Proteasome activity and expression of mammalian target of rapamycin signaling factors in skeletal muscle of dairy cows supplemented with conjugated linoleic acids during early lactation

Y. Yang,¹ H. Sadri,^{2*†} C. Prehn,³ J. Adamski,^{3,4,5,6} J. Rehage,⁷ S. Dänicke,⁸ D. von Soosten,⁸ C. C. Metges,⁹ M. H. Ghaffari,¹ and H. Sauerwein¹

¹Institute of Animal Science, Physiology and Hygiene Unit, University of Bonn, 53115 Bonn, Germany

²Department of Clinical Science, Faculty of Veterinary Medicine, University of Tabriz, Tabriz 5166616471, Iran

³Research Unit Molecular Endocrinology and Metabolism, Genome Analysis Center, Helmholtz Zentrum München, German Research Center for Environmental Health, 85764 Neuherberg, Germany

⁴Lehrstuhl für Experimentelle Genetik, Technische Universität München, 85350 Freising-Weihenstephan, Germany

⁵German Center for Diabetes Research (DZD), 85764 München-Neuherberg, Germany

⁶Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, 8 Medical Drive, Singapore 117597, Singapore

⁷University for Veterinary Medicine, Foundation, Clinic for Cattle, 30173 Hannover, Germany

⁸Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), 38116 Braunschweig, Germany

⁹Leibniz Institute for Farm Animal Biology (FBN), Institute of Nutritional Physiology “Oskar Kellner,” 18196 Dummerstorf, Germany

ABSTRACT

The mammalian target of rapamycin (mTOR) is a major regulator of protein synthesis via its main downstream effectors, ribosomal protein S6 kinase (S6K1) and eukaryotic initiation factor 4E binding protein (4EBP1). The ubiquitin-proteasome system (UPS) is the main proteolytic pathway in muscle, and the muscle-specific ligases tripartite motif containing 63 (TRIM63; also called muscle-specific ring-finger protein 1, MuRF-1) and F-box only protein 32 (FBXO32; also called atrogin-1) are important components of the UPS. We investigated 20S proteasome activity and mRNA expression of key components of mTOR signaling and UPS in skeletal muscle of dairy cows during late gestation and early lactation and tested the effects of dietary supplementation (from d 1 in milk) with conjugated linoleic acids (sCLA; 100 g/d; n = 11) compared with control fat-supplemented cows (CTR; n = 10). Blood and muscle tissue (semitendinosus) samples were collected on d –21, 1, 21, and 70 relative to parturition. Dry matter intake increased with time of lactation in both groups. It was lower in sCLA than in CTR on d 21, which resulted in a reduced calculated metabolizable protein balance. Most serum and muscle concentrations of AA followed time-related changes but were unaffected by CLA supplementation. In both groups,

serum and muscle 3-methylhistidine (3-MH) concentrations and the ratio of 3-MH:creatinine increased from d –21 to d 1, followed by a decline on d 21. The mRNA abundance of *MTOR* on d 21 and 70 was greater in sCLA than in CTR. The abundance of *4EBP1* mRNA did not differ between groups but was upregulated in both on d 1. The mRNA abundance of *S6K1* on d 70 was greater in CTR than in sCLA, but remained unchanged over time in both groups. The mRNA abundance of *FBXO32* (encoding atrogin-1) on d 21 was greater in sCLA than in CTR. The mRNA abundance of *TRIM63* (also known as *MuRF1*) showed a similar pattern as *FBXO32* in both groups: an increase from d –21 to d 1, followed by a decline. The mRNA for the α (*BCKDHA*) and β (*BCKDHB*) polypeptide of branched-chain α -keto acid dehydrogenase was elevated in sCLA and CTR cows on d 21, respectively, suggesting a role of CLA in determining the metabolic fate of branched-chain AA. For the mTOR protein, no group differences were observed. The abundance of S6K1 protein was greater across all time points in sCLA versus CTR. The antepartum 20S proteasome activity in muscle was elevated in both groups compared with postpartum, probably reflecting the start of protein mobilization before parturition. Plasma insulin concentrations decreased in both groups postpartum but to a greater extent in CTR than in sCLA, resulting in greater insulin concentrations in sCLA than in CTR. Thus, the greater abundance of *MTOR* mRNA and S6K1 protein in sCLA compared with CTR might be mediated by the greater plasma insulin postpartum. The upregulation of *MTOR* mRNA in sCLA cows on d 21, despite greater *FBXO32* mRNA abundance, may reflect a simultaneous activation of both anabolic and

Received July 11, 2019.

Accepted November 21, 2019.

*Corresponding author: sadri@tabrizu.ac.ir

†H. Sadri was a visiting scientist at the Institute of Animal Science, Physiology and Hygiene Unit, University of Bonn, 53115 Bonn, Germany, at the time the research was done.

catabolic signaling pathways, likely resulting in greater protein turnover.

Key words: conjugated linoleic acid, mammalian target of rapamycin, ubiquitin-proteasome system, muscle, dairy cow

INTRODUCTION

The periparturient period in dairy cows is characterized by extensive changes in metabolic, endocrine, and immune functions (Drackley, 1999). Insufficient nutrient intake during the first weeks of lactation and increased nutrient demand for milk synthesis at the onset of lactation commonly result in a negative nutrient balance. The success of adaptation to these physiological changes is highly variable among individual cows, and compromised adaptation may result in production diseases (Drackley, 1999; Sundrum, 2015). Besides fat, mobilization of body protein reserves is also necessary to overcome phases of negative nutrient balance. Excessive mobilization of body fat and protein can be associated with increased incidence of metabolic disorders, immune dysfunction, and poor reproductive performance (Ji and Dann, 2013). Mobilization of skeletal muscle protein, the main labile source of AA, in the adaptation to early lactation of dairy cows has rarely been studied (Komaragiri et al., 1998; Chibisa et al., 2008; Kuhla et al., 2011). Estimates of the amount of body protein loss in early lactation range between approximately 9 kg from 14 d prepartum to 15 d postpartum (Chibisa et al., 2008) and 21 to 24 kg from 14 d prepartum to 35 d postpartum (Komaragiri et al., 1998). Interestingly, the mobilization of body protein in most cows seems to start antepartum, even before the onset of lipid mobilization (van der Drift et al., 2012).

In recent decades, interest in the use of CLA supplements in human and animal nutrition has increased due to the many bioactive health-related properties of CLA. Supplementation with CLA represents a dietary strategy for reducing the milk fat content in dairy cows while improving energy balance, especially during early lactation (Sippel et al., 2009; Schlegel et al., 2012; Qin et al., 2018). The CLA effects comprise inhibition of de novo fatty acids (FA) synthesis in the mammary gland as well as reduction of FA uptake from triacylglycerol-rich lipoproteins by inhibiting lipoprotein lipase activity (Bauman et al., 2011). Prior research also showed that CLA reduces endogenous glucose production, which might indicate lower glucose utilization for milk fat synthesis (Hötger et al., 2013). The mechanisms of action are not entirely clear. Further studies have shown that *trans*-10,*cis*-12 CLA isomer is the most effective CLA isomer for milk fat reduction (Bauman et al., 2011). Among the various benefits

claimed for human health, only reductions in body fat and weight have supporting evidence, as reviewed by McCrorie et al. (2011). Although most studies in CLA have focused on body fat reduction, considerable evidence supports a concurrent increase in lean body mass, body proteins, or specific skeletal muscle mass (Kennedy et al., 2010; Dilzer and Park, 2012; Park, 2014). In mice, CLA was confirmed to lead to increased total protein content (not only %) as a representation of lean mass in mice (Park et al., 1997). Some researchers have suggested that CLA supplementation leads to repartitioning of nutrients in the body, with less body fat and more lean mass (Stangl, 2000). This conclusion is further supported by a study using CLA supplementation in a mouse model (Park et al., 1999), in which an increase in lean muscle mass preceded a reduction in fat mass. Together, these findings suggest a potentially significant role of muscle in the effects of CLA on body composition. The regulation of skeletal muscle mass depends on the balance between protein synthesis and degradation (Atherton and Smith, 2012). The mammalian target of rapamycin (**mTOR**) and ubiquitin-proteasome system (**UPS**) are considered the major regulators of protein synthesis and protein degradation (You et al., 2015; Bilodeau et al., 2016), which are proposed to be coordinately regulated (Schiaffino et al., 2013). The mTOR complex 1 (**mTORC1**) is a nutrient sensor, in particular of AA, and activates protein synthesis through its main downstream effectors, ribosomal protein S6 kinase (**S6K1**) and eukaryotic initiation factor 4E binding protein (**4EBP1**; You et al., 2015). Interestingly, previous studies have shown the effects of CLA on the signaling pathway mTOR in human adipocytes (Chung et al., 2005) and HepG2 cells (Go et al., 2013), although its potential effects on the mTOR pathway in muscle are not clear yet. The UPS-mediated catabolism is also essential to maintain free AA pools in acute starvation, and it contributes significantly to the degradation of defective proteins (Ciechanover, 2005). The UPS requires the coordinated reactions of 3 enzymes, including ubiquitin-activating enzyme (**E1**), ubiquitin-conjugating enzyme (**E2**), and ubiquitin ligases (**E3**; Nandi et al., 2006). The targeted proteins for degradation by the UPS are first “tagged” in the ubiquitination pathway and are later proteolyzed by the proteasome enzyme complex (Nandi et al., 2006; Bodine and Baehr, 2014). The proteasome enzyme complexes are formed by the combination of the catalytic 20S proteasome with 19S regulators in an ATP-dependent manner (Nandi et al., 2006).

Although CLA have attracted interest because of their health-related benefits, few studies have examined gene expression related to protein synthesis and degradation in skeletal muscle. To our knowledge, the question of

whether CLA supplements affect skeletal muscle protein metabolism in dairy cows has not been answered. In consideration of the CLA-induced decrease of body fat and evidence from human and laboratory animal studies supporting a concurrent increase in lean body mass, body protein, or specific skeletal muscle mass, we hypothesized that dietary CLA supplemented during early lactation may affect the regulation of specific signaling components in skeletal muscle of dairy cows in support of protein synthesis and decreased protein degradation. Therefore, the objective of this study was to investigate the effects of a rumen-protected CLA supplement on the 20S proteasome activity and mRNA expression of key components of mTOR signaling and UPS in skeletal muscle of dairy cows during late gestation and early lactation.

MATERIALS AND METHODS

Animals, Treatment, and Experimental Design

All animal experiments were conducted according to the European Community regulations on the protection of experimental animals and the guidelines of the LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Germany, File Number 33.14.42502-04-071/07). The current experiment was part of a larger study reported elsewhere (Pappritz et al., 2011). A subset of animals and samples from that study (21 multiparous Holstein cows) were used in the current study. Details of the experimental design, feed analyses, and feeding management of cows have been described previously (Pappritz et al., 2011; Supplemental Table S1, <https://doi.org/10.3168/jds.2019-17244>). In brief, cows were fed ad libitum with a partial mixed ration (PMR), according to the recommendations of the German Society of Nutrition Physiology (GfE, 2001). On a DM basis, the PMR (6.8 MJ of NE_L/kg of DM) contained 37.8% corn silage, 25.2% grass silage, and 37% concentrate. Cows were assigned to 2 groups on the day after calving: a control (CTR; n = 10) group and a supplemental CLA (sCLA; n = 11) group. The encapsulated rumen-protected CLA (100 g per cow/d; Lutrell Pure, BASF SE, Ludwigshafen, Germany) was added to the diet to supply 7.6 g of *cis*-9,*trans*-11 CLA and 7.6 g of *trans*-10,*cis*-12 CLA per day. The animals in the CTR group received a diet that included 100 g/d of rumen-protected control fat supplement (Silafat, BASF) in which CLA was substituted by stearic acid (C18:0) to form an isoenergetic control diet. From DIM 1 throughout the observation period, the fat supplements were given with 4 kg of additional concentrate (8.8 MJ of NE_L/kg of DM) consisting of the same components as the concentrate used in the PMR.

Composite PMR, corn silage, grass silage, and concentrate samples were analyzed for their AA contents by Evonik Nutrition & Care GmbH (Hanau, Germany) using ion-exchange chromatography with postcolumn derivatization with ninhydrin (AOAC International, 1995; European Commission, 2009). Amino acids were oxidized with performic acid, which was neutralized with sodium metabisulfite. Amino acids were liberated from the protein by hydrolysis with 6 N HCl for 24 h at 110°C and quantified with the internal standard by measuring the absorption of reaction products with ninhydrin at 570 nm. Tryptophan was determined by HPLC with fluorescence detection (extinction 280 nm, emission 356 nm) after alkaline hydrolysis with barium hydroxide octahydrate for 20 h at 110°C.

Blood and Muscle Tissue Sampling

Blood samples were collected via puncture of the jugular vein using evacuated tubes on d -21, 1, 21, and 70 relative to parturition. Blood was sampled after the morning milking before cows had access to the new fresh ration and before collecting muscle biopsies (see below). The centrifugation (at 4°C and 3,000 × g for 10 min) of serum and plasma (heparin and EDTA) was performed after sampling, and the samples were stored at -80°C until analyzed.

Muscle tissue (semitendinosus) samples were collected on d -21, 1, 21, and 70 relative to parturition. Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until time of analysis.

Determination of 3-Methylhistidine, Creatinine, and Free AA Concentrations in Serum and Skeletal Muscle

Serum and muscle free 3-methylhistidine (3-MH) concentrations were analyzed by HPLC as described previously (Kuhla et al., 2010, 2011). The AA profiles and creatinine in serum and skeletal muscle were determined by liquid chromatography-electrospray ionization-tandem MS profiling through targeted metabolomics using the AbsoluteIDQ p180 Kit (Biocrates Life Sciences AG, Innsbruck, Austria). This kit was validated according to the European Medicines Agency guidelines (EMA Quality Guidelines), which implies a proof of reproducibility within a given error range (CHMP, 2011). The analyses were performed in the Helmholtz Zentrum München (GmbH), German Research Center for Environmental Health, Genome Analysis Center. For serum, 10 µL of the thawed sample was applied directly to the assay. For muscle, 25 mg of each frozen sample was homogenized and extracted using homog-

enization tubes with ceramic beads (1.4 mm) and a Precellys 24 homogenizer with an integrated cooling unit (PEQLAB Biotechnology GmbH, Erlangen, Germany). Three microliters of a dry ice-cooled mixture of ethanol/phosphate buffer (85/15 vol/vol) was added to each milligram of frozen muscle tissue. After centrifugation, 10 μ L of the homogenate supernatant was applied to the well plate of the p180 Kit. The assay procedures of the AbsoluteIDQ p180 Kit, the tissue preparation, and the metabolite nomenclature were described in detail elsewhere (Zukunft et al., 2013, 2018). The limit of detection was set to 3 times the values of zero samples (PBS for serum, ethanol/phosphate buffer [85/15 vol/vol] for muscle). Sample handling was performed by a Hamilton Microlab STAR robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a graphyvap nitrogen evaporator (Porvair Sciences, Leatherhead, UK), besides standard laboratory equipment. Mass spectrometric analyses were done on an API 4,000 triple quadrupole system (Sciex Deutschland GmbH, Darmstadt, Germany) equipped with a 1,200 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) controlled by the software Analyst 1.6.1. Data evaluation for quantification of metabolite concentrations and quality assessment were performed with the MetIDQ software package, which is an integral part of the AbsoluteIDQ Kit. Internal standards were used as a reference for the calculation of metabolite concentrations. The concentrations of the metabolites in the serum samples are given in micromoles per liter, the concentrations of the metabolites in the tissue samples in picomoles per milligram of tissue, and the concentrations of the metabolites in the tissue homogenate in micromoles per liter.

Determination of Insulin and IGF-1 in Plasma

Plasma insulin was measured by a commercially available double antibody radioimmunoassay (DSL-1600, Diagnostic Systems Laboratories Inc., Webster, TX). The intra- and interassay CV were 6.3 and 8.8%, respectively (Singh et al., 2014). Plasma IGF-1 was analyzed using 2-site immunoradiometric assay (DSL-5600 Active IGF-IIRMA; Diagnostic Systems Laboratories Inc.). The intra- and interassay CV were between 3.4 and 8.2%, respectively (Singh et al., 2014).

RNA Extraction and Quantitative Real-Time Reverse-Transcription PCR

Protocols for RNA extraction from tissue samples as well as for cDNA preparation were described in detail

previously (Saremi et al., 2012a,b; Sadri et al., 2015). The sequences of the primer pairs and the real-time PCR conditions are shown in Table 1. Analysis by quantitative real-time reverse-transcription PCR was performed in an Mx3000P cycler (Agilent, Santa Clara, CA) and in accordance with the MIQE guidelines (Bustin et al., 2009).

The RT-PCR amplifications were set up in a total volume of 10 μ L consisting of 2 μ L of cDNA (diluted 1:4) as a template, 1 μ L of primer mix, 2 μ L of water, and 5 μ L of the DyNAmo ColorFlash SYBR Green qPCR Kit master mix (Thermo Scientific, Dreieich, Germany). Each PCR run contained a negative-template control for quantitative PCR, as well as a negative-template control and no-reverse-transcriptase control of cDNA. A standard curve was generated with a serial-diluted cDNA as the template to calculate efficiency-corrected relative quantities of the targets (run-specific target amplification efficiency). A set of 2 interrun calibrators was used for each PCR plate to correct interrun variation. Four reference genes (Saremi et al., 2012a)—emerin (*EMD*), RNA polymerase II (*POLR2A*), eukaryotic translation initiation factor 3 (*EIF3K*), and low-density lipoprotein receptor-related protein 10 (*LRP10*)—were used to normalize gene expression data with qBasePLUS 2.0 (Biogazelle, Ghent, Belgium). The output data from the software were calibrated normalized relative quantities.

Quantification of Target Proteins

The detailed description of the tissue preparation has been described in detail previously (Webb et al., 2019). Briefly, muscle tissue samples were homogenized in ice-cold Tris/HCl lysis buffer (pH 7.4) containing protease inhibitors cOmplete, Mini Protease Inhibitor Cocktail, 1 tablet per 10 mL (Roche Diagnostics, Mannheim, Germany); Pefabloc SC, also known as 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1 mM (Sigma Aldrich, St. Louis, MO) for inhibiting serine, cysteine, and metalloproteases in a tissue-to-buffer ratio of 1:4 using the Precellys 24 system (VWR/Peqlab Biotechnology, Erlangen, Germany). Total protein concentration was quantified using the Bradford assay (Roti Nanoquant K880, Carl Roth, Karlsruhe, Germany).

Protein quantification was performed using the Simple Western size-based protein assay (WES, ProteinSimple, San Jose, CA), an automated capillary-based electrophoretic immunoassay, according to the manufacturer's instructions (https://www.proteinsimple.com/technical_library.html). Briefly, samples were first diluted with 0.1 $\times$ sample buffer to adjust the protein concentration to a dilution within the linear

Table 1. Characteristics of primers and real-time PCR conditions

Gene ¹	Sequence (5'-3')	NCBI accession no.	Length (bp)	Annealing conditions ²	Mean efficiency (%)
<i>MTOR</i>					
Forward	CGAAACCTGGATGTCCCAA	XM_002694043.2	94	30 s, 61°C	96.1
Reverse	AGGACACCAGCCAATGTAGC				
<i>4EBP1</i>					
Forward	CCCTGGAGGTACCAGGATCA	NM_001077893.2	125	60 s, 62°C	103.3
Reverse	CATCGCCTGTAGGGCTAGTG				
<i>S6K1</i>					
Forward	CGGAACAGTCAACACACCT	NM_205816.1	97	30 s, 61°C	100.1
Reverse	ACTCCACCAATCCACAGCAC				
<i>UBA1</i>					
Forward	GGGGAACCGGCATTGATGT	NM_001102477.1	118	60 s, 59°C	92.2
Reverse	AGGGCACTTCGGACAATACG				
<i>UBE2G1</i>					
Forward	TATGCTGGCAGACCCCAATG	NM_001082458.1	109	60 s, 59°C	103.3
Reverse	TCTTACACAGCGGGCAACTT				
<i>UBE2G2</i>					
Forward	TCACCCCAACATCTACCCAGA	NM_001076328.1	129	60 s, 59°C	96.2
Reverse	AGGAGGATCTTCTCCACGCT				
<i>FBXO32</i>					
Forward	GAAACGCTTCTCGGACGAGA	NM_001046155.1	125	30 s, 59°C	92.7
Reverse	TCTTCTTGGCTGCAACGTCA				
<i>TRIM63</i>					
Forward	CCTGATCCAGGATGGAAACCC	NM_001046295.1	149	60 s, 59°C	89.6
Reverse	CAGCCTGCTGGAAGATGTCGT				
<i>BCKDHA</i>					
Forward	AGAACCAGCCCTTCTCATT	NM_174506.1	108	30 s, 61°C	89
Reverse	TGTCCCAGTAGTTGACCTCGT				
<i>BCKDHB</i>					
Forward	GGCCAAAGATCCTACGGCAGTAAT	NM_174507.2	129	30 s, 61°C	96.7
Reverse	CCTTGTTACACAGTGGGGT				
<i>LRP10</i>					
Forward	CCAGAGGATGAGGACGATGT	BC149232	125	45 s, 59°C	98.4
Reverse	ATAGGGTTGCTGTCCCTGTG				
<i>POL2RA</i>					
Forward	GAAGGGGGAGAGACAACTG	X63564	86	60 s, 60°C	100.4
Reverse	GGGAGGAAGAAGAAAAAGGG				
<i>EMD</i>					
Forward	GCCCTCAGCTTCACTCTCAGA	NM_203361	100	45 s, 59°C	95.5
Reverse	GAGGCGTTCCCGATCCTT				
<i>EIF3K</i>					
Forward	CCAGGCCCCACCAAGAAGAA	NM_001034489	180	60 s, 59°C	89.8
Reverse	TTATACCTTCCAGGAGGTCCATGT				

¹*MTOR* = mammalian target of rapamycin; *4EBP1* = eukaryotic translation initiation factor 4E binding protein 1; *S6K1* = ribosomal protein S6 kinase, polypeptide 1; *UBA1* = ubiquitin-like modifier activating enzyme 1; *UBE2G1* = ubiquitin-conjugating enzyme E2G 1; *UBE2G2* = ubiquitin-conjugating enzyme E2G 2; *FBXO32* = F-box only protein 32 (atrogin-1); *TRIM63* = tripartite motif containing 63 (also called muscle ring-finger protein-1, *MuRF1*); *BCKDHA* = branched-chain α -keto acid dehydrogenase E1, α polypeptide; *BCKDHB* = branched-chain α -keto acid dehydrogenase E1, β polypeptide; *LRP10* = lipoprotein receptor-related protein 10; *POL2RA* = RNA polymerase II; *EMD* = emerlin; *EIF3K* = eukaryotic translation initiation factor 3 subunit K.

²Initial denaturation for 10 min at 90°C; denaturation for 30 s at 95°C; extension for 30 s at 72°C, except for *4EBP1*, *UBA1*, *UBE2G1*, *UBE2G2*, *TRIM63* (60 s at 72°C), and *LRP10* (20 s at 72°C).

range (0.5 mg/mL), and then combined with a provided sample master mix consisting of 1× sample buffer, 1× fluorescent molecular weight markers, and 40 mmol/L dithiothreitol. The final samples were vortex mixed and heated for 5 min at 95°C, placed on ice for 5 min, briefly centrifuged twice (2,500 × *g*, 5 s, 20°C) with short vortex spins in between, and applied to the wells in duplicates. A control sample (bovine muscle) was also loaded on every plate to correct for interrun variations (accepted intra-assay CV <8%, interassay

CV <15%). Primary antibodies against total mTOR (CST2983; Cell Signaling Technologies, Danvers, MA), 4EBP1 (CST9452; Cell Signaling Technologies), S6K1 (CST2708; Cell Signaling Technologies), and TRIM63/MuRF-1 (AF5366, R&D Systems Inc., Minneapolis, MN) were diluted 1:100, 1:50, 1:10, and 1:25, respectively. All other reagents, including antibody diluent, secondary antibodies [except in case of FBXO32/atrogin-1 (goat IgG HRP-conjugated Antibody; HAF109, R&D Systems, Inc., diluted 1:50)], streptavidin-HRP,

luminol-S, and hydrogen peroxide, loaded on the plate were obtained from ProteinSimple and used according to instructions. Simple Western analysis was performed with multi-image exposures and otherwise instrument default settings at room temperature. Using Compass Software (WES, ProteinSimple), the area under the curve, which represents the signal intensity of the chemiluminescent immunoreaction and directly correlates with the abundance of the target protein, was assessed for each sample and normalized to the control. The analyses had to be limited to the total form of proteins and phosphorylated forms could not be assessed because the muscle samples had been used for other analyses and the amount of tissue remaining was insufficient for a reliable analysis.

20S Proteasome Activity

The muscle samples were homogenized in 4 volumes of ice-cold HEPES buffer (pH 7.5) consisting of 5 mM EDTA, 5 mM MgCl₂, and 5 mM dithiothreitol followed by centrifugation at $14,000 \times g$ for 30 min at 4°C as previously reported (Korzick et al., 2013). The proteasome enzymatic activity was measured by using the BML-AK470 kit (Enzo Life Sciences, Lausen, Switzerland) following the manufacturer's instructions. Briefly, determination of the chymotrypsin-like protease activity of the 20S proteasome in samples was initiated by adding a fluorogenic peptidyl substrate succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC) to homogenized muscle samples. This substrate was cleaved by the proteasome activity, and the subsequently released free AMC was then detected by a fluorometer (excitation = 360 nm, emission = 460 nm). The fluorescence signal was monitored before and at 10-min intervals for 2 h at 37°C. The changes in fluorescence signal were normalized to the amount of protein used in the assay and all assays were in the linear range. Results are expressed as nanomoles per minute per milligram of protein.

Calculations and Statistical Analysis

The diet used for the experimental cows was evaluated by the NittanyCow Dairy Ration Evaluator (Mifflinburg, PA) to estimate the supply of the different protein fractions and AA balances using actual DMI, milk yield, milk composition, BCS, and BW of the cows at days in milk 1, 21, and 70 according to the National Research Council (NRC) model (NRC, 2001), as follows. Dietary requirements are expressed as RDP and RUP, and metabolic requirements are expressed as MP. Small intestinal protein supply (MP) is equal to the di-

gestible fraction of microbial proteins plus the digestible fraction of undegraded feed proteins (RUP). Microbial protein is considered to contain 80% true protein with 80% intestinal digestibility. Ruminally undegraded feed protein is assumed to be 100% true protein with 50 to 100% intestinal digestibility (dependent on feed type). The MP requirement includes needs for maintenance [urinary endogenous N + scurf N (skin, skin secretions, and hair)] and production (conceptus, growth, and lactation).

Statistical analysis of the data was carried out using SAS software (version 9.3; SAS Institute Inc., Cary, NC). The data were tested for normality before analysis using the UNIVARIATE procedure. When the data were not normally distributed, they were transformed using a log₁₀ transformation before analysis. The data were analyzed using repeated measures in the MIXED procedure of SAS. The model included treatment, time, and interaction of treatment $\times$ time as the fixed effects and cow as the random effect. The first-order autoregressive covariance structure (AR1) was determined as the most appropriate covariance structure for all repeated statements according to the Akaike's information criterion and Bayesian information criterion. The Tukey-Kramer adjustment was applied to account for multiple comparisons. The threshold of significance was set at $P \leq 0.05$, and trends were declared at $0.05 < P \leq 0.10$.

RESULTS

DMI, BW, Milk Yield and Milk Components, BCS, and Calculated MP Balance

Dry matter intake, milk yield, milk protein percentage, BW, BCS (modified from Pappritz et al., 2011), and calculated MP balance are presented in Figure 1. During the postpartum period, DMI was 22.3 ± 0.24 kg/d (mean $\pm$ SEM) and 21.2 ± 0.24 kg/d in the CTR and sCLA groups, respectively. The DMI increased ($P < 0.01$) with time of lactation in both groups, and an interaction of treatment $\times$ time was observed ($P < 0.01$). The DMI on d 21 was lower in sCLA than in CTR ($P < 0.01$). Weekly milk yield (wk 1–10) did not differ between the groups, but changed over time ($P < 0.01$), and no treatment $\times$ time interaction occurred. The dietary supplementation of CLA during early lactation resulted in a gradual decrease in milk fat percentage and led to a significant 12% reduction from wk 8 of supplementation onward, thus confirming the effectiveness of the sCLA treatment, as described previously (Pappritz et al., 2011). Milk protein percentage was affected by treatment ($P = 0.05$) and time ($P < 0.01$),

Figure 1. Dry matter intake, BW, milk yield, MP balance, BCS, and milk protein percentage in dairy cows receiving CLA (sCLA) or a control fat supplement (CTR) from d 1 of lactation (modified from Pappritz et al., 2011). Asterisks indicate differences ($P < 0.01$) between sCLA and CTR at a given time point. Data are presented as means $\pm$ SEM.

and a trend was found for a treatment $\times$ time interaction ($P = 0.10$). The mean percentage of milk protein across all time points in sCLA was slightly lower than in CTR (3.02 ± 0.02 versus 3.04 ± 0.02 ; means $\pm$ SE). Body weight and BCS were not affected by treatment or by a treatment $\times$ time interaction, but they changed over time ($P < 0.01$) and followed a similar pattern in both groups. The BCS during the postpartum period

was lower than prepartum. The effect of CLA on MP balance depended on time in lactation ($P = 0.02$). The MP balance was estimated to be about -429 ± 74 g/d (mean $\pm$ SEM) and -201 ± 89 g/d on d 1 in sCLA and CTR, respectively. The sCLA group was estimated to be still deficient in MP intake on d 21 (-72 ± 56 g/d), whereas the animals of the CTR group reached a positive calculated MP intake balance on d 21 ($377 \pm$

decreased ($P < 0.05$) from d -21 to d 1. In case of Gln they remained unchanged thereafter in both groups, whereas increased Glu concentrations ($P < 0.05$) were observed on d 21 and 70 compared with d 1 in both groups. For the concentrations of Ala, Cit, Gly, Pro, Ser, and total AA in muscle tissue from both groups, no time-related differences were found during the study.

Amino Acid Profiles in Serum

As shown in Table 3, the AA concentrations in serum were not affected by CLA. For Ala, Asn (sCLA group only), Asp, Cit, Gly, Orn, Thr, Trp, Tyr, total AA, as well as Glu, Phe, and Val (CTR group only), changes over time ($P < 0.01$) were observed. In both groups, the concentrations of Arg, Gln, His, Ile, Leu, Lys, Met, Pro, Ser, BCAA, and total AA did not change with time during the course of the study.

As shown in Figure 2, the serum and muscle concentrations of 3-MH and the serum ratio 3-MH:creatinine

Item	Time (days relative to parturition)					
	−21		1		21	
	Pretreatment		sCLA		CTR	
Ala	938 ± 49.7	989 ± 67	1,166 ± 140	735 ± 46	1,125 ± 119	910 ± 115
Arg	29.2 ± 4.14 ^{B,b}	126 ± 11.9 ^{A,a}	35.3 ± 5.64 ^B	36.8 ± 5.65 ^b	34.2 ± 4.49 ^B	31.8 ± 11.4 ^b
Asn	58.6 ± 6.97 ^B	90.3 ± 4.93 ^A	79.9 ± 11.1 ^{AB}	75.0 ± 5.76	90.5 ± 11.9 ^{AB}	62.8 ± 11.06
Asp	110 ± 17.0 ^{B,b}	97.5 ± 9.34 ^{B,b}	275 ± 22.2 ^A	264 ± 20.0 ^a	198 ± 15.6 ^{A,y}	119 ± 17.5 ^{b,z}
Cit	17.1 ± 2.14	26.6 ± 1.62	19.5 ± 1.69	20.8 ± 3.14	28.1 ± 1.81	16.7 ± 1.93
Gln	4,573 ± 466 ^{A,a}	1,552 ± 188 ^{B,b}	1,216 ± 178 ^B	1,153 ± 206 ^b	2,304 ± 412 ^B	1,680 ± 290 ^b
Glu	1,287 ± 114 ^{AB,b}	1,017 ± 144 ^{B,b}	2,416 ± 413 ^A	3,309 ± 388 ^a	1,934 ± 261 ^A	1,748 ± 290 ^{ab}
Gly	1,233 ± 107	1,203 ± 64	1,226 ± 164	1,103 ± 55	1,430 ± 240	1,261 ± 310
His	42.2 ± 6.81 ^{C,c}	112 ± 8.83 ^{A,a}	95.1 ± 18.8 ^{AB}	95.6 ± 11.7 ^{ab}	49.5 ± 3.97 ^{BC}	50.5 ± 9.39 ^{bc}
Ile	81.0 ± 5.17 ^{B,b}	155 ± 9.01 ^{A,a}	178 ± 15.9 ^A	153 ± 14.6 ^a	105 ± 8.54 ^B	99.8 ± 4.3 ^b
Leu	95.1 ± 8.31 ^{B,b}	196 ± 13.4 ^{A,a}	170 ± 11.9 ^A	158 ± 13.5 ^a	104 ± 5.89 ^B	120 ± 9.33 ^{ab}
Lys	34.1 ± 7.05 ^{C,c}	251 ± 23.7 ^{A,a}	112 ± 13.1 ^{AB}	116 ± 11.7 ^{ab}	49.0 ± 7.91 ^{BC}	75.8 ± 24.9 ^{bc}
Met	24.1 ± 5.06 ^{B,c}	78.9 ± 6.21 ^{A,a}	54.2 ± 9.73 ^A	42.9 ± 4.54 ^{ab}	31.5 ± 9.95 ^B	31.4 ± 3.3 ^{bc}
Orn	29.7 ± 5.65 ^{A,a}	13.7 ± 3.67 ^{AB,b}	4.94 ± 1.21 ^B	6.4 ± 1.28 ^b	24.5 ± 5.39 ^a	12.4 ± 4.12 ^{ab}
Phe	40.0 ± 3.53 ^{B,b}	94.0 ± 5.78 ^{A,ab}	65.9 ± 5.4 ^A	59.3 ± 4.81 ^b	34.9 ± 1.76 ^B	49.8 ± 6.61 ^b
Pro	157 ± 11.4	197 ± 8.51	147 ± 11.6	129 ± 8.41	163 ± 21.9	130 ± 15.6
Ser	209 ± 18.3	262 ± 14.0	219 ± 35.3	209 ± 11.9	184 ± 9.31	211 ± 23.1
Thr	137 ± 8.99 ^B	186 ± 10.6 ^A	155 ± 16.5 ^{AB}	149 ± 8.38	193 ± 28.8 ^{AB}	169 ± 20.8
Trp	5.14 ± 1.16 ^{B,b}	15.7 ± 1.53 ^{A,a}	17.1 ± 2.09 ^A	16.2 ± 1.82 ^a	11.6 ± 1.59 ^A	11.6 ± 1.15 ^a
Tyr	46.5 ± 3.01 ^{C,c}	99.7 ± 6.38 ^{A,a}	73.4 ± 6.99 ^{AB}	73.0 ± 5.07 ^{ab}	50.2 ± 3.26 ^{BC}	61.0 ± 7.15 ^{bc}
Val	159 ± 9.74 ^{B,b}	238 ± 13.1 ^{A,ab}	252 ± 10.4 ^A	252 ± 16.0 ^a	231 ± 16.21 ^A	203 ± 12.3 ^{ab}
BCAA ¹	335 ± 21.9 ^{C,b}	590 ± 34.0 ^{AB,a}	600 ± 28.3 ^A	563 ± 41.1 ^a	440 ± 27.8 ^B	423 ± 18.7 ^{ab}
EAA	617 ± 39.7 ^{C,c}	1,327 ± 63.2 ^{A,a}	1,099 ± 68.6 ^B	1,041 ± 75.1 ^{ab}	804 ± 44.9 ^B	811 ± 61.3 ^{bc}
Total AA	9,306 ± 6,477	6,999 ± 295	7,978 ± 655	8,155 ± 538	8,368 ± 969	7,054 ± 797

¹BCAA = branched-chain amino acid, consisting of leucine, isoleucine, and valine.

were not affected by treatment or by a treatment $\times$ time interaction, but changed over time ($P < 0.01$), following a similar pattern in both groups: after an increase from d -21 to d 1 ($P < 0.01$), the values declined ($P < 0.01$) until d 21 and remained unchanged thereafter. The ratio 3-MH:creatinine in muscle did not differ between the groups, but showed a similar pattern across time as muscle 3-MH ($P < 0.01$) with an interaction between treatment and time ($P = 0.04$).

Plasma Insulin and IGF-1

The time courses of the plasma concentrations of insulin and IGF-1 are presented in Figure 3. Plasma insulin concentrations were affected by CLA supplementation ($P = 0.02$) and decreased ($P < 0.01$) in both groups postpartum but to a greater extent in CTR than in sCLA, resulting in lower ($P < 0.05$) insulin concentrations on d 21 and 70 in CTR than in sCLA. Plasma IGF-1 concentrations were not different between groups, but the concentrations decreased ($P < 0.01$) in both groups postpartum. No treatment $\times$ time interaction was observed for plasma IGF-1 concentrations.

mRNA Abundance of Genes in the Skeletal Muscle

The mRNA abundance of genes related to the mTOR signaling pathway in skeletal muscle is shown in Figure 4. The mRNA abundance of *MTOR* on d 21 and 70 was greater in sCLA than in CTR ($P < 0.01$) and was also greater ($P \leq 0.01$) on d 21 and 70 compared with d -21 and 1 in sCLA. Slightly lower values were observed on d 70 than on d 1 and 21 in CTR ($P < 0.05$). The mRNA abundance of *4EBP1* was not affected by CLA supplementation, but changed over time ($P < 0.01$) and followed a similar pattern in both groups, specifically, a 5.9-fold increase from d -21 to d 1, followed by a decline thereafter and approaching prepartum values by d 70. No treatment $\times$ time interaction was observed for *4EBP1* mRNA abundance. The abundance of *S6K1* mRNA on d 70 was greater ($P < 0.01$) in CTR than in sCLA. The mRNA abundance of *S6K1* remained unchanged over time in both groups.

The mRNA abundance of UPS-related factors in skeletal muscle is shown in Figure 5. The mRNA abundance of *UBA1* was not affected by treatment or time, and no treatment $\times$ time interaction was found. The mRNA abundance of *UBE2G1* on d 70 was lower (P

Table 3. Serum AA concentrations (μmol/L) in dairy cows during late gestation and early lactation; supplementation with CLA (sCLA) or control fat (CTR) was initiated at d 1 of lactation

Item	Time (days relative to parturition)					
	−21		1		21	
	Pretreatment		sCLA		CTR	
Ala	236 ± 10.7 ^{AB,b}	181 ± 6.14 ^{B,b}	252 ± 15.3 ^A	214 ± 15.3 ^{ab}	267 ± 15.3 ^A	227 ± 15.7 ^{ab}
Arg	153 ± 5.20	166 ± 6.85	173 ± 8.16	171 ± 9.54	178 ± 7.97	180 ± 4.82
Asn	25.2 ± 1.94 ^C	25.4 ± 2.03 ^{BC}	39.2 ± 2.42 ^{AB}	30.7 ± 3.53	43.9 ± 3.18 ^A	35.0 ± 3.88
Asp	12.3 ± 0.78 ^{B,b}	14.3 ± 0.96 ^{AB,b}	20.0 ± 0.82 ^A	22.7 ± 2.49 ^a	21.6 ± 2.68 ^A	16.5 ± 1.2 ^{ab}
Cit	65.7 ± 3.97 ^{AB,ab}	52.4 ± 2.54 ^{B,b}	68.5 ± 3.60 ^{AB}	80.7 ± 6.37 ^a	80.2 ± 6.16 ^A	67.1 ± 4.44 ^{ab}
Gln	294 ± 14.3	233 ± 15.0	251 ± 17.0	225 ± 22.4	248 ± 17.1	211 ± 13.1
Glu	92.2 ± 7.02 ^a	66.0 ± 6.66 ^{ab}	62.1 ± 3.89	72.7 ± 8.63 ^{ab}	61.2 ± 5.01	50.6 ± 2.09 ^b
Gly	439 ± 24.1 ^{B,b}	606 ± 37.3 ^{A,ab}	806 ± 45.4 ^A	767 ± 71.5 ^a	624 ± 45.9 ^A	611 ± 58.9 ^{ab}
His	56.0 ± 3.42	52.6 ± 4.83	48.0 ± 4.78	40.1 ± 6.11	47.0 ± 6.43	46.8 ± 6.94
Ile	121.5 ± 4.86	110 ± 8.60	169 ± 23.6	147 ± 9.26	132 ± 9.28	114 ± 9.98
Leu	141 ± 6.85	118 ± 7.48	128 ± 8.62	124 ± 8.97	118 ± 9.03	123 ± 6.88
Lys	86.4 ± 4.52	69.6 ± 4.34	86.3 ± 3.95	85.2 ± 6.05	86.0 ± 5.32	84.3 ± 7.06
Met	21.4 ± 2.13	26.5 ± 4.58	30.9 ± 6.46	16.2 ± 3.05	34.5 ± 12.7	24.6 ± 2.43
Orn	42.6 ± 2.34 ^{A,a}	19.7 ± 1.56 ^{B,b}	29.7 ± 1.66 ^A	32.4 ± 2.60 ^a	43.2 ± 3.93 ^A	39.0 ± 4.58 ^a
Phe	58.6 ± 2.18 ^a	52.0 ± 2.47 ^{ab}	49.7 ± 2.41	48.5 ± 2.40 ^b	46.8 ± 3.0	54.5 ± 3.06 ^{ab}
Pro	79.8 ± 4.13	75.8 ± 3.76	96.2 ± 3.27	93.8 ± 8.39	93.6 ± 4.08	88.8 ± 7.46
Ser	85.4 ± 3.9	110 ± 7.11	109 ± 7.23	118 ± 12.9	99.9 ± 5.78	100 ± 12.6
Thr	73.0 ± 3.93 ^{AB,ab}	62.5 ± 4.52 ^{B,b}	89.7 ± 9.43 ^{AB}	102 ± 7.42 ^a	116 ± 14.8 ^A	97.3 ± 14.7 ^{ab}
Trp	47.1 ± 2.12 ^{Aa}	21.3 ± 2.56 ^{B,b}	43.8 ± 3.51 ^A	43.2 ± 3.14 ^a	55.6 ± 2.61 ^A	50.0 ± 5.80 ^a
Tyr	49.4 ± 2.41 ^{AB,a}	33.4 ± 1.6 ^{B,b}	40.3 ± 3.55 ^{AB}	40.5 ± 3.96 ^{ab}	49.4 ± 4.21 ^A	45.8 ± 3.97 ^{ab}
Val	186 ± 8.12 ^{ab}	161 ± 10.2 ^b	219 ± 11.0	221 ± 11.9 ^a	217 ± 11.0	203 ± 15.7 ^{ab}
BCAA ¹	449 ± 19.0	390 ± 24.6	517 ± 39.0	492 ± 23.6	467 ± 27.3	440 ± 31.4
EAA	791 ± 31.9	674 ± 36.4	865 ± 43.1	827 ± 37.3	853 ± 58.5	797 ± 58.1
Total AA	2,366 ± 64.7 ^{B,ab}	2,258 ± 87.9 ^{AB,b}	2,812 ± 81.2 ^A	2,695 ± 132 ^a	2,661 ± 119 ^{AB}	2,469 ± 155 ^{ab}

^{a,b, A-C} Different letters indicate differences between time points in the CTR group (a,b) and in the sCLA (A-C) group, respectively ($P < 0.05$).

¹BCAA = branched-chain amino acid, consisting of leucine, isoleucine, and valine.

= 0.05) in CTR than in sCLA. Also, the abundance of *UBE2G1* mRNA in both groups was lower or tended to be lower (CTR: $P < 0.01$; sCLA: $P \leq 0.1$) on d 70 as compared with other time points. The abundance of *UBE2G2* mRNA did not differ between the groups, but changed over time and followed a similar pattern in both groups postpartum ($P < 0.01$). No treatment $\times$ time interaction was observed for *UBE2G2* mRNA abundance. The mRNA abundance of *FBXO32* on d 21 was greater ($P < 0.05$) in sCLA than in CTR, but the relationship reversed on d 70 ($P < 0.05$). Time-related changes in the mRNA abundance of *FBXO32* were noted in both experimental groups ($P < 0.01$); the abundance increased 2.6-fold from d -21 to d 1, followed by a decrease thereafter in both groups to almost prepartum values by d 70. The mRNA abundance of *TRIM63* was not affected by treatment or by a treatment $\times$ time interaction, but it changed over time ($P < 0.01$), following a similar pattern in both groups, that is, increasing 3.3-fold from d -21 to d 1, then declining in both groups to almost prepartum values by d 70.

The mRNA abundance of the different subunits of the branched-chain α -keto acid dehydrogenase complex (*BCKDH*) E1 component in skeletal muscle is shown in Figure 6. The mRNA abundance of both the α polypeptide (*BCKDHA*) and the β polypeptide (*BCKDHB*) was not affected by CLA supplementation, but changed over time ($P \leq 0.02$) and a treatment $\times$ time interaction was observed ($P < 0.01$). On d 21, the mRNA abundance of *BCKDHA* was elevated in sCLA cows, whereas that of *BCKDHB* was elevated in CTR cows.

Protein Abundance of mTOR Signaling and UPS-Related Factors in Skeletal Muscle

The protein abundance of factors related to the mTOR signaling pathway and UPS in skeletal muscle is shown in Figure 7. No group differences were observed for the mTOR protein, but a trend was observed for time ($P = 0.06$). No treatment $\times$ time interaction was observed for the mTOR protein abundance. The protein abundance of S6K1 was not affected by time or by a

Figure 2. Time course of the muscle and serum concentrations (means $\pm$ SEM) of 3-methylhistidine (3-MH) and of the ratio 3-MH:creatinine in dairy cows during late gestation and early lactation. Supplementation with CLA (sCLA) or control fat (CTR) was initiated at d 1 of lactation. Different letters indicate differences ($P < 0.05$) between the time points in the control (CTR) (a-c) and in the sCLA (A-C) group.

treatment $\times$ time interaction, but the overall treatment effect was significant ($P = 0.04$). The mean protein abundance of S6K1 across all time points in sCLA was greater than in CTR. The protein abundance of 4EBP1 and TRIM63 was not affected by treatment or time, and no treatment $\times$ time interaction was found.

20S Proteasome Activity in Skeletal Muscle

As shown in Figure 8, 20S proteasome activity in the skeletal muscle did not differ between the groups, but

Figure 3. Time course of the plasma concentrations (means $\pm$ SEM) of insulin and IGF-1 in dairy cows during late gestation and early lactation. Supplementation with CLA (sCLA) or control fat (CTR) was initiated at d 1 of lactation. Different letters indicate differences ($P < 0.05$) between the time points in the control (CTR; a, b) and in the sCLA (A, B) group. Data for insulin and IGF-1 are from Singh et al. (2014).

Figure 4. Time course of skeletal muscle mRNA abundance (means $\pm$ SEM) of genes related to the mammalian target of rapamycin (mTOR) pathway in dairy cows during late gestation and early lactation. Supplementation with CLA (sCLA) or control fat (CTR) was initiated at d 1 of lactation. Different letters indicate differences ($P < 0.05$) between the time points in the control (CTR) (a-c) and in the sCLA (A-C) group. Asterisks indicate differences ($P < 0.01$) between sCLA and CTR at a given time point. 4EBP1 = eukaryotic translation initiation factor 4E binding protein 1; S6k1 = ribosomal protein S6 kinase, polypeptide 1.

Figure 5. Time course of skeletal muscle mRNA abundance (means $\pm$ SEM) of genes related to the ubiquitin-proteasome system (UPS) in dairy cows during late gestation and early lactation. Supplementation with CLA (sCLA) or control fat (CTR) was initiated at d 1 of lactation. Different letters indicate differences ($P < 0.05$) between the time points in the control (CTR; a, b) and in the sCLA (A, B) group. Asterisks indicate differences ($P < 0.01$) between sCLA and CTR at a given time point. *UBA1* = ubiquitin-like modifier activating enzyme 1; *UBE2G1* and *UBE2G2* = ubiquitin-conjugating enzymes; *FBXO32* = F-box only protein 32 (atrogen-1); *TRIM63* = tripartite motif containing 63 (also known as muscle ring-finger protein-1, *MuRF1*).

decreased ($P \leq 0.01$) in both groups on d 21 and 70 compared with those of d -21 and 1, and an interaction of treatment $\times$ time was observed ($P < 0.01$).

DISCUSSION

The animals of the sCLA group reached a positive calculated MP balance on d 70, whereas the CTR group already had a positive calculated MP balance on d 21. This variation is most likely due to the lower DMI in the

sCLA group over the first 21 d of lactation because no significant differences were found in milk yield, BW, or BCS. In addition, this variation is supported by plasma NEFA and BHB concentrations as reported previously (Pappritz et al., 2011). In a study reported by Hötger et al. (2013), CLA-fed cows had greater negative energy balance and a tendency for lower DMI as compared with control cows. Nevertheless, other studies regarding CLA supplementation in dairy cow nutrition have yielded mostly consistent results, and in contrast to our findings, no influence on DMI in early lactation was observed (Moore et al., 2004; Castañeda-Gutiérrez et al., 2005; Piamphon et al., 2009). However, reduced feed intake by CLA feeding has been observed (but not consistently) in some previous studies in laboratory animals (e.g., Cao et al., 2007; So et al., 2009). It has been suggested that the feed intake reduction by CLA may be due in part to the decreased palatability of free fatty acid compared with the triglyceride form, which is supported by the reduced intake only being observed early in the CLA feeding period (Park and Pariza, 2007). This suggested mechanism is not the case in our study because the CLA-containing supplements (4 kg of additional concentrate) were completely consumed by all cows. Alternatively, the postingestive metabolic effects of CLA could modulate appetite without having aversive effects on the animals (So et al., 2009). So et al. (2009) reported that feed intake was reduced by 23.6% in mice fed a low-fat diet supplemented with *trans*-10,*cis*-12 CLA. They also found that gene expression ratio of proopiomelanocortin to neuropeptide Y (NPY) in the hypothalamus was lower in mice receiving *trans*-10,*cis*-12 CLA, indicating that CLA may affect hypothalamic appetite-regulating genes. This hypothesis was supported by Cao et al. (2007) who found that injection of mixed isomers of CLA into the rat hypothalamus was associated with reduction in the expression of NPY and agouti-related protein, neuropeptides that robustly increase feed intake. Whether CLA may act in the same manner in dairy cows is currently unknown; however, considering the data from the literature (human and laboratory animal studies) and our observations, we hypothesize that postingestive metabolic effects of CLA could have modulated appetite without affecting the palatability of the diet.

The finding that the abundance of *FBXO32* mRNA was 1.7-fold greater in sCLA compared with CTR on d 21 likely resulted from the reduced DMI and thus protein intake in this group. This result may indicate upregulation of the UPS system at the mRNA level. The expression level of *FBXO32*, which encodes a muscle-specific F-box protein, is tightly regulated at the transcriptional level in skeletal muscle and is strongly induced in catabolic states; thus, it plays a key role in

Figure 6. Time course of skeletal muscle mRNA abundance (means $\pm$ SEM) of the different subunits of the branched-chain α -keto acid dehydrogenase complex (*BCKDH*) E1 component in dairy cows during late gestation and early lactation. Supplementation with CLA (sCLA) or control fat (CTR) was initiated at d 1 of lactation. Different letters indicate differences ($P < 0.05$) between the time points in the control (CTR; a, b) and in the sCLA (A, B) group. Asterisks indicate differences ($P < 0.01$) between sCLA and CTR at a given time point. *BCKDHA* = BCKDH α polypeptide; *BCKDHB* = BCKDH β polypeptide.

muscle atrophy through promoting muscle protein degradation by the UPS (Bodine et al., 2001; Gomes et al., 2001; Foletta et al., 2011). The *FBXO32* gene appears to be expressed in an early stage before muscle loss is detectable (Sorrentino, 2019). However, the mRNA abundance of another important E3 ligase, TRIM63 (MuRF-1), was not affected by the treatment, likely pointing to the differential response of the 2 ligases to the CLA supplementation or to the decreased DMI during early lactation, or to a combination of both. Indeed, the downstream substrates of TRIM63 and FBXO32 are not similar (Foletta et al., 2011) and differential expression of *FBXO32* and *TRIM63* mRNA in certain experimental conditions has been previously reported in mice (Frost et al., 2007; Yoshida et al., 2010), neonatal pigs (Suryawan and Davis, 2014), and neonatal calves (Sadri et al., 2017). The myogenic transcription factors MyoD and myogenin are known as protein tar-

get substrates for FBXO32 (atrogin-1; Foletta et al., 2011), whereas TRIM63 preferentially binds to and degrades myofibrillar protein components such as titin (Centner et al., 2001) and myosin light chain (MLC)1, and MLC2 (Cohen et al., 2009). Foletta et al. (2011), in a review of published studies, proposed that FBXO32 may be involved in regulation of the substrate targets affecting protein synthesis and muscle growth, whereas TRIM63 (MuRF-1) may play a role in the control of protein degradation and probably also contributes to skeletal muscle metabolism.

Due to the reduced DMI in sCLA cows over the first days of lactation and the related lower calculated MP balance, we therefore reasoned that protein degradation would be increased in the skeletal muscle during this period. This assumption was partly confirmed by the 1.7-fold greater abundance of *FBXO32* mRNA on d 21. We unexpectedly found, however, that the abundance

Figure 7. Time course of skeletal muscle abundance (means $\pm$ SEM) of proteins related to the mammalian target of rapamycin (mTOR) pathway and tripartite motif containing 63 (TRIM63; also known as muscle ring-finger protein-1, MuRF-1) in dairy cows during late gestation and early lactation. Supplementation with CLA (sCLA) or control fat (CTR) was initiated at d 1 of lactation. 4EBP1 = eukaryotic translation initiation factor 4E binding protein 1; S6K1 = ribosomal protein S6 kinase.

of *MTOR* mRNA and S6K1 protein was upregulated in sCLA, likely pointing to the potential stimulatory effect of CLA on muscle mTOR signaling pathway. Interestingly, the abundance of *FBXO32* mRNA was upregulated early (on d 21) and apparently transiently with CLA, whereas *MTOR* mRNA and S6K1 protein seemed to be more sustained. Thus, we speculate that the upregulation of *MTOR* mRNA and S6K1 protein may represent a feedback mechanism that prevents excessive muscle protein degradation during early lactation in the sCLA cows. However, data on the interplay in both anabolic and catabolic cellular response to sCLA treatment, even in human studies, are lacking and remain to be defined experimentally.

Insulin and AA—in particular BCAA—are known to be key factors mediating the stimulation of protein synthesis in muscle in response to feeding (Davis et al., 2010). Several studies have suggested that mTOR integrates AA and insulin signals through multiple mechanisms including activation of S6K1 and 4E-BP1, the 2 best-characterized regulators of protein synthesis downstream of mTORC1 (Proud, 2006; Davis et al., 2010; You et al., 2015). Therefore, one limitation of our study is the absence of data for the phosphorylated abundance of the main downstream targets of mTORC1. The greater abundance of *MTOR* mRNA and S6K1 protein in sCLA cows on d 21 and 70 might

be mediated by the greater plasma insulin postpartum. Comparable effects on insulin concentrations by feeding a similar CLA mixture have also been reported in a mouse model, in which the results were explained by an increase in β -cell mass and number (Poirier et al., 2005). In contrast to our findings, Hötger et al. (2013) reported no increase of insulin concentrations in dairy cows during supplementation with CLA for 9 wk during their second lactation. In the current study, plasma IGF-1 concentrations were not different between groups; thus, the observed responses to feeding CLA cannot be attributed to circulating IGF-1.

The BCAA Leu, Ile, and Val are catabolized in muscle by transamination, followed by an irreversible oxidative decarboxylation by the action of BCKDH (EC 1.2.4.4), which is considered the rate-limiting step in the overall BCAA catabolic pathway (Harper et al., 1984). From the different subunits of the BCKDH E1 component, the mRNA for the α polypeptide (*BCKDHA*) was elevated in sCLA cows on d 21, whereas the β polypeptide (*BCKDHB*) was elevated in CTR cows. Most studies in mammals have focused on assessing the BCKDHA component (Harris et al., 1997; Herman et al., 2010; Nichols et al., 2016), assuming it to be one of the main subunits responsive to nutritional and hormonal modulation and therefore regulating enzymatic activity. In the current study, the differences observed in the abundance of *BCKDH* subunit mRNA might be due to the differential effects of CLA on the regulatory mechanisms modulating BCKDH subunit expression, which warrants further investigations. We therefore speculate that regulation of *BCKDH* gene expression by CLA might play a role in determining the metabolic fate of BCAA in muscle. Catabolism of BCAA leads predominantly to the release of Ala and Gln, which play important roles in transferring both carbon atoms and amino groups to be taken up by the liver for gluconeogenesis and urea synthesis. Thus, these data may suggest a relatively high oxidative potential of the muscle in sCLA cows on d 21. However, we cannot determine whether or not these observations were independent of lower feed intake in sCLA cows on d 21; thus, the changes observed in the abundance of *BCKDH* subunits are likely, at least in part, to represent a component of the adaptations of BCAA metabolism in muscle to compensate lower feed intake related to CLA in this study.

The loss of body protein during early lactation is due to increased skeletal muscle degradation, decreased protein synthesis, or both. In this regard, a proteomics study found coordinated downregulation of proteins involved in protein synthesis and stabilization as well as upregulation of the cell growth suppressor during the first weeks of lactation in dairy cows (Kuhla et

Figure 8. Time course of skeletal muscle 20S proteasome activity (means $\pm$ SEM) in dairy cows during late gestation and early lactation. Supplementation with CLA (sCLA) or control fat (CTR) was initiated at d 1 of lactation. Different letters indicate differences ($P < 0.05$) between the time points in the control (CTR; a, b) and in the sCLA (A, B) group.

al., 2011). Although protein breakdown rates were not measured in the current study, our data show that the 20S proteasome activities were elevated on d -21 and 1 compared with d 21 and 70 in both groups, probably reflecting the start of protein mobilization antepartum. However, this possibility was only supported by upregulated muscle atrogenes and greater serum and muscle 3-MH concentrations and the ratio of 3-MH:creatinine on d 1. The onset as well as the duration and extent of protein and energy mobilization from body reserves during the transition period is highly variable in dairy cows (van der Drift et al., 2012). Based on plasma 3-MH concentrations and muscle thickness profiles, the mobilization of protein reportedly starts before calving, interestingly even before the onset of lipid mobilization in most cows, and continues up to 4 wk after parturition (van der Drift et al., 2012). The latter authors speculated that mobilization of protein might result from a prepartum AA deficiency in the absence of a negative energy balance. In the current study, the MP intake balance was estimated to be deficient on d 1, whereas it exceeded the MP requirements on d 21 (CTR group only) and d 70 due to increased DMI. Although antepartum DMI was not recorded in this experiment, elevated 20S proteasome activity was likely related to the lower DMI because a decrease in DMI beginning 3 wk antepartum is well documented in dairy cows (Hayirli et al., 2002). Another model proposed by Kuhla et al. (2011) highlighted that muscle's catabolic state in early lactation counteracts the imbalanced plasma AA pattern driven by the substrate supply for hepatic gluconeogenesis and milk production.

CONCLUSIONS

For unknown reasons, supplementation of the diet with CLA resulted in a decreased DMI over the first days of lactation and consequently a reduced calculated MP balance. The elevated activity of 20S proteasome on d -21 and 1 compared with d 21 and 70 in both groups may point to the start of protein mobilization antepartum, although this possibility was only supported by upregulated muscle *TRIM63* and *FBXO32* mRNA and greater serum and muscle 3-MH concentrations and the ratio of 3-MH:creatinine on d 1. Dietary supplementation of CLA increased the mRNA abundance of *FBXO32* on d 21, likely resulting from the reduced DMI, which may be related to an upregulation of the UPS and consequent stimulation of protein degradation in the muscle tissue. The upregulation of *MTOR* mRNA and S6K1 protein in sCLA cows despite a greater *FBXO32* mRNA abundance might be a feedback mechanism that prevents excessive muscle protein degradation during early lactation in these cows. Such

effects seem to be related by the greater plasma insulin concentration postpartum. The observed regulation in the mRNA abundance of *BCKDH* subunits by CLA might play a role in determining the metabolic fate of BCAA in muscle and probably reflects a relatively high oxidative potential of the muscle in sCLA cows on d 21. Further studies that include phosphorylated abundance of the main downstream targets of mTORC1, 4E-BP1, and S6K1 may provide additional clues that could help discern muscle tissue responses in the context of the dietary CLA supplement in dairy cows.

ACKNOWLEDGMENTS

The authors express their appreciation to Inga Hofmann and Isabella Israel (Institute of Animal Science, Physiology and Hygiene Unit, University of Bonn, Germany) for their excellent laboratory assistance as well as to Evonik Nutrition & Care GmbH (Hanau, Germany) for partial financial support of this experiment. Y. Yang was recipient of a scholarship from the China Scholarship Council (CSC). This project was partially supported by a research grant from the University of Tabriz (S/797-05/03/1397). The authors have not stated any conflicts of interest.

REFERENCES

- AOAC International. 1995. Official Methods of Analysis. 15th ed. AOAC International, Arlington, VA.
- Atherton, P. J., and K. Smith. 2012. Muscle protein synthesis in response to nutrition and exercise. *J. Physiol.* 590:1049–1057. <https://doi.org/10.1113/jphysiol.2011.225003>.
- Bauman, D. E., K. J. Harvatine, and A. L. Lock. 2011. Nutrigenomics, rumen-derived bioactive fatty acids, and the regulation of milk fat synthesis. *Annu. Rev. Nutr.* 31:299–319. <https://doi.org/10.1146/annurev.nutr.012809.104648>.
- Bilodeau, P. A., E. S. Coyne, and S. S. Wing. 2016. The ubiquitin proteasome system in atrophying skeletal muscle: Roles and regulation. *Am. J. Physiol. Cell Physiol.* 311:C392–C403. <https://doi.org/10.1152/ajpcell.00125.2016>.
- Bodine, S. C., and L. M. Baehr. 2014. Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1 and MAFbx/atrogen-1. *Am. J. Physiol. Endocrinol. Metab.* 307:E469–E484. <https://doi.org/10.1152/ajpendo.00204.2014>.
- Bodine, S. C., E. Latres, S. Baumhueter, V. K. M. Lai, L. Nunez, B. A. Clarke, W. T. Poueymirou, F. J. Panaro, E. Na, K. Dharmarajan, Z.-Q. Pan, D. M. Valenzuela, T. M. DeChiara, T. N. Stitt, G. D. Yancopoulos, and D. J. Glass. 2001. Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* 294:1704–1708. <https://doi.org/10.1126/science.1065874>.
- Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, and C. T. Wittwer. 2009. The MIQE guidelines—Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55:611–622. <https://doi.org/10.1373/clinchem.2008.112797>.
- Cao, Z. P., F. Wang, X. S. Xiang, R. Cao, W. B. Zhang, and S. B. Gao. 2007. Intracerebroventricular administration of conjugated linoleic acid (CLA) inhibits food intake by decreasing gene expression of NPY and AgRP. *Neurosci. Lett.* 418:217–221. <https://doi.org/10.1016/j.neulet.2007.03.010>.

- Castañeda-Gutiérrez, E., T. R. Overton, W. R. Butler, and D. E. Bauman. 2005. Dietary supplements of two doses of calcium salts of conjugated linoleic acid during the transition period and early lactation. *J. Dairy Sci.* 88:1078–1089. [https://doi.org/10.3168/jds.S0022-0302\(05\)72775-2](https://doi.org/10.3168/jds.S0022-0302(05)72775-2).
- Centner, T., J. Yano, E. Kimura, A. S. McElhinny, K. Pelin, C. C. Witt, M.-L. Bang, K. Trombitas, H. Granzier, C. C. Gregorio, H. Sorimachi, and S. Labeit. 2001. Identification of muscle specific ring finger proteins as potential regulators of the titin kinase domain. *J. Mol. Biol.* 306:717–726. <https://doi.org/10.1006/jmbi.2001.4448>.
- Chibisa, G. E., G. N. Gozho, A. G. Van Kessel, A. A. Olkowski, and T. Mutsavangwa. 2008. Effects of peripartum propylene glycol supplementation on nitrogen metabolism, body composition, and gene expression for the major protein degradation pathways in skeletal muscle in dairy cows. *J. Dairy Sci.* 91:3512–3527. <https://doi.org/10.3168/jds.2007-0920>.
- CHMP (Committee for Medicinal Products for Human Use). 2011. Guideline on bioanalytical method validation. EMEA/CHMP/EWP/192217/2009 Rev. 1 Corr. 2, 21 July 2011. European Medicines Agency, London, UK.
- Chung, S., J. M. Brown, M. B. Sandberg, and M. McIntosh. 2005. *Trans*-10, *cis*-12 CLA increases adipocyte lipolysis and alters lipid droplet-associated proteins: Role of mTOR and ERK signaling. *J. Lipid Res.* 46:885–895. <https://doi.org/10.1194/jlr.M400476-JLR200>.
- Ciechanover, A. 2005. Proteolysis: From the lysosome to ubiquitin and the proteasome. *Nat. Rev. Mol. Cell Biol.* 6:79–87. <https://doi.org/10.1038/nrm1552>.
- Cohen, S., J. J. Brault, S. P. Gygi, D. J. Glass, D. M. Valenzuela, C. Gartner, E. Latres, and A. L. Goldberg. 2009. During muscle atrophy, thick, but not thin, filament components are degraded by MuRF1-dependent ubiquitylation. *J. Cell Biol.* 185:1083–1095. <https://doi.org/10.1083/jcb.200901052>.
- 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. <https://doi.org/10.1017/S1751731110000984>.
- Dilzer, A., and Y. Park. 2012. Implication of conjugated linoleic acid (CLA) in human health. *Crit. Rev. Food Sci. Nutr.* 52:488–513. <https://doi.org/10.1080/10408398.2010.501409>.
- Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: The final frontier? *J. Dairy Sci.* 82:2259–2273. [https://doi.org/10.3168/jds.S0022-0302\(99\)75474-3](https://doi.org/10.3168/jds.S0022-0302(99)75474-3).
- European Commission. 2009. 2009/150/EC Commission regulation laying down the methods of sampling and analysis for the official control of feed. *Off. J. Eur. Union L* 54:1–130.
- Foletta, V. C., L. J. White, A. E. Larsen, B. Léger, and A. P. Russell. 2011. The role and regulation of MAFbx/atrogen-1 and MuRF1 in skeletal muscle atrophy. *Pflügers Arch.* 461:325–335. <https://doi.org/10.1007/s00424-010-0919-9>.
- Frost, R. A., G. J. Nystrom, L. S. Jefferson, and C. H. Lang. 2007. Hormone, cytokine, and nutritional regulation of sepsis-induced increases in atrogen-1 and MuRF1 in skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* 292:E501–E512. <https://doi.org/10.1152/ajpendo.00359.2006>.
- GfE (German Society of Nutrition Physiology). 2001. Ausschuss für Bedarfsnormen der Gesellschaft für Ernährungsphysiologie. Nr. 8. Empfehlungen zur Energie- und Nährstoffversorgung der Milchkühe und Aufzuchttrinder (Recommendations of energy and nutrient supply for dairy cows and breeding cattle). DLG-Verlag, Frankfurt am Main, Germany.
- Go, G. W., S. Oh, M. Park, G. Gang, D. McLean, H. S. Yang, M. H. Song, and Y. Kim. 2013. t10, c12 conjugated linoleic acid up-regulates hepatic de novo lipogenesis and triglyceride synthesis via mTOR pathway activation. *J. Microbiol. Biotechnol.* 23:1569–1576. <https://doi.org/10.4014/jmb.1308.08008>.
- Gomes, M. D., S. H. Lecker, R. T. Jagoe, A. Navon, and A. L. Goldberg. 2001. Atrogen-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proc. Natl. Acad. Sci. USA* 98:14440–14445. <https://doi.org/10.1073/pnas.251541198>.
- Harper, A. E., R. H. Miller, and K. P. Block. 1984. Branched-chain amino acid metabolism. *Annu. Rev. Nutr.* 4:409–454. <https://doi.org/10.1146/annurev.nu.04.070184.002205>.
- Harris, R. A., J. W. Hawes, K. M. Popov, Y. Zhao, Y. Shimomura, J. Sato, J. Jaskiewicz, and T. D. Hurley. 1997. Studies on the regulation of the mitochondrial alpha-ketoacid dehydrogenase complexes and their kinases. *Adv. Enzyme Regul.* 37:271–293. [https://doi.org/10.1016/S0065-2571\(96\)00009-X](https://doi.org/10.1016/S0065-2571(96)00009-X).
- Hayirli, A., R. R. Grummer, E. V. Nordheim, and P. M. Crump. 2002. Animal and dietary factors affecting feed intake during the pre-fresh transition period in Holsteins. *J. Dairy Sci.* 85:3430–3443. [https://doi.org/10.3168/jds.S0022-0302\(02\)74431-7](https://doi.org/10.3168/jds.S0022-0302(02)74431-7).
- Herman, M. A., P. She, O. D. Peroni, C. J. Lynch, and B. B. Kahn. 2010. Adipose tissue branched chain amino acid (BCAA) metabolism modulates circulating BCAA levels. *J. Biol. Chem.* 285:11348–11356. <https://doi.org/10.1074/jbc.M109.075184>.
- Hötger, K., H. M. Hammon, C. Weber, S. Görs, A. Tröschner, R. M. Bruckmaier, and C. C. Metges. 2013. Supplementation of conjugated linoleic acid in dairy cows reduces endogenous glucose production during early lactation. *J. Dairy Sci.* 96:2258–2270. <https://doi.org/10.3168/jds.2012-6127>.
- Ji, P., and H. M. Dann. 2013. Negative protein balance: Implications for transition cows. Pages 101–112 in *Proc. Cornell Nutrition Conference*, Syracuse, NY. Cornell Univ., Ithaca, NY.
- Kennedy, A., K. Martinez, S. Schmidt, S. Mandrup, K. LaPoint, and M. McIntosh. 2010. Antiobesity mechanisms of action of conjugated linoleic acid. *J. Nutr. Biochem.* 21:171–179. <https://doi.org/10.1016/j.jnutbio.2009.08.003>.
- Komaragiri, M. V., D. P. Casper, and R. A. Erdman. 1998. Factors affecting body tissue mobilization in early lactation dairy cows. 2. Effect of dietary fat on mobilization of body fat and protein. *J. Dairy Sci.* 81:169–175. [https://doi.org/10.3168/jds.S0022-0302\(98\)75564-X](https://doi.org/10.3168/jds.S0022-0302(98)75564-X).
- Korzick, D. H., D. R. Sharda, A. M. Pruznak, and C. H. Lang. 2013. Aging accentuates alcohol-induced decrease in protein synthesis in gastrocnemius. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 304:R887–R898. <https://doi.org/10.1152/ajpregu.00083.2013>.
- Kuhla, B., M. Kucia, S. Görs, D. Albrecht, M. Langhammer, S. Kuhla, and C. C. Metges. 2010. Effect of a high-protein diet on food intake and liver metabolism during pregnancy, lactation and after weaning in mice. *Proteomics* 10:2573–2588. <https://doi.org/10.1002/pmic.200900789>.
- Kuhla, B., G. Nürnberg, D. Albrecht, S. Görs, H. M. Hammon, and C. C. Metges. 2011. Involvement of skeletal muscle protein, glycogen, and fat metabolism in the adaptation on early lactation of dairy cows. *J. Proteome Res.* 10:4252–4262. <https://doi.org/10.1021/pr200425h>.
- McCrorie, T. A., E. M. Keaveney, J. M. W. Wallace, N. Binns, and M. B. Livingstone. 2011. Human health effects of conjugated linoleic acid from milk and supplements. *Nutr. Res. Rev.* 24:206–227. <https://doi.org/10.1017/S0954422411000114>.
- Moore, C. E., H. C. Hafliger III, O. B. Mendivil, S. R. Sanders, D. E. Bauman, and L. H. Baumgard. 2004. Increasing amounts of conjugated linoleic acid (CLA) progressively reduces milk fat synthesis immediately postpartum. *J. Dairy Sci.* 87:1886–1895. [https://doi.org/10.3168/jds.S0022-0302\(04\)73347-0](https://doi.org/10.3168/jds.S0022-0302(04)73347-0).
- Nandi, D., P. Tahiliani, A. Kumar, and D. Chandu. 2006. The ubiquitin-proteasome system. *J. Biosci.* 31:137–155. <https://doi.org/10.1007/BF02705243>.
- Nichols, K., J. J. M. Kim, M. Carson, J. A. Metcalf, J. P. Cant, and J. Doelman. 2016. Glucose supplementation stimulates peripheral branched-chain amino acid catabolism in lactating dairy cows during essential amino acid infusions. *J. Dairy Sci.* 99:1145–1160. <https://doi.org/10.3168/jds.2015-9912>.
- NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Sci., Washington, DC.
- Pappritz, J., U. Meyer, R. Kramer, E. M. Weber, G. Jahreis, J. Rehage, G. Flachowsky, and S. Dänicke. 2011. Effects of long-term supplementation of dairy cow diets with rumen-protected conju-

- gated linoleic acids (CLA) on performance, metabolic parameters and fatty acid profile in milk fat. *Arch. Anim. Nutr.* 65:89–107. <https://doi.org/10.1080/1745039X.2011.552275>.
- Park, Y. 2014. Conjugated linoleic acid in human health effects on weight control. Pages 429–446 in *Nutrition in the Prevention and Treatment of Abdominal Obesity*. R. R. Watson, ed. Academic Press, London, UK.
- Park, Y., K. Albright, W. Liu, J. Storkson, M. Cook, and M. Pariza. 1997. Effect of conjugated linoleic acid on body composition in mice. *Lipids* 32:853–858. <https://doi.org/10.1007/s11745-997-0109-x>.
- Park, Y., K. J. Albright, J. M. Storkson, W. Liu, M. E. Cook, and M. W. Pariza. 1999. Changes in body composition in mice during feeding and withdrawal of conjugated linoleic acid. *Lipids* 34:243–248. <https://doi.org/10.1007/s11745-999-0359-7>.
- Park, Y., and M. W. Pariza. 2007. Mechanisms of body fat modulation by dietary conjugated linoleic acid (CLA). *Food Res. Int.* 40:311–323. <https://doi.org/10.1016/j.foodres.2006.11.002>.
- Piamphon, N., C. Wachirapakorn, M. Wanapat, and C. Navanukraw. 2009. Effects of protected conjugated linoleic acid supplementation on milk fatty acid in dairy cows. *Asian-Australas. J. Anim. Sci.* 22:49–56. <https://doi.org/10.5713/ajas.2009.70380>.
- Poirier, H., C. Rouault, L. Clément, I. Niot, M. C. Monnot, M. Guerre-Millo, and P. Besnard. 2005. Hyperinsulinaemia triggered by dietary conjugated linoleic acid is associated with a decrease in leptin and adiponectin plasma levels and pancreatic beta cell hyperplasia in the mouse. *Diabetologia* 48:1059–1065. <https://doi.org/10.1007/s00125-005-1765-8>.
- Proud, C. G. 2006. Regulation of protein synthesis by insulin. *Biochem. Soc. Trans.* 34:213–216. <https://doi.org/10.1042/BST0340213>.
- Qin, N., A. R. Bayat, E. Trevisi, A. Minuti, P. Kairenius, S. Viitala, M. Mutikainen, H. Leskinen, K. Elo, T. Kokkonen, and J. Vilkkii. 2018. Dietary supplement of conjugated linoleic acids or polyunsaturated fatty acids suppressed the mobilization of body fat reserves in dairy cows at early lactation through different pathways. *J. Dairy Sci.* 101:7954–7970. <https://doi.org/10.3168/jds.2017-14298>.
- Sadri, H., S. Dänicke, U. Meyer, J. Rehage, J. Frank, and H. Sauerwein. 2015. Tocopherols and tocotrienols in serum and liver of dairy cows receiving conjugated linoleic acids or a control fat supplement during early lactation. *J. Dairy Sci.* 98:7034–7043. <https://doi.org/10.3168/jds.2015-9710>.
- Sadri, H., J. Steinhoff-Wagner, H. M. Hammon, R. M. Bruckmaier, S. Gors, and H. Sauerwein. 2017. Mammalian target of rapamycin signaling and ubiquitin proteasome-related gene expression in 3 different skeletal muscles of colostrum- versus formula-fed calves. *J. Dairy Sci.* 100:9428–9441. <https://doi.org/10.3168/jds.2017-12857>.
- Saremi, B., A. Al-Dawood, S. Winand, U. Muller, J. Pappritz, D. von Soosten, J. Rehage, S. Dänicke, S. Haussler, M. Mielenz, and H. Sauerwein. 2012a. Bovine haptoglobin as an adipokine: Serum concentrations and tissue expression in dairy cows receiving a conjugated linoleic acids supplement throughout lactation. *Vet. Immunol. Immunopathol.* 146:201–211. <https://doi.org/10.1016/j.vetimm.2012.03.011>.
- Saremi, B., H. Sauerwein, S. Dänicke, and M. Mielenz. 2012b. Technical note: Identification of reference genes for gene expression studies in different bovine tissues focusing on different fat depots. *J. Dairy Sci.* 95:3131–3138. <https://doi.org/10.3168/jds.2011-4803>.
- Schiaffino, S., K. A. Dyar, S. Ciciliot, B. Blaauw, and M. Sandri. 2013. Mechanisms regulating skeletal muscle growth and atrophy. *FEBS J.* 280:4294–4314. <https://doi.org/10.1111/febs.12253>.
- Schlegel, G., R. Ringseis, W. Windisch, F. J. Schwarz, and K. Eder. 2012. Effects of a rumen-protected mixture of conjugated linoleic acids on hepatic expression of genes involved in lipid metabolism in dairy cows. *J. Dairy Sci.* 95:3905–3918. <https://doi.org/10.3168/jds.2011-4835>.
- Singh, S. P., S. Häussler, J. F. Heinz, B. Saremi, B. Mielenz, J. Rehage, S. Dänicke, M. Mielenz, and H. Sauerwein. 2014. Supplementation with conjugated linoleic acids extends the adiponectin deficit during early lactation in dairy cows. *Gen. Comp. Endocrinol.* 198:13–21. <https://doi.org/10.1016/j.ygcen.2013.12.008>.
- Sippel, M. A., R. S. Spratt, and J. P. Cant. 2009. Milk production responses of primiparous and multiparous dairy cows to dose of conjugated linoleic acid consumed in rumen inert form. *Can. J. Anim. Sci.* 89:393–399. <https://doi.org/10.4141/CJAS08104>.
- So, M. H., I. M. Tse, and E. T. Li. 2009. Dietary fat concentration influences the effects of trans-10, cis-12 conjugated linoleic acid on temporal patterns of energy intake and hypothalamic expression of appetite controlling genes in mice. *J. Nutr.* 139:145–151. <https://doi.org/10.3945/jn.108.093849>.
- Sorrentino, M. J. 2019. Statins and muscle damage. Pages 519–527 in *Nutrition and Skeletal Muscle*. S. Walrand, ed. Academic Press, London, UK.
- Stangl, G. I. 2000. Conjugated linoleic acids exhibit a strong fat-to-lean partitioning effect, reduce serum VLDL lipids and redistribute tissue lipids in food-restricted rats. *J. Nutr.* 130:1140–1146. <https://doi.org/10.1093/jn/130.5.1140>.
- Sundrum, A. 2015. Metabolic disorders in the transition period indicate that the dairy cows' ability to adapt is overstressed. *Animals (Basel)* 5:978–1020. <https://doi.org/10.3390/ani5040395>.
- Suryawan, A., and T. A. Davis. 2014. Regulation of protein degradation pathways by amino acids and insulin in skeletal muscle of neonatal pigs. *J. Anim. Sci. Biotechnol.* 5:8–18. <https://doi.org/10.1186/2049-1891-5-8>.
- van der Drift, S. G. A., M. Houweling, J. T. Schonewille, A. G. M. Tielens, and R. Jorritsma. 2012. Protein and fat mobilization and associations with serum beta-hydroxybutyrate concentrations in dairy cows. *J. Dairy Sci.* 95:4911–4920. <https://doi.org/10.3168/jds.2011-4771>.
- Webb, L. A., H. Sadri, D. von Soosten, S. Dänicke, S. Egert, P. Stehle, and H. Sauerwein. 2019. Changes in tissue abundance and activity of enzymes related to branched-chain amino acid catabolism in dairy cows during early lactation. *J. Dairy Sci.* 102:3556–3568. <https://doi.org/10.3168/jds.2018-14463>.
- Yoshida, T., L. Semprun-Prieto, S. Sukhanov, and P. Delafontaine. 2010. IGF-1 prevents ANG II-induced skeletal muscle atrophy via Akt- and Foxo-dependent inhibition of the ubiquitin ligase atrogin-1 expression. *Am. J. Physiol. Heart Circ. Physiol.* 298:H1565–H1570. <https://doi.org/10.1152/ajpheart.00146.2010>.
- You, J. S., G. B. Anderson, M. S. Dooley, and T. A. Hornberger. 2015. The role of mTOR signaling in the regulation of protein synthesis and muscle mass during immobilization in mice. *Dis. Model. Mech.* 8:1059–1069. <https://doi.org/10.1242/dmm.019414>.
- Zukunft, S., C. Prehn, C. Röhring, G. Möller, M. Hrabe de Angelis, J. Adamski, and J. Tokarz. 2018. High-throughput extraction and quantification method for targeted metabolomics in murine tissues. *Metabolomics* 14:18–29. <https://doi.org/10.1007/s11306-017-1312-x>.
- Zukunft, S., M. Sorgenfrei, C. Prehn, G. Möller, and J. Adamski. 2013. Targeted metabolomics of dried blood spot extracts. *Chromatographia* 76:1295–1305. <https://doi.org/10.1007/s10337-013-2429-3>.