Effects of level of nutrient intake and age on mammalian target of rapamycin, insulin, and insulin-like growth factor-1 gene network expression in skeletal muscle of young Holstein calves

P. Wang,*† J. K. Drackley,† J. A. Stamey-Lanier,†¹ D. Keisler,‡ and J. J. Loor†²

*College of Animal Science and Technology, Northwest A&F University, Yangling, 712100, China

†Department of Animal Sciences and Division of Nutritional Sciences, University of Illinois, Urbana 61801

‡Department of Animal Sciences, University of Missouri, Columbia 65211

ABSTRACT

The molecular mechanisms by which level of nutrient intake enhances skeletal muscle growth in young ruminants are not fully understood. We examined mammalian target of rapamycin (mTOR), insulin, and insulin-like growth factor-1 (*IGF-1*) gene network expression in semitendinosus muscle tissue of young male Holstein calves fed a conventional milk replacer plus conventional starter (CON) or an enhanced milk replacer plus high-protein starter (ENH) for 5 wk followed by a conventional starter or a high-protein starter until 10 wk of age. Feeding ENH led to greater concentration of plasma IGF-1 and leptin and greater carcass protein and fat mass throughout the study. Despite the greater plasma IGF-1 and protein mass at wk 5, calves fed ENH had lower expression of *IGF1R*, *INSR*, and *RPS6KB1* but greater expression of *IRS1* and *PDPK1* in muscle tissue. Except for *IGF1R* expression, which did not differ at wk 10, these differences persisted at wk 10, suggesting a long-term effect of greater nutrient intake on physiological and molecular mechanisms. Components of mTOR complex (mTORC)1 and mTORC2 (*RICTOR* and *RPTOR*) and *FOXO1* expression decreased by wk 10 regardless of diet. Overall, the present data revealed that greater nutrient intake throughout the milk-fed and early postweaning phase alters body mass composition partly by altering hormonal and molecular profiles of genes associated with glucose and amino acid signaling. Those networks may play a crucial role in coordinating neonatal muscle growth and metabolism in response to level of nutrient intake.

Key words: growth, metabolism, dairy calf, protein synthesis

INTRODUCTION

Physiological and molecular studies primarily in non-ruminants have provided evidence of higher growth rates in neonates attributed to the feeding-induced stimulation of protein synthesis in muscle via insulin- and AA-mediated signaling pathways (Davis and Fiorotto, 2009). The mammalian target of rapamycin (mTOR) signaling pathway, which is downstream of insulin and AA signaling, plays a central role in enhancing protein synthesis and cell growth (Wullschleger et al., 2006). Signaling through IGF-1 and the IGF-1 receptor also appears to play a role in skeletal muscle cell differentiation during the neonatal period (Dickson et al., 1991). Furthermore, hypertrophy of skeletal muscle myotubes is enhanced through the activation of the IGF-1–mTOR signaling pathways (Rommel et al., 2001).

Dietary protein ingestion affects the expression of growth hormone receptor (*GHR*) or *IGF1* in muscle (Brameld et al., 1996), and the concentration of both hormones in blood (Daniels et al., 2009). Therefore, the greater growth rate of dairy calves (Blome et al., 2003; Bartlett et al., 2006) fed higher-protein diets could involve not only hormonal changes but also alterations in mRNA expression of genes associated with mTOR, insulin, and IGF-1 signaling pathways. From a nutritional standpoint, however, alterations in expression of the negative feedback loop encompassing tuberous sclerosis 2 (*TSC2*)–mTOR–ribosome protein S6 kinase (*RPS6KB1*)–insulin receptor substrate 1 (*IRS1*) (Harrington et al., 2004; Um et al., 2006; Tremblay et al., 2007) are important. In humans, nutrient overload or obesity leads to altered insulin sensitivity (Um et al., 2006), with potential involvement of this feedback loop in coordinating such a response. Relatively little is known in livestock species about the expression pattern of mTOR, insulin, and IGF-1 signaling pathways and the feedback loop as it relates to level of nutrient intake and physiological state. Therefore, it is important to understand the molecular events whereby diet coordinates growth and metabolism.

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¹Current address: Prince Agri Products Inc., 229 Radio Road, Quincy, IL 62306.

²Corresponding author: jloor@illinois.edu

Figure 1. Model depicting the mammalian target of rapamycin (mTOR) signaling pathway in response to nutrients and growth factors. The mTOR complex (mTORC)1, including mTOR, regulatory-associated proteins of mTOR (RPTOR), and G protein β -subunit-like protein (G β L), plays central roles in modulating cell growth and metabolism in response to extracellular growth stimuli, including nutritional signals (amino acids and glucose) and growth factors. On one hand, the mTOR pathway responds to growth factors via the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway. Binding of insulin or IGF to their receptor (i.e., INSR or IGF1R) leads to recruitment and phosphorylation of insulin receptor substrate 1 (IRS-1) and subsequent recruitment of PI3K, and PI3K bound to IRS converts phosphatidylinositol-4,5-phosphate (PIP2) in the cell membrane to phosphatidylinositol-3,4,5-phosphate (PIP3). Accumulation of PIP3 is antagonized by the lipid phosphatase phosphatase and tensin homolog (PTEN). PIP3 co-recruits PDK1 and Akt to the membrane, resulting in the phosphorylation and activation of Akt by PDK1. mTOR is wired to the PI3K pathway through tuberous sclerosis complex (TSC)1/TSC2 (Wulschleger et al., 2006). The TSC1/TSC2 complex acts as a negative modulator of mTORC1 relaying growth factors signals in addition to energy status. Energy stress conditions leads to activation of AMP-activated protein kinase (AMPK), which phosphorylates TSC2 (Inoki et al., 2003). The TSC1/TSC2 complex is also required for maintaining insulin signaling to PI3K by repressing a negative feedback loop from mTOR/S6K (also known as ribosomal protein S6 kinase beta-1, RPS6KB1) to the adaptor molecule IRS-1 (Harrington et al., 2004). On the other hand, AA seem to be the most crucial signals for mTORC1 activation (see review by Jewell and Guan, 2013). Nutrients such as AA and glucose increases hVps 34 (also known as phosphatidylinositol 3-kinase, catalytic subunit type 3, PIK3C3) activity, stimulating the production of PIP3; PIP3, in turn, recruits proteins containing FYVE or PX domains to endosomal membranes (see review by Um et al., 2006). Additionally, Akt seems to play a pivotal role in the regulation of skeletal muscle hypertrophy and atrophy in rodents and cells through mTOR and FOXO1 (Léger et al., 2006). Moreover, Akt is suggested to be a downstream of mTORC2, which contains mTOR, rapamycin-insensitive companion of mTOR (RICTOR), and G β L (Sarbasov et al., 2005).

Components of cell growth and metabolism related to the mTOR, insulin, and IGF-1 signaling pathways that were measured in this study are shown in Figure 1. The figure was developed primarily based on the review of Wulschleger et al. (2006). The main objective of this study was to examine the effect of level of nutrient intake (Naeem et al., 2012; Stamey et al., 2012) on expression of genes with relevance to the mTOR, insulin, and IGF-1 signaling pathways in skeletal muscle. Our general hypothesis was that both plane of nutrition and stage of growth would alter mRNA expression of genes associated with these pathways. Quantitative PCR (qPCR) was used as a tool for in-depth study of the relationships among the 15 genes chosen and hormones in response to different plane of nutrition from birth through the first week after weaning. The present data provide evidence of coordination between growth and

metabolism when different levels of nutrition are provided to young calves.

MATERIALS AND METHODS

Experimental Animals and Sampling

Details of diets and general management have been published (Stamey et al., 2012). Briefly, male calves were fed 1 of 2 different diets: (1) control (CON), conventional milk replacer (MR; 20% CP, 20% fat, 4.95 Mcal/kg of DM) plus conventional starter (19.6% CP, 3.16 Mcal/kg of DM); and (2) enhanced (ENH) MR (28.5% CP, 15% fat, 4.72 Mcal/kg of DM) plus high-CP starter (25.5% CP, 3.21 Mcal/kg of DM). Conventional MR was reconstituted to 12.5% solids and fed at 1.25% of birth BW daily in 2 feedings from wk 1 to 5 and at

0.625% of birth BW once daily during wk 6. Enhanced MR was reconstituted to 15% solids and fed at 1.5% of BW during wk 1, and at 2% of BW during wk 2 to 5, divided into 2 daily feedings. Calves were offered starter for ad libitum intake starting on d 3. Calf starter intake was recorded once daily. After weaning (wk 7 through 10), calves in CON and ENH continued to receive the respective starter ad libitum.

Animals used in the present study came from a previous larger study (Stamey, 2008). Details of measurement and analysis of animal performance (i.e., ADG, carcass weight, carcass composition, empty BW, composition of empty BW) are reported elsewhere (Stamey, 2008; Naeem et al., 2012; Stamey et al., 2012). Five calves from each treatment were slaughtered at 43 d (wk 5) and 5 were slaughtered at 71 d (wk 10) of life at the Meat Sciences Laboratory at the University of Illinois, Urbana-Champaign, via captive bolt stunning followed by exsanguination. Calves slaughtered at 10 wk of age had been weaned at d 42. Semitendinosus muscle samples (~0.5 g) from a rear leg were collected and stored in liquid N₂ until RNA extraction.

Blood was collected each Monday at 0500 h, before the morning feeding, via jugular venipuncture. Samples were collected into evacuated serum tubes (SST serum separator tube; Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) containing clot activator. Serum was obtained by centrifugation at $1,300 \times g$ for 15 min. The serum and plasma recovered were aliquoted into polypropylene tubes and frozen (-20°C) until analyses. Concentrations of growth hormone (GH), IGF-1, and leptin were measured via RIA as described previously (Powell and Keisler, 1995; Lalman et al., 2000).

RNA Extraction, Real-Time Reverse Transcription-PCR, and Primer Design and Evaluation

The complete details of these procedures can be found in the supplemental file (<http://dx.doi.org/10.3168/jds.2013-7042>). Briefly, semitendinosus tissue was weighed (~0.3–0.5 g) and immediately subjected to RNA extraction with ice-cold Trizol (Invitrogen Corp., Carlsbad, CA) as described previously (Grauagnard et al., 2009). The quality of RNA was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The average RNA integrity number of the samples used was >8.0. A portion of the RNA was diluted to 100 mg/L using DNase-, RNase-free water before reverse transcription.

The cDNA were synthesized using 100 ng of RNA, 1 µg of dT18 (Operon Biotechnologies, Huntsville, AL), 1 µL of 10 mmol/L dNTP mix (Invitrogen Corp.), 1 µL of random primer p(dN)₆ (Roche cat. no. 11 034 731 001; Roche, Indianapolis, IN), and 10 µL of DNase-,

RNase-free water. The qPCR was performed using 4 µL of diluted cDNA (dilution 1:4) combined with 6 µL of a mixture composed of 5 µL of 1 × SYBR Green master mix (Applied Biosystems, Foster City, CA), 0.4 µL each of 10 µM forward and reverse primers, and 0.2 µL of DNase-, RNase-free water in a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems). Each sample was run in triplicate and a 6-point relative standard curve plus the nontemplate control were used (User Bulletin #2, Applied Biosystems). The cycle threshold (Ct) data were analyzed and transformed using the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems). The final data were normalized using the geometric mean of the 4 most-stable genes among the ones tested as internal controls, and the percentage of specific mRNA abundance among total mRNA from all genes quantified in present study was calculated as reported previously (Bionaz and Looor, 2007). This approach is particularly helpful for the biological interpretation of the change in expression of genes as it relates to the relative amount of mRNA in the biological sample. Sequence information of primers for the target genes of interest is listed in Supplemental Table S1 (<http://dx.doi.org/10.3168/jds.2013-7042>).

Selection and Evaluation of Internal Control Genes

The geometric mean of actin-β (*ACTB*), ELKS/RAB6-interacting/CAST family member 1 (*ERC1*), mitochondrial ribosomal protein L39 (*MRPL39*), and ubiquitously expressed transcript (*UXT*) was used to normalize gene expression data (Supplemental Figures S1 and S2; <http://dx.doi.org/10.3168/jds.2013-7042>).

Statistical Analysis

Data were analyzed using Proc MIXED in SAS as a repeated-measures ANOVA (SAS Institute Inc., Cary, NC). The qPCR data normalized with the internal control genes (ICG) were log-2 transformed before statistical analysis. The model consisted of diet, age, and the diet × age interaction as fixed effects, as well as calf nested within diet × age as a random effect. An autoregressive covariance structure was used. Least squares means were computed and were compared using the PDIF statement of SAS (SAS Institute Inc.). Significance was declared at $P \leq 0.10$.

RESULTS

Animal Performance

Calves fed ENH had greater ($P < 0.05$) final live BW, ADG, preharvest shrunk BW (SBW), and car-

cass weight (Table 1). Carcass as a percentage of SBW was not affected ($P = 15$) by diet but was less ($P < 0.05$) in calves at 10 wk than in those at 5 wk. Calves fed ENH had greater ($P < 0.05$) liver weight than those fed CON, and liver was heavier ($P < 0.05$) at 10 wk than at 5 wk. As a percentage of SBW, however, liver weight did not differ ($P = 0.17$) between diets but was greater ($P < 0.05$) at 10 wk than at 5 wk. Calves fed ENH had greater amounts of protein ($P < 0.01$), fat ($P = 0.10$), and ash ($P < 0.01$) in the carcass because of larger carcass mass, but percentages of protein, fat, and ash did not vary between diets. The percentage of fat in the carcass of 10-wk-old calves was greater than that in calves at 5 wk.

Concentrations of Hormones in Blood

An overall diet effect was observed for growth hormone ($P = 0.02$), IGF-1 ($P < 0.01$), and leptin ($P = 0.08$), with higher concentrations observed in calves fed ENH (Table 1). The concentration of GH ($P < 0.01$) and leptin ($P < 0.06$) was lower at wk 10 than at wk 5 regardless of diet.

Gene Expression

Among the genes measured, v-akt murine thymoma viral oncogene homolog 1 (*AKT1*), phosphoinositide-

3-kinase (*PIK3C3*), regulatory associated protein of mTOR (*RPTOR*), and *TSC2* had the greatest relative mRNA abundance and accounted for 12.4, 18.6, 11.9, and 18.8% among all mRNA quantified in present study, respectively (Table 2). We detected a diet $\times$ age ($P = 0.04$) interaction for *IGF1R* expression due to lower expression for calves fed ENH compared with CON at wk 5. A diet $\times$ age interaction was observed for *TSC2* ($P = 0.02$) due to higher expression of *TSC2* in calves fed ENH compared with CON at wk 10 or ENH at wk 5, whereas the opposite pattern of expression observed for tuberous sclerosis 1 (*TSC1*) reflected downregulated expression ($P = 0.10$) in calves fed ENH at wk 10 compared with wk 5.

Overall diet effects were observed for *IRS1* ($P = 0.02$), *RPS6KB1* ($P = 0.03$), insulin receptor (*INSR*, $P = 0.10$) and 3-phosphoinositide-dependent protein kinase (*PDPK1*, $P = 0.10$). Insulin receptor substrate 1 and *INSR* were downregulated, and *RPS6KB1* and *PDPK1* were upregulated in response to feeding ENH compared with CON. An overall age effect was observed for forkhead box O1 (*FOXO1*, $P = 0.04$), *INSR* ($P = 0.05$), RPTOR independent companion of mTOR, complex 2 (*RICTOR*, $P = 0.07$), and *RPTOR* ($P = 0.10$), all of which decreased in expression at wk 10 compared with wk 5.

Table 1. Carcass composition and hormones in blood of male Holstein calves fed a conventional milk replacer (20% CP, 20% fat) plus conventional starter (CON; 19.6% CP) or enhanced milk replacer (28.5% CP, 15% fat) plus high-CP starter (ENH; 25.5% CP) from d 3 to wk 6 of age followed by a 4-wk period of feeding a conventional starter (CON) or high-CP starter (ENH)

Item	Wk 5		Wk 10		SEM ¹	P-value ²		
	CON	ENH	CON	ENH		Diet	Age	D $\times$ A
Final live BW, kg	59.4	72.9	85.5	109.8	7.9	0.02	0.001	0.47
ADG, kg/d	0.32	0.72	0.51	0.86	0.087	0.001	0.054	0.74
Preharvest shrunk BW (SBW), kg	57.1	71.0	81.3	106.4	7.4	0.01	0.001	0.43
Carcass weight, kg	33.5	42.9	42.2	56.0	4.1	0.01	0.01	0.57
Carcass, % of SBW	58.7	60.4	51.7	52.7	0.9	0.15	0.001	0.67
Liver, kg	1.14	1.47	1.79	2.51	0.20	0.01	0.01	0.31
Liver, % of SBW	2.00	2.08	2.19	2.35	0.09	0.17	0.02	0.67
Carcass composition								
Protein, kg	5.87	7.51	7.37	10.1	0.81	0.01	0.01	0.46
Fat, kg	1.64	2.18	2.72	3.63	0.45	0.10	0.01	0.65
Ash, kg	1.54	1.89	1.90	2.45	0.19	0.01	0.01	0.57
Carcass composition								
Protein, %	17.54	17.48	17.32	18.05	0.31	0.26	0.57	0.19
Fat, %	4.90	5.07	6.18	6.35	0.47	0.70	0.01	0.99
Ash, %	4.60	4.42	4.55	4.36	0.19	0.32	0.77	0.99
Blood ³								
GH, ng/mL	11.8	3.52	2.71	2.13	2.05	0.02	0.01	0.47
IGF-1, ng/mL	93.3	191	119	159	23	0.01	0.98	0.18
Leptin, ng/mL	1.75	2.25	1.28	1.66	0.31	0.08	0.06	0.93

¹SEM = the largest standard error of the mean.

²P-value for the effect of diet (D), age (A), and diet $\times$ time (D $\times$ A).

³Concentrations of insulin, glucose, NEFA, total protein, urea-N, and hydroxybutyric acid have been published (Naeem et al., 2012). GH = growth hormone.

Table 2. Relative mRNA expression (log-2 scale) and the percentage of specific mRNA abundance among the total mRNA of genes quantified in present study in skeletal muscle of male Holstein calves fed a conventional milk replacer (20% CP, 20% fat) plus conventional starter (CON; 19.6% CP) or enhanced milk replacer (28.5% CP, 15% fat) plus high-CP starter (ENH; 25.5% CP) from d 3 of age to wk 6 of age followed by a 4-wk period of feeding a conventional starter (CON) or high-CP starter (ENH)

Gene (symbol)	% mRNA ¹	Wk 5		Wk 10		SEM ²	P-value ³		
		CON	ENH	CON	ENH		D	A	D × A
v-akt murine thymoma viral oncogene homolog 1 (<i>AKT1</i>)	12.4	4.23	4.35	4.30	4.28	0.06	0.45	0.94	0.22
Forkhead box O1 (<i>FOXO1</i>)	4.2	5.10	4.82	4.21	4.39	0.35	0.87	0.04	0.47
IGF-1 receptor (<i>IGF1R</i>)	3.5	4.83*	4.56*	4.62	4.79	0.11	0.60	0.95	0.04
Insulin receptor (<i>INSR</i>)	7.6	5.00	4.86	4.82	4.71	0.09	0.10	0.05	0.89
Insulin receptor substrate 1 (<i>IRS1</i>)	2.7	4.82	5.15	4.73	5.03	0.14	0.02	0.45	0.90
FKBP12-rapamycin complex-associated protein (<i>MTOR</i>)	1.9	5.11	5.04	5.07	4.93	0.09	0.23	0.39	0.72
3-Phosphoinositide-dependent protein kinase (<i>PDPK1</i>)	1.5	4.83	5.00	4.96	5.20	0.12	0.10	0.21	0.95
Phosphoinositide-3-kinase (<i>PIK3C3</i>)	18.6	5.24	5.19	5.04	5.15	0.09	0.73	0.15	0.36
Phosphatase and tensin homolog (<i>PTEN</i>)	4.2	4.73	4.94	4.99	4.97	0.11	0.36	0.15	0.27
RPTOR independent companion of mTOR (<i>RICTOR</i>)	4.6	5.08	5.02	4.95	4.79	0.11	0.27	0.07	0.63
Ribosomal protein S6 kinase (<i>RPS6KB1</i>)	1.5	5.06	4.82	5.09	4.94	0.10	0.03	0.41	0.65
Regulatory associated protein of mTOR (<i>RPTOR</i>)	11.9	4.79	4.87	4.61	4.77	0.09	0.19	0.10	0.69
Ras-related GTP binding C (<i>RRAGC</i>)	2.9	5.03	4.91	5.18	5.03	0.12	0.23	0.22	0.88
Tuberous sclerosis 1 (<i>TSC1</i>)	3.4	4.94	5.08 ^a	4.93	4.79 ^b	0.08	0.97	0.06	0.10
Tuberous sclerosis 2 (<i>TSC2</i>)	18.8	4.69	4.55 ^b	4.77*	4.97 ^{*a}	0.08	0.74	0.01	0.02

^{a,b}Means with different symbols denote significant interactions due to diet × age (D × A) between week ($P \leq 0.10$).

¹The % mRNA abundance was calculated as $\{[(1/E^{\Delta C_t}) \text{ specific gene} / \sum (1/E^{\Delta C_t}) \text{ all genes}] \times 100\}$, where $E = 10^{(-1/\text{Curve Slope})}$, and $\Delta C_t = C_t \text{ gene} - \text{geometric mean } C_t \text{ of 4 internal control genes}$.

²SEM is the largest standard error of the mean.

³P-value for the effect of diet (D), age (A), and diet × age (D × A).

*Means with symbols differ due to diet × age (D × A) within week ($P \leq 0.10$).

DISCUSSION

Gene Markers of Protein Metabolism in Skeletal Muscle

Physiological studies in piglets have demonstrated that the enhanced rate of protein synthesis induced by greater diet consumption is an effect of elevated postprandial signals such as insulin and AA (Davis et al., 2010). Similar effects on protein synthesis in muscle were also observed in hind-limb of sheep (Oddy et al., 1987; Wester et al., 2000) and rats (Garlick et al., 1983). Those responses to feeding, however, decreased with age in piglets (Davis et al., 1996) and horses (Wagner and Urschel, 2012). Molecular events, including the components involved in insulin and AA signaling pathways, as well as the mTOR signaling pathway, have provided evidence to support the above physiological effects (Davis and Fiorotto, 2009).

In the present study, downregulation of *INSR* at wk 10 compared with wk 5, combined with the downregulation of components of mTOR complex (mTORC) 1 and mTORC2 (i.e., *RICTOR* and *RPTOR*), which are implicated in the regulation of cell growth (Wulschleger et al., 2006), suggests a decrease in the rate of protein synthesis via insulin and AA signaling. The pattern of transcriptional alterations in skeletal muscle with age in the present study agrees with observations from a series of studies in neonatal pig muscle (Suryawan and Davis, 2003, 2005, 2010; Suryawan et al., 2001, 2004, 2006, 2007). Those studies reported that enhanced protein synthesis was paralleled by an activation of the insulin signaling cascade, including *INSR*, *IRS-1/2*, phosphatidylinositol 3-kinase (**PI3K**), *PDPK1*, and protein kinase B (**PKB** or **AKT**).

We cannot rule out the possibility, however, that AA availability mediates cell growth through activation of mTOR. For instance, it has been reported that AA are important regulators of mTORC1 activation and enhanced cell growth, a process suggested to occur in a class III PI3K (**hVps34**)-dependent manner (Nobukuni et al., 2005; Yoon et al., 2011). Moreover, AA may activate mTORC2 in a different way from their effect on mTORC1; for example, via activity of class I PI3K and of AKT, which is dependent on the presence of *RICTOR*, a specific component of mTORC2 (Tato et al., 2011). The selective activation of mTORC1 or mTORC2 by AA can occur under different starvation conditions (Tato et al., 2011). For instance, in overnight serum-starved cells, AA induced the phosphorylation of Akt through class I PI3K and mTORC2 (Tato et al., 2011). On the other hand, when the culture medium was devoid of AA, the stimulation of *RPS6KA1*, *S6* ribosomal protein (**RPS6**), and eIF4E binding protein

1 (**4E-BP1**) was inhibited by rapamycin, wortmannin, and the hVps34 inhibitor 3-methyladenine. Those results suggested that the effect of AA is mediated by the class III PI3K hVps34 and mTORC1 (Tato et al., 2011). Together with the high abundance of *PIK3C3*, *AKT1*, and *RPTOR* in skeletal muscle, the lower expression of *RPTOR* and *RICTOR* at wk 10 in our study suggests a developmentally reduced sensitivity of skeletal muscle to AA. Because of the complexity of mTORC1 and mTORC2 regulation, more studies are needed to improve our understanding of the role of AA in mTOR activation in young calves.

The downregulated expression of *FOXO1* coupled with the lack of change in expression of *AKT1* at wk 10 of age (Table 2) could be taken as an indication that protein degradation may have decreased during growth. Activation of AKT1 regulates both hypertrophy and atrophy through its effect on FOXO1 and mTOR (Léger et al., 2006). Phosphorylation of FOXO1 by AKT1 activates ubiquitin-protein ligases and leads to skeletal muscle atrophy (Stitt et al., 2004; Glass, 2010). In vitro, it has been shown that activation of FOXO1 in murine myoblasts blocked myogenic differentiation and led to proteasomal degradation of mTOR, *RPTOR*, *TSC2*, and *RPS6KB1* (Wu et al., 2008).

Gene Markers of Insulin Sensitivity in Skeletal Muscle

Previous work has underscored a critical role for the activation of *RPS6KB1*-*IRS1* signaling in compensating for a decline in sensitivity to insulin during metabolic abnormalities such as obesity and type II diabetes (Tremblay et al., 2005; Um et al., 2006). Increased insulin sensitivity was observed in *RPS6KB1*^{-/-} mice on a high-fat diet compared with wild-type mice, suggesting that the absence of *RPS6KB1* facilitates insulin signaling (Um et al., 2004). In the present study, the upregulation of *IRS1* and *PDPK1* in parallel with the downregulation of *RPS6KB1* due to feeding ENH would indicate greater insulin sensitivity.

The greater growth rate in calves fed ENH was due to the greater rate of carcass accumulation without changes in chemical composition (Table 1). Taken together with those data, the lower overall *INSR* expression coupled with a lower glucose-to-insulin ratio in the same calves as previously reported (Naeem et al., 2012), particularly after weaning (0.34 vs. 0.43 for CON and ENH; *P* < 0.10; Naeem et al., 2012), might argue against decreased insulin sensitivity for ENH calves after weaning. This agrees with the negative feedback action of *TSC2* on insulin sensitivity via regulation of *IRS1* (Harrington et al., 2004), which is discussed below.

Gene Markers of Energy Metabolism in Skeletal Muscle

The high mRNA abundance of *TSC2* among measured genes (and also relative to *TSC1*) underscores its essential role in integrating energy metabolism and muscle cell growth. The expression and function of *TSC2* is regulated by cellular energy level (Inoki et al., 2003). The marked increase of *TSC2* expression in ENH compared with CON after weaning seems to suggest a residual effect of the improved nutrient status before weaning on growth. The lower overall expression of *RP-S6KB1* in response to ENH supports this suggestion.

The opposite response in *TSC1* and *TSC2* expression agrees with the idea that both work in concert to regulate mTOR downstream (Tee et al., 2002). Although as a percentage of BW, it represented only a 0.5% increase, the greater overall mass of carcass protein at wk 10 in calves fed ENH compared with those fed CON (Table 1) suggests a positive effect of high-quality starter on muscle growth postweaning. Tuberous sclerosis 2, which is regulated by both AMPK and PI3K/Akt (Hay and Sonenberg, 2004), has been proposed as an integrator of insulin and energy status in the processes regulating cell growth.

In human cancers and tumor syndromes (e.g., tuberous sclerosis complex and Peutz-Jegher's syndrome), which are characterized by imbalance of intracellular energy, the lack of *TSC2* attenuates insulin sensitivity through a failure of inhibition of RPS6KB1 action on IRS1 (Harrington et al., 2004). Furthermore, AA or glucose stimulated Ser302 phosphorylation of IRS1, which is normally inhibited by wortmannin or rapamycin (Giraud et al., 2004), suggesting a positive role of nutrients (AA and glucose) on insulin signaling via the mTOR cascade.

If one assumes that the weaning transition encompasses a period of nutrient stress (Mieulet and Lamb, 2010), it could be possible that the postweaning increase in carcass mass (without changes in chemical composition) in response to high-quality dietary starter was driven, at least in part, by an improvement of insulin sensitivity via *TSC1/2* to maintain normal insulin signaling at the level of PI3K (Harrington et al., 2004). Furthermore, *TSC2* upregulation between wk 5 and 10 for calves fed ENH appears to suggest that a high plane of nutrition could curtail any negative effect of age on growth because previous work demonstrated that *TSC2* is a negative regulator of mTOR signaling during cell growth (Mieulet and Lamb, 2010).

Gene Markers of Muscle Growth in Young Calves

Postnatal muscle growth is partly attributed to hypertrophy (Brameld et al., 1998). The IGF-1/IGF-1

receptor signaling pathway, which regulates anabolic processes such as protein synthesis, plays a crucial role in muscle growth of the young animal due to the hypertrophic effects of local IGF-1 (Barton-Davis et al., 1999). The decrease in expression of *IGF1R* mRNA during postnatal development in the rat (Werner et al., 1989) is suggestive of an involvement of IGF-1 signaling in muscle growth of young animals. Accordingly, the more pronounced effect of ENH on calf growth (i.e., carcass accumulation, Table 1; Naeem et al., 2012) during the milk-fed period was associated with lower *IGF1R* expression (Table 2), which may be interpreted as a sign of greater protein accumulation due to enhanced hypertrophic effects by the improved plane of nutrition (Brameld et al., 1998). The observed changes in hormones support the benefit of ENH on protein accumulation; that is plasma concentration of IGF-1 reflected plane of nutrition and GH was inversely related to the plane of nutrition (Chelikani et al., 2009). In those mice, the impaired muscle growth was characterized by a decrease in fiber size, but not by a decrease in fiber number, suggesting that the activation of satellite cells is needed by, and provides structural basis for, the subsequent muscle hypertrophy during growth.

CONCLUSIONS

Although more molecular and cellular studies are needed, results from this study suggest 2 possible explanations for the adaptation of growth and metabolism due to changes in nutrition level in young calves: (1) enhanced growth in calves fed the ENH milk replacer during the preweaning period contributes to better responses to postweaning high level of nutrition; and (2) better quality of starter has the potential to improve insulin sensitivity due to upregulation of *TSC1/TSC2*, leading to lower inhibition of *RPS6KB1* on *IRS1*.

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REFERENCES

- Bartlett, K. S., F. K. McKeith, M. J. VandeHaar, G. E. Dahl, and J. K. Drackley. 2006. Growth and body composition of dairy calves fed milk replacers containing different amounts of protein at two feeding rates. *J. Anim. Sci.* 84:1454-1467.

- Barton-Davis, E. R., D. I. Shetrum, and H. L. Sweeney. 1999. Contribution of satellite cells to IGF-I induced hypertrophy of skeletal muscle. *Acta Physiol. Scand.* 167:301–305.
- Bionaz, M., and J. J. Loores. 2007. Identification of reference genes for quantitative real-time PCR in the bovine mammary gland during the lactation cycle. *Physiol. Genomics* 29:312–319.
- Blome, R. M., J. K. Drackley, F. K. McKeith, M. F. Hutjens, and G. C. McCoy. 2003. Growth, nutrient utilization, and body composition of dairy calves fed milk replacers containing different amounts of protein. *J. Anim. Sci.* 81:1641–1655.
- Brameld, J. M., J. L. Atkinson, J. C. Saunders, J. M. Pell, P. J. Buttery, and R. S. Gilmour. 1996. Effects of growth hormone administration and dietary protein intake on insulin-like growth factor I and growth hormone receptor mRNA Expression in porcine liver, skeletal muscle, and adipose tissue. *J. Anim. Sci.* 74:1832–1841.
- Brameld, J. M., P. J. Buttery, J. M. Dawson, and J. M. Harper. 1998. Nutritional and hormonal control of skeletal-muscle cell growth and differentiation. *Proc. Nutr. Soc.* 57:207–217.
- Chelikani, P. K., D. J. Ambrose, D. H. Keisler, and J. J. Kennelly. 2009. Effects of dietary energy and protein density on plasma concentrations of leptin and metabolic hormones in dairy heifers. *J. Dairy Sci.* 92:1430–1441.
- Daniels, K. M., A. V. Capuco, M. L. McGilliard, R. E. James, and R. M. Akers. 2009. Effects of milk replacer formulation on measures of mammary growth and composition in Holstein heifers. *J. Dairy Sci.* 92:5937–5950.
- Davis, T. A., D. G. Burrin, M. L. Fiorotto, and H. V. Nguyen. 1996. Protein synthesis in skeletal muscle and jejunum is more responsive to feeding in 7- than in 26-day-old pigs. *Am. J. Physiol.* 270:E802–E809.
- Davis, T. A., and M. L. Fiorotto. 2009. Regulation of muscle growth in neonates. *Curr. Opin. Clin. Nutr. Metab. Care* 12:78–85.
- Davis, T. A., A. Suryawan, R. A. Orellana, M. L. Fiorotto, and D. G. Burrin. 2010. Amino acids and insulin are regulators of muscle protein synthesis in neonatal pigs. *Animal* 4:1790–1796.
- Dickson, M. C., J. C. Saunders, and R. S. Gilmour. 1991. The ovine insulin-like growth factor-I gene: Characterization, expression and identification of a putative promoter. *J. Mol. Endocrinol.* 6:17–31.
- Garlick, P. J., M. Fern, and V. R. Preedy. 1983. The effect of insulin infusion and food intake on muscle protein synthesis in postabsorptive rats. *Biochem. J.* 210:669–676.
- Giraud, J., R. Leshan, Y.-H. Lee, and M. F. White. 2004. Nutrient-dependent and insulin-stimulated phosphorylation of insulin receptor substrate-1 on serine 302 correlates with increased insulin signaling. *J. Biol. Chem.* 279:3447–3454.
- Glass, D. J. 2010. PI3 kinase regulation of skeletal muscle hypertrophy and atrophy. *Curr. Top. Microbiol. Immunol.* 346:267–278.
- Graugnard, D., P. Piantoni, M. Bionaz, L. Berger, D. Faulkner, and J. Loores. 2009. Adipogenic and energy metabolism gene networks in longissimus lumborum during rapid post-weaning growth in Angus and Angus x Simmental cattle fed high-starch or low-starch diets. *BMC Genomics* 10:142.
- Harrington, L. S., G. M. Findlay, A. Gray, T. Tolkacheva, S. Wigfield, H. Rebholz, J. Barnett, N. R. Leslie, S. Cheng, P. R. Shepherd, I. Gout, C. P. Downes, and R. F. Lamb. 2004. The TSC1–2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. *J. Cell Biol.* 166:213–223.
- Hay, N., and N. Sonenberg. 2004. Upstream and downstream of mTOR. *Genes Dev.* 18:1926–1945.
- Inoki, K., Y. Li, T. Xu, and K. L. Guan. 2003. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev.* 17:1829–1834.
- Jewell, J. L., and K. Guan. 2013. Nutrient signaling to mTOR and cell growth. *Trends Biochem. Sci.* 38:233–242.
- Lalman, D. L., J. E. Williams, B. W. Hess, M. G. Thomas, and D. H. Keisler. 2000. Effect of dietary energy on milk production and metabolic hormones in thin, primiparous beef heifers. *J. Anim. Sci.* 78:530–538.
- Léger, B., R. Cartoni, M. Praz, S. Lamon, O. Deriaz, A. Crettenand, C. Gobelet, P. Rohmer, M. Konzelmann, F. Luthi, and A. P. Russell. 2006. Akt signalling through GSK-3 β , mTOR and Foxo1 is involved in human skeletal muscle hypertrophy and atrophy. *J. Physiol.* 576:923–933.
- Mieulet, V., and R. F. Lamb. 2010. Tuberous sclerosis complex: Linking cancer to metabolism. *Trends Mol. Med.* 16:329–335.
- Naeem, A., J. K. Drackley, J. Stamey, and J. J. Loores. 2012. Role of metabolic and cellular proliferation genes in ruminal development in response to enhanced plane of nutrition in neonatal Holstein calves. *J. Dairy Sci.* 95:1807–1820.
- Nobukuni, T., M. Joaquin, M. Rocco, S. G. Dann, S. Y. Kim, P. Gulati, M. P. Byfield, J. M. Backer, F. Natt, J. L. Bos, F. J. T. Zwartkruis, and G. Thomas. 2005. Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc. Natl. Acad. Sci. USA* 102:14238–14243.
- Oddy, V. H., D. B. Lindsay, P. J. Barker, and A. J. Northrop. 1987. Effect of insulin on hind-limb and whole-body leucine and protein metabolism in fed and fasted lambs. *Br. J. Nutr.* 58:437–452.
- Powell, M. R., and D. H. Keisler. 1995. A potential strategy for decreasing milk production in the ewe at weaning using a growth hormone release blocker. *J. Anim. Sci.* 73:1901–1905.
- Rommel, C., S. C. Bodine, B. A. Clarke, R. Rossman, L. Nunez, T. N. Stitt, G. D. Yancopoulos, and D. J. Glass. 2001. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat. Cell Biol.* 3:1009–1013.
- Sarbasov, D. D., D. A. Guertin, S. M. Ali, and D. M. Sabatini. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307:1098–1101.
- Stamey, J. A. 2008. Influence of starter protein content and plane of nutrition on growth and body composition of dairy calves. MS Thesis. University of Illinois, Urbana.
- Stamey, J. A., N. A. Janovick, A. F. Kertz, and J. K. Drackley. 2012. Influence of starter protein content on growth of dairy calves in an enhanced early nutrition program. *J. Dairy Sci.* 95:3327–3336.
- Stitt, T. N., D. Drujan, B. A. Clarke, F. Panaro, Y. Timofeyeva, W. O. Kline, M. Gonzalez, G. D. Yancopoulos, and D. J. Glass. 2004. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol. Cell* 14:395–403.
- Suryawan, A., and T. A. Davis. 2003. Protein-tyrosine-phosphatase 1B activation is regulated developmentally in muscle of neonatal pigs. *Am. J. Physiol. Endocrinol. Metab.* 284:E47–E54.
- Suryawan, A., and T. A. Davis. 2005. Developmental regulation of protein kinase B activation is isoform specific in skeletal muscle of neonatal pigs. *Pediatr. Res.* 58:719–724.
- Suryawan, A., and T. A. Davis. 2010. The abundance and activation of mTORC1 regulators in skeletal muscle of neonatal pigs are modulated by insulin, amino acids, and age. *J. Appl. Physiol.* 109:1448–1454.
- Suryawan, A., J. Escobar, J. W. Frank, H. V. Nguyen, and T. A. Davis. 2006. Developmental regulation of the activation of signaling components leading to translation initiation in skeletal muscle of neonatal pigs. *Am. J. Physiol. Endocrinol. Metab.* 291:E849–E859.
- Suryawan, A., H. V. Nguyen, J. A. Bush, and T. A. Davis. 2001. Developmental changes in the feeding-induced activation of the insulin-signaling pathway in neonatal pigs. *Am. J. Physiol. Endocrinol. Metab.* 281:E908–E915.
- Suryawan, A., P. M. J. O'Connor, S. R. Kimball, J. A. Bush, H. V. Nguyen, L. S. Jefferson, and T. A. Davis. 2004. Amino acids do not alter the insulin-induced activation of the insulin signaling pathway in neonatal pigs. *J. Nutr.* 134:24–30.
- Suryawan, A., R. A. Orellana, H. V. Nguyen, A. S. Jeyapalan, J. R. Fleming, and T. A. Davis. 2007. Activation by insulin and amino acids of signaling components leading to translation initiation in skeletal muscle of neonatal pigs is developmentally regulated. *Am. J. Physiol. Endocrinol. Metab.* 293:E1597–E1605.
- Tato, I., R. Bartrons, F. Ventura, and J. L. Rosa. 2011. Amino acids activate mammalian target of rapamycin complex 2 (mTORC2) via PI3K/Akt signaling. *J. Biol. Chem.* 286:6128–6142.
- Tee, A. R., D. C. Fingar, B. D. Manning, D. J. Kwiatkowski, L. C. Cantley, and J. Blenis. 2002. Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of

- rapamycin (mTOR)-mediated downstream signaling. *Proc. Natl. Acad. Sci. USA* 99:13571–13576.
- Tremblay, F., S. Brûlé, S. Hee Um, Y. Li, K. Masuda, M. Roden, X. J. Sun, M. Krebs, R. D. Polakiewicz, G. Thomas, and A. Marette. 2007. Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance. *Proc. Natl. Acad. Sci. USA* 104:14056–14061.
- Tremblay, F., M. Krebs, L. Dombrowski, A. Brehm, E. Bernroider, E. Roth, P. Nowotny, W. Waldhäusl, A. Marette, and M. Roden. 2005. Overactivation of S6 kinase 1 as a cause of human insulin resistance during increased amino acid availability. *Diabetes* 54:2674–2684.
- Um, S. H., D. D'Alessio, and G. Thomas. 2006. Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. *Cell Metab.* 3:393–402.
- Um, S. H., F. Frigerio, M. Watanabe, F. Picard, M. Joaquin, M. Sticker, S. Fumagalli, P. R. Allegrini, S. C. Kozma, J. Auwerx, and G. Thomas. 2004. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* 431:200–205.
- Wagner, A. L., and K. L. Urschel. 2012. Developmental regulation of the activation of translation initiation factors of skeletal muscle in response to feeding in horses. *Am. J. Vet. Res.* 73:1241–1251.
- Werner, H., M. Woloschak, M. Adamo, Z. Shen-Orr, C. T. Roberts, and D. LeRoith. 1989. Developmental regulation of the rat insulin-like growth factor I receptor gene. *Proc. Natl. Acad. Sci. USA* 86:7451–7455.
- Wester, T. J., G. E. Lobley, L. M. Birnie, and M. A. Lomax. 2000. Insulin stimulates phenylalanine uptake across the hind limb in fed lambs. *J. Nutr.* 130:608–611.
- Wu, A.-L., J.-H. Kim, C. Zhang, T. G. Unterman, and J. Chen. 2008. Forkhead box protein O1 negatively regulates skeletal myocyte differentiation through degradation of mammalian target of rapamycin pathway components. *Endocrinology* 149:1407–1414.
- Wullschlegel, S., R. Loewith, and M. N. Hall. 2006. TOR signaling in growth and metabolism. *Cell* 124:471–484.
- Yoon, M. S., G. Du, J. M. Backer, M. A. Frohman, and J. Chen. 2011. Class III PI-3-kinase activates phospholipase D in an amino acid-sensing mTORC1 pathway. *J. Cell Biol.* 195:435–447.