RAPID COMMUNICATION: Residual feed intake in beef cattle is associated with differences in protein turnover and nutrient transporters in ruminal epithelium

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ABSTRACT: Residual feed intake (RFI) is a widely used measure of feed efficiency in cattle. Although the precise biologic mechanisms associated with improved feed efficiency are not well-known, most-efficient steers (i.e., with low RFI coefficient) downregulate abundance of proteins controlling protein degradation in skeletal muscle. Whether cellular mechanisms controlling protein turnover in ruminal tissue differ by RFI classification is unknown. The aim was to investigate associations between RFI and signaling through the mechanistic target of rapamycin (MTOR) and ubiquitin-proteasome pathways in ruminal epithelium. One hundred and forty-nine Red Angus cattle were allocated to 3 contemporary groups according to sex and herd origin. Animals were offered a finishing diet for 70 d to calculate the RFI coefficient for each. Within each group, the 2 most-efficient ($n = 6$) and least-efficient animals ($n = 6$) were selected. Compared with least-efficient animals, the most-efficient animals consumed less feed ($P < 0.05$; 18.36 vs. 23.39 kg/d DMI). At day 70, plasma samples were collected for insulin concentration analysis. Ruminal epithelium was collected immediately after slaughter to determine abundance

and phosphorylation status of 29 proteins associated with MTOR, ubiquitin-proteasome, insulin signaling, and glucose and amino acid transport. Among the proteins involved in cellular protein synthesis, most-efficient animals had lower ($P \leq 0.05$) abundance of MTOR, p-MTOR, RPS6KB1, EIF2A, EEF2K, AKT1, and RPS6KB1, whereas MAPK3 tended ($P = 0.07$) to be lower. In contrast, abundance of p-EEF2K, p-EEF2K:EEF2K, and p-EIF2A:EIF2A in most-efficient animals was greater ($P \leq 0.05$). Among proteins catalyzing steps required for protein degradation, the abundance of UBA1, NEDD4, and STUB1 was lower ($P \leq 0.05$) and MDM2 tended ($P = 0.06$) to be lower in most-efficient cattle. Plasma insulin and ruminal epithelium insulin signaling proteins did not differ ($P > 0.05$) between RFI groups. However, abundance of the insulin-responsive glucose transporter SLC2A4 and the amino acid transporters SLC1A3 and SLC1A5 also was lower ($P \leq 0.05$) in most-efficient cattle. Overall, the data indicate that differences in signaling mechanisms controlling protein turnover and nutrient transport in ruminal epithelium are components of feed efficiency in beef cattle.

Key words: beef cattle, insulin signaling, nutrient transporters, protein turnover, residual feed intake, ruminal epithelium

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INTRODUCTION

Feed costs represent the major expense of beef cattle production and are the crucial factor in

determining profitability for producers (Clemmons et al., 2018). Therefore, reducing feed consumption on-farm without compromising animal performance to enhance feed efficiency is of utmost importance. Variability in feed efficiency among individuals is commonly measured via the residual feed intake (RFI) approach, defined as the difference between actual and predicted feed intake required for maintenance and growth (Koch et al., 1963). Residual feed intake is a popular index of feed efficiency because it is phenotypically independent from body weight (BW) and growth rate; hence, differences in RFI between individuals reflect variations in metabolic processes rather than differences in production level (Nkrumah et al., 2006). Despite the great economic importance of RFI, the biological mechanisms regulating RFI divergence in beef cattle are not well understood. It is estimated that in beef cattle, rumen-related functions such as feed digestion and heat increment of fermentation contribute to 23% of RFI variation among individuals (Herd et al., 2004). Therefore, RFI ranking may be due to differences in rumen “function” between most- and least-efficient animals. In support of this notion, in a recent study our group revealed that feed-efficient cattle are characterized by transcriptional adaptations in ruminal epithelium to enhance absorption and metabolism of volatile fatty acids (VFA) (Elolimy et al., 2018).

In nonruminants it is well-known that the overall process of cellular protein synthesis is controlled largely by the mechanistic target of rapamycin (MTOR) signaling pathway, including ribosome biogenesis and RNA translation (Saxton and Sabatini, 2017). Exogenous signals such as systemic insulin concentration (Di Camillo et al., 2016), the supply of glucose and amino acids, and intracellular energy status are key regulators of MTOR (Buller et al., 2011; Dyachok et al., 2016). In contrast, the ubiquitin-proteasome system is the major pathway for protein degradation; hence, it plays a role in cellular protein homeostasis (Coleman and Crews, 2018).

An intricate balance between synthesis and degradation of protein, i.e., protein turnover, is crucial for animal growth (Silva-Vignato et al., 2017). Richardson and Herd (2004) estimated that protein turnover and tissue metabolism account for the largest proportion of variation in RFI among animals, i.e., 37% of the difference in RFI for beef cattle. Compared with less-efficient steers, a decrease in protein degradation in skeletal muscle was detected

during the finishing period in most-efficient beef steers (Blank et al., 2017). Whether pathways of cellular protein synthesis and degradation and related metabolic processes such as insulin signaling and nutrient transport differ in ruminal epithelium due to divergence in RFI is unknown. Therefore, the objective of the present study was to measure protein abundance and phosphorylation status of major proteins known to regulate cellular protein turnover in ruminal epithelium in finishing Red Angus cattle with high or low RFI.

MATERIALS AND METHODS

Animals, Experimental Design, and Diets

Institutional Animal Care and Use Committee of the University of Illinois approved animal handling and all procedures under protocol no. 12009. Three contemporaneous groups of Red Angus cattle (Group 1: 35 steers; Group 2: 37 heifers; Group 3: 80 steers) were used in this study. Details of the experimental design and diet composition have been described previously (Elolimy et al., 2018). After 70 d on RFI testing, RFI values for all animals were calculated based on dry matter intake, average daily gain, metabolic body weight, and 12th rib fat thickness. All animals were ranked by RFI coefficient, then the 2 lowest RFI animals and 2 highest RFI animals from each group were selected to form 2 RFI groups: most-efficient animals with the lowest RFI coefficient ($n = 6$) and least-efficient animals ($n = 6$) with the highest RFI coefficient.

Ruminal Epithelium and Blood Collection

At day 70 of RFI evaluation period, blood samples were obtained by coccygeal vein puncture from the selected animals before feeding. Blood samples were collected into evacuated tubes containing lithium heparin (Becton Dickinson Vacutainer, BD). Samples were placed on ice until centrifuged at $1,900 \times g$ for 15 min at 4 °C. Isolated plasma was collected and stored at -80°C until analysis for insulin concentrations using a bovine-specific commercial ELISA kit (Cat. 10-1201-01; Mercodia, Sweden). The selected animals were slaughtered after the finishing RFI testing period. Ruminal papillae from the ventral sac was immediately sampled at slaughter, then placed into liquid nitrogen and stored at -80°C until further processing.

Table 1. Proteins measured by western blot in ruminal epithelium tissue of most- ($n = 6$) and least- ($n = 6$) efficient beef cattle after 70 d on RFI testing during the finishing period

Metabolic process and symbol	Protein name
Protein synthesis	
MTOR	Total mechanistic target of rapamycin kinase
p-MTOR	Phosphorylated mechanistic target of rapamycin kinase
RPS6KB1	Total ribosomal protein S6 kinase B1
p-RPS6KB1	Phosphorylated ribosomal protein S6 kinase B1
EIF4EBP1	Total eukaryotic translation initiation factor 4E binding protein 1
p-EIF4EBP1	Phosphorylated eukaryotic translation initiation factor 4E binding protein 1
EIF2A	Total eukaryotic translation initiation factor 2A
p-EIF2A	Phosphorylated eukaryotic translation initiation factor 2A
EEF2K	Total eukaryotic elongation factor 2 kinase
p-EEF2K	Phosphorylated eukaryotic elongation factor 2 kinase
AKT1	Total AKT serine/threonine kinase 1
p-AKT1	Phosphorylated AKT serine/threonine kinase 1
PRKAA2	Total protein kinase AMP-activated catalytic subunit B2
RRAGA and RRAGB	Total ras related GTP binding A and B
MAPK3	Total mitogen-activated protein kinase 3
p-MAPK3	Phosphorylated mitogen-activated protein kinase 3
Ketogenesis	
ACAT1	Total acetyl-CoA acetyltransferase 1
HMGCS2	Total 3-hydroxy-3-methylglutaryl-CoA synthase 2
Protein degradation (ubiquitin-proteasome)	
MDM2	Total MDM2 proto-oncogene
UBA1	Total ubiquitin like modifier activating enzyme 1
NEDD4	Total neural precursor cell expressed, developmentally downregulated 4, E3 ubiquitin protein ligase
STUB1	Total STIP1 homology and U-box containing protein 1
Insulin signaling	
IRS1	Total insulin receptor substrate 1
p-IRS1	Phosphorylated insulin receptor substrate 1
INSR	Total insulin receptor
p-INSR	Phosphorylated insulin receptor
Insulin-induced glucose transport	
SLC2A4	Total solute carrier family 2 member 4
p-SLC2A4	Phosphorylated solute carrier family 2 member 4
Amino acid transport	
SLC1A3	Total solute carrier family 1 member 3
SLC1A5	Total solute carrier family 1 member 5
SLC38A1	Total solute carrier family 38 member 1
Internal controls	
TUBA1A	Total tubulin alpha 1A
ACTB	Total actin B

Western Blot Analysis

The relative abundance of proteins involved in protein synthesis, protein degradation, insulin signaling, and glucose and amino acid transporters in ruminal epithelium (Table 1) were detected using western blotting in the same fashion as described in one of our previous publications (Batistel et al., 2017). Briefly, a portion of ruminal epithelium was homogenized in T-PER tissue protein extraction reagent (Cat. 78510, Thermo Scientific). The lysate was centrifuged at $10,000 \times g$ for 10 min at 4 °C. The protein concentration of the supernatant was determined using pierce bicinchoninic acid (BCA) protein assay kit (Cat. 23227, Thermo Scientific). Fifty micrograms protein were separated using 4% to 20% Mini-Protean TGX precast gels (Cat. 456-1096, Bio-Rad) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Cat. 1620261, Bio-Rad). After blocking with 5% nonfat dry milk in tris-buffered saline at room temperature for 2 h, the membrane was washed and incubated with primary antibodies listed in Supplementary Table S1, and HRP affiniPure goat anti-rabbit IgG as the secondary antibody (ab6721, Abcam, diluted to 1:10,000). Visualization of the target proteins was performed using the Clarity Western ECL Substrate (Cat. 170-5060, Bio-Rad). Each protein was normalized against the geometric mean of tubulin alpha 1A (TUBA1A) and actin beta (ACTB), and data reported as relative expression. The ECL signals were recorded using an imaging system (ChemiDoc MP, Bio-Rad), and analyzed using Image lab 5.2.1 (Bio-Rad).

Statistical Analysis

Plasma insulin and ruminal protein expression data were analyzed using the MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC) where individual animal was the experimental unit. Model included the fixed effects of RFI category, sex, and $\text{RFI} \times \text{sex}$. Significance was declared at a false discovery rate-corrected $P \leq 0.05$, and trends toward significance were discussed at $0.05 < P \leq 0.10$. Because sex had no effect on plasma insulin and protein abundance ($P > 0.05$) it was removed from the model.

RESULTS

No differences ($P = 0.73$) for plasma insulin concentration were observed between most- and least-efficient groups after 70 d on RFI testing

Table 2. Plasma insulin concentration and relative abundance of ruminal epithelium proteins in most- ($n = 6$) and least- ($n = 6$) efficient beef cattle after 70 d on RFI testing during the finishing period

Protein	Least-efficient group	Most-efficient group	SEM ¹	<i>P</i> -value
Plasma insulin ($\mu\text{g/liter}$)	0.68	0.77	0.18	0.73
Ruminal epithelium proteins				
Protein synthesis ²				
MTOR	0.88 ^a	0.64 ^b	0.04	<0.01
p-MTOR	0.43 ^a	0.22 ^b	0.06	0.05
p-MTOR:MTOR	0.44	0.30	0.05	0.29
RPS6KB1	0.97 ^a	0.48 ^b	0.08	<0.01
p-RPS6KB1	0.42	0.36	0.06	0.46
p-RPS6KB1:RPS6KB1	0.49	0.74	0.10	0.10
EIF4EBP1	0.41	0.48	0.08	0.53
p-EIF4EBP1	0.18	0.16	0.02	0.46
p-EIF4EBP1:EIF4EBP1	0.27	0.34	0.08	0.54
EIF2A	1.64 ^a	1.13 ^b	0.08	<0.01
p-EIF2A	0.60	0.60	0.11	0.96
p-EIF2A:EIF2A	0.32 ^b	0.54 ^a	0.06	0.04
EEF2K	1.44 ^a	0.99 ^b	0.12	0.03
p-EEF2K	0.03 ^b	0.08 ^a	0.01	0.02
p-EEF2K:EEF2K	0.02 ^b	0.07 ^a	0.01	<0.01
AKT1	1.28 ^a	0.95 ^b	0.09	0.02
p-AKT1	0.04	0.03	0.01	0.66
p-AKT1:AKT1	0.03	0.03	0.01	0.40
PRKAA2	0.69	0.81	0.09	0.35
RRAGA/GB	1.66	1.47	0.15	0.41
MAPK3	1.98	1.55	0.15	0.07
p-MAPK3	0.40	0.62	0.16	0.33
p-MAPK3:MAPK3	0.24	0.40	0.10	0.10
Ketogenesis				
ACAT1	0.61	0.56	0.06	0.55
HMGCS2	1.68	1.71	0.09	0.19
Protein degradation (ubiquitin-proteasome)				
MDM2	0.19	0.11	0.03	0.06
UBA1	0.65 ^a	0.38 ^b	0.05	<0.01
NEDD4	0.26 ^a	0.05 ^b	0.05	0.01
STUB1	0.67 ^a	0.41 ^b	0.05	0.01
Insulin signaling				
IRS1	0.50	0.47	0.04	0.51
p-IRS1	0.13	0.13	0.03	0.97
p-IRS1:IRS1	0.24	0.28	0.05	0.62
Insulin-induced glucose transport				
SLC2A4	0.28 ^a	0.06 ^b	0.04	<0.01
Amino acid transport				
SLC1A3	0.52 ^a	0.37 ^b	0.04	0.01
SLC1A5	0.60 ^a	0.37 ^b	0.05	<0.01
SLC38A1	1.13	0.96	0.10	0.27

^{a,b}Means differ ($P \leq 0.05$).¹SEM = standard error of the mean.²p-SLC2A4, INSR, and p-INSR were not detectable with the antibodies used.

(Table 2). Among the proteins involved in cellular protein synthesis, MTOR ($P < 0.01$), p-MTOR ($P = 0.05$), RPS6KB1 ($P < 0.01$), EIF2A ($P < 0.01$), EEF2K ($P = 0.03$), AKT1 ($P = 0.02$), and RPS6KB1 ($P < 0.01$) were downregulated, whereas MAPK3

($P = 0.07$) tended to be downregulated in most-efficient animals (Table 2). In contrast, p-EEF2K ($P = 0.02$), p-EEF2K:EEF2K ($P < 0.01$), and p-EIF2A:EIF2A ($P = 0.04$) were upregulated in most-efficient animals (Table 2).

Among proteins catalyzing steps required for marking proteins for degradation, the abundance of UBA1 ($P < 0.01$), NEDD4 ($P = 0.01$), and STUB1 ($P = 0.01$) was lower and MDM2 tended to be lower ($P = 0.06$) in most-efficient cattle (Table 2). Among glucose transporters, the insulin-induced transporter SLC2A4 had lower abundance ($P < 0.01$) in most-efficient animals (Table 2). Similarly, abundance of the Na-dependent, high-affinity amino acid transporters SLC1A3 ($P = 0.01$) and SLC1A5 ($P < 0.01$) was lower in most-efficient cattle (Table 2).

DISCUSSION

In dairy cattle, greater dry matter intake (DMI) increases energy and amino acid (AA) levels in the circulation, resulting in the stimulation of protein synthesis in skeletal muscle and milk partly through the upregulation of MTOR signaling (Sadri et al., 2017; Wang et al., 2019). As most-efficient beef cattle with low RFI coefficient consume less feed (Koch et al., 1963), one could expect that those animals harvest less energy and nutrients. However, recent studies revealed that most-efficient beef cattle may possess several mechanisms to maximize the capture of dietary energy and nutrients from lower amounts of feed. For example, most-efficient animals shift rumen microbiome profiles in favor of species that can maximize digestibility and VFA production particularly butyrate and propionate (Shabat et al., 2016). In addition, transcriptome profiles of ruminal epithelium in most-efficient cattle suggested a greater functional and metabolic activity consistent with the notion those animals are better able to transport/absorb and metabolize VFA than less-efficient animals (Li and Guan, 2017; Elolimy et al., 2018). In addition to differences in cellular pathways within ruminal epithelium, research with sheep revealed that most-efficient animals had a longer duodenum (Zhang et al., 2017). Such feature would allow for a longer residence time of nutrients such as AA in the small intestine, which can lead to greater absorption and availability to peripheral tissues of most-efficient animals (Merlot et al., 2016).

In nonruminants, among MTOR signaling proteins, RPS6KB1 and EIF2A are important for stimulating mRNA translation (Jefferies et al., 1997; Kolitz and Lorsch, 2010), an event that sets the stage for further assembly of mature proteins in the endoplasmic reticulum in mammalian cells (Holcik and Sonenberg, 2005). The kinase EEF2K catalyzes the guanosine triphosphate-dependent translocation of

peptidyl-transfer RNA in the ribosome during translation elongation; therefore, it is an essential component of the protein synthesis machinery (Li et al., 2016). The kinase MAPK is responsible for the regulation of fundamental cellular processes including surfactant proteins biosynthesis, proliferation, and development. As such, it has been speculated that MAPK is crucial for enhancing the functional capacity of the ruminal epithelium (Yan et al., 2018).

In the present study, the greater ratio of phosphorylated to total protein abundance for RPS6KB1, EIF2A, EEF2K, and MAPK3 in the most-efficient animals suggests those pathways likely were more active in ruminal epithelium. As such, we speculate that those animals could have had more cell layers in ruminal epithelium to allow absorption of nutrients, e.g., butyrate (Lam et al., 2017; Elolimy et al., 2018). Coupled with lower abundance of SLC2A4 and AA transporters, the fact that MTOR activation did not differ in the most-efficient group seems to suggest that efficiency of protein synthesis rather than nutrient uptake per se is a key feature of ruminal epithelium in those animals. Although we did not measure glucose or AA in plasma, previous studies reported no change in plasma glucose in beef cattle divergent for RFI (Kelly et al., 2010; Bourgon et al., 2017). It is also possible that lower abundance of SLC2A4 and AA transporters in most-efficient animals is an inherent feature that favors utilization of these nutrients by peripheral tissues such as skeletal muscle and adipose (Clare et al., 2018). The lack of difference in abundance of key enzymes associated with ketogenesis (ACAT1, HMGCS2) and intracellular ATP sensing (PRKAA2) suggest a similar capacity for oxidative metabolism regardless of RFI. From that perspective, an important feature of ruminal epithelium in most-efficient animals seems to be a greater capacity for uptake of VFA through alterations in abundance of key transporters (Elolimy et al., 2018).

Among key regulators of intracellular protein degradation, MDM2 and UBA1 trigger the first step of the ubiquitin-proteasome pathway, which can induce apoptosis (Galli et al., 2010; Jiang et al., 2010). In rodents and humans, the STUB1 protein binds unfolded and misfolded proteins, whereas NEDD4 binds membrane proteins for proteasomal degradation (Wang et al., 2012; Luan et al., 2018). Thus, the activation of protein degradation when NEDD4 and STUB1 are induced could promote cellular apoptosis. Although it is unclear if the ubiquitin-proteasome pathway has a role in apoptosis in bovine gut, the fact that feed-efficient cattle have more cell layers in ruminal epithelium

(Lam et al., 2017) suggests that downregulation of these proteins favors cellular proliferation. Furthermore, the apparent lower degree of cellular protein degradation in the most-efficient group agrees with the upregulation of calpastatin in skeletal muscle of feed-efficient cattle (Blank et al., 2017). Clearly, a reduction in cellular protein degradation could dictate the overall state of protein turnover in a tissue such as rumen epithelium that has a high mitogenic potential (Sakata and Tamate, 1978). It is well-known that protein synthesis and protein degradation are energy-costly processes (Poos et al., 1999). Therefore, it can be speculated that a reduction in protein degradation in actively proliferating tissues like the ruminal epithelium may be important to minimize energy expenditure in favor of directing energy toward protein synthesis (de Figueiredo et al., 2018). In doing so, feed-efficient cattle can maximize the utilization of energy harvested from less DMI.

In conclusion, translation initiation and elongation during cellular protein synthesis coupled with lower protein degradation are important mechanisms in ruminal epithelium of feed-efficient cattle that allow efficient harvest of nutrients from less feed digested. By maintaining a lower abundance of insulin-sensitive nutrient transporters in ruminal epithelium, glucose and amino acids are readily available for peripheral tissue accretion in feed-efficient cattle.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

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