Protein and energy intakes affected amino acid concentrations in plasma, muscle, and liver, and cell signaling in the liver of growing dairy calves

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ABSTRACT

The nutrient content of and feeding recommendations for milk replacers (MR) vary widely in North America, and acceleration of growth through manipulation of protein and energy intakes can reduce rearing costs of dairy operations. The effects of varying the protein and energy intake of MR on metabolite concentrations in plasma, liver, and muscle and the phosphorylation activity of protein kinase B (AKT) and ribosomal protein S6 (rpS6) cell signals in liver and muscle were assessed. Twenty-four newborn Holstein calves were fed 1 of 4 MR for 9 wk ($n = 6/\text{treatment}$): (1) a 20% crude protein (CP), 20% fat MR fed at 441 g of dry matter (DM)/d (CON); (2) a high-protein, medium-fat MR (HPMF; 28% CP, 20% fat) fed at 951 g of DM/d; (3) a high-protein, high-fat MR (HPHF; 27% CP, 28% fat) fed at 951 g of DM/d; and (4) HPHF fed at 1,431 g of DM/d (HPHF+). Water and starter (20% CP, 1.43% fat) were offered ad libitum and calves were fed MR twice daily. Plasma samples were obtained at 1, 5, and 9 wk of age. Calves were not weaned and were slaughtered after the last blood sampling. Liver and muscle tissues were collected and analyzed for metabolite concentrations and cell signaling activity. Calves fed all treatments had lower plasma concentrations of Phe and Tyr, and a trend for lower Leu, but greater concentrations of Thr relative to calves fed CON. Calves fed all treatments had increased muscle concentrations of Met and muscle to plasma ratios of Phe, Tyr, and branched-chain amino acids compared with CON. All treatments increased liver to plasma ratios of Phe and Tyr but diminished the ratios of Met compared with CON. Phosphorylation of protein kinase B was not affected by treatment; however, relative to calves fed HPHF, HPMF and HPHF+ diets increased phosphorylation ratios of ribosomal protein S6 in the liver. Therefore, the changes in plasma and tissue concentrations and

plasma to tissue ratios of amino acids were associated with enhanced growth rates. However, cell signaling activity was not consistent with accelerated growth in calves fed treatments with increased contents of energy and protein possibly due to confounding effects of diet (MR + starter) or fasting before tissue harvesting. Muscle concentrations of Met might have a regulatory role in protein synthesis in rapidly growing calves fed high levels of CP and energy.

Key words: milk replacer, ribosomal protein S6 (rpS6), protein kinase B (AKT), calf

INTRODUCTION

Several studies have been conducted to elucidate the effects of feeding varying amounts of energy and protein in milk replacer (MR) on lean growth and ADG in dairy calves (Bartlett et al., 2006; Hill et al., 2009, 2010). Nutritional guidelines have been proposed to reduce age at first calving, maximize lean growth, and maximize future milk production (NRC, 2001). Managing rearing to accelerate lean growth hastened the onset of puberty, reduced age at first calving, and tended to increase milk production during the first lactation (Rius and Dahl, 2006). Modifying the fat and protein content of MR and increasing total MR intake are potential approaches to accelerate growth in dairy calves (Blome et al., 2003; Bartlett et al., 2006; Hill et al., 2010). However, the effect of composition and intake of MR on concentrations of AA and energy-yielding metabolites in blood and tissues and signaling factors involved in the regulation of protein synthesis have not been described.

Amino acid and energy intake promoted protein synthesis in growing piglets (Davis et al., 1996), and the molecular mechanisms mediating these nutrient responses include the effects of AA and hormones (e.g., insulin, IGF-I) on phosphorylation of protein initiation factors in the mammalian target of rapamycin (mTOR) signaling pathway (Davis et al., 1996; O'Connor et al., 2003; Escobar et al., 2006). Leucine and insulin enhance mTOR signaling activity, which leads to increased

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rates of protein synthesis and growth in cultured cells (O'Connor et al., 2003). Infusion of a total AA mixture increased phosphorylation of the mTOR signaling factors in muscle and liver of growing piglets (Escobar et al., 2006). Although these mechanisms have been well explored in other species and cell culture systems (O'Connor et al., 2003), they have not been explored in growing cattle. We hypothesized that increased nutrient intake associated with feeding MR with varying compositions of fat and protein and at varying amounts would increase blood and tissue AA concentrations and stimulate mTOR signaling in the liver and muscle. The objectives of this experiment were to evaluate the effect of MR composition and intake on AA and metabolite concentrations and cell signaling activity in muscle and liver of growing dairy calves.

MATERIALS AND METHODS

Animals, Diets, and Feeding

All animal research procedures and protocols were approved by the Virginia Tech Animal Care and Use Committee. Animal management, housing, feeding, and treatments are described in Daniels et al. (2008) and Hill et al. (2008). Three groups of 8 calves were purchased from a commercial dairy farm as described in Daniels et al. (2008). Briefly, 24 Holstein heifer calves (starting age = 4 ± 1 d; starting BW = 40.4 ± 2.2 kg) were subjected to 1 of 4 MR treatments until slaughter ($n = 6$ per treatment). Treatments were a control MR that contained 20% CP and 21% fat fed at 441 g of DM/d (**CON**); a high-protein, medium-fat MR (**HPMF**) containing 28% CP and 20% fat fed at 951 g of DM/d; a high-protein, high-fat MR (**HPHF**) containing 27% CP and 28% fat fed at 951 g of DM/d; and the HPHF MR fed at 1,431 g of DM/d (**HPHF+**). Land O'Lakes Animal Products Co. (Arden Hills, MN) manufactured the MR with whey protein and animal fat to produce varying contents of protein and fat. Increased protein and fat concentrations resulted in reduced lactose concentrations. Ad libitum water and calf starter was offered at all times throughout the experiment. The ingredients and nutrient composition of the starter was presented in Hill et al. (2008). Because one calf died at 6 wk of age, data from this animal were removed from the analysis.

Sample Collection and Metabolite Analysis

Blood samples were collected from the jugular vein into tubes containing sodium heparin and sodium heparin plus sodium fluoride twice (Tuesday and Thursday) in wk 1, 4, and 9 of the experiment. Plasma was im-

mediately harvested following centrifugation at $2,000 \times g$ for 20 min at 4°C and stored at -20°C until analyzed for AA and metabolites. The 2 weekly samples were pooled for wk 1, 4, and 9; therefore, 3 samples per calf were used for plasma AA and metabolite analyses.

Calves were slaughtered at 65 ± 1 d of age following a 12-h fast. Tissue samples were collected immediately from the liver and muscle (semimembranosus) and snap-frozen in liquid N_2 . Samples were stored at -80°C and later subjected to analysis of metabolites, AA, and cell signaling activity. Plasma and tissue samples were analyzed for glucose, acetate, BHBA, and lactate using nuclear magnetic resonance spectroscopy (**NMR**) as described previously by Rius et al. (2010a). Briefly, 0.5 mL of plasma was deproteinized by addition of 0.5 mL of acetonitrile. Subsequently, 2,2-dimethyl-2-silapentane-5-sulfonic acid was added as an internal standard, and the sample was vortexed and centrifuged at $14,000 \times g$ for 20 min at 4°C . The supernatant was removed and freeze-dried (Savant SpeedVac SC 110, GMI Inc., Ramsey, MN). Dried samples were resuspended in 0.5 mL of phosphate-buffered (100 mM) deuterated water (D_2O , Sigma Aldrich, St. Louis, MO) with 0.5% Na azide. Tissue samples were pulverized using an impact grinder (Freezer Mill 6850, Fisher Scientific, Waltham, MA), and 50 mg was subsampled and mixed with 100 μL of chloroform and 100 μL of methanol. Samples were vortexed and centrifuged at $10,000 \times g$ for 10 min at 4°C , and the supernatant was freeze-dried. Dried samples were resuspended in 550 μL of phosphate-buffered D_2O and submitted for NMR analysis.

Proton NMR (Varian 400; Varian, Palo Alto, CA) was performed on the protein-free supernatant from blood and tissue samples, and the spectra were baseline adjusted and phase corrected, and the peak areas were integrated using the Know-It-All Informatics System (Bio-Rad, Hercules, CA). Free AA in tissues and plasma samples were determined by isotope dilution using a GC-MS according to the methods of El-Kadi et al. (2004) and Calder et al. (1999).

Cell Signaling Analysis

A 0.1-g aliquot of tissue was processed for Western blotting analysis as described previously by Rius et al. (2010b). Briefly, tissue samples were mixed 1:7 with homogenizing buffer containing a protease inhibitor cocktail (P8340, Sigma Chemical Company), homogenized (Power Gen 1000, Fisher Scientific), and centrifuged at $10,000 \times g$ for 10 min at 4°C . The supernatant was mixed with an equal volume of $2\times$ Laemmli SDS sample buffer, boiled for 5 min, and stored at -80°C until analysis. Proteins were electrophoretically separated in 12% SDS polyacrylamide gels and transferred to a

PVDF membrane (Bio-Rad), which was incubated with appropriate antibodies, as described by Escobar et al. (2006). Membranes were probed for the phosphorylated forms of protein kinase B (**AKT**; Ser⁴⁷³) or ribosomal protein S6 (**rpS6**; Ser^{235/236} and Ser^{240/244}). Membranes were subsequently stripped and incubated with antibodies that recognized the total form of each protein. Numerous attempts were conducted to determine rpS6 in muscle but low abundance of this protein limited its detection and quantification. All antibodies were from Cell Signaling Technology (Danvers, MA). Protein bands were visualized using an enhanced chemiluminescence kit (ECL Plus, Amersham, Piscataway, NJ) with ECL film and developed with a medical film processor (SRX 101A, Konica Minolta, Wayne, NJ). The films were scanned and bands quantified by densitometry. Band intensities were used to calculate the ratio of phosphorylated to total protein, which is indicative of the phosphorylation state of that particular factor.

Statistical Analysis

Statistical computations were performed using the Proc Mixed procedure in SAS (SAS Institute, 2001). Treatment, time (wk 1, 4, and 9), purchase group (1, 2, and 3), and their interactions were defined as fixed effects, and calf within treatment and group were defined as random effects for the analyses of AA and metabolites. Western blotting, tissue metabolite and AA concentrations, and tissue to plasma AA concentrations at wk 9 were analyzed using a model that included treatment and purchase group and the interaction as fixed effect.

The following model was used for analysis of the variables of interest:

$$Y_{ijkl} = \mu + \tau_i + \kappa_j + \omega_k + H_{(ij)l} + (\tau\kappa)_{ij} + (\tau\omega)_{ik} + E_{(ijk)l},$$

where Y_{ijkl} = dependent variable; μ = overall mean; τ_i = fixed effect of treatment ($i = 1, \dots, 4$); κ_j = fixed effect of group ($j = 1, 2, \text{ and } 3$); ω_k = fixed effect of experimental week ($k = 1, 4, \text{ and } 9$); $H_{(ij)l}$ = random effect of calf within treatment and group; $(\tau\kappa)_{ij}$ = fixed interaction of treatment and group; $(\tau\omega)_{ik}$ = fixed interaction of treatment and week; and $E_{(ijk)l}$ = residual error.

The interactions of group $\times$ week and group $\times$ week $\times$ treatment were tested when appropriate, but removed from the model because they were not significant. Preplanned nonorthogonal contrasts were used to test CON versus all treatments, HPMF versus HPHF, and HPHF versus HPHF+. The Bonferroni method

was used to adjust means comparisons. Unless otherwise stated, significance was declared at $P \leq 0.05$.

RESULTS

AA Concentrations

Plasma concentrations of AA are reported in Table 1. A treatment by group interaction was observed for Ile, Leu, Met, and Val ($P \leq 0.04$). Treatment affected Leu, Met, Phe, Thr, Ala, Glu, and Ser concentrations ($P \leq 0.05$). Relative to CON, all treatments reduced concentrations of Phe, Ser, and Tyr ($P = 0.01$), and tended to reduce concentrations of Leu ($P = 0.07$) but increased concentrations of Thr ($P = 0.01$). Relative to HPMF, calves fed HPHF had a trend to increase Ala concentrations ($P = 0.09$).

Liver concentrations of AA are reported in Table 2. Concentrations of His, Ala, and Ser were affected by treatments ($P \leq 0.05$). Relative to CON, all treatments reduced Ala and Ser ($P = 0.01$) and tended to reduce Glu concentrations ($P = 0.06$). Compared with HPMF, calves fed HPHF had reduced His concentrations ($P = 0.05$).

Muscle concentrations of AA are reported in Table 3. Treatment affected concentrations of Leu, Lys, Met, and Val ($P \leq 0.03$), and a treatment by group interaction was found for Pro ($P = 0.05$). Relative to CON, all treatments increased Met ($P = 0.01$) and tended to reduce Ser concentrations ($P = 0.07$). Compared with HPMF, HPMF increased muscle concentrations of Leu and Val ($P \leq 0.02$). Relative to HPHF, HPHF+ tended to increase Lys concentrations ($P = 0.09$).

Ratios of liver to plasma AA are reported in Table 4. A treatment by group interaction was found for Ala, Pro, and Tyr ($P \leq 0.03$) and a trend was observed for Ile and Glu ratios ($P \leq 0.08$). Treatments affected Phe, Ala, Pro, Ser, and Tyr ($P \leq 0.04$) and tended to affect Glu ratios ($P = 0.06$). Relative to CON, all treatments decreased Phe, Pro and Tyr ratios ($P \leq 0.05$) and there was a tendency to reduce Met ratio ($P = 0.06$). Compared with animals fed HPMF, calves fed HPHF increased Ala, Pro, and Ser ratios ($P \leq 0.03$).

Ratios of muscle to plasma AA are reported in Table 5. A treatment by group interaction was observed for Met, Pro, and Tyr ratios ($P \leq 0.04$). Treatments affected Met, Phe, and Tyr ratios ($P = 0.01$) and tended to affect Val and Glu ratios ($P \leq 0.09$). Compared with CON, all treatments increased Val, Phe, and Tyr ratios ($P \leq 0.04$) and tended to increase Ile, Leu, and Met ratios ($P \leq 0.09$). Calves fed HPHF had greater ratio of Tyr ($P = 0.01$) than calves fed HPMF. The ratio of Tyr was greater for HPHF than for HPHF+ ($P = 0.01$).

Table 1. Jugular plasma AA concentrations (μM) in dairy calves fed milk replacer with varying amounts of protein and fat for 9 wk ($n = 6/\text{treatment}$)

AA	Experimental treatment ¹					Effect, ² $P =$					
	CON	HPMF	HPHF	HPHF+	SEM	T	G	T $\times$ G	CON vs. all	HPMF vs. HPHF	HPHF vs. HPHF+
Essential AA											
Ile	59.4	47.0	47.4	52.3	4.1	0.22	0.92	0.04	0.19	0.99	0.82
Leu	101.0	77.5	78.6	81.1	4.7	0.01	0.16	0.01	0.07	0.96	0.87
Lys	88.4	84.0	74.6	98.8	9.0	0.22	0.37	0.53	0.98	0.89	0.21
Met	10.5	12.9	10.9	12.6	1.1	0.01	0.01	0.01	0.11	0.59	0.71
Phe	30.4	17.5	16.8	19.0	2.6	0.01	0.01	0.88	0.01	0.89	0.93
Thr	81.3	137	145	141	11.8	0.01	0.01	0.22	0.01	0.95	0.99
Val	150	124	127	140	8.1	0.31	0.98	0.04	0.13	0.89	0.66
Nonessential AA											
Ala	192	146	195	194	13.0	0.05	0.77	0.86	0.61	0.09	1.00
Glu	63.7	69.0	71.4	72.6	5.0	0.04	0.01	0.34	0.89	0.88	0.99
Pro	61.0	55.5	58.7	64.7	3.2	0.05	0.85	0.89	0.62	0.90	0.56
Ser	101	69.8	82.8	92.6	5.1	0.01	0.04	0.34	0.01	0.32	0.59
Tyr	28.4	11.7	5.2	7.8	2.0	0.08	0.62	0.81	0.01	0.17	0.78

¹CON = control milk replacer (MR) with 20% CP and 21% fat, fed at 441 g of DM/d; HPMF = high-protein, medium-fat MR with 28% CP and 20% fat, fed at 951 g of DM/d; HPHF = high-protein, high-fat MR with 27% CP and 28% fat, fed at 951 g of DM/d; HPHF+ = HPHF fed at 1,431 g of DM/d. Because one calf died (HPMF), data from this animal were removed from the analysis.

²Effects of treatment (T), group (G), and treatment by group (T $\times$ G). The effect of time (wk 1, 4, and 9) and its interactions were not significant ($P < 0.05$) and were removed from the model.

Metabolite Concentrations

Plasma, liver, and muscle concentrations of metabolites are reported in Table 6. Plasma concentrations of glucose were affected by treatment and, compared with calves fed CON, all treatments increased plasma glucose concentrations ($P = 0.01$). Plasma concentrations of BHBA were affected by treatments ($P =$

0.01), but the contrasts used were not able to identify differences among treatments. A significant treatment by group interaction was observed for liver concentrations of glucose, lactate, and BHBA ($P = 0.01$), and treatment effects on glucose, lactate, and BHBA concentrations ($P \leq 0.04$). Treatments did not affect glucose, lactate, propionate, or BHBA concentrations in muscle.

Table 2. Liver AA concentrations (μM) in dairy calves fed milk replacer with varying amounts of protein and fat for 9 wk ($n = 6/\text{treatment}$)

AA	Experimental treatment ¹					Effect, ² $P =$					
	CON	HPMF	HPHF	HPHF+	SEM	T	G	T $\times$ G	CON vs. all	HPMF vs. HPHF	HPHF vs. HPHF+
Essential AA											
His	436	489	344	388	31	0.05	0.04	0.14	0.69	0.05	0.75
Ile	161	149	127	159	10	0.16	0.01	0.30	0.39	0.34	0.25
Leu	299	278	234	304	20	0.12	0.01	0.21	0.48	0.52	0.13
Lys	234	270	210	277	22	0.20	0.02	0.60	0.73	0.36	0.22
Met	49.8	52.9	43.8	56.5	4.3	0.33	0.07	0.41	0.98	0.66	0.27
Phe	106	97.0	87.3	117	11.0	0.31	0.01	0.24	0.87	0.93	0.28
Thr	372	291	288	331	60	0.75	0.71	0.59	0.57	0.95	0.95
Val	521	502	407	494	37	0.20	0.05	0.32	0.42	0.38	0.37
Nonessential AA											
Ala	3,330	2,492	1,848	1,901	348	0.04	0.30	0.61	0.01	0.63	0.89
Asp	1,247	1,054	1,222	1,321	148	0.70	0.01	0.66	0.95	0.87	0.93
Glu	5,735	4,781	4,240	3,860	483	0.08	0.30	0.58	0.06	0.88	0.94
Gln	1,997	2,027	1,709	2,014	175	0.50	0.22	0.57	0.92	0.64	0.62
Pro	247	242	204	251	21.0	0.44	0.01	0.97	0.91	0.90	0.45
Ser	1,915	1,514	1,124	1,184	153	0.01	0.86	0.49	0.01	0.38	0.89
Tyr	86.8	69.8	62.5	82.0	7.7	0.13	0.01	0.26	0.19	0.91	0.28

¹CON = control milk replacer (MR) with 20% CP and 21% fat, fed at 441 g of DM/d; HPMF = high-protein, medium-fat MR with 28% CP and 20% fat, fed at 951 g of DM/d; HPHF = high-protein, high-fat MR with 27% CP and 28% fat, fed at 951 g of DM/d; HPHF+ = HPHF fed at 1,431 g of DM/d. Because one calf died (HPMF), data from this animal were removed from the analysis.

²Effects of treatment (T), group (G), and treatment by group (T $\times$ G).

Table 3. Muscle AA concentrations in dairy calves fed milk replacer with varying amounts of protein and fat for 9 wk (n = 6/treatment)

AA (μ M)	Experimental treatment ¹					Effect, ² P =					
	CON	HPMF	HPHF	HPHF+	SEM	T	G	T $\times$ G	CON vs. all	HPMF vs. HPHF	HPHF vs. HPHF+
Essential AA											
His	90.5	100	84.6	98.3	17.3	0.92	0.83	0.67	0.97	0.92	0.95
Ile	81.0	95.6	72.3	73.0	7.9	0.28	0.09	0.70	0.99	0.28	0.99
Leu	108	148	98.8	102	6.5	0.01	0.01	0.07	0.74	0.01	0.98
Lys	277	185	226	304	21.0	0.01	0.01	0.21	0.98	0.51	0.09
Met	17.0	35.2	28.6	34.8	3.4	0.01	0.01	0.13	0.01	0.49	0.57
Phe	60.3	71.6	62.2	62.0	5.5	0.58	0.95	0.44	0.71	0.68	1.00
Thr	241	238	222	323	42.0	0.46	0.08	0.48	0.90	0.99	0.44
Val	184	231	159	169	14.0	0.03	0.05	0.08	0.96	0.02	0.94
Nonessential AA											
Ala	1,141	862	907	854	112	0.68	0.26	0.58	0.11	0.99	0.98
Asp	128	79.5	99.1	126	16.3	0.99	0.25	0.94	0.21	0.87	0.73
Glu	2,240	1,994	1,806	1,581	244	0.37	0.79	0.94	0.28	0.95	0.92
Gln	2,351	2,653	1,948	2,673	365	0.56	0.06	0.36	0.97	0.68	0.56
Pro	174	173	153	228	18.0	0.05	0.16	0.05	0.81	0.85	0.04
Ser	369	238	269	292	36.7	0.16	0.23	0.81	0.07	0.94	0.97
Tyr	64.1	67.1	55.5	59.6	8.1	0.71	0.33	0.48	0.89	0.69	0.98

¹CON = control milk replacer (MR) with 20% CP and 21% fat, fed at 441 g of DM/d; HPMF = high-protein, medium-fat MR with 28% CP and 20% fat, fed at 951 g of DM/d; HPHF = high-protein, high-fat MR with 27% CP and 28% fat, fed at 951 g of DM/d; HPHF+ = HPHF fed at 1,431 g of DM/d. Because one calf died (HPMF), data from this animal were removed from the analysis.

²Effects of treatment (T), group (G), and treatment by group (T $\times$ G).

Cell Signaling Analysis

Phosphorylation ratios for AKT and rpS6 are presented in Table 7. Treatments did not affect AKT activity in the liver and muscle. The liver of calves fed HPHF had lower phosphorylation ratios of rpS6 than calves fed HPMF and HPHF+.

DISCUSSION

Treatments affected AA concentrations, tissue to plasma ratios of AA, energy-yielding metabolite concentrations, and cell signaling. Lower plasma concentrations of Phe, Tyr, and a trend for Leu suggest that these AA were removed by the peripheral tissue (e.g., muscle) to support body growth in calves fed all treatments compared with CON (Table 1). This is further supported by the greater muscle to plasma ratios of Phe, Tyr, and branched-chain AA in calves fed all treatments (Table 5). Conversely, it is likely that greater plasma concentrations of Thr were the response to excess of Thr in the peripheral tissue, which increased Thr recycled to the liver. It was reported that AA that were not removed by the peripheral tissue increased plasma concentrations and recycling to the liver to be removed by mass action kinetics (Hanigan et al., 1998). Moreover, Wray-Cahen et al. (1997) infused AA in the mesenteric vein of dry cows and reported that the liver removed Thr to a greater extent than Leu, Val, and Lys for catabolism and protein synthesis. Therefore, the changes in plasma concentrations of AA in calves

fed all treatments could be the result of AA removal by the peripheral and hepatic tissues in response to accelerated growth.

Relative to CON, all treatments increased muscle concentration of Met. It is possible that these calves increased protein synthesis because Met regulates protein synthesis signaling in bovine mammary cells (Appuhamy et al., 2010, 2011). However, in the current experiment, it was not possible to separate the effects of single AA, CP, and energy intakes on protein synthesis. The muscle to plasma ratio of AA generally resulted in a combination of reductions in plasma and increases in tissue concentrations suggesting that transport and uptake from plasma could have been enhanced in calves fed all treatments. Indeed, results from the current study (discussed below; reported by Hill et al., 2008) indicate that calves fed all treatments had greater total CP and energy intakes (MR + starter), which resulted in greater nitrogen retention. The moderate fat diet (HPMF) promoted Leu and Val muscle concentrations and lean tissue growth relative to high fat diets, which suggests that moderate fat diets could be better than high fat diets to promote lean tissue growth. Collectively, these results suggest that greater intake of nutrients increased transport activity and net uptake of AA to support rapid body growth.

It was surprising that liver concentrations of glucose were approximately 10-fold greater than concentrations in plasma, particularly because muscle concentrations were similar to plasma. However, an upregulation in gluconeogenesis and glucose accumulation could ex-

Table 4. Liver to plasma AA ratios in dairy calves fed milk replacer with varying amounts of protein and fat for 9 wk (n = 6/treatment)

AA	Experimental treatment ¹					Effect, ² P =					
	CON	HPMF	HPHF	HPHF+	SEM	T	G	T × G	CON vs. all	HPMF vs. HPHF	HPHF vs. HPHF+
Essential AA											
Ile	3.01	4.16	3.50	4.66	0.56	0.21	0.04	0.08	0.12	0.51	0.22
Leu	3.16	3.83	3.15	4.83	0.52	0.17	0.15	0.10	0.21	0.65	0.24
Lys	3.83	5.58	4.01	5.15	1.03	0.55	0.06	0.68	0.35	0.81	0.93
Met	13.5	7.66	7.31	9.56	2.19	0.16	0.15	0.13	0.06	0.91	0.98
Phe	2.51	3.16	3.83	6.00	0.71	0.01	0.03	0.16	0.02	0.88	0.08
Thr	6.33	13.8	4.16	4.17	5.41	0.39	0.13	0.29	0.76	0.42	0.97
Val	3.51	9.04	3.51	4.16	2.20	0.40	0.31	0.42	0.46	0.43	0.96
Nonessential AA											
Ala	25.3	34.8	17.3	19.0	2.25	0.04	0.03	0.03	0.67	0.03	0.92
Glu	73.3	110.1	74.2	61.1	11.2	0.06	0.05	0.06	0.52	0.14	0.89
Pro	5.33	9.00	5.39	6.86	0.84	0.03	0.01	0.01	0.05	0.02	0.51
Ser	26.3	44.6	21.5	19.8	4.3	0.02	0.05	0.14	0.59	0.02	0.85
Tyr	3.11	6.21	12.5	15.69	2.79	0.02	0.04	0.03	0.02	0.18	0.92

¹CON = control milk replacer (MR) with 20% CP and 21% fat, fed at 441 g of DM/d; HPMF = high-protein, medium-fat MR with 28% CP and 20% fat, fed at 951 g of DM/d; HPHF = high-protein, high-fat MR with 27% CP and 28% fat, fed at 951 g of DM/d; HPHF+ = HPHF fed at 1,431 g of DM/d. Because one calf died (HPMF), data from this animal were removed from the analysis. Plasma concentrations of AA during the last week of treatments were used to calculate the ratios.

²Effects of treatment (T), group (G), and treatment by group (T × G).

plain some of our results because gluconeogenesis is up-regulated due to insufficient lactose intake in calves fed colostrum or milk (Girard et al., 1992; Liggins, 1994). It is possible that gluconeogenesis is even more relevant to maintain glucose homeostasis in calves fed low lactose diets as used in the current experiment (e.g., high intakes of MR with high fat and protein and low lactose content). It seems unlikely that these observations were due to measurement error as concentrations were normal in muscle and propionate and butyrate concentra-

tions in liver were similar to blood concentrations. The presence of another 6-carbon free sugar with similar NMR spectra as glucose cannot be ruled out. However, it seems likely this compound would have to be actively transported or synthesized within the liver given the large concentration difference between blood and liver. Because hepatic glucose, fructose, and galactose transport are all by facilitated diffusion (Hocquette et al., 1996), fructose and galactose would not appear to be probable causes. The large concentration difference

Table 5. Muscle to plasma AA ratios in dairy calves fed milk replacer with varying amounts of protein and fat for 9 wk (n = 6/treatment)

AA	Experimental treatment ¹					Effect, ² P =					
	CON	HPMF	HPHF	HPHF+	SEM	T	G	T × G	CON vs. all	HPMF vs. HPHF	HPHF vs. HPHF+
Essential AA											
Ile	1.33	2.17	2.01	2.00	0.35	0.19	0.58	0.49	0.08	0.83	0.93
Leu	1.16	1.83	1.50	1.51	0.22	0.21	0.62	0.30	0.08	0.72	0.99
Lys	4.01	3.66	4.31	4.51	0.94	0.94	0.71	0.72	0.88	0.94	0.89
Met	6.83	5.34	6.00	19.8	1.90	0.01	0.01	0.02	0.09	0.95	0.01
Phe	1.33	2.51	2.57	3.16	0.37	0.01	0.48	0.55	0.01	0.99	0.46
Thr	4.00	4.10	3.00	3.83	0.43	0.71	0.61	0.31	0.64	0.80	0.84
Val	1.01	1.83	1.31	1.38	0.19	0.07	0.56	0.34	0.04	0.31	0.96
Nonessential AA											
Ala	8.33	8.81	8.01	8.83	1.41	0.95	0.83	0.56	0.87	0.97	0.96
Glu	34.1	44.3	31.6	23.5	4.40	0.09	0.10	0.15	0.86	0.29	0.63
Pro	4.01	5.54	4.16	6.60	0.87	0.21	0.27	0.04	0.14	0.20	0.71
Ser	5.16	6.33	4.61	5.63	1.06	0.95	0.76	0.98	0.78	0.61	0.94
Tyr	2.09	5.18	12.4	9.41	1.04	0.01	0.02	0.02	0.01	0.01	0.01

¹CON = control milk replacer (MR) with 20% CP and 21% fat, fed at 441 g of DM/d; HPMF = high-protein, medium-fat MR with 28% CP and 20% fat, fed at 951 g of DM/d; HPHF = high-protein, high-fat MR with 27% CP and 28% fat, fed at 951 g of DM/d; HPHF+ = HPHF fed at 1,431 g of DM/d. Because one calf died (HPMF), data from this animal were removed from the analysis. Plasma concentrations of AA during the last week of treatments were used to calculate the ratios.

²Effects of treatment (T), group (G), and treatment by group (T × G).

Table 6. Plasma and tissue metabolite concentrations (mmol/L) in dairy calves fed milk replacer with varying amounts of protein and fat for 9 wk (n = 6/treatment)

Item	Experimental treatment ¹					Effect, ² P =					
	CON	HPMF	HPHF	HPHF+	SEM	T	G	T × G	CON vs. all	HPMF vs. HPHF	HPHF vs. HPHF+
Plasma											
Glucose	3.79	4.16	4.16	4.48	0.21	0.01	0.01	0.99	0.01	0.39	0.54
Lactate	2.97	3.05	3.08	3.15	0.43	0.85	0.18	0.38	0.45	0.42	0.42
Propionate	0.406	0.393	0.363	0.560	0.061	0.17	0.02	0.58	0.71	0.26	0.69
BHBA	0.106	0.078	0.088	0.108	0.007	0.01	0.01	0.24	0.23	0.25	0.52
Liver											
Glucose	30.7	24.6	29.1	49.8	3.21	0.01	0.01	0.01	0.71	0.49	0.01
Lactate	21.1	15.4	15.0	28.8	2.62	0.01	0.01	0.01	0.78	0.67	0.02
Propionate	0.323	0.354	0.252	0.184	0.063	0.27	0.06	0.27	0.37	0.68	0.87
BHBA	0.186	0.384	0.234	0.170	0.039	0.01	0.01	0.01	0.71	0.93	0.19
Muscle											
Glucose	2.34	3.00	3.45	2.53	0.47	0.37	0.06	0.89	0.71	0.98	0.39
Lactate	19.7	24.6	31.2	22.9	5.74	0.53	0.04	0.18	0.51	0.63	0.45
Propionate	0.163	0.165	0.126	0.059	0.030	0.10	0.34	0.44	0.27	0.89	0.32
BHBA	0.087	0.071	0.088	0.082	0.006	0.33	0.82	0.12	0.82	0.94	0.19

¹CON = control milk replacer (MR) with 20% CP and 21% fat, fed at 441 g of DM/d; HPMF = high-protein, medium-fat MR with 28% CP and 20% fat, fed at 951 g of DM/d; HPHF = high-protein, high-fat MR with 27% CP and 28% fat, fed at 951 g of DM/d; HPHF+ = HPHF fed at 1,431 g of DM/d. Because one calf died (HPMF), data from this animal were removed from the analysis.

²Effects of treatment (T), group (G), and treatment by group (T × G). The effect of time (wk 1, 4, and 9) and its interactions on plasma metabolite concentrations were not significant ($P < 0.05$) and were removed from the model.

could simply reflect the natural concentration gradient between cell and plasma due to glucose synthesis, which would be consistent with the lack of such a difference in muscle tissue.

Treatment had no effect on AKT activity in liver or muscle. The protein initiation factor AKT was chosen because it is upstream in the mTOR cascade and is regulated by hormones and nutrients associated with energy supply; however, we have previously observed no effect of EAA on phosphorylation of AKT in bovine tissue (Appuhamy et al., 2011). Insulin and IGF-I plasma concentrations changed among treatments (reported by Daniels et al., 2008), which should have increased the likelihood of finding changes in the phosphorylation of

AKT. However, it is possible that 12-h fasting before slaughter prevented detection of differences on AKT activity. Therefore, although changes in energy supply and hormone concentrations should have affected AKT, we were unable to detect changes in this study.

Phosphorylation of rpS6 in the liver was increased for the HPHF+ treatment compared with HPHF. Compared with HPHF, HPMF also increased phosphorylation ratios of rpS6, but this effect was confounded because starter intake increased more for the HPMF calves than for the HPHF calves during the last week of the trial (see Hill et al., 2008). Changes in insulin, IGF-I, and IGF-binding proteins reported by Daniels et al. (2008) do not appear to be consistent with the ob-

Table 7. Protein initiation factor ratios (phosphorylated to total abundance ratio of arbitrary density units) in liver and muscle of dairy calves fed milk replacer with varying amounts of protein and fat for 9 wk (n = 6/treatment)

Item ¹	Experimental treatment ²					Effect, ³ P =					
	CON	HPMF	HPHF	HPHF+	SEM	T	G	T × G	CON vs. all	HPMF vs. HPHF	HPHF vs. HPHF+
Liver											
AKT	1.57	1.36	1.14	0.82	0.47	0.65	0.19	0.24	0.41	0.51	0.47
rpS6	2.58	2.80	1.49	2.88	0.33	0.02	0.01	0.35	0.72	0.05	0.02
Muscle											
AKT	0.37	0.13	0.38	0.38	0.12	0.58	0.24	0.44	0.87	0.91	0.89

¹AKT = protein kinase B; rpS6 = ribosomal protein S6.

²CON = control milk replacer (MR) with 20% CP and 21% fat, fed at 441 g of DM/d; HPMF = high-protein, medium-fat MR with 28% CP and 20% fat, fed at 951 g of DM/d; HPHF = high-protein, high-fat MR with 27% CP and 28% fat, fed at 951 g of DM/d; HPHF+ = HPHF fed at 1,431 g of DM/d. Because one calf died (HPMF), data from this animal were not collected.

³Effects of treatment (T), group (G), and treatment by group (T × G).

served changes in phosphorylation. Nonetheless, some interactions among them and nutrient supply cannot be ruled out. Appuhamy et al. (2009) indicated that acetate supply increased rpS6 phosphorylation to a lesser degree than EAA. We cannot rule out that greater starter intake during the last week of the trial for CON and HPMF calves or 12 h of fasting before slaughter of the calves could have been partially responsible for the altered rpS6 phosphorylation activity. Collectively, changes in nutrient supply affected rpS6 phosphorylation activity; however, the role of hormones and other key metabolites on cell signaling activity in growing dairy calves remains unclear.

Muscle total and phosphorylated rpS6 levels were too low to be reliably detected. The latter may be due to low synthesis of rpS6 in muscle or loss of activity in processed samples during storage as we could reliably measure phosphorylated rpS6 in fresh muscle from mice. Another explanation is the overnight fast, which would have reduced primarily phosphorylation levels of the cell signals in muscle.

We have no clear explanation for the effect of purchase group on the variables analyzed in this study. Daniels et al. (2008) reported greater serum total protein concentrations in the last group of calves purchased compared with the second group of calves. However, no differences were found between the first and second group of calves purchased. Plasma serum concentrations reflect nutritional and health status of the calves, and these factors might have been responsible for some of the findings or lack of findings in this study.

It should be pointed out that tissues were harvested in calves after an approximately 12-h fast, which could have affected metabolism and signaling in the target tissues. This is the most likely explanation for the lack of consistency in cell signaling activity. Second, because increasing MR content of fat and protein was matched by reduced lactose, the change in lactose could also explain the changes in metabolites in this study. Third, the difference in total DM intake (MR + starter) among treatments was reduced during the last week of the trial due to greater starter intake in calves fed CON and HPMF (Hill et al., 2008). During the last week of the trial, daily nutrient intakes were 300 g of CP, 102 g of fat, and 4.08 Mcal of gross energy for CON calves; 475 g of CP, 199 g of fat, and 6.25 Mcal of gross energy for HPMF calves; 354 g of CP, 282 g of fat, and 6.31 Mcal of gross energy for HPHF calves; and 465 g of CP, 414 g of fat, and 8.27 Mcal of gross energy for HPHF+ calves. Compared with all treatments, CON calves had greater starter intake, which in turn reduced apparent digestibility of DM from 79 to 62% (all treatments vs. CON; Hill et al., 2008). It is possible that calves fed the CON diet increased rumen development as suggested

by changes in metabolite concentrations in plasma and liver. Therefore, it is not possible to separate the effect of starter from that of MR; however, nutrient intakes still reflected the dietary changes in MR and thus the treatments were effective in altering nutrient intakes despite differences in starter intake.

CONCLUSIONS

Changes in plasma and tissue concentrations and ratios of AA suggest that transport activity was affected by treatments and these changes were consistent with enhanced growth. Greater plasma concentrations of Thr in treatments providing increased nutrients relative to CON suggest that Thr was in excess of requirements for accelerated growth in those diets. Low Phe, Tyr, and Leu plasma concentrations suggests greater removal of these AA by muscle in calves fed all treatments providing increased nutrient intakes. Greater muscle concentrations of Met could be part of a regulatory mechanism of protein synthesis in calves fed all treatments. Changes in nutrients supply did not affect AKT signaling activity in the muscle and liver. However, increasing MR intake increased phosphorylation of rpS6 in the liver (HPHF+ vs. HPHF). Therefore, interactions between AA, metabolites, and signaling activity remain to be investigated to identify regulatory responses in the initiation of protein synthesis. The muscle concentrations of Met and its role in cell signaling and protein accretion in the carcass of calves fed high levels of nutrients warrant further investigations.

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