18S rRNA gene was provided by Richard Torzynski (Cytoclonal Pharmaceutics Inc., Dallas, TX).

The cDNA inserts were excised from plasmids by restriction enzyme digestion followed by separation in low-melting temperature agarose gel and purified using the Wizard DNA purification system (Promega, Madison, WI). Radiolabelled cDNA probes were generated using ^{32}P[dCTP] and the Ready-to-go DNA labeling kit (Pharmacia, Piscataway, NY) by oligonucleotide priming. The specific activity of cDNA probes was approximately 10^9 cpm/μg of DNA.

Northern Blotting

Total RNA was extracted from liver biopsy samples (Chomczynski and Sacchi, 1987), and a 20-μg aliquot was separated by electrophoresis through a 1% agarose gel (Tsang et al., 1993) and transferred to Genescreen membrane (NEN Life Science Products, Boston, MA) by capillary action. The RNA was crosslinked using UV light, and the membrane was baked at 80°C for 2 h to remove any residual formaldehyde as per manufacturer's instructions. Membranes were prehybridized for 12 h in 50% deionized formamide, 5× SSPE, 5× Denhardt's, 10% dextran sulfate, and 200 μg/mL denatured herring sperm DNA at 42°C for 6 to 18 h. Hybridization was performed in the same solution with the addition of ^{32}P-labeled cDNA (2×10^6 cpm/mL) for 16 h at 42°C. Following hybridization, membranes were washed twice in 2× SSC (0.3 *M* NaCl, 0.03 *M* sodium citrate; pH = 7.0) for 5 min at room temperature, twice in 2× SSC, 1% SDS sodium dodecyl sulfate at 65°C for 30 min, and twice in 0.1× SSC for 30 min at room temperature.

Expression of mRNA was visualized by exposing membranes to Kodak X-Omat AR film and quantified

using Kodak Digital Science 1-D Image Analysis software (Eastman Kodak Co., Rochester, NY). Multiple sets of combs within a gel were necessary to accommodate all samples for the experiment. To account for possible differences between sets of samples, a pooled sample (20 μg) of bovine liver RNA was added to outside lanes within each comb and was used to adjust for variation in transfer of RNA and hybridization conditions. Variations in sample loading were adjusted using18S rRNA within each sample.

Plasma Analysis

Glucose concentrations were determined by a glucose oxidase method (Sigma kit #510-A; Sigma Diagnostics, St. Louis, MO). Plasma NEFA was measured using the NEFA C kit (Wako Chemical Co., Dallas, TX). Plasma urea nitrogen (**PUN**) concentrations were determined by a colorimetric method (Sigma #535; Sigma Diagnostics). Plasma glucagon and insulin were determined using radioimmunoassay kits and the human standards supplied (Diagnostic Products Corporation, Los Angeles, CA). Variation within assay for insulin averaged 5.7%, and variation between assays was 5.1%. Variation within assay for glucagon averaged 4.9%, and variation between assays was 2.4%.

Preparation of Liver Slices and Measures of Gluconeogenesis

Liver biopsy samples from cows in Experiment 2 were obtained using a 9-mm diameter biopsy needle (Greenfield et al., 2000b), transported on ice to the laboratory in Dulbecco's Modified Eagles medium containing 1% BSA. Within 40 min of removal from the animal, the biopsy core was sliced into 3- to 4-mm uniform sections weighing 30 to 50 mg; slices for each cow were incubated in Dulbecco's Modified Eagles medium containing 1% BSA and 2.5 m*M* propionate, 1.0 m*M* pyruvate, and 1.0 m*M* lactate as outlined previously for bovine hepatocytes (Donkin and Armentano, 1993). The metabolism of [2-^{14}C]propionate and [U-^{14}C]lactate to glucose was determined over a 2-h interval as previously described (Donkin and Armentano, 1993). Measurements were performed in triplicate for 1-h and 2-h incubations. Incubations were terminated by the addition of 0.5 mL of 5 *N* H_2SO_4 to the media. Explant cultures were removed from the flasks, homogenized in 1 mL of DNA buffer, and assayed for DNA content as described previously (Donkin and Armentano, 1993). The conversion of radiolabelled precursor to glucose was determined in the media as described previously (Donkin and Armentano, 1993). Data for 1- and 2-h incubations were used to test for linearity of rates of gluconeogenesis, which are expressed as nanomoles of precursor converted to glucose per microgram of DNA per hour.

Statistics

Data for experiment 1 were analyzed using PROC MIXED of SAS (1999). For repeated measurements, the model included the fixed effects of treatment and time, random effects of cow within treatment by time, the interactions of fixed effects, and the residual error. Time represented for d 5, 10, and 20. The covariance structure was determined for anti-dependence, simple, unstructured, and autoregressive models. The model that yielded the minimum range of values for Akaike information criterion and Bayesian information criterion for each variable was used for data analysis. Comparisons between means for control and FR groups were determined using single degree of freedom contrasts. For experiment 2, data were analyzed using PROC MIXED (1999). The model accounted for the effects of treatment (feed restriction or ad libitum feeding) and cow within treatment. Data for mRNA and gluconeogenic activity were combined for both sampling times (ad libitum and restricted feeding), and Pearson correlation coefficients were obtained using the PROC CORR of SAS (1999). Data are presented as least squares means and standard errors.

RESULTS AND DISCUSSION

There were no differences in DMI or milk production for the 2 groups during the first 5 d of experiment 1 (Table 2). Dry matter intake for the FR cows averaged 43% less than control cows during the period of imposed feed restriction. Average feed intake and milk production for the control cows were not different for d 1 through 5 compared with d 6 through 10 of the experiment.

Milk production for the FR cows decreased during the feed restriction period and averaged 78% of the values for the control cows during d 6 through 10 of the trial. These results were expected in the design of the trial. Energy balance, calculated as the difference between energy intake and calculated energy requirements for milk, fat, and protein yields and maintenance costs as a function of live weight (NRC, 2001), averaged –9.11 Mcal/d for the FR cows during d 6 through 10 of the trial compared with 12.93 Mcal/d for the control cows (Table 2; Figure 1). The negative energy balance for the restricted cows represents a potential loss of 1.94 kg/d, whereas the ad libitum fed cows had a potential gain of 2.42 kg/d given a starting BCS of 3.5 (NRC, 2001). The imposed reduction in feed intake in the absence of an offsetting reduction in milk production pro-

Table 2. Effects of feed restriction on feed intake, calculated energy balance, milk production, and milk composition (Experiment 1).

	Day of experiment[1]		
	d 1 to 5	d 6 to 10	d 11 to 20
Feed intake, kg[2,3,4]			
Feed restricted	25.30 ± 0.54	12.33 ± 0.60	23.91 ± 0.51
Control	26.56 ± 0.54	28.89 ± 0.60*	26.11 ± 0.51*
Energy balance, Mcal/d[2,3,4]			
Feed restricted	8.54 ± 1.15	–9.11 ± 1.23	10.44 ± 1.11
Control	7.94 ± 1.15	12.93 ± 1.23*	11.29 ± 1.11
FCM, kg/d[2,3]			
Feed restricted	31.07 ± 1.96	23.87 ± 1.96	25.29 ± 1.96
Control	32.16 ± 1.96	33.27 ± 1.96*	29.52 ± 1.96
Milk, kg/d[2,3,4]			
Feed restricted	31.50 ± 1.21	23.69 ± 1.26	29.65 ± 2.82
Control	32.84 ± 1.21	30.25 ± 1.26	32.01 ± 2.82
Milk fat, kg/d[2,4]			
Feed restricted	1.23 ± 0.07	0.96 ± 0.11	0.88 ± 0.13
Control	1.27 ± 0.07	1.41 ± 0.11*	1.11 ± 0.13
Milk protein, kg/d[3]			
Feed restricted	0.96 ± 0.06	0.69 ± 0.06	0.74 ± 0.06
Control	1.00 ± 0.06	0.89 ± 0.06*	0.85 ± 0.06
Milk lactose, kg/d[2,3]			
Feed restricted	1.50 ± 0.07	1.08 ± 0.06	1.36 ± 0.15
Control	1.63 ± 0.07	1.44 ± 0.06*	1.70 ± 0.15
Milk SNF, kg/d[2,3]			
Feed restricted	2.74 ± 0.11	1.97 ± 0.12	2.33 ± 0.26
Control	2.93 ± 0.11	2.60 ± 0.12*	2.82 ± 0.26
SCC, cells/mL[2]			
Feed restricted	120.27 ± 27.93	217.28 ± 76.78	231.05 ± 59.44
Control	32.22 ± 27.93	50.03 ± 76.78	30.59 ± 59.44*
Milk fat, %[3]			
Feed restricted	3.91 ± 0.25	4.05 ± 0.25	3.25 ± 0.25
Control	3.88 ± 0.25	4.58 ± 0.25	3.52 ± 0.25
Milk protein, %[3]			
Feed restricted	3.04 ± 0.08	2.89 ± 0.10	2.70 ± 0.09
Control	3.05 ± 0.08	2.93 ± 0.11	2.66 ± 0.09*
Milk lactose, %[2,3]			
Feed restricted	4.74 ± 0.08	4.56 ± 0.08	5.01 ± 0.08
Control	4.95 ± 0.08	4.78 ± 0.08	5.30 ± 0.08*
Milk solids, %[3]			
Feed restricted	8.69 ± 0.17	8.30 ± 0.17	8.53 ± 0.17
Control	8.92 ± 0.17	8.59 ± 0.17	8.79 ± 0.17
Milk urea N, mg/dL[2,3]			
Feed restricted	14.83 ± 0.62	12.00 ± 0.62	15.51 ± 0.62
Control	16.25 ± 0.62	14.25 ± 0.62*	16.88 ± 0.62

[1]Mean and standard error.

[2]Treatment effect ($P < 0.05$).

[3]Time effect ($P < 0.05$).

[4]Treatment × time effect ($P < 0.05$).

*Denotes differences ($P < 0.05$) within column for feed restricted vs. control.

vided a model to investigate changes in hepatic gene expression when energy intake was limiting.

Experimentally imposed complete nutrient deprivation caused a steady decline in milk yield. Milk production decreased 25% after 24 h and 44% following a 48-h starvation period (Baird et al., 1972; Athanasiou and Phillips, 1978). Imposed feed intake reduction results in negative energy balance provided that energy expenditure exceeds energy intake. Energy balance of FR cows in this study declined dramatically during the first day of feed restriction but became less negative and was stabilized during the rest of the feed restriction interval (Figure 1) likely because of compensatory decreases in milk production.

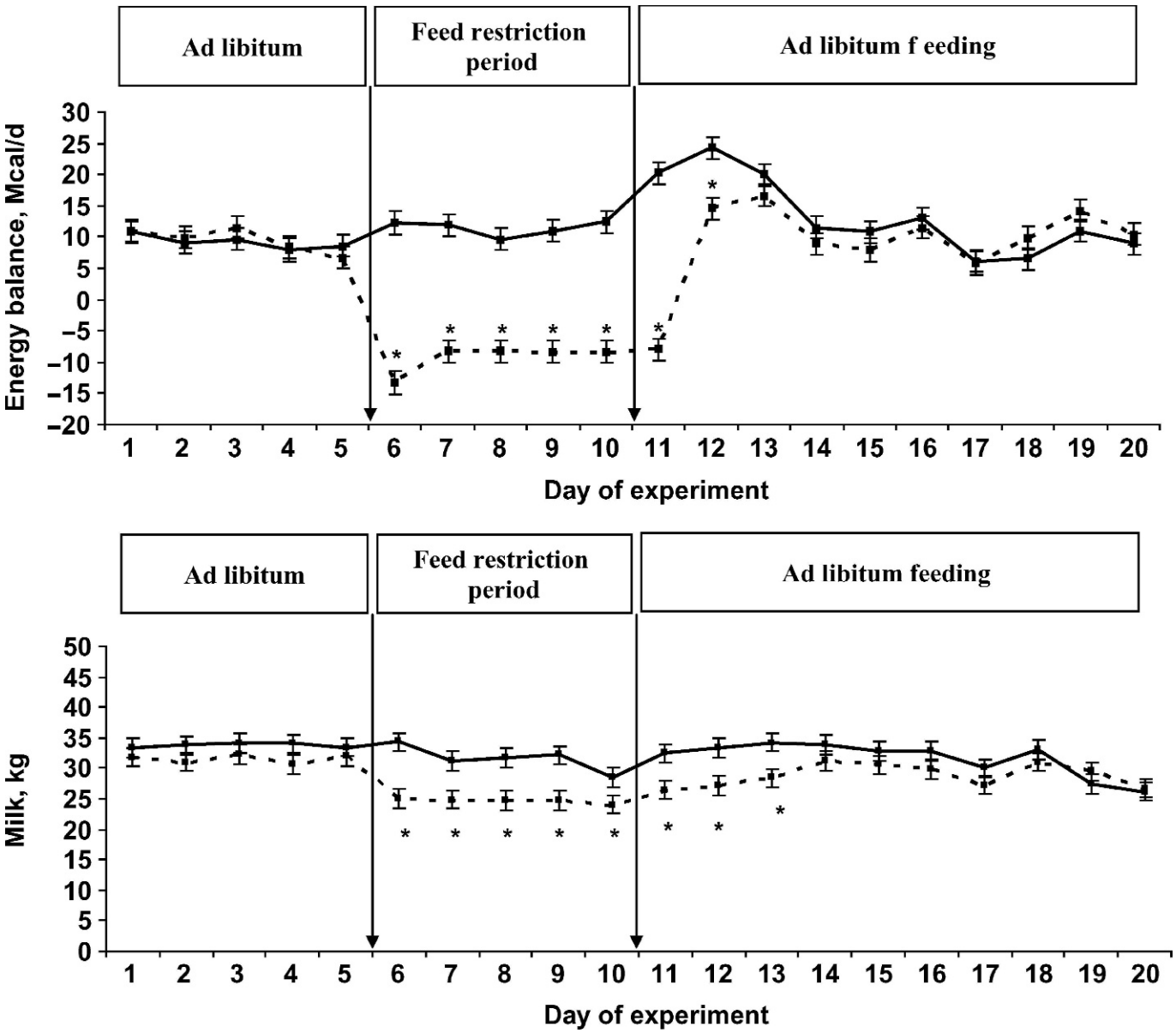

Figure 1. Calculated net energy balance (upper panel) and daily milk production (lower panel) for control cows (—) and cows subjected to feed restriction (– – –). Data represent least squares means and SEM. Statistics: treatment effect ($P < 0.05$), time effect ($P < 0.05$), treatment × time effect ($P < 0.05$). Differences ($P < 0.05$) between treatments within day of experiment are indicated by the symbol (*).

Glucose concentrations (mg/dL) were not different for the 2 groups of cows during d 6 through 10 d of the trial (Table 3). Blood glucose levels in FR cows with feed withdrawal for 24 to 48 h were previously observed to decrease (Baird et al., 1972; Reid et al., 1977; Athanasiou and Phillips, 1978), but in other instances a 6-d feed withdrawal period had relatively little effect on blood glucose (Lomax and Baird, 1983). The lack of change in blood glucose in the present trial suggests an effect of imposed feed restriction that permitted compensatory changes in glucose synthesis, glucose utilization, or both and maintenance of normal glycemia.

Plasma NEFA (μmol/L) concentrations were similar for the 2 groups of cows during the first 5 d of the trial (Table 3). Nonesterified fatty acid levels were elevated for FR cows during d 6 through 10 of trial; values for control cows remained unchanged compared with samples taken on d 5. Plasma NEFA declined below prerestriction values during d 11 through 20 of the trial. Both groups were at 40% of values obtained during d

Table 3. Effects of feed restriction on insulin, glucagon, and plasma metabolite concentrations.

	Day of experiment[1]		
	d 1 to 5	d 6 to 10	d 11 to 20
Glucose, mg/dL[2]			
Feed restricted	62.5 ± 1.8	58.5 ± 1.8	60.4 ± 1.8
Control	60.4 ± 1.8	57.6 ± 1.8	57.5 ± 1.8
NEFA, μM[2]			
Feed restricted	326 ± 58	531 ± 107	153 ± 21
Control	429 ± 58	467 ± 107	187 ± 21
Urea nitrogen, mg/dL[2,3]			
Feed restricted	20.1 ± 1.2	15.7 ± 1.2	19.1 ± 1.2
Control	22.3 ± 1.2	19.3 ± 1.2*	23.3 ± 1.2*
Glucagon, pg/mL			
Feed restricted	108 ± 8	114 ± 8	125 ± 8
Control	118 ± 8	118 ± 8	126 ± 8
Insulin, pg/mL			
Feed restricted	285 ± 45	321 ± 48	326 ± 45
Control	222 ± 45	330 ± 45	261 ± 45
Insulin:glucagon, molar ratio			
Feed restricted	1.83 ± 0.33	1.82 ± 0.35	1.63 ± 0.33
Control	1.56 ± 0.33	1.69 ± 0.33	1.40 ± 0.33

[1]Mean and standard error.
[2]Treatment effect ($P < 0.05$).
[3]Time effect ($P < 0.05$).
[4]Treatment × time effect ($P < 0.05$).
*Denotes differences ($P < 0.05$) within column for feed restricted vs. control.

1 through 5. The differences between NEFA during these phases of the experiment are not readily apparent. The change in NEFA during partial feed restriction is less than values reported in the literature for feed restriction (Drackley et al., 1991), and it is also considerably less than changes in NEFA measured at calving (Greenfield et al., 2000b). Feed withdrawal increased NEFA by 25% within 24 h in dairy cattle, and concentrations declined during realimentation but failed to match levels prior to feed restriction after 48 h (Athanasiou and Phillips, 1978). An imposed reduction in feed intake of 80% caused a 2-fold increase in blood NEFA when compared with control cows (Drackley et al., 1991). In addition, net splanchnic uptakes of FFA were increased 400% during a 6-d feed withdrawal period for dairy cows (Lomax and Baird et al., 1983). The rise in NEFA is due to the lipid mobilization triggered by an imposed energy deficit. Decreased NEFA serum levels during d 11 through 20 of the present study suggest restoration to energy equilibrium.

Plasma urea nitrogen was similar for the 2 groups of cows during d 1 through 5 of trial. However, PUN levels tended to be lower for the FR cows compared with control cows during d 6 through 10 of the trial. Activity of the urea cycle, in nonruminants, increases during starvation and in response to high-protein diets (Morris, 1992). Levels of MUN decreased ($P < 0.05$) for FR cows during d 6 through 10 when compared with the control group (Table 2). Plasma urea nitrogen is directly proportional to MUN levels, and both are responsive to several factors in lactating cows (Roseler et al., 1993; Jonker et al., 1998). Changes in PUN and MUN during the trial suggest differences in protein supply during d 6 through 10. Excessive ruminal protein degradation results in increased urea in blood, milk, and urine. The decrease in PUN and MUN with feed restriction in the present study suggests a reduction in ammonia load from the rumen with reduced N intake as noted previously (Jonker et al., 1998) or more efficient use of absorbed protein.

The concentrations of insulin and glucagon were not altered by feed restriction (Table 3). Similarly, the molar ratios of these hormones were not changed in response to feed restriction. A similar lack of response to feed restriction has been previously observed for peripheral insulin and glucagon (Drackley et al., 1991). Plasma insulin and glucagon concentrations were not altered in lactating cows given 50% of ad libitum feed intake (De Boer et al., 1986). In rats, peripheral glucagon concentrations are relatively constant during feed deprivation, despite elevated hepatic portal blood concentrations of glucagon (Balks and Jungermann, 1984). In beef steers, peripheral insulin and glucagon are elevated with increased feed intake because of increased splanchnic flux of these hormones, which exceeds their hepatic removal (Lapierre et al., 2000). The severity of

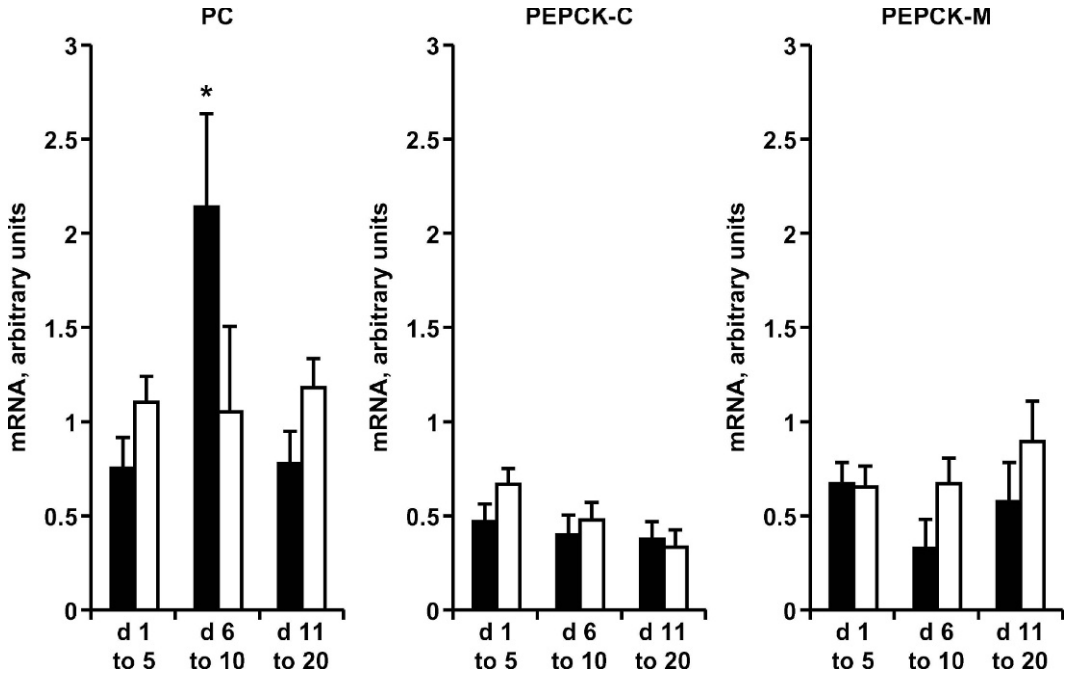

Figure 2. Effects of feed restriction on expression of hepatic pyruvate carboxylase (PC), cytosolic phophoenolpyruvate carboxykinase (PEPCK-C), and mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M) mRNA. Solid bars represent cows subjected to feed restriction, and closed bars are controls. Data are least squares means and standard errors. Differences ($P < 0.05$) between d 1 to 5 and d 6 to 10 are denoted by the symbol (*). Main effects: for PC, treatment, $P = 0.63$; time, $P = 0.17$, and treatment × time, $P = 0.12$; for PEPCK-C, treatment, $P = 0.45$, time, $P < 0.05$, and treatment × time, $P = 0.32$; and for PEPCK-M, treatment, $P = 0.29$, time, $P = 0.40$, and treatment × time, $P = 0.48$.

feed restriction in the present study might not have been adequate to alter insulin and glucagon release. Alternatively, changes in splanchnic flux of insulin and glucagon, in response to reduced feed intake, might have been offset by compensatory changes in hepatic extraction of these hormones.

Expression of PC mRNA (Figure 2) was similar between the 2 groups during the first 5 d of trial ($P > 0.05$). Conversely, during d 6 through 10, abundance of PC mRNA increased ($P < 0.05$) for FR cows compared with the previous 5-d period. Expression of PC mRNA for control cows was not different between d 1 through 5 and d 6 through 10 of the experiment. Elevated expression of PC and other gluconeogenic enzymes during feed withdrawal and starvation have been reported in the rat (Jitrapakdee and Wallace, 1999). There is an obligatory requirement for glucose as an oxidizable substrate for brain, erythrocytes, kidney medulla, and mammary tissue (Mayes, 1996). Therefore, feed restriction results in increased gluconeogenesis from lactate, amino acids, and glycerol to meet glucose needs (Baird et al., 1980). An increase in PC activity in feed deprived sheep supports increased use of lactate and alanine during feed withdrawal (Filsell et al., 1969). A strong correlation between PC mRNA and PC activity in bovine liver (Greenfield et al., 2000a) supports an increase in the synthesis of oxaloacetate from pyruvate during feed restriction because of increased PC activity.

In Experiment 2, we directly tested the hypothesis that the changes in PC mRNA reflect altered capacity for gluconeogenesis from lactate. Biopsy samples from cows under ad libitum and FR conditions were used for mRNA analysis and separate measures of gluconeogenesis from lactate and propionate. Consistent with observations from experiment 1, the data (Table 4) indicate an increase in PC mRNA abundance ($P = 0.06$) during the feed restriction period. The changes in PC mRNA are mirrored by an increase ($P < 0.05$) in lactate conversion to glucose in the same biopsy samples. Furthermore, the Pearson correlation coefficient for the PC mRNA and lactate conversion to glucose was 0.64 ($P < 0.05$; n = 10). Taken together, these data indicate that changes in PC expression are indicative of a change in gluconeogenic capacity from lactate.

The abundance of mRNA for PEPCK-M and PEPCK-C were not responsive ($P > 0.05$) to feed restriction or realimentation (Figure 2). Expression of PEPCK, a key gluconeogenic enzyme in nonruminants, is upregulated in response to feed deprivation, glucagon, and glucocorticoids and is reduced when feeding a carbohydrate-

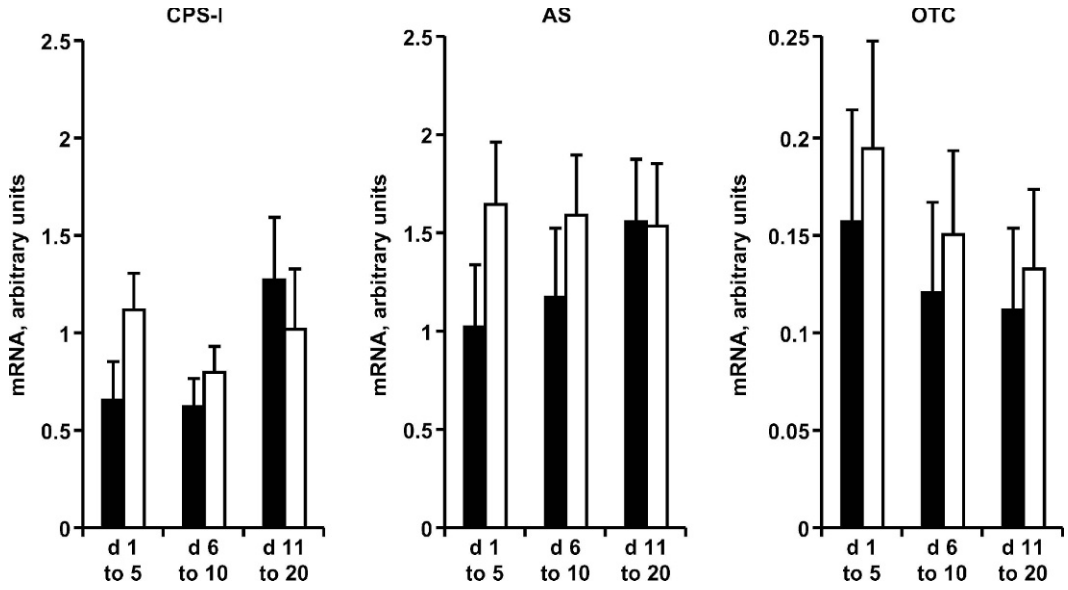

Figure 3. Effects of feed restriction on expression of carbamoyl phosphate synthetase (CPS-I), argininosuccinate synthetase (AS), and ornithine transcarbamylase (OTC) mRNA. The solid bars represent samples from cows subjected to feed restriction, and open bars are controls. Main effects: for CPS-I, treatment, $P = 0.60$, and time, $P < 0.05$; for AS, treatment, $P = 0.33$, time, $P = 0.70$, and time × treatment, $P = 0.35$; and for OTC, treatment, $P = 0.59$, time, $P = 0.27$, and time × treatment, $P = 0.98$.

rich diet or by elevated insulin (Pilkis and Granner, 1992). In nonruminants, the activity of PEPCK is primarily regulated at the level of transcription of the gene (O'Brien and Granner, 1991). The activity of PEPCK did not change during starvation in dairy cows but was induced in lactating compared with non-lactating cows (Ballard et al., 1969) and as cows approach peak milk production (Greenfield et al., 2000a). The lack of simultaneous changes of mRNA expression for PC and PEPCK during feed restriction suggests a change in capacity for pyruvate and lactate metabolism that may not be matched by increased capacity for glucose synthesis from precursors that contribute to the oxaloacetate pool in liver.

An increase in gluconeogenesis from lactate coupled with lack of change in gluconeogenesis from propionate during feed restriction indicates specific changes in gluconeogenesis prior to the cytosolic phosphoenolpyruvate step of gluconeogenesis. Compartmentalization of oxaloacetate metabolism cannot be determined from these experiments; however, to maintain a net balance of NADH in glucose synthesis propionate and lactate, carbon would preferentially utilize PEPCK-C and PEPCK-M, respectively. Mitochondrial PEPCK is usually considered as unregulated and constitutive. These data suggest that the activity of PEPCK-M in dairy cattle is adequate to accommodate increased gluconeogenesis from lactate and points to PC as a controlling step for gluconeogenesis from lactate.

Expression of CPS-I mRNA abundance (Figure 3) during d 6 through 10 did not change ($P > 0.05$) for FR cows compared with controls. Expression of mRNA for argininosuccinate synthetase and OTC was not different between d 1 through 5 and d 6 through 10 for control and FR cows. Enzymes of the urea cycle catalyze the conversion of ammonia and carbon dioxide into urea, which serves to detoxify ammonia, the primary nitrogenous waste product derived from protein catabolism (Christowitz et al., 1981). It is important to note that induction of urea enzymes is slow, requiring 5 to 7 d to achieve a new steady state, and, in the case of arginase, the half-life of the enzyme is 5 d (Schimke, 1962, 1964). Previous work with rats suggests a slight increase in urea cycle enzyme activity after a 48-h feed withdrawal (Snodgrass et al., 1978). Urea cycle enzyme activity is elevated in response to starvation and high-protein diets (Morris, 1992). The current data from dairy cows would indicate that feed restriction reduces urea formation without a change in expression of urea cycle enzymes. Although feed restriction did not alter urea cycle enzyme mRNA compared with samples taken prior to feed restriction, there was a tendency ($P = 0.08$) for expression of CPS-I mRNA to increase in FR cows during the realimentation period. Elevated levels of CPS-I mRNA during the realimentation phase might be due to a delayed effect in response to feed restriction.

Increased expression of PC mRNA during feed restriction is one of the metabolic adaptations that occurs

Table 4. Effects of feed restriction on propionate and lactate metabolism to glucose and phosphoenolpyruvate carboxykinase and pyruvate carboxylase mRNA (Experiment 2).

	Nutritional status			
	Ad libitum feeding		Feed restriction	
	Mean	SE	Mean	SE
PEPCK mRNA, arbitrary units	0.49	0.08	0.37	0.08
PC mRNA, arbitrary units	1.50[a]	0.17	2.12[b]	0.17
Propionate converted to glucose, nmol/μg of DNA per h	3.82	0.67	4.59	0.67
Lactate converted to glucose, nmol/μg of DNA per h	0.91[a]	0.69	3.50[b]	0.69

[a,b]Means within a row with different superscripts differ ($P < 0.10$).

in liver of lactating dairy cows to meet the high demands of glucose for metabolism and milk synthesis. The lack of change in expression of urea cycle in conjunction with a lack of corresponding change in PUN and MUN of FR cows may reflect an already abundant capacity for ammonia detoxification in liver of healthy dairy cows. Alternatively, a reduction in feed intake is likely to reduce rumen ammonia despite an increase in amino acid catabolism, thereby reducing the total ammonia load to liver for detoxification through the urea cycle. In either case, feed restriction, as used in these experiments with lactating dairy cattle, does not appear to be effective model to evoke changes in urea cycle enzyme mRNA expression. Increased expression of PC mRNA and the lack of a parallel response of PEPCK mRNA imply a greater capacity for metabolism of lactate to oxaloacetate during feed restriction. Elevated levels of PC mRNA during feed restriction may be coupled to increased protein turnover during feed restriction and enhanced metabolism of lactate, alanine, and other amino acids. Conversely, an increase in PC in the absence of changes in PEPCK activity may provide oxalacetate for NEFA oxidation in the TCA cycle. The latter is likely to be critical only if oxalacetate is consumed by ancillary processes that occur simultaneously with an increased need for NEFA oxidation. The lack of changes in the expression of PEPCK mRNA but increased expression of PC with feed restriction indicates adaptations in the profile of precursors used to maintain hepatic gluconeogenesis.

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