Response to Conjugated Linoleic Acid in Dairy Cows Differing in Energy and Protein Status¹

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ABSTRACT

The *trans*-10, *cis*-12 conjugated linoleic acid (CLA) isomer inhibits milk fat synthesis, whereas milk yield and synthesis of other milk components generally remain unchanged in established lactation. However, in some CLA studies increases in milk yield, milk protein yield, or both have been observed in cows limited in energy, either in early lactation or when grazing pasture. Our objective was to evaluate the performance and monitor peripheral tissue responses to homeostatic signals regulating lipolysis and glucose uptake with CLA supplementation when cows were limited in metabolizable energy in combination with moderate or excess metabolizable protein supply. Holstein cows ($n = 48$; 112 ± 5 d in milk; mean $\pm$ SE) were provided ad libitum access to a diet that met energy and protein requirements for a 16-d standardization interval. Based on performance during this interval, the Cornell Net Carbohydrate and Protein System was used to design energy-limiting rations that provided 80% of metabolizable energy requirements, and these were fed throughout the treatment periods. Cows were randomly allocated to 4 treatments, in a 2-period crossover design. Treatments were 1) moderate metabolizable protein (MP) supply, 2) moderate MP supply + CLA, 3) excess MP supply, and 4) excess MP supply + CLA. Moderate and excess MP supply were at 88 and 117%, respectively, of the MP requirement established during the standardization period, as estimated by the Cornell Net Carbohydrate and Protein System. Each experimental period comprised 16 d, with crossover of CLA within each protein level. The lipid-encapsulated CLA supplement provided 12 g/d of *trans*-10, *cis*-12 CLA. Conjugated linoleic acid treatment reduced milk fat yield by 21% but increased milk yield and milk protein yield by

2.6 and 2.8%, respectively. Milk yield and content and yield of both milk protein and fat were unaltered by either protein treatment alone or in combination with CLA. Basal concentrations of glucose, insulin, and non-esterified fatty acids were unaffected by CLA supplementation. The fractional rate of glucose clearance in response to an insulin challenge and the nonesterified fatty acid response to an epinephrine challenge were also not altered by either CLA treatment or MP supply. Overall, the results demonstrate that CLA supplementation when cows are energy-limited allows for repartitioning of nutrients, resulting in increased yields of milk and milk protein, and this can occur without changes in whole-body glucose homeostasis and adipose tissue response to lipolytic stimuli.

Key words: conjugated linoleic acid, milk fat, milk protein, insulin sensitivity

INTRODUCTION

Dietary supplements of conjugated linoleic acid (CLA) that contain the isomers *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA have a number of biological effects in nonruminant species, including anticarcinogenesis, immunomodulation, antiatherosclerosis, and alterations in body composition (Pariza et al., 2001). Investigations of these biological effects in ruminants have been limited, but it has been clearly established that CLA supplements reduce milk fat synthesis in lactating cows and sheep. Baumgard et al. (2000) first showed that the *trans*-10, *cis*-12 CLA isomer is a potent inhibitor of milk fat synthesis in lactating dairy cows, with this CLA isomer reducing milk fat in a dose-dependent manner (de Veth et al., 2004).

Initial short-term studies (3 to 5 d) with dairy cows in which CLA was infused abomasally, either as a mixture of isomers or as pure *trans*-10, *cis*-12 CLA, observed that the lactational effects of CLA were specific to the mammary gland and milk fat (Bauman et al., 2003). Likewise, the first long-term study (20 wk) used a mixture of CLA isomers, and a sustained reduction in milk fat yield was observed, with no effects on feed intake, milk yield, BW gain, or net energy balance (Per-

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field et al., 2002). The idea that CLA specifically affects milk fat synthesis in lactating dairy cows was supported by Baumgard et al. (2002) when they observed that CLA treatment had no effect on the response of tissues to homeostatic signals regulating lipolysis and glucose homeostasis. All of these initial studies were conducted with cows in mid- to late-lactation that were fed diets in excess of energy and protein requirements.

Interestingly, recent studies provide evidence that in situations in which lactating cows are limited by energy supply, the energy spared by CLA-induced milk fat depression (MFD) may be repartitioned toward the synthesis of milk protein and milk lactose. One such situation is early lactation, and studies of CLA supplementation during this period of negative energy balance have sometimes shown increases in milk and milk protein yields (Bernal-Santos et al., 2003; Shingfield et al., 2004; de Veth et al., 2005b). Moreover, responses have not been consistent, and Castañeda-Gutiérrez et al. (2005) have suggested that cows that are in a more negative energy balance may be more likely to have a milk yield response when energy is spared by CLA-induced MFD. A second situation in which the energy intake of cows is less than that required to meet their productive potential is when pasture is fed (Kolver and Muller, 1998). When the diets of cows grazing pasture in early lactation were supplemented with CLA, increases in both milk and milk protein yield were reported, as reviewed by Griinari and Bauman (2003). Although pasture-fed cows in early lactation are typically consuming MP in excess of that required for milk production, the role of the amount and quality of this excess protein in determining a milk protein response has not been determined.

The primary objective of the current study was to examine production responses to CLA supplementation when the ME supply was limiting (characteristic of the early-lactation cow) and also when the MP supply was in excess (characteristic of the pasture-fed dairy cow). Additionally, peripheral tissue responses to homeostatic signals regulating lipolysis and glucose uptake were examined as potential mechanisms by which CLA may alter nutrient partitioning.

MATERIALS AND METHODS

Animals, Design, and Sampling

All procedures involving animals were approved by the Cornell University Institutional Animal Care and Use Committee. Prior to the initiation of treatments, a 16-d standardization period was conducted in which 48 Holstein cows (112 ± 5 DIM; mean $\pm$ SE) were provided ad libitum access to a TMR diet that met or exceeded their energy and protein requirements (Fox et al.,

2004). Cows were selected from the Cornell University Dairy and Teaching Research facility and were an equal mix of primiparous and multiparous cows. Body weight and BCS were determined on d 4 and 7 of the standardization period and feed samples were collected for analysis on d 7. Milk yield and content of milk fat and protein were determined on d 7 and 8 of the standardization period. By using the Cornell Net Carbohydrate and Protein System (CNCPS) model (Fox et al., 2004) and the feed analysis results and performance data from the standardization period, 2 diets were formulated (Table 1). The diets were either moderate or high in CP content, with both having a similar proportion of soluble protein, energy content, NDF content, and crude fat content.

Throughout both treatment periods each cow received 1 of the 2 formulated diets, with intake set on an individual cow basis to provide 80% of the ME required for milk production based on CNCPS predictions using her data from the standardization period. This was achieved using the performance measurements and feed analysis as well as animal, environment, and management factors recorded during d 15 and 16 of the standardization period. The extent of the restriction in ME-allowable milk is similar to the level observed in lactating dairy cows when a TMR diet is substituted with pasture (78%), when accounting for the reduction in DMI (Kolver and Muller, 1998).

The experiment was conducted as a 2×2 factorial, 2-period crossover design, with 1 factor being MP supply and the other factor being inclusion or omission of a CLA supplement. Each of the 2 experimental periods was 16 d. At initiation of the experiment, cows were blocked by parity, DIM, and current yield of milk and milk fat content. Cows within each block were randomly assigned to 1 of 4 treatments. Treatments were 1) a moderate MP supply, 2) a moderate MP supply + CLA, 3) an excess MP supply, and 4) an excess MP supply + CLA. Each treatment period comprised 16 d, with crossover of CLA within each level of protein. The moderate and excess MP supply were achieved by feeding the moderate and excess CP diets, respectively, and were predicted to supply 88 and 117% of the MP-allowable milk, as estimated by the CNCPS model based on their performance during the 16-d standardization interval. The CLA supplement was in a lipid-encapsulated form (Balchem Encapsulates, New Hampton, NY). Cows not supplemented with CLA received calcium salts of palm fatty acid distillate (Megalac; Church and Dwight Co., Princeton, NJ) so that the diets would be isoenergetic.

The lipid-encapsulated CLA and Megalac contained 64 and 83% fat, respectively. Supplements provided 73.8 g/d of fat and were top-dressed once daily on the

Table 1. Ingredient and chemical composition of diets fed in the standardization and treatment periods and their estimated NE_L

Diet composition	Diet		
	Standardization period	Low-energy treatment period ¹	
		Moderate protein	Excess protein
DM, %	48.5	47.5	49.3
Ingredient, % of DM			
Corn silage	23.7	25.1	19.9
Alfalfa haylage	26.1	25.7	28.1
Timothy hay	3.8	3.8	3.4
High-moisture shelled corn	16.6	17.4	9.6
Wheat straw	—	0.4	0.4
Soybean hulls	2.7	2.9	3.2
Wheat middlings	2.8	2.9	3.2
Corn gluten feed	5.0	4.8	5.2
Soybean meal (48% CP)	8.5	6.8	10.8
Amino Plus ²	4.1	3.8	7.4
Blood meal	—	0.3	1.4
Corn gluten meal	0.5	0.4	0.8
Provaal Elite ³	2.4	2.0	2.3
Cane molasses	—	0.6	0.7
Urea	0.3	0.3	0.9
Megalac Plus	0.3	—	—
Limestone	1.2	1.1	1.2
Salt	0.4	0.4	0.4
Mineral and vitamin mix ⁴	0.3	0.2	0.3
Sodium bicarbonate	1.0	0.9	1.0
MgO	0.2	0.1	0.2
Megalac	0.5	—	—
Chemical composition, % of DM			
CP	16.0	18.1	23.3
Soluble CP	6.6	7.0	9.6
NDF	28.1	33.5	34.1
ADF	20.1	19.1	20.4
Crude fat	3.9	3.2	3.0
Ash	6.2	6.6	6.9
NE _L , Mcal/kg of DM	1.81	1.65	1.63

¹The low-energy diets were designed using the Cornell Net Carbohydrate and Protein System and performance data from the standardization period. The ingredient and chemical composition values do not include either the conjugated linoleic acid (Balchem Encapsulates, New Hampton, NY) or the Megalac (control; Church and Dwight Co., Princeton, NJ) supplements, which provided an additional 73.8 g/d of fat.

²Obtained from AgProcessing Inc., Omaha, NE.

³Provaal Elite is a selected blood meal-based animal protein blend combined with Met sources (produced by Venture Milling Co., Seaford, DE).

⁴Contained 19.5% Ca, 17.0% S, 1.25% Zn, 1.72% Mn, 0.44% Cu, 0.057% I, 0.019% Co, and 0.0012% Se. In addition, the vitamin content per gram of mix was 597 IU of vitamin A, 111 IU of vitamin D, and 2,896 IU of vitamin E.

TMR at the morning feeding. The CLA supplement contained the methyl ester of 2 CLA isomers, *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA; these comprised 16.4 and 16.3% of the total fat content in the CLA supplement. Other major fatty acids in the CLA supplement were palmitic, stearic, and oleic acid (8.8, 34.9, and 16.5% of the total fat content, respectively). The major fatty acids in the Megalac supplement were palmitic, stearic, oleic, and linoleic acids, which made up 43.9, 4.9, 34.9, and 8.9% of the total fatty acid content, respectively.

Cows were housed in individual tie stalls and were milked, and their yields were recorded daily at 0030,

0830, and 1630 h. Milk samples were taken at each milking from d 12 to 16 of each period and composited by day. One aliquot was stored at 4°C with a preservative (bronopol tablet; D&F Control Systems, San Ramon, CA) until analyzed for fat and true protein content (Method 972.160, AOAC, 2000; Dairy One Cooperative, Inc., Ithaca, NY) and SCC (Method 978.26, AOAC, 2000) by methods previously described (Bernal-Santos et al., 2003). Milk urea nitrogen was measured by automated infrared analysis (MilkoScan 4000; Foss Electric, Hillerød, Denmark; by Dairy One Cooperative, Inc.). A second aliquot of milk was composited by period and

stored at -20°C until analyzed for fatty acid composition.

All diets were prepared and fed once daily at 0900 h. Water was provided at all times from automatic water bowls. Feed samples were taken on d 12 to 16 and composited by period. Dry matter content was determined by drying at 54°C until constant weight and then analyzed by wet chemistry for CP, soluble protein, NDF, ADF, and crude fat (Dairy One Cooperative, Inc.).

Blood samples were taken from all cows on d 12 and 14 of each period to determine basal levels of glucose, NEFA, and insulin. Blood was obtained via coccygeal venipuncture and collected in Vacutainer tubes (Becton Dickinson, Franklin, NJ) containing sodium heparin (100 U/mL of blood). Plasma was harvested following centrifugation ($2,800 \times g$, 15 min at 4°C) and stored at -20°C until analyzed for blood metabolites and hormones.

Data from 2 cows were completely excluded from the analysis because 1 cow on the moderate MP treatment developed chronic mastitis during period 1 and the other cow on the excess MP + CLA treatment developed a gastrointestinal sickness and her performance did not recover. Additionally, 1 cow in each of the moderate MP + CLA and excess MP + CLA treatments developed chronic mastitis during period 2 and their data were not included for that period.

Metabolic Challenges

After the 0830 h milking on d 13 of each experimental period, 12 randomly selected cows (3 per treatment) were fitted with an indwelling jugular catheter. On d 15 and 16, all cows were subjected to an epinephrine challenge and an insulin challenge. Six cows received an insulin challenge 1 h after the morning milking on d 15 and an epinephrine challenge 1 h after the afternoon milking on d 16. The remaining 6 cows received an insulin challenge and an epinephrine challenge at the same times on d 16 and 15, respectively. Epinephrine HCl (1 mg/mL; International Medical Systems, Ltd., So. El Monte, CA) was diluted 50:50 with sterile saline. Bovine insulin (26.6 U/mg, lot no. 615-70N-80; Eli Lilly and Company, Indianapolis, IN) was initially dissolved to 2 mg/mL in 0.005 M HCl and then dissolved to 0.2 mg/mL in sterile saline. Epinephrine (1.4 $\mu\text{g}/\text{kg}$ of BW) and insulin (1.0 $\mu\text{g}/\text{kg}$ of BW) challenges were administered via the jugular catheter and were followed with 10 mL of sterile saline. Blood samples were collected at -45, -40, -30, -20, -10, -5, 0, 2.5, 5, 7.5, 10, 15, 20, 30, 45, 60, 120, 125, and 130 min relative to the challenge. Blood (10 mL) was sampled via the jugular catheter and collected into sodium heparinized tubes

(100 IU/mL). Plasma was harvested and stored as described for basal blood samples.

Fatty Acid Analysis

The fat content of Megalac and lipid-encapsulated CLA was determined using acid hydrolysis (Method 954.02; AOAC, 2000) and ether extraction (Foss Tecator, Application Subnote AN 3414; Foss), respectively (Dairy One Cooperative, Inc.). Milk fatty acids were extracted using the method of Hara and Radin (1978). Fatty acid methyl esters from the lipids extracted from the CLA supplement and milk fatty acids were prepared by base-catalyzed transmethylation as detailed by Perfield et al. (2002). Methyl esters were prepared for the FFA from Megalac using 1% methanolic sulfuric acid as described by Christie (1989).

The fatty acid methyl esters were quantified using a gas chromatograph (GCD system HP 6890+; Hewlett-Packard, Avondale, PA) equipped with a CP-SIL 88 fused-silica capillary column [100 m $\times$ 0.25 mm (i.d.) with 0.2- μm film thickness; Varian, Inc. Walnut Creek, CA] and a flame-ionization detector with hydrogen as the carrier gas. Gas chromatographic conditions for separation of the fatty acid methyl esters were as described by Perfield et al. (2002). Fatty acid peaks were identified using pure methyl ester standards (Nu-Chek Prep, Elysian, MN). A butter oil reference standard (CRM 164; Commission of the European Community Bureau of References, Brussels, Belgium) was also analyzed periodically to control for column performance and the calculation of recoveries and correction factors for individual fatty acids.

Metabolite and Hormone Analysis

Plasma concentrations of NEFA were determined by enzymatic colorimetric analysis (NEFA-C kit; Wako Chemicals, Richmond, VA) as previously described (Sechen et al., 1990). Plasma glucose was determined by enzymatic analysis (Procedure No. 510-A; Sigma Diagnostics, St. Louis, MO). Plasma concentrations of insulin were quantified by double-antibody RIA using bovine insulin (lot no. 615-70N-80; Eli Lilly and Company) for iodination and standards as previously described by McGuire et al. (1995).

Statistical Analysis

Milk production values were reduced to means of d 12 to 16 of each period. Basal plasma concentrations of glucose, insulin, and NEFA were analyzed as mean values of d 12 and 14. Nonesterified fatty acid and glucose responses to the epinephrine and insulin chal-

lenges were calculated as the area under the curve using PROC EXPAND command (SAS Institute, 2001) with the trapezoid rule method. To minimize the contribution of clearance and counter-regulatory effects, the response areas of both plasma NEFA and glucose were calculated from the time of epinephrine and insulin challenge to 30 min postchallenge. Response areas were corrected for differences in baseline concentration (mean of concentrations at -30, -20, -10, -5, 0, 45, 60, 120, 125, and 130 min from the time of the respective challenge). The rate of glucose clearance in response to insulin was determined using glucose concentrations over the initial decline phase of the response (0 to 20 min postchallenge). This was expressed as the fractional rate of change as determined from the slope of the natural logarithm of glucose concentration vs. time (Shipley and Clark, 1972).

All experimental data were analyzed as a 2×2 factorial, 2-period crossover design using PROC MIXED procedure of (SAS Institute, 2001). The statistical model used was

$$Y_{ijkl} = \mu + \theta_i + \alpha_j + \beta_{jk} + \gamma_l + \tau_g + \rho_h + (\tau\rho)_{gh} + \varepsilon_{ijkl}$$

where Y_{ijkl} is observations for dependent variables; μ is the overall mean; θ_i is the effect of the i th block; α_j is the effect of the j th treatment sequence; β_{jk} is the effect of the k th cow on the j th treatment sequence; γ_l is the effect of the l th period; τ_g is the effect of the g th protein level; ρ_h is the effect of the h th CLA level; $(\tau\rho)_{gh}$ is the effect of the interaction between the g th protein level and the h th CLA level; and ε_{ijkl} is the random error. Cow was considered a random effect and the remaining factors were considered fixed effects. All data are presented as least squares means with accompanying standard error. Orthogonal contrasts were conducted to determine treatment effects by comparing 1) no CLA vs. CLA treatment (combined protein levels), 2) moderate protein vs. excess protein (combined CLA levels), and 3) the interaction between CLA level and protein level.

RESULTS

The DMI, milk yield, and content of milk fat and milk protein over the last 2 d of the standardization period was 22.8 ± 0.9 kg/d, 44.1 ± 1.7 kg/d, $3.16 \pm 0.08\%$ and $2.87 \pm 0.05\%$ (mean $\pm$ SE), respectively. Based on the cow's performance during the standardization period, the CNCPS model was used on an individual cow basis to estimate the feed allowance that would supply, on average, 80% of the ME required for lactation, and this was the amount fed during the treatment periods. Cows received 1 of 2 diets that supplied either moderate or

excess levels of MP, with the CNCPS model predicting that the diets would supply, on average, 88 and 117%, respectively, of the MP required for the standardization period production. By design, the DM allowance during the treatment periods for each individual cow was lower than its DMI during the standardization period, and as a result, there were noorts during the treatment periods and the desired intake to obtain 80% ME supply was achieved. The DMI of the cows on the moderate and excess protein diets were 19.8 ± 0.7 and 20.9 ± 0.7 kg/d, respectively.

During the treatment periods, CLA supplementation reduced the yield and content of milk fat by 21 and 23%, respectively (Table 2). Milk yield and the yield of protein and lactose were increased with CLA supplementation, although the increases were small (2.6, 2.8, and 2.4%, respectively). Conjugated linoleic acid treatment had no direct effect on the milk content of protein and lactose, MUN, or SCC. Milk yield, SCC, and the yield and content of milk fat, protein, and lactose were not altered by the dietary protein level. Milk urea nitrogen was increased when cows received the high-protein diet, and there was a CLA by protein interaction for both MUN and milk lactose content.

The CLA supplement resulted in a shift in the pattern of fatty acids in milk fat, although the changes were minor (Table 3). Overall, CLA supplementation reduced the proportion of short- and medium-chain fatty acids (4 to 14 carbons) and increased the proportion of long-chain fatty acids (>16 carbons). The two main CLA isomers in the CLA supplement were *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA, and their proportion in milk fat increased slightly when cows received the CLA supplement. Although 3 of the pairs of fatty acids that serve as a proxy for Δ^9 -desaturase activity were altered by the CLA treatments, the changes were minimal (Table 3). Protein level increased the proportion of long-chain fatty acids in milk fat; however, this increase was not observed for all long-chain fatty acids.

Milk fatty acids can be grouped based on their origin as either de novo synthesized fatty acids (<16 carbons), preformed fatty acids taken up from circulation (>16 carbons), or fatty acids derived from both sources (16-carbon fatty acids). Conjugated linoleic acid treatments reduced the yield, on a molar basis, of each of these 3 groups, with the extent of the reduction relatively similar for milk fatty acids derived from the 2 sources (24 and 17% for $<C_{16}$ and $>C_{16}$, respectively; Figure 1). Protein supply did not alter the yield of milk fatty acids when grouped based on their source (data not presented). The yield of all individual milk fatty acids was reduced by CLA supplementation, except for *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA (Table 4). Milk fat secretion of the *cis*-9, *trans*-11 CLA was maintained,

Table 2. Milk variable results of cows at different levels of nutrient intake and conjugated linoleic acid (CLA) supplementation¹

Milk variable	Low ME ²				SEM	<i>P</i>		
	Moderate MP		Excess MP			CLA ³	Protein ⁴	CLA × protein ⁵
	Control	CLA	Control	CLA				
Milk yield, kg	36.7	37.9	37.0	37.8	0.6	0.03	0.92	0.61
Milk fat								
%	3.50	2.73	3.60	2.72	0.08	<0.001	0.66	0.27
g/d	1,273	1,024	1,331	1,039	37	<0.001	0.45	0.28
Milk protein								
%	2.88	2.88	2.84	2.86	0.04	0.50	0.53	0.57
g/d	1,053	1,080	1,047	1,080	20	0.01	0.93	0.79
Milk lactose								
%	4.75	4.76	4.85	4.82	0.03	0.09	0.07	0.03
g/d	1,742	1,800	1,790	1,817	30	0.05	0.37	0.46
MUN, mg/dL	12.6	13.4	19.5	19.2	0.3	0.34	<0.001	0.05
SCC, × 1,000	58	47	57	104	23	0.24	0.31	0.07

¹Cows received a lipid-encapsulated CLA supplement that supplied 12 g/d of *trans*-10, *cis*-12 CLA (+CLA treatment) or an isoenergetic equivalent supplement of Megalac (control). Results represent mean values from d 12 to 16 of each treatment period.

²Low ME and different MP treatments (moderate and excess) were established using the Cornell Net Carbohydrate and Protein System and performance data from the standardization period.

³Main effect of CLA.

⁴Main effect of protein.

⁵Interaction of CLA × protein.

whereas *trans*-10, *cis*-12 CLA was increased by CLA supplementation. The apparent transfer efficiency of *trans*-10, *cis*-12 CLA into milk was calculated by comparing the amount of the isomer in the dietary supplement with incremental changes in the amount secreted in milk fat. The transfer efficiency was similar at both protein levels ($P = 0.09$), and averaged 2.4 and 2.7% at the moderate and excess protein levels, respectively ($SE = 0.09$).

Basal plasma concentrations of glucose, insulin, and NEFA were unaffected by CLA treatment (Table 5). In contrast, as the protein supply was increased, both basal glucose and NEFA plasma concentrations were increased to a small extent, whereas basal insulin concentrations were lower. Both the glucose response area to an insulin challenge and the fractional rate of decrease in plasma glucose were unaltered by either the level of protein supply or the CLA treatment (Figure 2). There were also no differences in lipolytic response to the epinephrine challenge related to either CLA treatment or protein supply (Table 5).

DISCUSSION

The major objective of the present study was to investigate the effect of CLA supplementation on production responses of lactating dairy cows when nutrient status was characteristic of the early-lactation cow (inadequate energy and moderate protein) and the pasture-fed dairy cow (inadequate energy and excess protein).

Some earlier studies of CLA supplementation in early lactation and pasture-fed cows demonstrated the expected reduction in milk fat yield, but increases in milk and milk protein yield also were observed (Bernal-Santos et al., 2003; Mackle et al., 2003; de Veth et al., 2005b). However, in early lactation increases in milk production have not always been observed (e.g., Moore et al., 2004; Castañeda-Gutiérrez et al., 2005). Castañeda-Gutiérrez et al. (2005) supplied the same amount of CLA as that supplemented in the study by Bernal-Santos et al. (2003), but a comparison of plasma NEFA levels and ME balance predicted by the CNCPS for the control groups in these studies suggested that cows in the latter study were in a more negative energy balance.

Assessing the importance of nutrient status in early lactation is difficult because energy balance is dynamic during this period and is largely dependent on the progressive increase in milk yield. In contrast, the period of lactation immediately following peak milk production is characterized by a relatively constant energy balance because of minimal changes in milk production and DMI. Therefore, the present study used mid-lactation cows and used the CNCPS model to devise diets and feed allowances that would supply 80% of ME requirements for each individual cow. The set level of energy restriction was similar to reported energy intake limitations observed in early lactation (~25%; Bell, 1995) and for grazing dairy cows (~22%; Kolver and Muller, 1998).

Table 3. Composition of milk fat of cows at different levels of nutrient intake and conjugated linoleic acid (CLA) supplementation¹

Fatty acid, %	Low ME ²				SEM	<i>P</i>		
	Moderate MP		Excess MP			CLA ³	Protein ⁴	CLA × protein ⁵
	Control	CLA	Control	CLA				
4:0	3.80	3.71	3.50	3.55	0.09	<0.001	0.89	0.28
6:0	1.85	1.57	1.84	1.60	0.04	<0.001	0.88	0.40
8:0	0.97	0.81	0.97	0.83	0.02	<0.001	0.70	0.55
10:0	2.11	1.82	2.10	1.86	0.05	<0.001	0.76	0.54
12:0	2.38	2.22	2.35	2.24	0.05	0.001	0.97	0.46
14:0	9.24	9.26	8.85	8.93	0.15	0.69	0.06	0.79
14:1	0.90	1.03	0.85	0.90	0.04	0.003	0.05	0.25
15:0	0.71	0.74	0.73	0.74	0.24	0.33	0.74	0.63
16:0	29.07	27.43	27.73	25.72	0.36	<0.001	<0.001	0.49
16:1	2.02	2.17	1.96	1.94	0.08	0.15	0.13	0.08
17:0	0.54	0.54	0.56	0.57	<0.01	0.08	0.005	0.41
18:0	7.96	8.16	8.42	8.82	0.17	0.07	0.003	0.53
18:1								
<i>cis</i> -9	28.70	30.15	29.87	31.24	0.49	<0.001	0.07	0.90
<i>trans</i> -6 to 8	0.29	0.32	0.26	0.30	<0.01	<0.001	0.01	0.39
<i>trans</i> -9	0.25	0.27	0.24	0.26	<0.01	<0.001	0.11	0.43
<i>trans</i> -10	0.48	0.53	0.38	0.44	0.02	<0.001	0.001	0.85
<i>trans</i> -11	1.21	1.40	1.27	1.53	0.04	<0.001	0.03	0.29
<i>trans</i> -12	0.43	0.45	0.37	0.40	0.01	0.01	<0.001	0.39
18:2								
<i>cis</i> -9, <i>cis</i> -12	2.88	3.02	3.18	3.40	0.06	<0.001	<0.001	0.005
<i>cis</i> -9, <i>trans</i> -11	0.60	0.74	0.62	0.79	0.02	<0.001	0.15	0.31
<i>trans</i> -10, <i>cis</i> -12	<0.01	0.03	<0.01	0.04	<0.01	<0.001	0.32	0.10
18:3	0.40	0.42	0.44	0.50	<0.01	<0.001	<0.001	<0.001
20:0	0.07	0.07	0.07	0.08	<0.01	0.66	<0.001	0.14
Other	3.14	3.28	3.23	3.24	0.07	0.29	0.71	0.31
Summation								
<16 carbons	21.96	20.94	21.40	20.65	0.32	<0.001	0.25	0.55
16 carbons	31.08	29.59	29.69	27.66	0.38	<0.001	<0.001	0.37
>16 carbons	43.81	46.16	45.69	48.44	0.58	<0.001	0.003	0.64
Desaturase ratio ⁶								
14:1/14:0	0.10	0.11	0.10	0.10	<0.01	0.001	0.19	0.13
16:1/16:0	0.07	0.08	0.07	0.08	<0.01	<0.001	0.69	0.08
18:1/18:0	3.64	3.78	3.58	3.61	0.09	0.25	0.24	0.45
<i>cis</i> -9, <i>trans</i> -11 18:2/ <i>trans</i> -11 18:1	0.50	0.54	0.49	0.52	0.01	<0.001	0.41	0.83

¹Cows received a lipid-encapsulated CLA supplement that supplied 12 g/d of *trans*-10, *cis*-12 CLA (+CLA treatment) or an isoenergetic equivalent supplement of Megalac (control). Results represent mean values from d 12 to 16 of each treatment period.

²Low ME and different MP treatments (moderate and excess) were established using the Cornell Net Carbohydrate and Protein System and performance data from the standardization period.

³Main effect of CLA.

⁴Main effect of protein.

⁵Interaction of CLA × protein.

⁶Ratio of fatty acid pairs that represent product:substrate for Δ^9 -desaturase.

The CNCPS model was used because it allows more precise control of the restriction in ME supply, which is achievable only through modeling the dynamics of rumen fermentation. The restriction in ME supply was successfully met through reducing the feed allocation on an individual cow basis, and there were no refusals throughout the treatment periods. The reduction in milk yield in the treatment periods from that of the standardization period averaged 16% across the 2 protein levels (SE = 1.0%), which compared closely to our

target goal of imposing a 20% reduction in ME supply for milk production.

The reduction in milk fat yield and content in the present study (21 and 23%, respectively) is similar to that observed by Perfield et al. (2004) when a lipid-encapsulated CLA supplement containing 10 g/d of *trans*-10, *cis*-12 CLA was administered intraruminally to cows in established lactation. In contrast, when CLA was supplemented in early lactation, the reduction in milk fat synthesis was minimal until a few weeks post-

Figure 1. Secretion of milk fatty acids classified according to origin. All animals received an energy-limited diet that supplied 80% of ME requirements on an individual cow basis. The treatment period had 2 factors at 2 levels, MP supply at either moderate or excess levels (88 and 117% of requirements, respectively) and supplements of either conjugated linoleic acid (CLA) or an isoenergetic equivalent amount of Megalac (control). Experimental periods were for 16 d and values represent least squares means averaged over d 12 to 16 (SEM for fatty acid yield ranged from 43 to 67 mmol/d). Different letters above the bars within each grouping of fatty acids indicate treatment differences ($P < 0.05$).

partum; thus, the extent of MFD observed was less than at comparable doses of *trans*-10, *cis*-12 CLA in established lactation (Bernal-Santos et al., 2003; Moore et al., 2004; Castañeda-Gutiérrez et al., 2005).

In the present study, cows whose diets were supplemented with CLA in combination with a restricted energy intake had increased milk and milk protein yields. These results indicate that when milk energy is spared through CLA-induced MFD, energy could be repartitioned to the synthesis of milk protein and lactose. However, the increase of ~3% in milk yield was much smaller than that observed previously during early lactation (average 8%; Bernal-Santos et al., 2003; de Veth et al., 2005b) or with pasture feeding (11%; Mackle et al., 2003). A difference in physiological state may form a basis for the greater milk yield response in the early studies. Early lactation is characterized by a number of dramatic metabolic adaptations that occur in the mammary and nonmammary tissues of the lactating dairy cow, and many are coordinated via attenuation in the sensitivity of the liver, adipose tissue, and skeletal muscle to insulin (Bauman, 2000). These adaptations persist through the early lactation period but diminish as the cow's energy intake begins to approach her energetic requirements for lactation. Because the cows in the present study were beyond their peak in milk production, the metabolic adaptations that occurred in early lactation would be attenuated. Therefore, the cur-

rent study design may more accurately simulate the grazing cow that confronts periods of limited energy supply because of decreases in pasture availability, pasture digestibility, or both by reducing milk yield rather than attempting to meet inherent productive capacity by mobilizing NEFA from adipose tissue, as does the early-lactation cow. Although in such grazing situations the metabolic adaptations differ from early lactation, we anticipate that cows in the present study would have the potential to respond to spared milk fat energy. Kolver and Muller (1998) observed that dairy cows grazing pasture were first limited by energy supply, and when they were switched to a higher energy-dense TMR diet, they were able to increase their milk yield by almost 30% within 2 wk.

The small milk response in the present study may relate to the degree of the energy limitation that was imposed, with a more negative ME balance in early lactation resulting in a greater milk yield response, as suggested by Castañeda-Gutiérrez et al. (2005). For pasture-fed dairy cows, the severity of the limitation in energy from that required to meet their potential milk production can be even greater (36%) than that typically seen in early lactation (Kolver and Muller, 1998). Therefore, the greater milk yield response with CLA supplementation observed by Mackle et al. (2003) may be a result of cows being better able to respond to the energy spared by the CLA-induced MFD.

Table 4. Secretion of fatty acids in milk fat of cows at different levels of nutrient intake and conjugated linoleic acid (CLA) supplementation¹

Fatty acid, g/d	Low ME ²				SEM	<i>P</i>		
	Moderate MP		Excess MP			CLA ³	Protein ⁴	CLA × protein ⁵
	Control	CLA	Control	CLA				
4:0	37.6	27.9	38.1	28.6	1.2	<0.001	0.63	0.91
6:0	19.3	13.3	20.1	13.9	0.7	<0.001	0.43	0.73
8:0	10.4	7.1	11.1	7.6	0.4	<0.001	0.33	0.65
10:0	23.2	16.3	24.5	17.3	1.0	<0.001	0.35	0.76
12:0	26.5	20.0	27.6	20.9	1.0	<0.001	0.47	0.85
14:0	103.9	84.1	103.8	82.2	3.1	<0.001	0.81	0.60
14:1	10.1	9.2	9.7	8.2	0.5	<0.001	0.23	0.12
15:0	7.9	6.8	8.5	6.8	0.3	<0.001	0.41	0.22
16:0	328.1	249.9	326.4	238.3	9.5	<0.001	0.60	0.26
16:1	22.7	19.5	22.9	17.9	1.0	<0.001	0.59	0.04
17:0	6.1	4.9	6.6	5.2	0.2	<0.001	0.11	0.46
18:0	90.8	75.0	101.0	81.8	3.9	<0.001	0.10	0.46
18:1								
<i>cis</i> -9	324.1	273.1	353.0	286.7	11.7	<0.001	0.15	0.31
<i>trans</i> -6 to 8	3.2	2.9	3.1	2.8	0.1	<0.001	0.15	0.68
<i>trans</i> -9	2.9	2.4	2.8	2.4	0.1	<0.001	0.83	0.77
<i>trans</i> -10	5.4	4.8	4.4	4.0	0.3	<0.001	0.01	0.69
<i>trans</i> -11	13.8	12.7	14.9	13.8	0.5	0.001	0.07	0.88
<i>trans</i> -12	4.9	4.1	4.4	3.7	0.1	<0.001	0.01	0.43
18:2								
<i>cis</i> -9, <i>cis</i> -12	32.7	27.5	37.7	32.0	1.0	<0.001	<0.001	0.71
<i>cis</i> -9, <i>trans</i> -11	6.7	6.7	7.2	7.1	0.2	0.75	0.11	0.81
<i>trans</i> -10, <i>cis</i> -12	<0.1	0.3	<0.1	0.3	<0.1	<0.001	0.73	0.19
18:3	4.5	3.8	5.2	4.6	0.1	<0.001	<0.001	0.78
20:0	0.8	0.6	0.9	0.7	<0.1	<0.001	0.04	0.91
Other	35.6	30.3	38.0	29.9	1.1	<0.001	0.45	0.09

¹Cows received a lipid-encapsulated CLA supplement that supplied 12 g/d of *trans*-10, *cis*-12 CLA (+CLA treatment) or an isoenergetic equivalent supplement of Megalac (control). Results represent mean values from d 12 to 16 of each treatment period.

²Low ME and different MP treatments (moderate and excess) were established using the Cornell Net Carbohydrate and Protein System and performance data from the standardization period.

³Main effect of CLA.

⁴Main effect of protein.

⁵Interaction of CLA × protein.

In addition to increases in milk yield with CLA supplementation, studies with pasture-fed cows have shown increases in milk protein yield (Mackle et al., 2003; Kay et al., 2006). Because of the high CP content in high-quality pasture, grazing cows are typically consuming protein well in excess of their energy requirements for milk synthesis. The presence of a milk protein response with pasture suggests that the level of protein may be important when determining the production response with CLA supplementation. The present study evaluated the effect of 2 levels of MP supply as predicted by the CNCPS model. Diets supplied either moderate amounts of MP for milk production (88% of MP requirements) to replicate cows in early lactation or excess amounts of MP (117% of MP requirements) to resemble a pasture-based system. Results indicated that changes in the level of MP supply had no effect on any of the production variables in CLA-supplemented cows.

To evaluate the relationship between protein quantity and cow performance during early and mid-lactation, Nocek and Russell (1988) combined data from 21 studies and found no correlation for either milk yield or protein yield with content of CP, soluble protein, or RDP in the diet. They did note that in instances in which protein influenced milk yield and milk protein content, this was largely driven by increased DMI. In the present study cows on the excess MP treatment were not able to adjust their DMI because their daily feed allowance was set throughout the treatment period. The absence of an effect of CLA in combination with excess protein indicates that simply increasing the quantity of protein available for absorption is insufficient to elicit a milk protein response with CLA supplementation. The milk protein response that has been seen with CLA supplementation on pasture may relate to the fact that the quality of protein supplied can differ

Table 5. Changes in plasma variables of cows at different levels of nutrient intake and conjugated linoleic acid (CLA) supplementation¹

Plasma variable	Low ME				SEM	<i>P</i>		
	Moderate MP		Excess MP			CLA ²	Protein ³	CLA × protein ⁴
	Control	CLA	Control	CLA				
Basal ⁵								
Glucose, mg/dL	55.8	55.4	58.8	59.2	1.1	0.99	0.02	0.63
Insulin, ng/mL	0.82	0.80	0.60	0.61	0.06	0.95	0.005	0.73
NEFA, μ <i>M</i>	102	117	157	137	9	0.73	<0.001	0.03
Insulin challenge ⁶								
Glucose response area, mg/dL·min	−213	−197	−169	−182	24	0.95	0.25	0.43
Glucose FRC, min × 10 ^{−3}	−9.9	−9.1	−8.0	−8.4	1.0	0.86	0.24	0.53
Epinephrine challenge ⁷								
NEFA response area, μ <i>M</i> ·min	3,406	2,246	4,692	4,554	1,024	0.29	0.13	0.40

¹Cows received a lipid-encapsulated CLA supplement that supplied 12 g/d of *trans*-10, *cis*-12 CLA (+CLA treatment) or an isoenergetic equivalent supplement of Megalac (control). Low ME and different MP treatments (moderate and excess) were established using the Cornell Net Carbohydrate and Protein System and performance data from the standardization period.

²Main effect of CLA.

³Main effect of protein.

⁴Effect of CLA × protein.

⁵Average concentrations determined from samples taken from all cows on d 12 and 14 of each treatment period.

⁶Glucose response to the insulin challenge (1.0 μ g/kg of BW) in cows on d 15 and 16 of each treatment period (n = 24). Response area values represent the change in plasma glucose concentrations over the interval of 0 to 30 min following insulin administration. Values for the fractional rate of clearance (FRC) of glucose concentrations for samples 0 to 20 min after the insulin challenge; FRC values represent the slope of natural logarithms of glucose concentrations (mg/dL).

⁷Lipolytic response to the epinephrine challenge (1.4 μ g/kg of BW) in cows on d 15 and 16 of each treatment period (n = 24). Values represent area for the change in plasma NEFA concentrations over the interval of 0 to 30 min following epinephrine administration.

from TMR-based diets in the supply of individual amino acids for absorption by the small intestine (Kolver et al., 1999).

Figure 2. Glucose clearance in response to the insulin challenge (1.0 μ g/kg of BW). All animals received an energy-limited diet that supplied 80% of ME requirements. Cows received a supplement of either conjugated linoleic acid (CLA) or an isoenergetic equivalent amount of Megalac (control). Fractional rates of glucose clearance are based on the decline in plasma glucose (log basis) from insulin challenges administered on d 15 and 16 of the treatment period. The 2 MP levels were combined and values represent means (n = 24); SEM for the log of glucose values averaged 0.03 log mg/dL.

Milk urea nitrogen is closely associated with changes in dietary CP content (Nousiainen et al., 2004), and this was observed in the present study where MUN concentrations were higher for cows on the excess MP treatment (Table 2). Nousiainen et al. (2004) compiled data from 50 studies involving 305 treatment means and developed an equation to predict MUN from the content of CP in the diet. With their equation, the predicted proportional increase in MUN (~50%) was similar to that observed in the present study based on the difference in dietary CP content between the 2 diets. Moreover, the predicted concentrations of MUN for the moderate and excess protein diets were higher than we observed (16.6 vs. 13.0 mg/dL and 25.4 vs. 19.3 mg/dL, respectively), which is likely related to the high RDP in the pasture-based diets that are part of the database.

The similarity in the magnitude of decline in the yield of de novo synthesized fatty acids and preformed fatty acids in response to CLA supplementation, as well as the minimal changes in Δ^9 -desaturase activity, are consistent with results from previous studies that abomasally infused low doses of *trans*-10, *cis*-12 CLA (de Veth et al., 2004) or fed rumen-protected CLA supplements (Castañeda-Gutiérrez et al., 2005; de Veth et al., 2005a). The milk fat output of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA in CLA-supplemented cows is consistent with the direct transfer of these isomers from the dietary supplement. The transfer of *trans*-10, *cis*-12 CLA from the dietary supplement into milk fat was lower

(average 2.6%; SE = 0.09) than was found in the only previously published study (7.9% transfer) in which a lipid-encapsulated CLA formulation was supplemented for 7 d (Perfield et al., 2004). A primary factor that may have contributed to the difference in transfer efficiency is the reduction in the proportion of protective lipid coating in the formulation used in the present study.

In the present study the absence of an effect of CLA treatment on basal levels of glucose, insulin, and NEFA is consistent with previous studies with cows fed a TMR both in early and established lactation (Perfield et al., 2002; Bernal-Santos et al., 2003; Castañeda-Gutiérrez et al., 2005). In addition, Baumgard et al. (2002) found no difference in the plasma glucose response to an insulin challenge and only a minimal decrease in the adipose tissue response to an epinephrine challenge when cows in established lactation were administered abomasally infused CLA for 5 d. Although they concluded that CLA effects are specific to the mammary gland, whole-body glucose homeostasis and adipose tissue responsiveness to homeostatic signals regulating lipolysis had not been evaluated in a situation in which CLA supplementation resulted in a milk production response. In the current study, although CLA treatment resulted in a small increase in milk synthesis, there was no effect on the plasma glucose response to an insulin challenge or the NEFA response to an epinephrine challenge. These results suggest that the effects of CLA on milk production were predominantly due to coordinated adaptations by the mammary gland.

Basal concentrations of glucose and NEFA in plasma were slightly elevated, whereas plasma insulin was reduced with the excess MP treatment. Although the entry rate of glucose was not determined, the increase in plasma glucose may be related to the role of amino acids as precursors of gluconeogenesis in ruminants, of which their contribution is largely dependent on supply (Drackley et al., 2001). Although changes in plasma insulin and NEFA concentrations were observed with changing levels of MP supply, these changes were small and are expected to be of little biological significance.

Assuming that the effect of CLA was specific to the mammary gland, the observed milk yield response is likely to have resulted from repartitioning of the spared milk fat energy in a manner allowing for increased milk protein and lactose synthesis. Using the milk composition in the present study and the NRC equation for NE_L (NRC, 2001), we estimated that the milk energy output of those cows receiving CLA was 2.2 Mcal/d lower than that of cows not receiving CLA (25.1 and 22.9 Mcal/d, respectively). If all of this difference in milk energy output were repartitioned to milk synthesis, an additional 3.7 kg of milk could have been produced. Therefore, the increase in milk yield observed in the

cows receiving CLA supplements would represent less than one-third of the spared milk fat energy. The treatment periods in the present study would be expected to be too short to detect changes in BW or BCS. However, Shingfield et al. (2004) examined the partitioning of gross energy in early-lactation dairy cows by using excreta collection and respiratory calorimetry over the first 15 wk of lactation. In their study, supplementation of CLA had no impact on estimated heat energy, energy excreted in methane, or energy excreted in feces. As a result of a 35% reduction in milk fat yield, the partitioning of energy toward milk energy decreased and the proportion of gross energy retained in body tissues was increased, indicating that the remaining spared energy with CLA-induced MFD may be partitioned toward body tissues and improving overall energy status.

CONCLUSIONS

The present study demonstrated that dietary CLA supplementation in cows that had a restricted energy intake resulted in the expected reduction in milk fat yield, but also that the increased yields of milk and milk protein were energetically equivalent to about one-third of the reduction in milk fat. This shift in milk synthesis indicates that in some situations in which energy is spared through CLA-induced reductions in milk fat, energy may be repartitioned to allow for increased synthesis of milk protein and lactose. When cows were fed an energy-limiting diet that contained excess protein, no additional milk response was observed with CLA supplementation above that observed when protein in the diet was at a moderate level. The repartitioning of energy toward milk synthesis with CLA supplementation appeared to predominantly involve coordinated adaptations by the mammary gland because peripheral tissue responses to homeostatic signals regulating lipolysis and glucose uptake were unchanged. The increases in milk yield and milk protein were small, and additional studies are required to establish to what extent milk synthesis may be increased when energy status is limiting in early lactation.

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